

Reproductive ecology of an intertidal red seaweed, *Gracilaria gracilis*: influence of high and low tides on fertilization success

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Patterns of *in situ* fertilization success with respect to high and low tide periods are reported for the intertidal red seaweed *Gracilaria gracilis* which inhabits rock pools. Cystocarp (zygote) production was experimentally compared at high tide and low tide in both high- and low-shore tide pools at two sites using ten virgin female plants. High- and low-shore pools showed different patterns with respect to tide period. High-shore pools showed significantly greater numbers of cystocarps at low tide than at high tide whereas cystocarp production did not differ between high and low tide periods in low shore pools. The possible mechanisms of gamete release are discussed in light of these results.

INTRODUCTION

Theoretical models and empirical studies indicate that free-spawning marine species (i.e. those that release sperm into the water column) are confronted with the potential problem of low probability of gamete encounters since sperm may be rapidly and greatly diluted in the sea (for review, see Levitan & Petersen, 1995). These studies generally suggest that the availability of male gametes limits female fertilization success (reviewed in Levitan, 1998).

Most red seaweeds are dioecious and only male plants release their gametes (spermatia) since fertilization and subsequent zygote development take place on the female plant. While sperm limitation may be reduced in species that retain female gametes (Denny & Shibata, 1989; Bishop, 1998; Levitan, 1998), several characteristics of the reproductive biology of red seaweeds suggest that spermatia could be limiting. First, spermatia are not flagellated, leading Searles (1980) to speculate that fertilization is a rare event. Second, contrary to some internally-fertilizing invertebrates (e.g. Bishop, 1998), spermatia are short-lived in at least one species, being viable for less than 6 h after release (Destombe et al., 1990). Finally, there are no known pheromones or other active mechanisms of gamete attraction in red seaweeds. Despite these potential setbacks, female fertilization success does not appear to be spermatia-limited in the dioecious red seaweed, *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine & Farnham (ex. *G. verrucosa*) (Engel et al., 1999).

Reproductive strategies, reproductive systems and/or reproductive structures may increase the probability of gamete encounters and ensure high fertilization rates (for review, see Yund, 2000). For example, in fucoid seaweeds, high fertilization success is achieved by coordinating gamete release with environmental cues to maximize gamete encounters: gamete release only occurs in calm

hydrodynamic conditions, i.e. in low current speeds and/or during low tide (Pearson & Brawley, 1996; Serrão et al., 1996; for review, see Brawley et al., 1999). Similarly, seaweeds that occupy intertidal rock pools may in general benefit from low-tide conditions to maximize gamete encounters. For example, *G. gracilis* occupies rock pools distributed throughout the intertidal zone. At low tide, plants remain immersed but pools are isolated from wave action. The limited volume of seawater and relatively calm conditions at low tide could promote gamete encounters by maximizing spermatia concentrations in rock pools. In contrast, high-tide conditions potentially dilute, diffuse and disperse spermatia (Destombe et al., 1990). Here is tested the hypothesis that fertilization success is higher at low tide than at high tide in *G. gracilis*.

MATERIALS AND METHODS

Gracilaria gracilis is characterized by a typical red seaweed life history whereby female gametes, once fertilized, develop into cystocarps, macroscopic fruiting bodies on the surface of a female thallus. Fertilization success in each of the experimental treatments was thus evaluated by the presence of cystocarps, easily visible to the naked eye four weeks after fertilization when they reach maturity. About two months before the experiment, female thalli were collected in the field and cultivated as described in Engel et al. (in press) to obtain virgin, new growth. In *G. gracilis*, reproductive structures (i.e. gametangia) are produced throughout the growing season; accordingly, the reproductive season occurs essentially from March through September (Jones, 1959; Destombe et al., 1989).

The experiment was carried out in the field on the French coast of the Strait of Dover during the new moon spring tides in May 1998 and replicated at two sites sepa-

Table 1. *Field methods. (A) Topographic and demographic characteristics of the tide pools employed in this study; and (B) times of emersion and immersion of each pool and experimental outplant and retrieval times as well as the total duration of the experiment in each tide period.*

Date	Site	Pool	A. Tide pool characteristics				B. Exposure times							
			Height (m)	Area (m ²)	Density		Low tide				High tide			
					(ind m ⁻²)	Freq. males	Emersion (h:min)	Outplant (h:min)	Retrieval (h:min)	Duration (h:min)	Immersion (h:min)	Outplant (h:min)	Retrieval (h:min)	Duration (h:min)
26 May 1998	GN	High	2.8	5.0	33.8 ¹	0.32 ¹	5:44	7:05	9:40	2:35	9:59	10:15	18:05	7:50
		Low	1.5	26.0	7.5 ²	0.18 ²	6:48	6:50	9:25	2:35	9:22	9:35	19:10	9:35
27 May 1998	Aud	High	4.9	17.5	7.4 ¹	0.28 ¹	5:04	8:10	10:35	2:25	11:28	11:50	17:10	5:20
		Low	1.5	7.5	8.6 ³	0.28 ³	7:36	7:55	10:05	2:10	10:11	10:20	19:55	9:35

GN, Cape Gris-Nez; Aud, Audresselles; Height, estimated height on the shore with respect to the zero tide level; Area, approximate surface area of the tide pool; Density, based on the number of censused individuals; Freq. males, proportion of individuals identified as males. Census information taken from ¹Engel et al. (2001); ²Wattier et al. (1997); ³unpublished data. Emersion and immersion times calculated based on estimated tidal height using the French Marine Hydrography and Oceanography Service (SHOM) available at http://www.shom.fr/fr_page/fr_serv_prediction/ann_marees.htm. Outplant and retrieval times are actual experimental times. Times given are GMT+2.

rated by 5 km, Cape Gris-Nez and Audresselles. In the English Channel, *G. gracilis* typically occupies intertidal rock pools found below the mean tide level (e.g. below 4.9 m in the Strait of Dover). At each site, the experiment was performed in two rock pools occupied by *G. gracilis* individuals, one situated low on the shore (1.5 m above the 0 tide level, exposed only during spring tides) and one situated high on the shore (>2.8 m, exposed daily). Topographic and demographic characteristics of the tide pools employed in this study are given in Table 1A.

Ten females coming from both sites were each divided into eight clonal branches, or replicates (two sites×2 shore heights×two tide periods). One branch (bearing at least 20 cm of new growth) of each of the ten females was fixed in a foam-lined PVC clip (barrette) and placed in each rock pool at low tide and at high tide. For the low tide treatment, since low tide lasts longer for pools situated higher on the shore, to avoid a confounding time effect, the barrette was placed in the high-shore pool for the same duration of low tide in the low-shore pool (i.e. ~2.5 h) (see Table 1B). For the high tide treatment, since pools are inaccessible at high tide, the barrette was introduced when the first waves of the rising tide broke in each pool. Immersion time varies with height on the shore, ~9.5 h for low-shore pools and 5.3–7.8 h for high-shore pools (Table 1B). Current speeds, measured in each pool at low tide using fluorescein dye, ranged from 0.01 to 0.03 ms⁻¹ and were comparable in high- and low-shore pools (one-way analysis of variance (ANOVA), $F_{1,3}=0.55$, $P=0.53$).

A three-way factorial ANOVA was employed to detect differences in cystocarp production according to tide period, height on the shore, female identity and all possible interactions. To standardize cystocarp production, the number of cystocarps produced was divided by the total length of new growth. The dependent variable, cystocarps·cm⁻¹, was fourth-root transformed to homogenize variances.

RESULTS AND DISCUSSION

A total of 566 cystocarps were produced in this experiment. The results of the ANOVA indicate that three terms

were significant sources of variation of cystocarp production: female identity, tide period and the interaction between shore-height and tide period (Table 2).

First, some females bore more cystocarps per cm thallus than others. Variation in female reproductive success has been observed in natural populations of *Gracilaria gracilis* (Engel et al., 1999). Controlled crosses suggested that this variation could be attributed to several factors, including genetic-based differences in fertility, receptivity and/or putative incompatibility reactions, but not to population origin (Engel et al., in press). Although the different female branches are not independent replicates of the experimental conditions, including the same females in all of the experimental conditions allowed us to account for intrinsic variation in female fertilization success.

Second, cystocarp production varied with tidal period; however, the significant interaction term indicates that the patterns of cystocarp production varied according to height on the shore. Indeed, although there were no significant overall differences in fertilization success between high- and low-shore pools, timing of fertilization differed at the two shore levels (Table 2). Cystocarp production in high-shore pools behaved as predicted, with the vast majority of mating events taking place at low tide. In contrast, cystocarp production in low-shore pools did not differ between the two tide periods (Figure 1). Nonetheless,

Table 2. *Three-way factorial ANOVA on cystocarp production (cystocarps cm⁻¹). Analysis performed on fourth-root transformed data. The factor 'female identity' was designated as random.*

Source	df	SS	MS	F	P
Height on the shore	1	0.055	0.055	1.17	0.307
Tide period	1	0.378	0.378	18.67	0.002
Female identity	9	2.623	0.291	7.79	<0.001
Height×tide	1	1.168	1.168	48.55	<0.001
Height×female	9	0.419	0.047	1.25	0.296
Tide×female	9	0.182	0.020	10.54	0.835
Height×tide×female	9	0.216	0.024	0.64	0.753
Error	40	1.500	0.037		

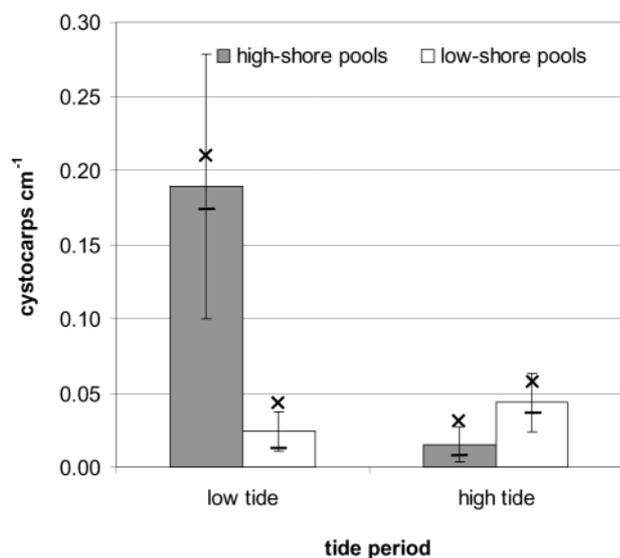


Figure 1. Cystocarp production according to tide period. Shaded bars, high-shore pools; white bars, low-shore pools. Raw means and their standard errors are given. Average cystocarp production in each pool at each tide period is also given, crosses, Cape Gris-Nez, dashes, Audresselles.

in neither high nor low shore pools was spermatia efficacy restricted to low tide periods, as a non-negligible number of fertilizations occurred at high tide even in high-shore pools (6.3% of high-shore mating events; see also Figure 1). On the other hand, considering the long immersion times, the efficiency of fertilizations at high tide was greatly reduced with respect to that at low tide.

The duration of low tide increases with increasing height on the shore. This relationship between height and emersion may result in substantially greater concentrations of spermatia in higher-shore pools. The high-shore pools in our experiment were isolated at least 1.3 h before the low-shore pools; thus, the high-shore pools were emersed more than 1 h longer than low-shore pools. By restricting the exposure of female branches to the emersion time of the low-shore pool, released spermatia may have accumulated in high-shore pools, enhancing cystocarp production during low tide in high-shore pools. Nonetheless, correcting cystocarp production for actual pool emersion and immersion times revealed the same differences in response between high- and low-shore pools. Substituting cystocarps·cm⁻¹·h⁻¹ for the dependent variable in the three-way factorial ANOVA in Table 2 showed the same three significant sources of variation, namely female identity, tide period and interaction of tide period with shore height (all three terms, $P < 0.001$, all other terms, $P > 0.30$; results not shown). Thus, the differences in the high- and low-shore exposure times at high and low tide do not account for the different patterns observed in high and low pools.

Although, since this experiment was performed in the field, tide pools varied in volume and male density (Table 1A), the same patterns were detected at both sites (Figure 1). Further, these patterns were uncovered in spite of our small sample size (only two replicates of the experimental conditions) which afforded only limited statistical power, suggesting that the difference in response between high and low pools had a real ecological basis. Nevertheless, it

is clearly necessary to repeat this type of experiment on larger spatial and temporal scales to determine the generality of the patterns revealed here.

It is not yet possible to suggest a mechanism of gamete release in this species. In furoid seaweeds, gamete release is actively triggered by environmental signals related to calm conditions (Serrão et al., 1996; Pearson et al., 1998). Although, the difference in response in high and low pools does not lend support to a similar type of active mechanism, the relatively short exposure times at low tide may have precluded detection of activated gamete release. Indeed, 2–4 h of photosynthesis in calm conditions are required to trigger gamete release in furoids (Pearson et al., 1998). While delayed activation of gamete release may explain the high fertilization success in high-shore pools compared to low-shore pools, long elicitation periods would severely restrict spermatia release in lower-shore pools. For example, at a tidal level of 1.5 m, emersion during equinox spring tides never last longer than 3.5 h. However, total female fertilization success, measured at the end of the reproductive season, does not differ between high- and low-shore pools in this region (Engel, 2000). Thus, if release is activated only in high-shore pools, cystocarps produced in low-shore pools are sired mainly by males from higher-shore populations. On the other hand, low-tide conditions may also maximize gamete encounters by passive mechanisms. Spermatia may in fact be continually released; in this case, fertilization rates would simply be a function of spermatia concentration. Clearly, relating variation in cystocarp production to differences in spermatia concentration, resulting from active or passive processes, would require evaluation of fertilization success in the different pools throughout the tide periods. For example, one set of females could be outplanted for the entire duration of low tide, a second set at the beginning of low tide, retrieved at mid-emersion time relayed by a third set outplanted at mid-emersion until the end of low tide. Finally, as the small ($\sim 5 \mu\text{m}$), colourless, non-flagellated spermatia can be easily confused with microorganisms and/or particles in non-sterile seawater, it was not possible to measure actual spermatia concentration. Relating variation in cystocarp production with spermatia concentration—due to continuous or triggered release or to differences in male density and spermatia production—would thus greatly benefit from the development of a probe or assay method to quantify *G. gracilis* spermatia *in situ*.

The differences in the timing of fertilization periods according to the location of a pool in the intertidal zone revealed in this study have implications for gene flow. Limited gene flow into high-shore pools would promote local adaptation to high-shore ecophysiological conditions (e.g. large, daily fluctuations in temperature, salinity, luminosity, etc.) by maintaining adaptive gene combinations. In isolated, exposed pools, immigration of extra-pool spermatia is limited while immersed pools, subject to greater water motion and mixing, may receive a substantial proportion of immigrant spermatia. According to the patterns discerned in this study, gene flow (via spermatia) would be low in high-shore pools compared to low-shore pools. Indeed, a paternity analysis in a high-shore pool showed that less than 10% of the cystocarps produced during one reproductive season were attributed to extra-

pool males (Engel et al., 1999). A paternity analysis is underway to evaluate the spermatia flow in a low-shore population.

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