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Phenology and thallus size in a non-native population of *Gracilaria vermiculophylla*

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Abstract
Phenology, or seasonal variation in life cycle events, is poorly described for many macroalgal species. We describe the phenology of a non-native population of *Gracilaria vermiculophylla* whose thalli are free-living or anchored by decorating polychaetes to tube caps. At a site in South Carolina, USA, we sampled 100 thalli approximately every month from January 2014 to January 2015. We assessed the reproductive state and measured thallus size based on wet weight, thallus length, and thallus surface area from herbarium mounts. Because life cycle stage cannot be assigned using morphology, we implemented a PCR assay to determine the life cycle stage—tetrasporophyte, female gametophyte, or male gametophyte—of each thallus. Tetrasporophytes dominated throughout the year, making up 81%–100% of thalli sampled per month. Reproductive tetrasporophytes varied between 0% and 65% of monthly samples and were most common in warm summer months (July through September) when thalli also tended to be larger. The vast majority of the reproductive thalli were worm-anchored and not fixed to hard substratum via a holdfast. Thus, free-living thalli can be reproductive and potentially seed new non-native populations. Given *G. vermiculophylla* reproduction seems tied closely to temperature, our work suggests phenology may change with climate-related changes in seawater temperatures. We also highlight the importance of understanding the natural history of macroalgae to better understand the consequence of range expansions on population dynamics.

KEYWORDS
invasion, life cycle, mudflat, seaweed, sex

INTRODUCTION

Life cycle events are critical for survival and reproduction and therefore the establishment and persistence of populations. Organisms respond to a wide variety of environmental parameters, including temperature, photoperiod, and precipitation across seasons. Climate change has shifted the timing and magnitude of air and water temperatures in many locations (Chmura et al., 2019), and climate change related shifts

in phenology vary in direction and magnitude across taxa (e.g., Thackeray et al., 2016). When these life cycle events are integral to community interactions (e.g., flowering or seed set in angiosperms), mismatches can arise, and these will likely increase with climate change (Thackeray et al., 2010). As more and more studies attempt to develop species distribution models, there is increasing recognition of the need to incorporate phenological patterns in projections for more accurate model predictions (Ponti & Sannolo, 2022). Phenology is, therefore, a potentially influential but often overlooked aspect in the functioning of communities (Cleland et al., 2007).

For macroalgae, phenological studies can be time consuming when reproductive structures are not easily observed, in contrast with when there are readily observed leaves and flowers on plants or striking animal behaviors. Moreover, many thalli may be vegetative throughout the year, rendering it challenging or impossible to assign a life cycle stage (i.e., generation: sporophyte vs. gametophyte, sensu Drew, 1955) or sex growth rates (Kain & Norton, 1995), and the onset of gametogenesis and sporogenesis is closely tied to thermal regimes (Lüning, 1990). For example, Novaczek et al. (1987) observed reproductive thalli were restricted to during the period of maximum temperature in the summer across four species of red algae in Nova Scotia. By contrast, other red algal populations have thalli that are reproductive in all seasons (e.g., *Chondracanthus pectinatus*, Pacheco-Ruíz & Zertuche-González, 1999), show temporal restrictions without defined seasonality (e.g., *Gelidium sesquipedale*, Santos & Duarte, 1996), or have peak periods of reproduction in winter with temporal shifts between tetrasporophytes and cystocarpic female gametophytes (Dyck & DeWreede, 2006).

The phenology of species in the Gracilariales has been the subject of many studies, largely because of their economic (Popper et al., 2011; Valero et al., 2017) and ecological importance (Kain & Destombe, 1995). There is some evidence that with increasing latitude, there is a seasonal reproductive peak in summer (see figure 2 in Kain & Destombe, 1995). However, Kain and Destombe (1995) note that there is likely strong ascertainment bias in the populations selected for phenological studies whereby populations were selected because reproductive thalli had been observed and because there were ecological conditions conducive to sporic recruitment, such as hard substratum. Conversely, there are many examples of sites in which *Gracilaria* spp. commonly drift as free-floating thalli above soft sediments that are often vegetative and are thought to be sterile (e.g., Simonetti et al., 1970; Stokke, 1957).

Here, we focus on the red alga *Gracilaria vermiculophylla*, in which populations are observed in both hard and soft substratum habitats (Krueger-Hadfield

et al., 2016). In its native range in the northwest Pacific, thalli are largely fixed to hard substratum and populations display genetic signatures of sexual reproduction (Krueger-Hadfield et al., 2016, 2017). In Japan, Terada et al. (2000) documented a summer peak in reproductive thalli at a site in Hokkaido (northern Japan, ~4° N), while the peak occurred in winter at a site in Okinawa (subtropical, southern Japan, ~26° N; Terada et al., 2010), likely due to latitudinal differences in thermal regime. In Hokkaido, most thalli were vegetative throughout the sampling period from April to February, but in Okinawa, thalli were only present in winter, disappearing as temperatures increased. Curiously, at a site on Kyushu Island (~33° N) in southern Japan, Muangmai et al. (2014) observed reproductive tetrasporophytes overwhelmingly dominated the population throughout the year. Temperatures at this site were in the optimal range for tetrasporophytes (see Yokoya et al., 1999), suggesting abiotic conditions favoring tetrasporophytes and facilitating year-round reproduction. Yet, these three Japanese sites had sufficient hard substratum and all thalli were fixed (sensu Krueger-Hadfield et al., 2018) to hard substratum via a holdfast (Figure 1a).

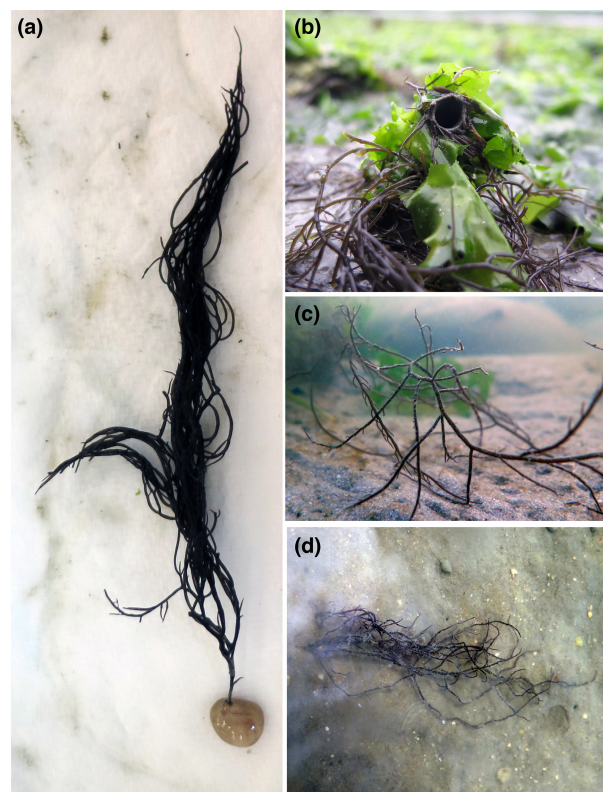**FIGURE 1** (a) A *Gracilaria vermiculophylla* thallus fixed to a small pebble via a holdfast. (b) Free-living *Ulva* sp. and *G. vermiculophylla* thalli glued to the tube cap and anchored in the photic zone by *Diopatra cuprea*. (c) Free-living *G. vermiculophylla* thalli free-floating above soft sediment. (d) A *G. vermiculophylla* thallus buried in soft sediment (Image credits: S.A. Krueger-Hadfield).

Over the last 100 years, *Gracilaria vermiculophylla* thalli were introduced throughout the Northern Hemisphere to coastal high salinity marshes dominated by soft-sediment habitats (e.g., Kim et al., 2010; Krueger-Hadfield et al., 2017, but see Krueger-Hadfield et al., 2018). *Gracilaria vermiculophylla*, to the best of our knowledge, does not "reattach" via secondary attachments or discs as observed in other macroalgae (e.g., *Plocamium* sp., Heiser, Amsler, et al., 2023). Once detached from the substrate, *G. vermiculophylla* thalli are free-living and persist indefinitely in the environment. Confusion arises though in terminology, as other studies (e.g., Freshwater et al., 2006) have used the term attached to refer to both thalli that are fixed to hard-substratum and those that are *worm-anchored* (Figure 1b–d). While all *G. vermiculophylla* thalli originate from a spore, fixed and worm-anchored thalli do not have the same immediate history. We also note that fixed and worm-anchored thalli will not experience the same abiotic conditions. For example, the process of being incorporated into a worm tube cap likely fragments the thallus. Thus, we have clarified and developed additional terminology to reflect algal biology and ecology (Table 1).

In soft sediment sites in both the native and non-native ranges, free-living thalli exhibit multiple signatures of asexual reproduction through thallus fragmentation (e.g., repeated genotypes or heterozygote excess, Krueger-Hadfield et al., 2016, 2017; Krueger-Hadfield, Guillemin, et al., 2021). Free-floating thalli drift in the water column at high tide and rest on the benthos at low tide. In some North Atlantic regions, *Gracilaria vermiculophylla* thalli are often incorporated into the mucus tube caps of the polychaete genus *Diopatra* (Abreu et al., 2011; Thomsen & McGlathery, 2005). The thalli are kept in the photic zone and not lost to either the supralittoral or the turbid subtidal (Kollars et al., 2016; Thomsen & McGlathery, 2005). Asexual reproduction in haploid-diploid species typically leads to the dominance of one stage (see review in Krueger-Hadfield, 2020). In *G. vermiculophylla*, there is a predictable pattern of tetrasporophytic dominance in these

TABLE 1   Glossary of terms used in this study and proposed for attached and unattached macroalgae.

Term	Definition	Notes
Attached	To fasten or fix something in position[a]	The term attached likely originates from Old French *atachier* or *estachier* meaning "to fasten, fix." Algal attachment traditionally refers to adherence on the substratum, typically the benthos, via a holdfast. In other words, a spore settled, germinated, and grew into an adult thallus.
Free-living	For macroalgae, thalli that were once fixed to a substrate via a holdfast and now are detached (sensu Norton & Mathieson, 1983)	This contrasts with communities of unattached macroalgae. These *free-living* macroalgae have become detached from a substrate and are no longer connected via holdfast. *Free-living* macroalgae will be subjected to different environmental conditions than those "attached to the ancestral rock" (Norton & Mathieson, 1983).
		Some algae can regrow secondary attachment discs (e.g., *Chondracanthus*, Macchiavello et al., 2003; Oyarzo et al., 2021; Pacheco-Ruíz et al., 2005) or from secondary attachments (e.g., *Plocamium*, e.g., Heiser, Amsler, et al., 2023) following detachment from the substrate. However, this is also a distinct phenomenon to that observed when invertebrates decorate with macroalgae, such as the polychaete genus *Diopatra* (e.g., Thomsen & McGlathery, 2005).
Fixed	A thallus that is attached to hard substratum via a holdfast, indicating sporic recruitment and germination.	This term originates from the French *fixé*. Krueger-Hadfield et al. (2018) used the term fixed to refer to algae attached by a holdfast to differentiate hard and soft sediment sites where *G. vermiculophylla* populations are observed. Fixed thalli originate from a spore that settled, germinated, and produced an adult thallus.
Free-floating	A thallus that has become detached from hard substratum; free-living	Free-floating is a term previously used to describe non-native sites in which *G. vermiculophylla* is observed as a catch-all term for any thalli detached from hard substratum (Krueger-Hadfield et al., 2016). Here, we suggest *free-floating* and *drifting* are synonyms and a specific type of *free-living* macroalgae. These terms should be used to refer to detached thalli that are either floating in the water column at high tide or rest on the benthos at low tide.
Drifting	A thallus that has become detached from hard substratum; free-living	
Worm-anchored	A thallus anchored and glued to the tube cap of a decorator worm; free-living	*Worm-anchored* thalli are free-living but are anchored in the photic zone by decorator worms in the genus *Diopatra* (Abreu et al., 2011; Thomsen & McGlathery, 2005). We suggest using this term rather than attached to be clear that the thallus is not adhered to the tube cap through sporic recruitment.

[a]Cambridge Dictionary https://dictionary.cambridge.org/us/dictionary/english/attach, Accessed 25 March 2023.

soft sediment habitats, though due to the aims of these surveys, we note sampling usually only happened once and in the boreal summer (Flanagan et al., 2021; Krueger-Hadfield et al., 2016, 2017). When compared to gametophytes, tetrasporophytes can be stronger (Lees et al., 2018) and have greater survival (Krueger-Hadfield & Ryan, 2020), possibly contributing to their abundance in soft sediment habitats.

In a review focused on free-living algae, Norton and Mathieson (1983) concluded that most modified forms of unattached algae were only capable of vegetative fragmentation. Early work in *Gracilaria* species observed few reproductive thalli in free-living sites (e.g., Stokke, 1957). However, at some sites, Krueger-Hadfield et al. (2016, 2017) observed reproductive *G. vermiculophylla* tetrasporophytes, which contradicted previously described patterns in the genus *Gracilaria* (see also Kain & Destombe, 1995) and those in Norton and Mathieson (1983). As Norton and Mathieson (1983) concluded, we still require a great deal of basic biological knowledge to understand "the processes responsible for the formation and maintenance of unattached seaweeds" (p. 374).

To expand our knowledge of free-floating *Gracilaria vermiculophylla* populations from which we have many one-off sampling efforts, we explored the phenology and thallus size of *G. vermiclophylla* at a site near Charleston, South Carolina. As with many non-native sites, reproductive gametophytes are rare (Gerstenmaier et al., 2016; Krueger-Hadfield et al., 2016, 2017). For example, Gerstenmaier et al. (2016) surveyed all the thallus fragments glued to each of 20 *Diopatra cuprea* tube caps in February (N = 187 thallus fragments) and May (N = 341 thallus fragments). They observed a single haploid thallus each in February and May (<1% of thallus fragment genotyped), assayed through fixed homozygosity at the microsatellite loci used, as the thalli were vegetative. With the advent of sex-linked molecular markers (Flanagan et al., 2021), we can now explore the monthly frequencies of tetrasporophytes, female gametophytes, and male gametophytes and couple our observations with abiotic conditions.

In this study, we determined the reproductive state, generation, and size (wet weight, thallus length, and thallus surface area) of ~100 *Gracilaria vermiculophylla* thalli collected monthly for one calendar year to determine (i) whether the Fort Johnson population is dominated by tetrasporophytes year-round, (ii) if there is a pattern of reproductive periodicity despite thalli being predominantly free-living and worm-anchored, and (iii) if there are patterns of thallus size as measured by wet weight, length, and surface area. This builds on our earlier work and enhances our knowledge of the natural history of free-floating algal populations (see also Norton & Mathieson, 1983).

MATERIALS AND METHODS

2014–2015 Fort Johnson collections

We sampled *Gracilaria vermiculophylla* thalli at a mudflat near Fort Johnson, South Carolina, USA (32°45′6.552″ N, 79°54′1.944″ W; Appendix S1: Table S1 in the Supporting Information). This site is composed of predominantly free-living thalli drifting at high tide or glued to the tube caps of the polychaete *Diopatra cuprea* (see Table 1 as well as Kollars et al., 2016; Krueger-Hadfield et al., 2016). A small proportion of thalli are observed fixed to hard substratum (e.g., shell hash; S. A. Krueger-Hadfield, personal observation). Thalli were considered diploid based on reproductive structures, the presence of one or more heterozygous microsatellite loci, or both in past sampling efforts (see also Kollars et al., 2015). No reproductive tetrasporophytes collected at Fort Johnson, to date, have been homozygous at every locus genotyped as has been observed in other red macroalgae (see Heiser, Shilling, et al., 2023; Krueger-Hadfield et al., 2013).

Every meter along a 100-m transect, we collected a single thallus at 0.3 m above mean lower low water during approximately every month from January 2014 to January 2015. For each thallus, we noted whether it was free-floating, worm-anchored, fixed, or buried. A thallus that was buried might have been fixed to hard substratum or free-floating, but we could not distinguish between these two possibilities with certainty. Each thallus was individually bagged and kept in spatial sampling order along the transect before processing immediately following collection. We were careful not to artificially break thalli during collection. As thalli are free-floating or incorporated into *Diopatra cuprea* tube caps, they can vary in size due to the environmental conditions of the site. In the laboratory, if multiple thalli were collected or possibly fragmented during collection, we haphazardly selected a single, contiguous piece of thallus. Thus, our estimates of thallus size and biomass are representative of the *Gracilaria vermiculophylla* at this site.

Identification of reproductive state

For each contiguous piece of thallus sampled, we noted whether a holdfast was present, even if the thallus had not been fixed to hard substratum at the time of collection. The presence of a holdfast would indicate that the thallus was fixed recently to hard substratum (see also Martín et al., 2011). We determined reproductive state using a dissecting microscope (40×; see Krueger-Hadfield et al., 2018). If a thallus had no visible reproductive structures, it was considered vegetative. If a thallus was putatively reproductive, we

noted the possible life cycle stage but considered the thallus as vegetative in downstream analyses, as we could not be certain about its reproductive state.

Algal mass and morphometrics

In the laboratory following field collection, we briefly re-hydrated each thallus in sterile seawater and then patted the thallus dry to obtain a wet weight (g) using a flatbed balance (0.01 g). After weighing, a small piece of each thallus (~5 cm) was preserved in silica gel for subsequent DNA extraction. We mounted each thallus on herbarium paper (University of California-type Herbarium Mounting Paper, Herbarium Supply, Bozeman, MT).

Each herbarium specimen was scanned using a photomicrographic scale (ABFO No. 2, 5 cm) and a color card (DGK Digital Kolor Kard, Digital Image Flow). Images were analyzed by modifying methods of WinRhizo (Regent Instruments), which was developed for plant roots. The methods are useful here as the *Gracilaria vermiculophylla* thallus resembles terrestrial plant roots (see also Lees et al., 2018; Murren et al., 2022). For each scanned herbarium specimen, we retained the measurements calculated by WinRhizo of total thallus length (cm) and surface area (cm^2), which is calculated by length and diameter.

As pressed thalli may be dorsoventrally flattened, we sampled 15 thalli from Fort Johnson and photographed them live following the methods described in Lees et al. (2018). Briefly, thalli were floated in sterile seawater and photographed in a uniform light environment. We mounted these thalli on herbarium paper and scanned as described above. Each corresponding image of live thallus and herbarium specimens were analyzed with WinRhizo as described above to determine length (cm) and surface area (cm^2). We compared live thallus and herbarium specimen lengths and surface areas using paired *t*-tests in R version 3.5.2 (R Core Team, 2018).

Sex-linked marker assay

As many thalli were vegetative at the time of sampling, we used the sex-linked genetic markers developed by Krueger-Hadfield, Flanagan et al. (2021) to determine life cycle stage. We adapted a Chelex DNA extraction protocol from Simon et al. (2020). We placed ~1 cm of each silica gel-dried thallus into a Fisherbrand™ 96-well non-skirted PCR plate and then added 200 µL of pre-heated (95°C) 5% Chelex solution (Bio-Rad Laboratories). Each plate was vortexed for 30 s, then incubated at 95°C for 15 min with a brief vortex every 5 min. Each plate was centrifuged for 3 min at 5600 × *g* after which 180 µL of the supernatant was transferred

to a new 96-well plate. Each plate was centrifuged again for 3 min at 5600 × *g*, and 150 µL of the supernatant was transferred to a final 96-well plate. For each plate, we had two female gametophytic, two male gametophytic, and two tetrasporophytic positive controls; one negative DNA extraction control; and one negative PCR control.

We modified the PCR assay from Krueger-Hadfield, Flanagan et al. (2021) and amplified the fem_o03 and mal_n09 in a 10-µL duplex PCR containing 2 µL of DNA template, 0.5 U GoTAQ Flexi-DNA Polymerase (Promega), 1× green reaction buffer, 100 µM of each dNTP, 1.5 mM of MgCl$_2$, 1 mg · mL^{-1} bovine serum albumin, 250 nM female forward and reverse primers, and 350 nM male forward and reverse primers, using the cycling profile: 95°C for 10 min, 35 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s, followed by a final extension of 72°C for 5 min. We visualized 3 µL of each PCR product on a 1.5% agarose gel stained with 6 µL GelRed (Biotium). We scored each thallus blind and then matched the life cycle stage based on the sex-linked marker (female: 73 bp; male: 270 bp; tetrasporophyte: both 73 and 270 bp) with the reproductive state identified at the time of collection.

Gametophyte to tetrasporophyte ratios and ploidy diversity

Using a binomial distribution, we tested whether the gametophyte to tetrasporophyte ratio differed from the predicted ratio of √2:1 that has been hypothesized if there are no fitness differences between life cycle stages (Destombe et al., 1989; Thornber & Gaines, 2004). We chose this prediction of ploidy ratios to compare the current findings with previous work at this same site (e.g., Krueger-Hadfield et al., 2016). We also calculated ploidy diversity (P_{HD}, Krueger-Hadfield et al., 2019). Since the proportion of tetrasporophytes was greater than 0.41 at all sampling periods, we used the equation $\frac{1-x}{0.59}$ where x is the proportion of tetrasporophytes at a site. When $P_{HD} = 1$, the ratio of gametophytes to tetrasporophytes is the predicted √2:1, but when $P_{HD} = 0$, there is only one life cycle stage—gametophyte or sporophyte. In our study, as P_{HD} approaches 0, there are only tetrasporophytes present at a site.

Abiotic data

We obtained the following abiotic datasets for December 2013–January 2015: (i) precipitation data from the National Weather Service (collected every 24 h; https://www.weather.gov/chs/climate) for downtown Charleston (CXM; Figure S1a in the Supporting Information); (ii) sea surface temperature data from the NOAA's Charleston Cooper River Entrance station 8665530 (collected every

6 min; 32°46′51″ N 79°55′25″ W, https://www.ndbc.noaa.gov/station_page.php?station=chts1; Figure S1b); and (iii) specific conductance from the Customs House from the USGS National Water Information System Web Interface (collected every 15 min; https://waterdata.usgs.gov/nwis/dv?referred_module=sw&site_no=02172 0710). We converted the conductivity measurements into salinity (Figure S1c).

For statistical analyses, we averaged the precipitation, temperatures, and salinity over the 30 d prior to each sample collection day. The total number of data points for precipitation, temperature, or salinity measurements that we averaged varied as some data were missing. This allowed us to use the values that would have influenced growth and reproduction of the thalli, rather than the environmental conditions that happened to occur on the sample collection day.

Data analyses

All statistical analyses were completed in the program R version 3.5.2 (R Core Team, 2018). We compared the size of live and herbarium-preserved thalli using a paired *t*-test. We used Spearman rank correlations to understand the covariation of monthly averages in precipitation, temperature, and salinity. We used Pearson correlations for our three metrics of thallus size (wet weight [g], length [cm], and surface area [cm^2]) among the tetrasporophytes only. We did not assess correlations among gametophytes because of low samples sizes. All metrics were log transformed to meet the assumption of normality for Pearson correlations. We used a set of general linear models with a binomial distribution to determine the effect of abiotic factors on the frequency of vegetative versus reproductive states. This was followed by running a standard linear model on the effect of reproductive state (vegetative or reproductive) on thallus wet weight, thallus length, and thallus surface area. We ran a multiple linear regression looking at the effect of all abiotic factors and reproductive state on thallus size. Finally, we explored if there were significant effects of month of sample collection, as well as the different abiotic factors on gametophyte size using a general linear model with a binomial distribution.

RESULTS

Thallus size in live and pressed specimens

Live thalli were longer (live = 1528.2 ± 266.5 cm; herbarium = 720.6 ± 89.1 cm; t_{28} = 4.08, $p < 0.001$) and with larger surface areas (live = 403.6 ± 69.9 cm^2; herbarium = 109.6 ± 14.0 cm^2; t_{28} = 5.09, $p < 0.0002$) as

compared to herbarium preserved thalli. However, all comparisons were in the same general direction.

Habitat characteristics

Most thalli were anchored by *Diopatra cuprea* throughout the year (75%–96% per monthly collection, Table 2). Smaller proportions of thalli were free-floating at the time of collection (4%–20%, Table 2). Only eight thalli of 1,189 collected (<1%) were fixed to hard substratum at the time of collection, and only 10 thalli (<1%) were partially buried in the sediment.

Over the course of the measured year, water temperature ranged from 9.9 to 29.4°C, rainfall ranged from 0.09 cm to 0.64 cm · d^{-1} on average, and salinity varied from 23.6 to 26.9 (Appendix S1: Figure S1). There were no significant correlations among monthly averages for these three abiotic factors (Spearman rank $p > 0.224$ for all three pairwise comparisons).

Patterns of reproductive state, ploidy ratios, and thallus size

Between 0% and 65% of thalli collected in a month were reproductive (Table 2; Figure 2). There were no reproductive thalli in January, March, or December 2014. The highest frequency of reproductive thalli was in September (65%).

All positive control thalli amplified consistently across PCRs. Only four thalli of 1189 did not match the reproductive state identified under the microscope (<1%). One thallus was identified as a putative tetrasporophyte in the December collection but with PCR assay data was categorized as a male gametophyte. Three thalli had a small number of cystocarps visible or excised but were tetrasporophytes based on the sex-linked marker (one each in May 2014, December 2014, and January 2015). We rehydrated small pieces of all three thalli and observed them under the microscope. There were no visible terasporangial sori for the two thalli collected in December and January. However, one cystocarp and tetrasporangial sori were clearly visible for the thallus material collected in May (Figure 3). We note that we either preserved female thallus with no visible cystocarps or, if this was not possible, we removed cystocarps through excision prior to preservation in silica gel, so this is unlikely to be contamination by carpospores.

Tetrasporophytes dominated the population year-round (81%–100%, Table 1; Figure 2; Appendix S1: Figure S2). The greatest frequency of gametophytes (19%: 6% females and 13% males) was in January 2014. From May 2014 to January 2015, gametophytes made up less than 7% of the sampled thalli. A total of eight cystocarpic females and 17 reproductive males were observed. All other gametophytes were vegetative at

TABLE 2 The proportion of different fixation types, reproductive state, and life cycle stages in the population of *Gracilaria vermiculophylla* present at the Fort Johnson mudflat from January 2014 to January 2015.

| Date | Julian day | n | Fixation type | | | | Reproductive state | | Life cycle stage | | | P_{HD} |
			Free-floating	Fixed	Diopatra-anchored	Buried	Vegetative	Reproductive	Female gametophytes	Male gametophytes	Tetrasporophytes	
31-Jan-14	31	96	0.15	0.02	0.80	0.03	1.00	0	0.06	0.13	0.81	0.32
1-Mar-14	60	100	0.04	0.01	0.94	0.01	1.00	0	0.01	0.08	0.91	0.15
30-Mar-14	89	100	0.09	–	0.90	0.01	0.92	0.08	0.07	0.07	0.86	0.24
25-Apr-14	115	100	0.12	0.01	0.87	–	0.63	0.37	0.05	0.07	0.88	0.20
25-May-14	145	99	0.20	0.01	0.75	0.03	0.76	0.24	0.03	0.04	0.93	0.12
27-Jun-14	178	100	0.05	–	0.95	–	0.63	0.37	0	0.01	0.99	0.02
25-Jul-14	206	100	0.09	–	0.90	0.01	0.59	0.41	0.01	0.01	0.98	0.03
8-Sept-14	251	94	0.05	–	0.88	0.01	0.35	0.65	0	0	1.00	0
11-Oct-14	284	100	0.15	0.01	0.84	–	0.70	0.30	0	0	1.00	0
8-Nov-14	312	100	0.12	0.01	0.87	–	0.85	0.15	0.02	0.01	0.97	0.05
19-Dec-14	353	100	0.04	–	0.96	–	1.00	0	0.02	0.04	0.94	0.10
17-Jan-15	17	100	0.09	0.01	0.90	–	0.99	0.01	0	0.05	0.95	0.08

Note: Free-floating thalli were drifting without fixation to hard substratum or anchored to a *Diopatra* tube cap. Fixed thalli had a holdfast connecting them to hard substratum (sensu Krueger-Hadfield et al., 2018). Worm-anchored thalli were incorporated in the tube cap of the predatory polychaete *Diopatra cuprea*. Buried thalli were partially in the muddy sediment, and we could not be certain if they were fixed to hard substratum or free-floating. We determined reproductive state under a dissecting microscope as vegetative or reproductive. We then determined life cycle stage for all thalli, including reproductive thalli, using a sex-linked marker (Krueger-Hadfield, Flanagan, et al., 2021). P_{HD} is the ploidy diversity metric that describes the amount of life cycle stage diversity at each sampling point (Krueger-Hadfield et al., 2019).
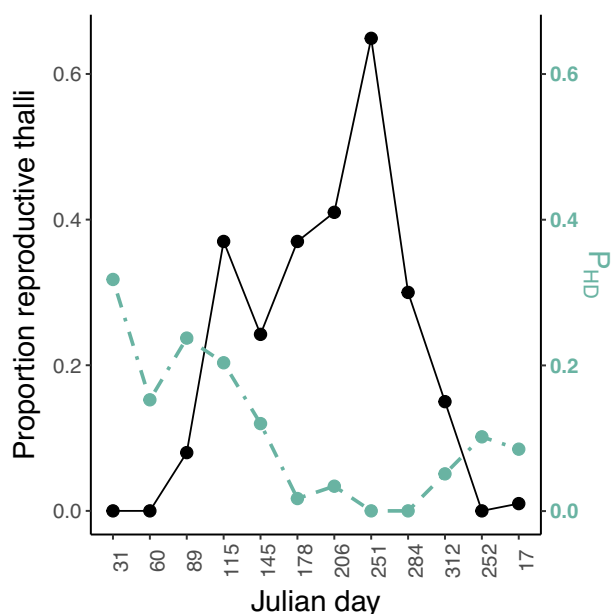
FIGURE 2 The proportion of reproductive *Gracilaria vermiculophylla* thalli sampled at the Fort Johnson mudflat from January 2014 to January 2015 on the left axis (shown as a solid line). On the right axis, is the ploidy diversity (P_{HD}) for each sampling point (shown as a dashed line). Julian day is shown on the *x*-axis (see also Table 1).

FIGURE 3 Possible mixed phase thallus collected in May 2014. White arrows point to two of the tetrasporangial sori. The inset image is the excised cystocarp showing a mass of putative carpospores. (Photo credit: S.A. Krueger-Hadfield).

the time of sampling and were identified using the sex-linked PCR assay.

Temperature ($p = 2.8 \times 10^{-30}$) was highly associated with reproductive state, in which warmer temperatures were associated with times of sampling more reproductive thalli (Appendix S1: Table S2a). Similarly, more precipitation ($p = 0.04$) was moderately associated with increases in reproductive thalli (Table S2b). Salinity, while highly associated with overall thallus size measured by wet weight and surface area (see below), was not associated with reproductive state (Table S2c). For gametophytes, lower temperatures were associated

with increased numbers of sampled gametophytes ($p = 1.2 \times 10^{-8}$, Table S2d).

For tetrasporophytes, we observed that all three size measurements (wet weight, surface area, and length) were highly correlated with each other (Appendix S1: Figure S3). In general, thalli tended to be largest in July through September (see Figure 4 for visualization with wet weight). For our three measures of size, reproductive thalli were larger ($p < 8.7 \times 10^{-5}$ for all comparisons, Table S2e–g).

We lastly ran a combined model with tetrasporophytes to determine the role of reproductive state and all the environmental factors combined. For thallus length and wet weight, all abiotic factors were positively associated with these two measures of size, as was being reproductive (Table S2h,i). Only reproductive state and higher salinity were associated with larger surface area (Table S2j).

DISCUSSION

Previous studies have suggested that many free-floating, unattached macroalgae are largely sterile (Norton & Mathieson, 1983), including *Gracilaria* spp. (e.g., Simonetti et al., 1970; Stokke, 195). In a free-living, worm-anchored population of *G. vermiculophylla*, we observed high frequency of reproductive tetrasporophytes (up to 59%) and a reproductive peak in warmer months (July through September). Thalli were largest in the boreal summer when temperatures were highest. We also used a sex-linked PCR assay to determine that tetrasporophytes dominated the samples from this site, regardless of the sampling month. Below, we discuss the factors that might lead to tetrasporophytic dominance, the abiotic factors associated with reproductive state and thallus size, and the utility of sex-linked PCR assays for studying haploid-diploid algal populations.

Tetrasporophytic dominance

The dominance of tetrasporophytes reported here is consistent with previous efforts using microsatellites or SNPs (Flanagan et al., 2021; Gerstenmaier et al., 2016; Krueger-Hadfield et al., 2016, 2017). We extended the temporal sampling to demonstrate that tetrasporophytes account for the majority of biomass at this site, regardless of season.

Most thalli were anchored to *Diopatra cuprea* tube caps, with few thalli fixed to hard substratum at the time of collection. Of the eight fixed thalli, there was a mix of each of the three stages, suggesting the recruitment of both gametophytes from tetraspores and tetrasporophytes from carpospores occurs. Indeed, when artificial substrates were placed on this same mudflat, all
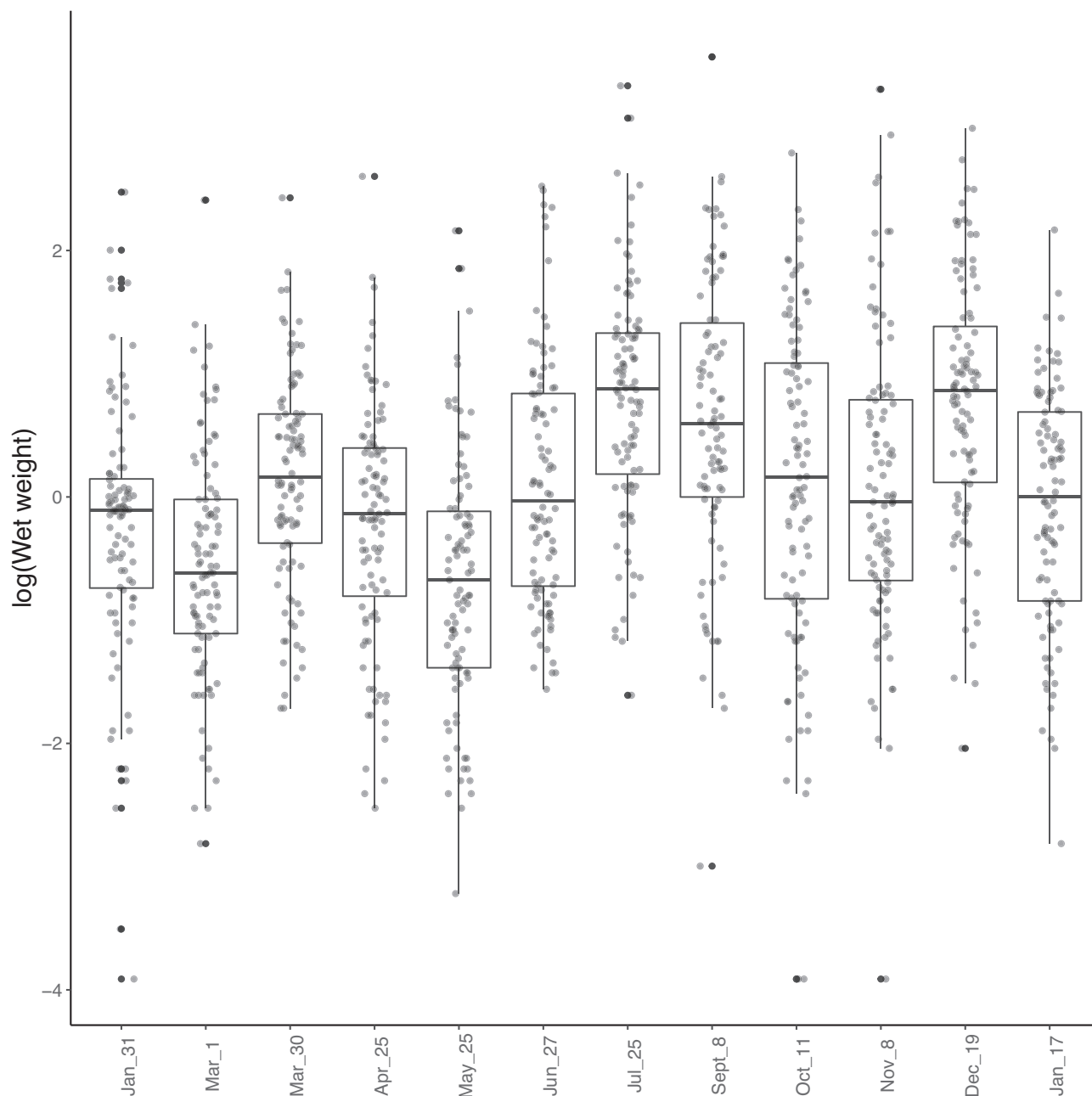
FIGURE 4　The log of thallus wet weight (g) for each sampling date from January 2014 to January 2015 from left to right on the *x*-axis (sample sizes are provided in Table 1).

three *Gracilaria vermiculophylla* stages were present after 1 year (see Lees et al., 2018). Though *D. cuprea* likely facilitates thallus fragmentation during decoration, Krueger-Hadfield et al. (2016) reported high genotypic richness (*R* = 0.74) in a study that followed the same strategy of sampling a single thallus every meter. Reproductive thalli drifting into the site could not have wholly replaced existing vegetative biomass. Future studies should explore genetic connectivity between free-living (see again Table 1) and fixed thalli as well as determining the relative contributions to biomass of recently detached thalli as compared to the standing stock of free-floating thalli.

Our results are distinct from previous work on the phenology of *Gracilaria vermiculophylla* at a site in Portugal (Abreu et al., 2011). At the Ria de Aviero lagoon, thalli were fixed, free-floating, or worm-anchored (by *Diopatra neopolitana*). Reproductive thalli were most abundant in January yet were observed throughout the year. In contrast to our study, Abreu et al. (2011) observed cystocarpic female gametophytes were the most abundant throughout the year, and tetrasporophytes were frequent; male gametophytes were never observed. The abundance of hard substratum at this site might explain the presence of cystocarpic females, but these patterns are

different from all previous work in the native range as well (Muangmai et al., 2014; Terada et al., 2000, 2010). Future studies should explore the phenological and demographic patterns at soft sediment sites with varying levels of hard substratum to examine this potential agent of selection.

Environmental variables influencing reproduction and thallus size

The onset of reproduction in *Gracilaria vermiculophylla* at Fort Johnson was related to temperature, slightly less strongly related to rainfall, and not strongly influenced by salinity. The summer peak in reproductive thalli and biomass matches previous work in other *Gracilaria* spp. (Kain & Destombe, 1995; Martín et al., 2011; Nelson, 1989,). The average temperatures from July through September 2014 were ~28°C through 32°C, similar to temperatures recorded by Terada et al. (2010) in Okinawa where *G. vermiculophylla* thalli had disappeared. Non-native tetrasporophytes exhibit greater tolerance to higher water temperatures relative to native thalli (Sotka et al., 2018), which may help to explain persistence of *G. vermiculophylla* in South Carolina. We note that the range of salinities as measured by the submerged station across Charleston Harbor may be an underestimate of the variation present on the mudflat at Fort Johnson and, as such, salinity patterns should be interpreted with caution.

Contrary to conclusions by Norton and Mathieson (1983), there was strong reproductive periodicity, which correlated with water temperature. Future studies are needed both in the native and non-native ranges focusing on both fixed and free-living thalli (see Krueger-Hadfield et al., 2017, as free-living thalli were observed in the native range as well) to determine phenological patterns and if these patterns persist across years.

Diopatra cuprea likely increases thallus fragmentation during the gluing or anchoring of thalli to its tube cap, either through direct manipulation of the thallus or due to different biomechanical forces on the thallus following anchoring. While fragmentation rates by *D. cuprea* on *Gracilaria vermiculophylla* have not been quantified, free-living thallus size did increase and was greatest in the boreal summer, when wet weight, length, and surface area were largest. Curiously, the largest tetrasporophytes were also reproductive during the July and September sampling dates. Guillemin et al. (2014) demonstrated a decrease in photosynthetic activity in reproductive *G. chilensis* tetrasporophytes, suggesting a strong physiological effect of reproduction, which influenced survival and growth rates. Future work should assess these patterns in *G. vermiculophylla*, although it is likely that free-floating tetrasporophytes have passed through some ecological sieve selecting for traits that facilitate a free-living lifestyle (Krueger-Hadfield et al., 2016; Krueger-Hadfield & Ryan, 2020; Sotka et al., 2018).

Mixed phases

We obtained evidence of three thalli that simultaneously bore infrequent cystocarps and tetrasporangial sori (Figure 3). Mixed phase thalli are not uncommon in *Gracilaria* spp. (Kain & Destombe, 1995). van der Meer (1977, 1981, 1986) pioneered much of the early work on bisexual variants, notably in *G. tikvahiae*, and is responsible for the early understanding of red algal recombination and genetic patterns. In a series of crosses with bisexual thalli, cystocarps appeared on the resultant tetasporophytic thalli after males were added to the culture (see figure 3 in van der Meer, 1986). These results are very similar to the three thalli from our study that bore tetrasporangial sori as well as cystocarps. Though we did not culture the spores from the cystocarps, each excised cystocarp was full of spores that superficially looked like those found in a cystocarp on a female gametophyte. van der Meer (1986) demonstrated that the spores from the *G. tikvahiae* variant germinated, but he did not follow them further. The origin of these observed mixed phases in our sample is unclear, but it might have been from similar bisexual mutants as described by van der Meer (1986).

Martín et al. (2011) also observed cystocarpic branches on tetrasporophytes in *Gracilaria gracilis*. Kain and Destombe (1995) described this as in situ germination of tetraspores epiphytically on the parental tetrasporophytic thallus. Similarly, Abreu et al. (2011) also noted cystocarpic and tetrasporangial branches "emerging from the same tuft of *Gracilaria* axes" (p. 457), although it is unclear if they were describing a collection of holdfasts. On some hard substrata, *G. vermiculophylla* holdfasts can be close together, and reproductive gametophytes and tetrasporophytes can appear as though they emerge from the same holdfast (S.A. Krueger-Hadfield, personal observations). We would argue that the two instances of mixed phases described by Martín et al. (2011) and Abreu et al. (2011) arise from different phenomena. We did not observe in situ germination of tetraspores leading to female or male gametophytes growing epiphytically on tetrasporophytes.

Genetic tools in phycological phenology

Overwhelmingly, thalli were vegetative at this site during our sampling period, but the use of a sex-linked genetic assay (Flanagan et al., 2021) enabled us to document tetrasporophytic dominance throughout the calendar

year. With the increase in red algal genomic resources (Lipinska et al., 2023), the development of sex-linked markers for other species will likely become easier (as an example, see Lipinska et al., 2015 as regards brown algae). Thus, there is an opportunity to revitalize red algal phenological studies and make use of efficient PCR-based assays to determine stage regardless of reproductive state. Moreover, while our measures of thallus length and surface area are likely underestimates of the live size based on our live to herbarium thallus comparisons, these data nevertheless show the utility of herbarium specimens for studying morphological patterns in large sample collections as well as verifying mixed phases.

In the specific case of *Gracilaria vermiculophylla*, these tools revealed that most free-living thalli are tetrasporophytes and that a substantial proportion of these thalli are reproductive depending on the season, contrary to the predictions of Norton and Mathieson (1983). The strong seasonal patterns of reproduction we have documented likely have implications for the demography and on-going invasion of this macroalga. For example, these free-living tetrasporophytes can "seed" a new population with the production of tetraspores and allow completion of the life cycle under certain ecological conditions (i.e., hard substratum). We also note that given *G. vermiculophylla* reproduction seems tied closely to temperature, phenology may change with climate-related changes in seawater temperatures and thus may play an important role in new invasions or range extensions (e.g., Cleland et al., 2007; Ponti & Sannolo, 2022). These patterns would not have been revealed without phenological observations, and we underscore the importance of such studies in macroalgal populations.

AUTHOR CONTRIBUTIONS

Stacy A. Krueger-Hadfield: Conceptualization (lead); data curation (lead); formal analysis (equal); funding acquisition (equal); investigation (lead); methodology (lead); visualization (lead); writing – original draft (lead); writing – review and editing (equal). **Alexis P. Oetterer:** Investigation (supporting); methodology (supporting); writing – review and editing (supporting). **Lauren E. Lees:** Investigation (supporting); methodology (supporting); writing – review and editing (supporting). **Jessica M. Hoffman:** Formal analysis (equal); writing – review and editing (supporting). **Erik E. Sotka:** Funding acquisition (equal); writing – review and editing (equal). **Courtney J. Murren:** Funding acquisition (equal); investigation (supporting); methodology (supporting); writing – review and editing (equal).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1: Phenology of a free-living, non-native *Gracilaria vermiculophylla* population: Ploidy ratios, reproductive state, and thallus size (includes Tables S1 and S2; Figures S1–S3).