# NONNATIVE *GRACILARIA VERMICULOPHYLLA* TETRASPOROPHYTES ARE MORE DIFFICULT TO DEBRANCH AND ARE LESS NUTRITIOUS THAN GAMETOPHYTES[1]

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Theory predicts that the maintenance of haplo-diplontic life cycles requires ecological differences between the haploid gametophytes and diploid sporophytes, yet evidence of such differences remain scarce. The haplodiplontic red seaweed *Gracilaria vermiculophylla* has invaded the temperate estuaries of the Northern Hemisphere, where it commonly modifies detrital and trophic pathways. In native populations, abundant hard substratum enables spore settlement, and gametophyte:tetrasporophyte ratios are ~40:60. In contrast, many non-native populations persist in soft-sediment habitats without abundant hard substratum, and can be 90%–100% tetrasporophytic. To test for ecologically relevant phenotypic differences, we measured thallus morphology, protein content, organic content, "debranching resistance" (i.e., tensile force required to remove a branch from its main axis node), and material properties between male gametophytes, female gametophytes, and tetrasporophytes from a single, nonnative site in Charleston Harbor, South Carolina, USA in 2015 and 2016. Thallus length and surface area to volume ratio differed between years, but were not significantly different between ploidies. Tetrasporophytes had lower protein content than gametophytes, suggesting the latter may be more attractive to consumers. More force was required to pull a branch from the main axis of tetrasporophytes relative to gametophytes. A difference in debranching resistance may help to maintain tetrasporophyte thallus durability relative to gametophytes, providing a potential advantage in free-floating populations. These data may shed light on the invasion ecology of an important ecosystem engineer, and may advance our understanding of life cycle evolution and the maintenance of life cycle diversity.

*Key index words:* asexual reproduction; biomechanics; ecological differences; gametophyte; life cycle evolution; tetrasporophyte

*Abbreviations:* MLG, multilocus genotype; PVC, polyvinyl chloride; SCDNR, South Carolina Department of Natural Resources

The alternation between haploid and diploid phases varies widely across eukaryotic taxa, but the majority of theoretical and empirical research to date has focused on the advantages of diploidy (Valero et al. 1992, Mable and Otto 1998, Coelho et al. 2007). Nevertheless, haplodiplontic life cycles are found in mosses, ferns, foraminiferans, fungi, and algae (Bell 1992), suggesting haplodiplonty is remarkably stable, and not a transitory state (Valero et al. 1992, Klinger 1993). Many algal life cycles are considered isomorphic, in which there are few distinguishing macroscopic features between haploid

gametophytic and diploid tetrasporophytic phases (Valero et al. 1992, Klinger 1993), but where there may be subtle differences in morphology, survival, or resource partitioning (Hughes and Otto 1999). Hughes and Otto (1999) showed that subtle differences in the ecological niches between haploid and diploid phases can maintain haplodiplontic life cycles. However, relatively few empirical studies have attempted to quantify predicted differences in ecologically relevant phenotypes between gametophytes and sporophytes (Valero et al. 1992, Thornber 2006).

Ploidy and mating system variation are well-characterized for several species in the red algal order Gracilariales (e.g., Destombe et al. 1992, Engel et al. 2004, Krueger-Hadfield et al. 2016a), making them good candidates for testing the role of ecological differentiation in maintaining haplodiplontic life cycles. Typically, meiosis occurs within the tetrasporangia on the tetrasporophyte, producing haploid tetraspores. The tetraspores settle onto hard substratum and germinate into morphologically similar haploid male or female gametophytes. Gametes are, then, produced by mitosis, and fertilization occurs on the female gametophyte. The zygote develops into the carposporophyte, and it is mitotically copied into thousands of genetically identical, diploid carpospores. Each carpospore, in theory, can develop into an adult tetrasporophyte after settlement and germination, resulting in many genetically identical tetrasporophytes, though few studies have addressed this phenomenon (but see Engel et al. 2004, Krueger-Hadfield et al. 2013). Gametophytes and tetrasporophytes are also capable of vegetative fragmentation, and these free-floating thalli can become detached from the holdfast and grow indefinitely (Norton and Mathieson 1983, Santelices et al. 1984). Despite tetrasporophytic dominance across multiple gracilarioid taxa (Kain and Destombe 1995), there are few empirical tests of differentiation between the ploidy stages.

*Gracilaria vermiculophylla* (Gracilariales) is native to the northwest Pacific, and has invaded temperate estuaries along the coastlines of North America, Europe, and northern Africa (Bellorin et al. 2004, Rueness 2005, Guillemin et al. 2008a, Saunders 2009, Kim et al. 2010, Krueger-Hadfield et al. 2017). It is a novel ecosystem engineer in habitats that were historically devoid of macroalgae (Byers et al. 2012), with both positive (e.g., Thomsen and McGlathery 2005, Thomsen 2010, Kollars et al. 2016) and negative ecological impacts (e.g., Freshwater et al. 2006).

There was a profound life cycle shift associated with this invasion. In the native range, both gametophytic (~40%) and tetrasporophytic (~60%) thalli are common, and are fixed to hard substrata via holdfasts (Terada et al. 2000, Muangmai et al. 2012, Krueger-Hadfield et al. 2016a). In contrast, many nonnative populations are composed of free-floating thalli that lack holdfasts, and are maintained by extensive asexual fragmentation. Nonnative populations can be upwards of 90%–100% tetrasporophytic (Krueger-Hadfield et al. 2016a), and in ~40% of the nonnative sites surveyed, no gametophytic thalli have been found along sampled transects (Krueger-Hadfield et al. 2016a, 2017). While haploid-dominated populations in other *Gracilaria* species have been documented (Kain and Destombe 1995, Arakaki et al. 2015), we have not yet encountered a gametophyte-dominated population in the nonnative range of *Gracilaria vermiculophylla* (Krueger-Hadfield et al. 2016a, 2017). Thus, the trend of tetrasporophytic bias in the native range coupled with dominance in nonnative soft-sediment habitats suggest, at a minimum, that tetrasporophytes and gametophytes differ in their phenotypes and ecological performance (Kain and Destombe 1995, Guillemin et al. 2013, Krueger-Hadfield et al. 2016a).

In this study, we hypothesized that differences in morphology, nutritional content, or biomechanics may contribute to tetrasporophytic dominance in *Gracilaria vermiculophylla*. Previous studies have hinted at differences in the morphology of *G. vermiculophylla* gametophytes and tetrasporophytes (Sfriso et al. 2012), and associated growth rates (Abreu et al. 2011). While differences in palatability to herbivores have been detected between native and nonnative thalli (Hammann et al. 2013), differences in nutritional content have not been evaluated between life history stages in *G. vermiculophylla*, but may also influence niche partitioning among ploidies (e.g., Lubchenco and Cubit 1980). Though algal biomechanics studies typically focus on interspecific variation (reviewed in Thomsen and Wernberg 2005), they have emphasized the influence of structural and material properties on hydrodynamic performance (e.g., Charters et al. 1969, Koehl and Wainwright 1977, Koehl 1984, Armstrong 1987, Lowell et al. 1991, Johnson and Koehl 1994, Shaughnessy et al. 1996, Gaylord and Denny 1997, Harder et al. 2006, Boller and Carrington 2007, Demes et al. 2013). In *Chondrus crispus*, one of the species for which life history stages have been compared, gametophytes and tetrasporophytes differed in material properties, such as tensile strength, stiffness, and extensibility (Carrington et al. 2001). Though *G. vermiculophylla* thalli are free-floating in many nonnative, soft-sediment habitats, they will nonetheless encounter a similar, dynamic habitat as rocky intertidal macroalgae (Koehl 1984, Denny et al. 1989). *Gracilaria vermiculophylla* thalli will experience a suite of deformations that incur tension, despite not being fixed to the substratum. Along the eastern coast of the United States of America, *G. vermiculophylla* thalli are often glued to the mucus tubes of the polychaete *Diopatra cuprea* (Thomsen and McGlathery 2005, Gerstenmaier et al. 2016, Kollars et al. 2016). Thalli become intertwined, and are effectively anchored to the substratum. The thallus,

particularly the node at which an algal branch articulates with the thallus, of both free-floating and worm-anchored thalli, can become damaged (e.g., wave action can force an algal thallus to collide with abrasive surface), and, subsequently, be more prone to breakage. The differences between material properties of *G. vermiculophylla* haploid and diploid thalli may, in part, explain the absence of gametophytes in many soft-sediment, nonnative habitats if the gametophytes are more often damaged resulting in smaller and smaller thalli that are less able to persist in the free-floating populations.

Given the dominance of *Gracilaria vermiculophylla* tetrasporophytes in the nonnative range, we hypothesized tetrasporophytes should be larger, less nutritious, more resistant to debranching at nodes, and less brittle (e.g., stronger, stiffer, and tougher) than gametophytes. These traits may help maintain tetrasporophytic dominance in the nonnative range if: (i) larger individuals can outcompete smaller individuals for resources (e.g., Schwinning and Weiner 1998); (ii) macroalgal protein content alters the feeding rates of marine consumers (e.g., Cruz-Rivera and Hay 2001, Raubenheimer et al. 2005, Clements et al. 2009); and (iii) stronger, stiffer, and tougher thalli with greater resistance to debranching are less likely to be damaged and maintain thallus integrity while free-floating or anchored to worm tubes in the intertidal zone.

## MATERIALS AND METHODS

*Sampling methodology.* We sampled *Gracilaria vermiculophylla* (Fig. 1) thalli from the Fort Johnson mudflat in the Charleston Harbor, South Carolina, United States (32°45′4.68″ N, 79°54′2.7″ W). At this site, the majority of the *G. vermiculophylla* biomass is tetrasporophytic, and either free-floating devoid of holdfasts or anchored in the mid-intertidal by the tube-building polychaete *Diopatra cuprea* (Gerstenmaier et al. 2016, Kollars et al. 2016, Krueger-Hadfield et al. 2016a). A small fraction of the *G. vermiculophylla* biomass (<2%) is fixed via holdfasts to pebbles in runoff from the South Carolina Department of Natural Resources (SCDNR), or on other flotsam and shells (S.A. Krueger-Hadfield, pers. obs.).

On 21 April 2015 and over two dates in 2016 (22 March, 6 April), we sampled reproductive thalli: 20 male gametophytes, 20 female gametophytes, and 20 diploid tetrasporophytes total, or 10 per life history phase per year. We confirmed reproductive state (i.e., the presence tetrasporangial sori, spermatangial sori, or cystocarps) using a dissecting microscope (40×). Ploidy was also verified using 10 microsatellite loci (Kollars et al. 2015, Krueger-Hadfield et al. 2016a; see also Genotypic diversity below).

In 2015, we sampled the 30 thalli from PVC posts that were deployed on the mudflat between January and April 2014 in order to obtain *Gracilaria vermiculophylla* recruits. The posts were checked monthly. *Gracilaria vermiculophylla* recruits were several centimeters in length by August 2014 (S.A. Krueger-Hadfield, unpub. data). When we sampled thalli in April 2015 for this study, the recruits were, at maximum, 1-year old. In 2016, we sampled the 30 thalli from the SCDNR runoff fixed via holdfasts (i.e., sporic recruits) to pebbles in the mid-intertidal. Therefore, we do not know the approximate age of the 2016 thalli, or how long they were present in the Fort Johnson mudflat.

*Thallus morphometrics.* Digital photographs were taken of all 60 thalli from both years using a Canon G16 (Canon, Tokyo, Japan) mounted on a camera stand with a white background and size standard. Each thallus was placed in a 245 mm petri dish (Corning, New York, USA) containing seawater. Large thalli were split across multiple photographs to minimize overlapping of thallus branches. Digital images were analyzed with WinRHIZO software (Regent Instruments, Quebec City, Canada), with modified methods for root analyses (see Bouma et al. 2000) as *G. vermiculophylla* thalli are similar to plant roots in overall structure. Using the size-calibration function in WinRHIZO, we collected data for each thallus on projected surface area, volume, total length, and average diameter.

*Mass measurements.* We obtained the following mass measurements for all 60 morphometric samples using an analytical balance (±0.1 mg): wet mass, dry mass, and ash-free dry mass. We patted the thalli dry, and placed them into preweighed foil envelopes for measuring wet mass. Thalli were dried at 70°C in a drying oven to a constant mass. To estimate inorganic content, we placed the dried tissue in a combustion oven at 500°C for 6 h. Following combustion, samples were weighed for ash weight (i.e., inorganic content). Organic content was estimated from the difference between the dry weight and ash weight.

*Protein assay.* We assayed protein concentrations for 2015 thalli only ($N = 28$, nine tetrasporophytes and 19 gametophytes). We subsampled 10 pieces of each thallus per life history stage, standardized at 10 cm in total length. We, then, dried the thalli to a constant weight at 70°C, followed by grinding in a 1.5 mL centrifuge tube using a pestle. We used 10 mg of the ground tissue, and followed the Bradford
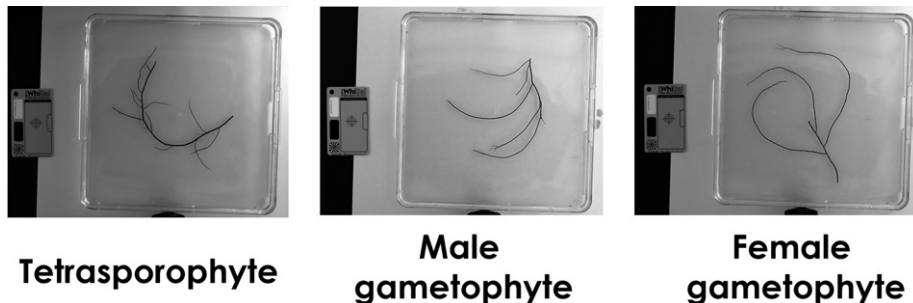


FIG. 1. A tetrasporophyte, male gametophyte, and female gametophyte of *Gracilaria vermiculophylla* identified by the presence of reproductive structures and sampled from the Fort Johnson mudflat in 2015. Thalli are in the Petri dishes used for morphological analyses in WinRhizo. (photo credit: S.A. Krueger-Hadfield).

Method (Bradford 1976). We added 0.5 mL of 1 M NaOH to each sample, vortexed and left the samples for 24 h at room temperature. We, then, added 20 μL of the extract and 1 mL of Bradford reagent to a polystyrene cuvette and allowed the mixture to sit at room temperature for 5 min. We read the absorbance of the samples at 595 nm and compared the samples to a standard curve based on bovine serum albumin standards prepared by following the Bradford Quick Start Guide (Bio-Rad, Berkeley, CA, USA). All samples were extracted in triplicate to account for instrumentation fluctuation. We calculated protein per gram dry mass as:

$$P = \frac{C}{W} \times 100$$

where $P$ is the percent protein per gram dry mass, $C$ is the concentration of the thallus sample ($\mu g \cdot mL^{-1}$), and $W$ is dry mass (mg).

*Debranching resistance.* We tested the susceptibility of a branch to breaking off a main axis (hereafter, node), consistent with vegetative reproduction through fragmentation, from five male gametophytic, five female gametophytic, and five tetrasporophytic thalli collected in 2015. Here, we simply refer to this characteristic as "debranching resistance." For each thallus, we ran 4–5 trials of debranching resistance for the branch-main axis nodes.

We employed quasi-static uniaxial tensile testing approaches using a motorized vertical testing stand (Imada EMS-275, Northbrook, IL, USA) with attached force and height gauges (Imada ZP-11 and Mitutoyo Digimatic height gauge, Auroria, IL, USA) to determine debranching resistance (Fig. 2, a and b). We clamped a randomly selected sample of an algal thallus between a pair of standard, serrated grips (one stationary grip or base, and one actuating grip attached to the force gauge). Nodes were clamped forming an inverse T-shape where the two pieces of the main axis were clamped at the stationary base and the branch apex was fixed to the mobile clamp. The applied uniaxial tension of all test samples involved the upward movement of the force gauge and connected height gauge at a strain rate of $1.5 mm \cdot min^{-1}$ until mechanical failure. During each tensile test, the applied force and distance data were sampled at 2.0 Hz using a custom software package (Imada SW2X).

We evaluated the peak forces (N) required to induce mechanical failure at the nodes. The debranching resistance was the amount of applied tensile force required to pull branches apart at their nodes.

*Material properties.* Using the approaches described above for recording force and distance, we performed tensile tests on thalli from five male gametophytes, five female gametophytes, and five tetrasporophytes collected in 2015. We clamped one end of the main longitudinal axis (hereafter, main axis) to the stationary base and the other end to the mobile clamp. We, then, applied mechanical tension along the main axis (Fig. 2c). For each main axis analysis, we ran 4–5 trials of each thallus (see below for statistical treatment of these technical replicates).

To determine the material properties of *Gracilaria vermiculophylla* main axes, we measured the length (initial grip separation) and diameter of the thin cylindrical thallus sample before applying tension. Cross-sectional area (CSA) of each sample was calculated as:

$$CSA = \pi R^2$$

where $R$ (radius) equals one half of the measured diameter.

With these dimensional data sets from the main axis samples, we converted our force and length data into stress–strain data. Stress ($\sigma$) was calculated as:

$$\sigma = \frac{F}{CSA}$$

where $F$ is force in Newtons (N) immediately prior to failure; and strain ($\varepsilon$) equals:

$$\varepsilon = \frac{\Delta L}{L_0}$$

where $L_0$ was the initial length of the algal sample exposed between the stationary and actuating grips prior to testing, and $\Delta L$ was the change in length that occurred during testing.

From the stress–strain curves, we measured the following material properties: breaking stress (or strength), peak strain (or extensibility), stiffness (or modulus), and strain energy storage (or toughness; or work of extension; Fig. 2d). Strength was defined as the maximum applied stress prior to failure, and the applied strain at the peak stress equaled the peak strain (extensibility). Here, the stiffness (or modulus) equaled the ratio of stress to strain in the steepest linear region of the stress–strain curve (Fig. 2d). We, also, measured the area under the stress–strain curves to determine the material's strain energy storage, which is also referred to as "breaking energy," "work of extension," or "toughness" (Vogel 2013). The integrals of the stress–strain curves were calculated using MagicPlot Student (Magicplot Systems LLC, St Petersburg, Russia).

In order to compare the material properties of *Gracilaria vermiculophylla* thalli to other macroalgal tissues, we performed a SCOPUS search (from 1920-present; 10 January 2018) with the key terms "alga*" and "biomecha*," and used extracted data from papers which reported similar material properties as in our study.

*Genotypic diversity.* Total genomic DNA was isolated from each thallus using 5–10 mg of dried tissue and the Nucleospin® 96 plant kit according to the manufacturer's instructions except for the cell lysis step in which lysate was left at room temperature for 1 h and DNA was eluted in 100 μL of $dH_2O$ (Krueger-Hadfield et al. 2013). Simplex PCRs, fragment analysis and genotyping are described elsewhere (see Kollars et al. 2015, Krueger-Hadfield et al. 2016a).

The number of repeated identical multilocus microsatellite genotypes (MLGs) was computed using *RClone* ver. 1.0 (Bailleul et al. 2016) implemented in R, ver. 3.4.0 (R Core Team, 2017) separately for each life history phase and for each collection year (Krueger-Hadfield et al. 2011, 2013, Krueger-Hadfield and Hoban 2016). $P_{sex}$, or the probability for a given MLG to be observed in $N$ samples as a consequence of different sexual reproductive events, was calculated for each repeated MLG. If the $P_{sex}$ $P$-value was greater than 0.05, duplicated MLGs were considered as different genets. If the $P_{sex}$ $P$-value was less than 0.05, the duplicated MLGs were considered as ramets (or clones) of the same genet. We calculated genotypic richness, $R$, as (G-1)/(N-1), where $G$ is the number of unique MLGs and $N$ is the total number of studied thalli (Dorken and Eckert 2001). We, also, calculated genotypic evenness, E.5, using *poppr*, ver. 2.2.0 (Kamvar et al. 2014, 2015) implemented in R, ver. 3.4.0 (R Core Team 2017).

*Data analyses.* All data satisfied assumptions of normality of residuals and homogeneity of variance for the analyses of variance, described below. As we did not detect significant differences between male and female gametophytes, we pooled them. We, then, analyzed differences between tetrasporophytes and pooled gametophytes. For response variables measured in 2 years, and for which we detected a significant year effect, we analyzed standardized variables, where
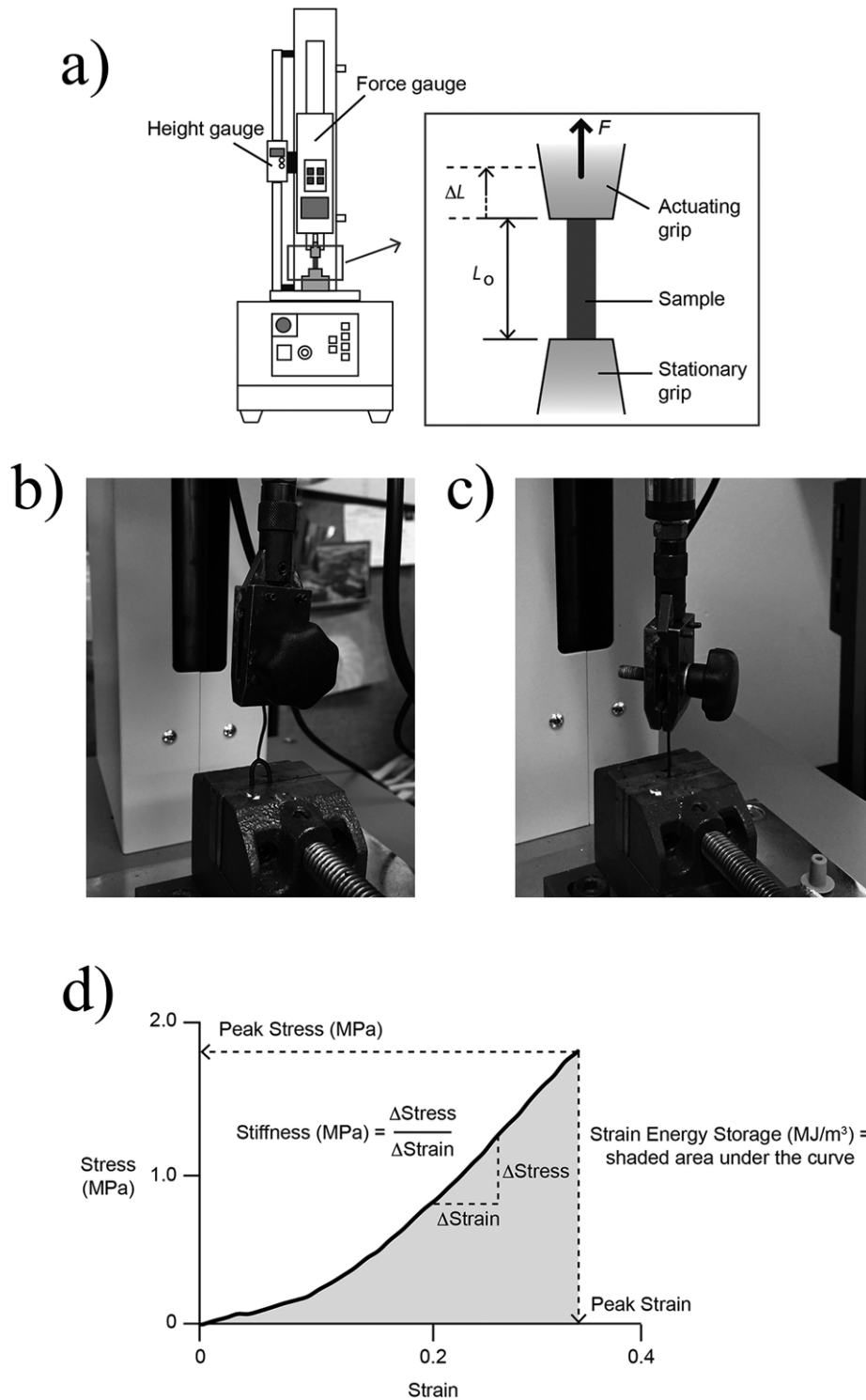
FIG. 2. Methods used for testing and analyzing thallus samples. (a) Illustration of motorized vertical materials testing rig, with the inset to the right showing how the initial length ($L_0$) and change in length ($\Delta L$) were measured from a test sample clamped between a stationary base and mobile grip from which the tensile force ($F$) was applied to the sample (illustration credit: A.J. Clark). (b) Image of a branch-main axis node test sample (photo credit: S.A. Krueger-Hadfield). (c) Image of a main axis test sample (photo credit: S.A. Krueger-Hadfield). (d) Representative stress–strain curve from a main axis thallus sample (tetrasporophyte) uniaxially stretched to failure, included with methods for determining the material properties: peak stress (strength), peak strain (extensibility), stiffness (modulus), and strain energy storage (toughness or work of extension).

the mean of the corresponding year was subtracted from each ploidy value, and subsequently divided by the standard deviation of that year. We generated a series of analyses of variance (ANOVA) in R, ver. 3.4.0 (R Core Team 2017) in which ploidy group (diploid tetrasporophyte or haploid gametophyte) was treated as a fixed, independent factor. Because protein, node integrity, and material property data included technical replicate assays per thallus, we analyzed these using a mixed model approach, where thallus was a random intercept, and we generated $P$-values via a Satterthwaite approximation in the *lmer* package (Kuznetsova et al. 2016) in R.

## RESULTS

*Thallus size and nutritional content.* Thalli collected in 2016 exhibited greater length to volume ratios than those sampled in 2015 (ANOVA, $F_{1,53} = 24.20$, $P < 0.0001$). Standardized ratios, in which 2015 and 2016 samples were combined and account for year variation, revealed no significant difference between ploidies ($F_{1,58} = 0.286$, $P = 0.595$; Fig. 3a).

Surface area to volume ratios were also larger in 2016 thalli compared to 2015 thalli (ANOVA, $F_{1,35} = 25.88$, $P < 0.001$). An analysis of the standardized surface area to volume ratios showed no difference between ploidies ($F_{1,58} = 0.191$, $P = 0.664$; Fig. 3b). Similarly, 2016 thalli had higher wet mass to dry mass ratios than 2015 individuals ($F_{1,53} = 36.89$, $P < 0.001$). Standardized wet mass to dry mass ratios for all samples were not found to be significantly different between ploidies ($F_{1,57} = 0.373$, $P = 0.544$; Fig. 3c, Table S1 in the Supporting Information).

Tetrasporophytes had significantly lower percent protein of dry mass than gametophytes ($P = 0.012$). Tetrasporophytes, also, had a lower percent protein of wet mass than gametophytes (ANOVA, $F_{1,26} = 20.179$, $P < 0.001$; Fig. 4). No differences were detected between ploidies in percent ash-free dry mass ($F_{1,58} = 0.027$, $P = 0.871$) or organic content per unit volume ($F_{1,58} = 0.027$, $P = 0.868$).

*Tensile mechanics.* Approximately 50% more force was required to pull the branch from the main axis of tetrasporophytes ($0.967 \pm 0.088$ N) compared to gametophytes ($0.651 \pm 0.047$ N; $P = 0.036$; Fig. 5). Because differences could be due to the node diameter, we compared the diameter of gametophytic and tetrasporophytic thalli. We found no difference in diameters generated with digital calipers for samples studied for material properties (tetrasporophytes: $0.728 \pm 0.039$ mm; gametophytes: $0.860 \pm 0.046$ mm; $P = 0.099$). We, also, found no differences examining average diameter values from images analyzed with WinRhizo, then standardized ($z$-scores) to account for between-year variation (tetrasporophytes $z$-score: $0.193 \pm 0.203$; gametophytes $z$-score: $-0.076 \pm 0.166$ mm; ANOVA, $F_{1,58} = 1.139$, $P = 0.290$).

By contrast, we found no difference between ploidies in the strength ($P = 0.532$), extensibility ($P = 0.308$), stiffness ($P = 0.282$), or strain energy storage ($P = 0.354$; Table 1). Overall, the material properties of *Gracilaria vermiculophylla* main axes were comparable to many macroalgal thalli previously examined (Table S2 in the Supporting Information).

*Genotypic diversity.* Genotypic richness ($R$: 0.7–1.0) and evenness (E.5: 0.866–0.952) were high, regardless of the sampling year, ploidy, or sex (Table 2, Table S3 in the Supporting Information). We detected two pairs of repeated tetrasporophytic MLGs; one pair in 2015 and another in 2016. However, only the pair sampled in 2015 was considered clones based on $P_{sex}$ ($P < 0.001$). For the female gametophytes, we sampled three pairs in 2015 and
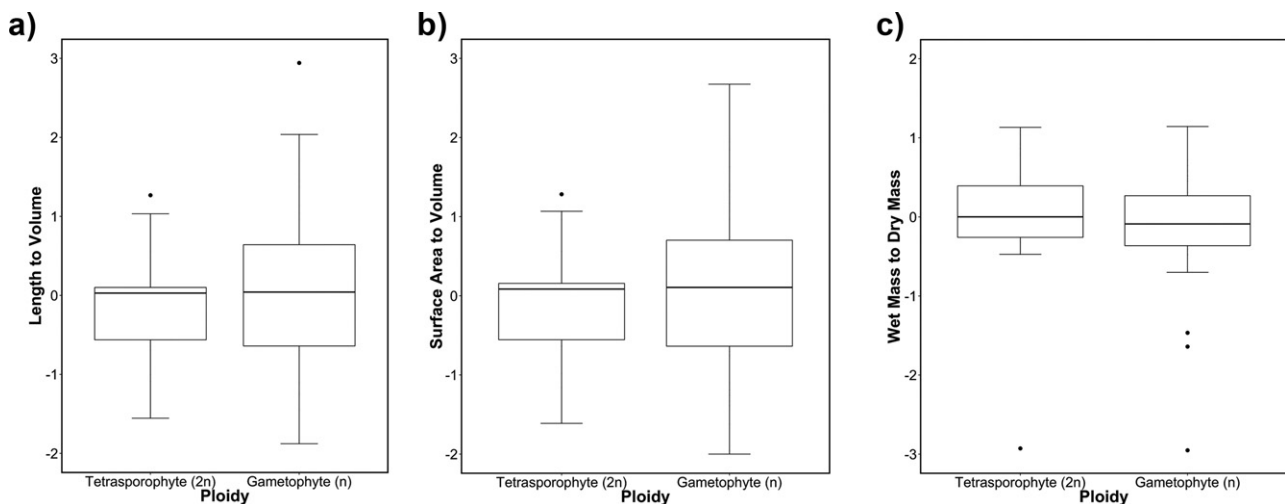


FIG. 3. Boxplots of (a) standardized length to volume, (b) standardized surface area to volume and (c) standardized wet mass to dry mass for tetrasporophytic (2n) and gametophytic (n) thalli sampled in 2015 and 2016. Sample sizes were 20 and 39 for tetrasporophytes and gametophytes, respectively.
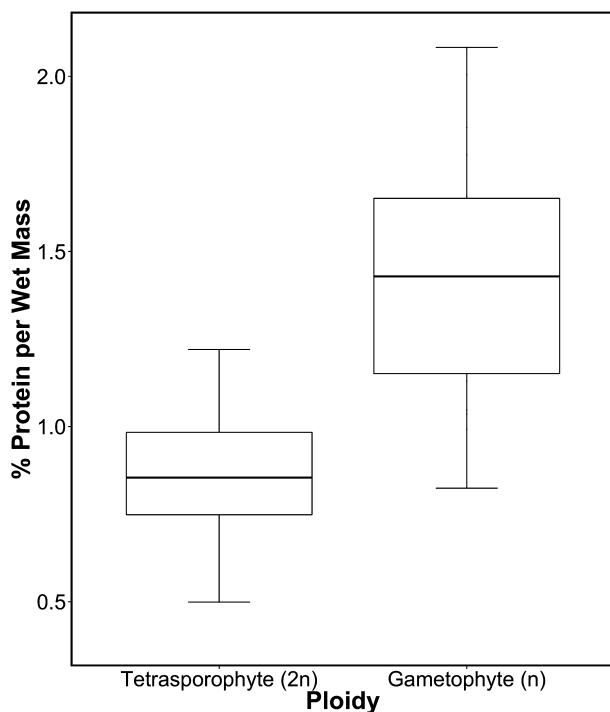
FIG. 4. Boxplot of average percent protein per wet mass for tetrasporophytic (2n) and gametophytic (n) thalli sampled in 2015. The difference between ploidies was significant ($P < 0.001$). Sample sizes were nine and 19 for tetrasporophytes and gametophytes, respectively.
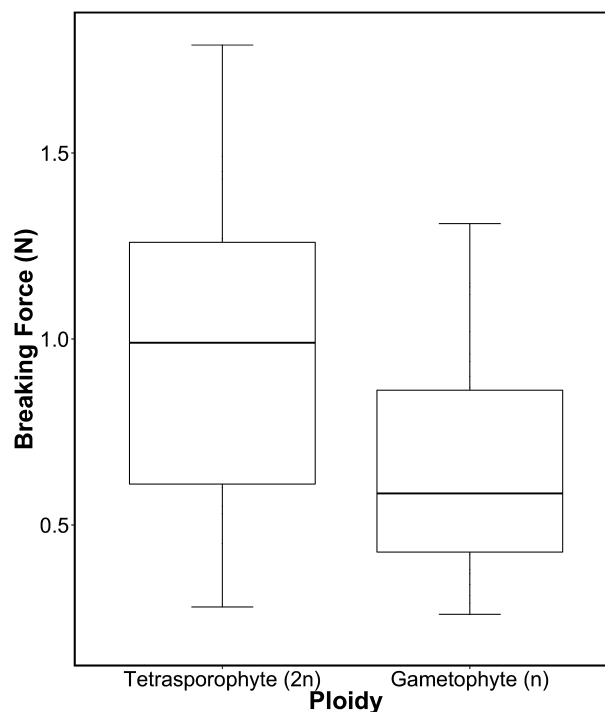


FIG. 5. Boxplot of the peak force (N) required to remove a branch from the main axis-branch node for tetrasporophytic (2n) and gametophytic (n) thalli sampled in 2015. Sample sizes were 5 and 10 for tetrasporophytes and gametophytes, respectively, with 3–4 technical replicates per thallus.

two pairs in 2016 of repeated MLGs, but all $P_{sex}$ *P*-values were greater than 0.1. As a result, we considered these repeated female MLGs as different genets. Finally, we encountered several repeated male gametophytic MLGs in both 2015 and 2016, in which $P_{sex}$ *P*-values were <0.001. While some replicates used in our study could be considered clones based on $P_{sex}$, overall, we considered the thalli as unique genotypes because the repeated haploid thalli were likely the result of low polymorphism across the 10 microsatellite loci and relatively low sample size. Moreover, each of these male and female gametophytic thalli was sampled from different holdfasts on settling posts deployed in 2015.

## DISCUSSION

We provide empirical support for potential ecological differentiation between gametophytic and tetrasporophytic thalli of *G. vermiculophylla* from a nonnative site. Specifically, tetrasporophytes had greater debranching resistance (i.e., resistance to mechanical failure at the branch-main axis node) and lower nutritional content (i.e., protein content). In this nonnative population, tetrasporophytes and gametophytes were statistically indistinguishable in thallus size, shape, and other proxies for nutritional content. Though there were no differences in material properties (i.e., tensile strength, extensibility, stiffness, and strain energy storage) between ploidies, *G. vermiculophylla* thalli were extensible, but, nevertheless, brittle, with relatively weak (low breaking stress), compliant (low stiffness) thalli that required little energy to break. Considering both plodies together, the material properties of *G. vermiculophylla* fell within the reported ranges of macroalgal tensile strength (0.91 MPa [Sheath and Hambrook 1988] to 25.9 MPa [Dudgeon and Johnson 1992]) and stiffness (0.77 MPa [Wolcott 2007] to 60.0 MPa [Biedka et al. 1987] of 46 species; Table S2).

The dominance of a particular ploidy phase has attracted attention in the phycological literature (reviewed in Coelho et al. 2007); however, empirical tests of differences between ploidies or sexes are rare. In the Gracilariales, tetrasporophytes seem to dominate soft-sediment habitats in which sporic recruitment is severely limited or nonexistent (Kain and Destombe 1995, Guillemin et al. 2008b, Krueger-Hadfield et al. 2016a, but see Arakaki et al. 2015). In the nonnative populations of *Gracilaria vermiculophylla*, tetrasporophytes dominate (Krueger-Hadfield et al. 2016a), including Fort Johnson (Gerstenmaier et al. 2016, Kollars et al. 2016, Krueger-Hadfield et al. 2016a). These observations strongly suggest tetrasporophytes have a fitness advantage in soft-sediment habitats that lack abundant hard substratum, thereby limiting sporic

TABLE 1. Material properties of the thalli in *Gracilaria vermiculophylla* from various life histories. Data sets include the mean (±SE) strength (peak stress), extensibility (peak strain), Stiffness (modulus determined from the peak slope), and toughness (strain energy storage or work of extension).

| Material properties | Strength (MPa) (±SE) | Extensibility (±SE) | Stiffness (MPa) (±SE) | Toughness (MJ · m$^{-3}$) (±SE) |
|---|---|---|---|---|
| Tetrasporophyte | 2.08 ± 0.38 | 0.27 ± 0.02 | 9.25 ± 1.3 | 0.29 ± 0.06 |
| Gametophyte | 1.67 ± 0.17 | 0.28 ± 0.02 | 7.68 ± 0.69 | 0.22 ± 0.03 |

TABLE 2. Genotypic richness, $R$, and evenness, E.5, by life history phase and year ($n = 10$ thalli each).

| Life history phase | Year | $R$ | E.5 |
|---|---|---|---|
| Tetrasporophyte | 2015 | 0.9 | 0.952 |
| | 2016 | 1.0 | 0.952 |
| Male gametophyte | 2015 | 0.8 | 0.866 |
| | 2016 | 0.7 | 0.866 |
| Female gametophyte | 2015 | 1.0 | 0.932 |
| | 2016 | 1.0 | 0.932 |

recruitment. Our results indicate two potential mechanisms for this fitness advantage.

First, greater tensile force was required to pull apart a tetrasporophytic thallus compared to a gametophyte at the branch-main axis node. Since we did not find any differences in the material properties of the main axes across ploidies, and assuming the material composition is conserved at the branches and nodes, the differences in debranching resistance must be attributed to structural differences in the node (e.g., node diameter, volume, or thickness). However, we did not specifically measure the diameter of the branch-main axis nodes in this study. Even though we detected no differences between tetrasporophytes and gametophytes in the diameter of the main axes used for the main axis materials analyses, or as calculated across the entire thallus using WinRhizo, it remains possible that branch-main axis node structural differences were present. If this were the case, it is possible that one, or multiple structural features, of the tetrasporophytic node might incur greater debranching resistance than gametophytic nodes. One possible means of achieving more resistance to debranching is by simply having a large node (e.g., a large node diameter to thallus diameter ratio). A large-diameter node will have a concomitantly large cross-sectional area, which effectively reduces the stresses applied to the node. Conversely, nodes with small diameters would be more prone to failure under mechanically identical situations because the applied forces would be distributed over a smaller cross-sectional area. Under the assumption that the material properties, thallus size, branch size, and water flow velocities are the same across ploidies, we hypothesize that greater debranching resistance results from a larger diameter node, or large node diameter to thallus diameter ratio.

In macroalgae, biomechanical failure often occurs at the stipe which connects the blade or fronds to the holdfast (Carrington 1990, Shaughnessy et al. 1996, Carrington et al. 2001). While the stipe-holdfast junction in *G. vermiculophylla* is a site of failure, holdfasts are rare in many soft-sediment sites, particularly in the nonnative range (Krueger-Hadfield et al. 2016a). Branch-main axis nodes may be more ecologically relevant for testing mechanical failure points of free-floating thalli lacking holdfasts, such as the dominant biomass at Fort Johnson. This is also relevant because the majority of these free-floating thalli are incorporated into polychaete mucus tubes on this mudflat (see also Thomsen 2004, Thomsen and McGlathery 2005, Kollars et al. 2016). As *G. vermiculophylla* tetrasporophytic branchlets were more firmly attached to the main axis, tetrasporophytes might maintain thallus integrity while drifting in the intertidal zone, or once glued to mucus tubes. In a survey of *G. vermiculophylla* thalli anchored to 20 different *Diopatra cuprea* tubes at the Fort Johnson mudflat, only two out of a total 528 (0.3%) thalli genotyped were haploid based on the microsatellite MLG (Gerstenmaier et al. 2016, see also Krueger-Hadfield et al. 2016a). The scarcity of free-floating and worm-tube anchored haploid gametophytic thalli (Gerstenmaier et al. 2016, S. A. Krueger-Hadfield, L. E. Lees, E. E. Sotka and C. J. Murren, unpub. data), coupled with data from the current study, supports the hypothesis that the ecological patterns of tetrasporophytic dominance may be due in part to differences at the branch-main axis node between gametophytes and tetrasporophytes. Future studies should explore differences in survival and growth rates of tetrasporophytes and gametophytes under a variety of scenarios (e.g., salinity or temperature) to further elucidate the mechanisms underlying diploid dominance (see also Guillemin et al. 2013).

In other red algal families, such as the Gigartinaceae, gametophytes and tetraporophytes are hypothesized to exhibit differences in structural properties because of the different gelling strength of their phycocolloids (e.g., Harvey and McLachlan 1973). In *Chondrus crispus*, for example, the stronger-gelling kappa carrageenans may provide gametophytes with a structural advantage in response to desiccation or herbivory (Carrington et al. 2001), but also are known to prevent infection by endophytic pathogens (Correa and McLachlan 1992, Krueger-Hadfield 2011). In the agarophytes, including *Gracilaria vermiculophylla*, the cell walls and extracellular matrix are composed of agar. Agar yield and

gel strength vary across species (e.g., Mollet et al. 1998, Marinho-Soriano 2001), but there are conflicting results as to whether there is a strong link between life history stage and agar quality (e.g., gelling strength; Whyte et al. 1981, Pickering et al. 1990, Marinho-Soriano et al. 1999). For example, female gracilarioid gametophytes have been found to have weaker gel strength than tetrasporophytes (e.g., Marinho-Soriano et al. 1999), whereas other studies found no differences between reproductive gametophytes and tetrasporophytes (e.g., Hoyle 1978). Though we did not measure gelling properties in our study, the similarity in the material properties of reproductive gametophytic and tetrasporophytic main axes hints that gel strength may be similar in the ploidy stages of *G. vermiculophylla*. Future studies should assess the ecophysiological role that phycocolloids, such as agar, may play in structuring haplodiplontic algal populations (see Carrington et al. 2001).

The second mechanism that may favor tetrasporophytes was suggested by their tissue quality with respect to herbivores. Intraspecific variation in resistance to herbivory among ploidies or sexes can also significantly alter the ecological consequences of algal–herbivore interactions (Lubchenco and Gaines 1981), particularly if one phase is more susceptible to mechanical failure than another. Most work has focused on differential herbivory between ploidy stages in heteromorphic life cycles (e.g., Lubchenco and Cubit 1980). Nevertheless, there are some tantalizing patterns in some isomorphic ploidy stages. For example, *Chondrus crispus* tetrasporophytes can become more heavily infected by a green algal endophyte, whereby herbivores preferred infected tissue to uninfected tissue, possibly contributing to greater incidence of herbivory in the more heavily infected tetrasporophytes (Correa and McLachlan 1992). Moreover, the infections become concentrated at the stipe, leading to higher incidences of herbivory and mechanical failure as compared to the rarely infected (Correa and McLachlan 1992). In *Gracilaria vermiculophylla*, gametophytes had higher protein concentrations, suggesting they are more nutritious and, therefore, may be more attractive to herbivores (Horn and Neighbors 1984). Increased grazing of gametophytes may decrease haploid thallus integrity (see also Lubchenco and Gaines 1981), further contributing to tetrasporophytic dominance. This hypothesis remains to be tested.

All the thalli in our study were reproductive. In other studies, reproductive structures decreased both the force to break and the fatigue strength of haploid and diploid thalli (Shaughnessy et al. 1996, Mach et al. 2011). Similarly, other studies have found differences between vegetative and reproductive thalli in terms of agar gel strengths with putative structural effects (e.g., Marinho-Soriano et al. 1999). At Fort Johnson, the *Gracilaria vermiculophylla*

biomass is composed of less than 20%, on average, reproductive thalli throughout the year (S. A. Krueger-Hadfield, L. E. Lees, E. E. Sotka and C. J. Murren, unpub. data). Reproductive structures may affect life history stages differently (Hannach and Santelices 1985, Shaughnessy et al. 1996, Mach et al. 2011), including *Gracilaria* thalli (Guillemin et al. 2014). Future work should compare nonreproductive tissue as vegetative tetrasporophytes could be even stronger as than reproductive diploid thalli, thereby affecting ploidy dominance and population structure.

Though genotypic richness and evenness was high, we did encounter several repeated MLGs based on $P_{sex}$. Repeated MLGs may be common in natural populations due to thallus fragmentation (e.g., Guillemin et al. 2008b, Krueger-Hadfield et al. 2016a) or, for diploids, cystocarpic amplification (but see, Engel et al. 2004, Krueger-Hadfield et al. 2013). Previous studies have shown high genotypic diversity in the Fort Johnson tetrasporophytic subpopulation over multiple years (Gerstenmaier et al. 2016, Krueger-Hadfield et al. 2016a), but gametophytes were not studied. We re-analyzed the gametophytic MLGs from Krueger-Hadfield et al. (2016a), and found ~25% and ~40% repeated haploid MLGs in native and nonnative sites, respectively. Guillemin et al. (2008b) also found repeated gametophytic MLGs in natural populations of *Gracilaria chilensis*, and suggested these repeated MLGs may be the result of insufficient polymorphism at the loci used (M. L. Guillemin, pers. comm.; see also Arnaud-Haond et al. 2005, 2007). Nevertheless, the thalli used in this study were ecologically representative of the Fort Johnson *G. vermiculophylla* biomass, which includes repeated MLGs.

In order to better understand the long-term eco-evolutionary impacts of the *Gracilaria vermiculophylla* invasion, and the life history pattern of tetrasporophytic dominance, in particular, it will be necessary to explore differences in growth and survival rates of spores, juveniles, and adults as has been done in *G. chilensis* (Guillemin et al. 2013). Tetraspores and carpospores released from Fort Johnson thalli germinated (S. A. Krueger-Hadfield, unpub. data), but more extensive culturing experiments are necessary in order to determine if the absence of ecological substratum is the only factor limiting sporic recruitment, and the persistence of gametophytes in primarily free-floating populations. Since Norton and Mathieson's (1983) seminal review on unattached algal populations, very little work has addressed these populations. Gracilarioid species are excellent models with which to empirically test these hypotheses as previously undocumented populations, and even species, are rapidly being found and recorded inhabiting soft-sediment habitats (e.g., Arakaki et al. 2015, Krueger-Hadfield et al. 2016a,b, 2017). These data will shed light on the invasion ecology of important ecosystem engineers, and advance our

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### Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

**Table S1.** The results of an ANOVA investigating differences between tetrasporophytes and gametophytes of standardized morphometric indices ($N = 60$ across 2 years). Means are displayed for each life history phase $\pm$ SE.

**Table S2.** Macroalgal material properties reported in the literature, including this study. We performed a SCOPUS search (10 January 2018) with the key terms "alga*" and "biomecha*," and used extracted material property data.

**Table S3.** Genotypes of the tetrasporophytes, male gametophytes, and female gametophytes from the Fort Johnson mudflat.