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Influence of nutrients on ploidy-specific performance in an invasive, haplodiplontic red macroalga

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ABSTRACT

Worldwide, macroalgae have invaded near-shore marine ecosystems. However, their haplodiplontic life cycles have complicated efforts to predict patterns of growth and spread, particularly since most theoretical predictions are derived from diplontic taxa (i.e., animals). To complete one revolution of the life cycle, two separate ploidy stages, often including separate haploid sexes, must pass through development and reproduction. In the case of the invasive, red macroalga *Agarophyton vermiculophyllum*, during the invasion of soft-sediment estuaries throughout the Northern Hemisphere, diploid tetrasporophytes came to dominate all free-floating populations and haploid gametophytes were consistently lost. The ecological hypothesis of nutrient limitation might contribute to an explanation of this pattern of tetrasporophytic dominance in free-floating populations. Under this hypothesis, gametophytes should outperform tetrasporophytes under nutrient limited conditions, but tetrasporophytes should be better able to exploit optimal or even abundant nutrient conditions, such as in eutrophic estuaries. We sampled tetrasporophytes, male gametophytes, and female gametophytes from two sites each located on either side of the Delmarva Peninsula that separates the Chesapeake Bay from the Atlantic Ocean. We subjected apices excised from multiple thalli from each life cycle stage to a nutrient-enriched and a nutrient-poor seawater treatment and assessed growth and survival. While nutrient addition increased growth rates, there was no significant difference among ploidies or sexes. Gametophytes did, however, suffer higher mortality than tetrasporophytes. We discuss how nutrient-dependent differences in growth and survival may contribute to observed patterns of tetrasporophytic dominance in soft-sediment *A. vermiculophyllum* populations.

KEY WORDS: algae, biological invasion, estuaries, Gracilaria, life cycle, nutrients, ploidy

ABBREVIATIONS: PES, Provasoli's Enriched Solution; GLMER, generalized linear mixed model; LMER, linear mixed model; AICc, corrected Akaike information criterion

The need to predict the growth and spread of populations takes on a particular urgency when studying non-native species. Yet, we often lack necessary data for making informed predictions about the population dynamics of organisms with complex life cycles, including common marine invaders, such as macroalgae (Krueger-Hadfield 2020). Life cycle characteristics, such as the number of free-living stages and whether these stages share the same genome (e.g., invertebrates with larval stages versus haplodiplontic algae), are predicted to alter the demographic states (i.e., age, spatial location, sex) under which persistence and expansion are expected (Caswell 1982). Predicting patterns in natural systems requires detailed knowledge of the energetic and demographic trade-offs that shape transition rates among life cycle stages.

For haplodiplontic macroalgae, free-living haploid gametophytes (often with separate sexes) and diploid sporophytes are linked through spatially and temporally separated alternations of meiosis and fertilization (Fig. 1; for a more detailed review see Bell 1994). The persistence of these life cycles is predicted by niche differentiation among life stages (Hughes and Otto 1999). While ecological differentiation is much more intuitive for heteromorphic life cycles (Lubchenco and Cubitt 1980, Valero et al. 1992, Klinger 1993, Bessho and Iwasa 2009), isomorphic life cycles are stabilized through much more subtle forms of differentiation (Hughes and Otto 1999, Scott and Rescan 2016). Cryptic differences in survival (e.g., due to herbivory or salinity) and resource use (e.g., nutrients) between stages in isomorphic algae have been found to make them as ecologically distinct as those found in heteromorphic life cycles (e.g., Destombe et al. 1992, Gonzales and Meneses 1996, Thornber and Gaines 2004, Carmona and Santos 2006, Krueger-Hadfield 2011, Guillemin et al. 2013, Lees et al. 2018, Neill et al. 2018, Vieira et al. 2018a,b). If different life cycle stages exhibit different responses to stressors or changing conditions, then trait differentiation may render a species vulnerable to selection against the “weakest link” (Istock 1967, Pandori and Sorte 2018, Krueger-Hadfield 2020). Following a perturbation, demographic consequences on one stage may cascade through the entire life cycle rendering such species particularly vulnerable. As many haplodiplontic macroalgae are introduced through the world’s oceans (Williams and Smith 2007), predicting how they may

respond to as a consequence of demographic shifts during invasions warrants further attention (e.g., loss of a free-living stage; Krueger-Hadfield et al. 2016).

The red, haplodiplontic macroalga *Agarophyton vermiculophyllum* (commonly known under the synonym *Gracilaria vermiculophylla*) has invaded virtually every temperate estuary, which are often anthropogenically modified and disturbed habitats, in the Northern Hemisphere over the last 100 years (Kim et al. 2010, Krueger-Hadfield et al. 2017, 2018) and has transformed these ecosystems (e.g., Nyberg et al. 2009, Byers et al. 2012). Its rapid spread was likely facilitated by its capacity for asexual fragmentation and indefinite survival as free-floating thalli (Krueger-Hadfield et al. 2016). Across much of the non-native range, the estuaries into which this alga was introduced lack sufficient hard substratum for the settlement of propagules. If *A. vermiculophyllum* gametophytes and tetrasporophytes performed equally well in these free-floating conditions, we would expect to find populations dominated by male gametophytes, female gametophytes, tetrasporophytes, or some combination based on the invasion history of the site (i.e., what type of thallus/thalli were introduced and propagated by fragmentation). Yet, tetrasporophytes dominate at all free-floating sites thus far studied (Krueger-Hadfield et al. 2016, 2017, 2018). This pattern has been mirrored in soft-sediment habitats in multiple species in the Gracilariales (e.g., Guillemain et al. 2008, also reviewed in Kain and Destombe 1995), suggesting a selective bias for tetrasporophytic thalli, despite morphological similarity among the free-living stages (Fig. 1).

Here, we investigated one ecological hypothesis in order to better understand the role of abiotic variation on tetrasporophytic dominance in *Agarophyton vermiculophyllum*. Lewis (1985) postulated that haploidy can confer an ecological advantage under nutrient limitation as haploids spend fewer resources on producing and maintaining DNA content and are, thus, better able to grow under conditions in which nutrients, such as phosphorus or nitrogen, are limited. Conversely, diploids have numerous genetic advantages, such as genetic buffering or heterosis (reviewed in Valero et al. 1992, Bell 1994), which may contribute to higher growth rates when nutrients are abundant (e.g., why diploid diatoms are early successional species amongst the unicellular algae; Lewis 1985). However, Lewis

(1985) and Bell (1994), among others, questioned whether the nutrient limitation hypothesis applies to multicellular taxa, because the efficiency gained by producing small, nutrient-conserving cells is offset by the need to produce more cells to reach the same thallus size as a diploid. Vieira et al. (2018a,b) advocated for the explicit testing of the nutrient limitation hypothesis building on suggestions by Richerd et al. (1993) about the role of resource availability in the evolutionary stability of haplodiplontic life cycles. Destombe et al. (1993) found juvenile gametophytic holdfasts in *Gracilaria gracilis* (as *G. verrucosa*) grew better than tetrasporophytic holdfasts when nutrients were limiting whereas diploids had the advantage in nutrient replete conditions, providing support for Lewis' (1985) hypothesis in a multicellular alga. However, for *A. vermiculophyllum*, holdfasts are rare in non-native soft, sediment habitats, begging the question of whether Lewis' (1985) hypothesis is relevant for adult thalli. As these populations are often propagated through asexual fragmentation, the growth patterns of adult tissue is critical to measure.

To test the nutrient limitation hypothesis, we sampled *Agarophyton vermiculophyllum* male gametophytes, female gametophytes, and tetrasporophytes at two sites in October 2018, where reproductive individuals are known to be present and genotypic diversity is high (Krueger-Hadfield et al. 2016). Ape Hole Creek (near Crisfield, Maryland; 37.9581, -75.8233) and Magotha Road (near Townsend, Virginia, 37.1762, -75.9318) are located along the Delmarva Peninsula that divides the Chesapeake Bay from the Atlantic Ocean (Fig. S1 in the Supporting Information). The Chesapeake Bay Estuary is a nutrient rich environment, which has also been under anthropogenic enrichment for hundreds of years (Kemp et al. 2005). In keeping with Lewis' (1985) hypothesis and based upon previous work by Destombe et al. (1993), enhanced diploid performance in eutrophic waters may help explain the success of tetrasporophytes in this region. While these two sites differed in substrate composition, temperature, and salinity (S.A. Krueger-Hadfield and W.H. Ryan, unpub. data), we had no a priori expectation that site of origin would influence the degree of response to nutrient levels among life stages. Our experimental design also allowed us to diagnose the relative importance of stage-specific responses to nutrients compared to the influence of genotype or site of origin. If there are differences in the response to nutrients, then

regardless of the site of origin, we expect to see gametophytic thalli grow and/or survive better in low nutrient conditions whereas tetrasporophytes will grow better under nutrient-replete conditions.

Thalli were collected by hand from the intertidal zone during low tide with at least 1 meter separating each putative individual. Each thallus was observed using a dissecting microscope (40x) for reproductive structures (see Krueger-Hadfield et al. 2018). In order to ensure we had a balanced design of male gametophytes, female gametophytes, and tetrasporophytes, and to eliminate differences that may arise from comparing reproductive versus non-reproductive genets, all thalli used in the subsequent experiment were reproductive at the time of collection. From five haphazardly selected thalli (putative genets) from each life cycle stage and site (30 genets total), we excised at least 40- 1 cm apices. We, then, selected 10 apices per thallus that were not bleached and looked healthy, yielding 300 total apices in the experiment. Half the apices were assigned to a nutrient enriched treatment and the other half to an unenriched treatment. We chose to replicate the experiment at the level of apices within genet in order to estimate the effect of genotype within stage and site of origin on growth rate and the probability of bleaching. Given the importance of asexual fragmentation for this species, understanding the relative importance of within- versus between-genet variation is also important for predicting the species performance in these habitats.

Culture conditions were chosen based on the combination of temperature and salinity with the lowest mortality and fastest growth rates in previous experiments with this species (Rueness 2005; W.H. Ryan and S.A. Krueger-Hadfield, unpub. data). Each apex was placed individually into wells of 12-well tissue culture plates (1.5-2.2 mL capacity per well, Corning Inc., New York, NY, USA) filled with sterile artificial seawater (salinity of 20; Amsler and Neushul 1989) kept at 20°C with at 12:12 h light:dark cycle (170:0 Lux) in an environmental control chamber (Percival I-36LL, Percival Scientific, Perry, IA, USA). For the enriched treatment, 1X PES was diluted in 1000 mL of artificial seawater (Provasoli 1968). Once per week, for four weeks in total, thalli were photographed for size measurements and the seawater was replaced using Pasteur pipets.

Growth was measured as the change in thallus length over the four weeks of the experiment. Initial and final thallus length were measured from photographs with *Image J* v. 1.51 (Schneider et al. 2012). Bleaching was scored visually for each thallus using photographs from each time point. Each apex was initially scored as unbleached (i.e., no evidence of any bleaching), partially bleached (i.e., evidence of partial bleaching along the apex), or fully bleached (i.e., thallus was completely bleached; see also Sotka et al. 2018). For analysis, the bleached status of each apex was treated as a binary proxy for survival, where partially and fully bleached thalli were both treated as dead and only unbleached thalli were marked alive. Over the course of the experiment, no apex was observed to recover to an unbleached state after any appearance of bleaching (Sotka et al. 2018). Thalli generally progressed to fully bleached and fragile (i.e., dead) within one week of the appearance of partial bleaching, indicating that this is a reliable correlate of mortality.

The influence of site, life cycle stage, and nutrient level on the probability of bleaching were evaluated with a generalized linear mixed model (GLMER) with a binomial distribution. Results are plotted as the genet-level probability of survival (one minus the probability of bleaching), which reflects the proportion of apices from each genet that remained unbleached for the duration of the experiment. For the subset of apices that showed no bleaching at the end of the experiment, the influence of site, stage, and nutrient level on growth was evaluated with a linear mixed model (LMER) as growth was normally distributed. In both cases, genotype (genet ID) was included as a random factor to account for similarity due to thallus of origin. Model selection allowed us to identify the combination of factors and interaction terms that explained the most variance while favoring parsimony, a balance quantified by the corrected Akaike information criterion (AICc). Stepwise model selection based on AICc was performed with the dredge function, and goodness of fit was estimated with the *r.squareGLMM* function, both in the package 'MuMIn' (Barton 2018) in *R* ver. 3.5.1 (R Core Team 2018).

We found that nutrient treatment had no effect on survival, but did influence growth, though not in a way that consistently discriminated among life cycle stages. The best-fit model for survival included genet identity, site, and life cycle stage, but not nutrient level as

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predictors (marginal $R^2 = 0.24$; Fig. 2A). Thalli sampled at Magotha Road had a significantly higher probability of survival than those from Ape Hole Creek (0.97 vs. 0.67; $X^2 = 31.68$, $df = 1$, $p < 0.001$). The probability of survival differed significantly among life cycle stages ($X^2 = 7.87$, $df = 2$, $p = 0.020$). Tetrasporophytes had a higher probability of survival (.90) than both male and female gametophytes (0.79 and 0.77, respectively; see Table S1 in the Supporting Information for model selection details). Although there was no apparent difference in mortality among stages at Magotha Road, no interactions among the factors were retained in the best-fit model.

All but one of the surviving apices grew, regardless of treatment. The best-fit model of growth included nutrient level, site, and life cycle stage, as well as an interaction between site and life cycle stage ($R^2 = 0.18$; Fig. 2B). Genet identity was not included in the best fit model, as variation within genets was high compared to variation among genets (Fig. S2B in the Supporting Information). Thalli grew significantly more in enriched versus unenriched water ($F_{1, 239} = 17.21$, $p < 0.001$). Thalli from Magotha Road grew significantly more than those from Ape Hole Creek ($F_{1, 239} = 10.69$, $p = 0.001$). There was no difference among life stages overall ($F_{2, 239} = 0.16$, $p = 0.922$). There was a significant interaction between site and stage ($F_{2, 239} = 9.70$, $p = 0.007$) driven by female thalli having lower growth than tetrasporophytes or male gametophytes at Magotha Road, but similar growth among stages at Ape Hole Creek (Fig. 2B). Average growth in thalli from Ape Hole Creek was higher under nutrient enrichment compared to Magotha Road, though the interaction between site and nutrient level was not retained in the best-fit model (Table S2 in the Supporting Information; Fig. S2).

While the addition of nutrients increased growth rates in *Agarophyton vermiculophyllum* apices, the life cycle stage-specific effects of nutrients are more complex than suggested solely by the nutrient limitation hypothesis (Lewis 1985) and as previously shown by Destombe et al. (1993) in another gracilarioid species, *G. gracilis*. Studies in yeast have shown that whether haploids or diploids perform better under nutrient limiting conditions depends on the taxon and the experiment (e.g., Adams and Hansche 1974,

Mable 2001, Otto and Gerstein 2008, Bessho et al. 2015). Thus, evidence for the nutrient limitation hypothesis is mixed for both uni- and multicellular taxa.

In our study, both the tetrasporophytes and male gametophytes from Magotha Road showed accelerated growth in nutrient-rich conditions, while female gametophytes did not. Previous studies (e.g., Zhang and van der Meer 1987, Guillemain et al. 2013, but see Hoyle 1978) have found females appear to grow faster due to senescence of male thalli following reproduction (Kain and Destombe 1995). Destombe et al. (1993) did not distinguish between male and female gametophytes, but under high nutrient conditions, the variance in gametophytic growth was low, suggesting the pooling of gametophytes did not obscure sex differences. In our study, the response to nutrients was highly variable among female genets (Fig. S2), particularly at Magotha Road where females, on average, had the lowest growth and did not differ among nutrient treatments. Females from Ape Hole Creek similarly showed high variability among genets, though the average response to nutrient treatments did not differ from male gametophytes or tetrasporophytes.

One limitation of our study is the reliance on reproductive material as we have no other method to distinguish life cycle stage in *Agarophyton vermiculophyllum* at present. Reproduction may decrease growth rates (see Guillemain et al. 2013), even if non-reproductive apices are excised from reproductive material as in our study. Guillemain et al. (2013) also found that the responses to stressors were much more subtle in older developmental stages, specifically in adult apices versus spores and juveniles (i.e., holdfasts). The observed differences among stages could also be due to unmeasured factors, such as an increase in energetic investment by females in reproduction (see e.g., Hannah and Santelices 1985, Thornber and Stachowicz 2006, Vergés et al. 2008), though we did not see any reproductive structures at the conclusion of our experiment. However, it is also important to note that differences in the life history stage (juvenile vs. adult), the media (natural seawater vs. artificial seawater), and the species (including native vs. non-native populations) used in previous work on species with isomorphic, haplodiplontic life cycles may all contribute to discrepancies in the response to a given stressor among experiments (e.g., Destombe et al. 1993, Guillemain et al. 2013, Neill et al. 2018, this study)

as stage-specific responses may be extremely subtle (Hughes and Otto 1999, Scott and Rescan 2016).

In the Gracilariales, tetrasporophytes overwhelmingly dominate in soft-sediment, free-floating populations (but see Guillemin et al. 2008, Robitzch et al. 2019). Guillemin et al. (2013) found vegetative tetrasporophytes grew faster and also had more branches developing along their main axes in *Agarophyton chilense*, which may contribute to tetrasporophyte dominance in free-floating populations. However, Vieira et al. (2018a) showed that gametophytes had greater survival in long-term monitoring of the three life cycle stages in the field. Even subtle tradeoffs between growth, reproduction, and survival may contribute to large shifts in population composition when novel habitats are encountered. It is also possible that the free-floating thalli that dominate soft sediment habitats represent a specialized subset of tetrasporophytic genets. No studies have yet compared tetrasporophytes and gametophytes from hard substratum sites with free-floating tetrasporophytes. There are likely strong selective filters that determine *which* tetrasporophytes thrive in a free-floating state. The effect of differential patterns of nutrient use among life cycle stages, developmental stages, or populations may add an additional layer of complexity to the problem of understanding tetrasporophytic dominance. Future studies will need to consider the relative importance of each of these factors in explaining the observed patterns as well as the role of heterosis (Zohary 2004) or over-dominance which has been suggested previously for these patterns (Guillemin et al. 2008).

Predicting patterns of species persistence and spread under the dual challenges of climate change and species invasion has revealed critical deficits in our understanding of population dynamics in organisms with complex life cycles. Many such organisms, including haplodiplontic macroalgae, are increasingly being introduced throughout the world. Experiments, such as this study, that examine sex- and ploidy-specific responses to potential stressors are necessary to understand which facets of a life cycle may act as the “weakest link” (Istock 1967, Pandori and Sorte 2018, Krueger-Hadfield 2020). Without such information, we are unable to make accurate predictions about the persistence and spread of species in an era when it is increasingly important to do so. Moreover, the conflicting

results of our study in light of other work on macroalgae suggests the need to conduct more comprehensive experiments that incorporate all life history stages within a life cycle in order to determine the role of different abiotic stressors in stabilizing or weakening complex life cycles.

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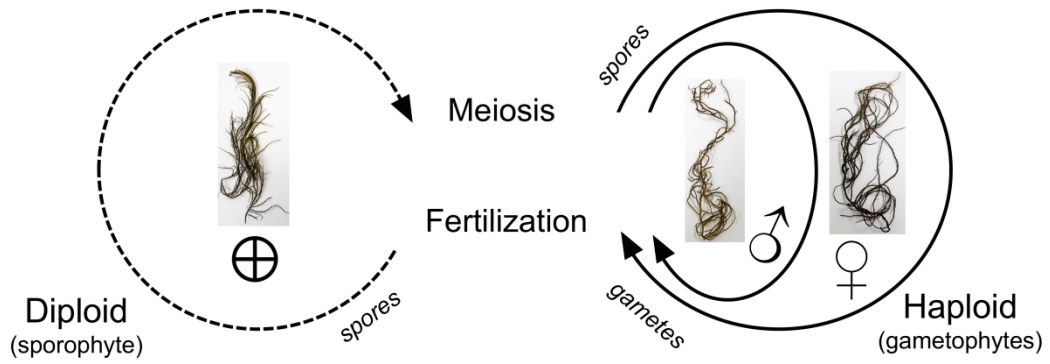
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Fig. 1. A simplified depiction of the isomorphic, haplodiplontic life cycle of *Agarophyton vermiculophyllum* in which haploid and diploid free-living stages are morphologically very similar. The diploid stage is shown with dashed lines and the haploid stages are shown with solid lines. For an example of the complete life cycle of *A. vermiculophyllum*, please see Krueger-Hadfield and Hoban (2016; photo credits: S.A. Krueger-Hadfield).

Fig 2. (a) Probability of survival after four weeks depending on site of origin, life history stage, and nutrient treatment. (b) Mean \pm standard error change in apex length (mm) after four weeks in culture depending on site of origin, life history stage, and nutrient treatment. Best-fit model indicated by either solid or dashed lines for haploid or diploid thalli, respectively.

Fig S1. Map of the Delmarva Peninsula on the eastern shore of Maryland and Virginia showing site locations and images at Ape Hole Creek in the Chesapeake Bay and Magotha Road on the Atlantic Ocean. (photo credit: S.A. Krueger-Hadfield)

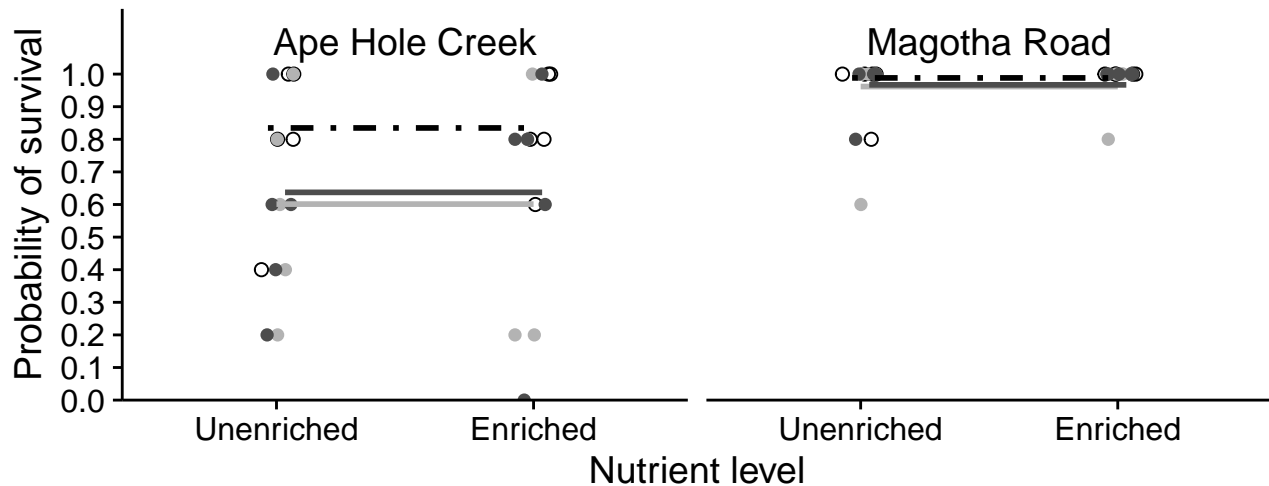
Fig. S2. Genet-level norms of reaction for (a) the probability of survival and (b) mean \pm standard error change in apex length (mm) after four weeks in common garden culture depending on site of origin, life history stage, and nutrient treatment. Bold line shows the average response across all genets.



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A.

Stage ○ Diploid ● Haploid Female ● Haploid Male

**B.**

Stage - - Diploid — Haploid Female — Haploid Male

