Angelika Graiff*, Marie Dankworth, Martin Wahl, Ulf Karsten and Inka Bartsch

Seasonal variations of *Fucus vesiculosus* fertility under ocean acidification and warming in the western Baltic Sea

Abstract: Ocean warming and acidification may substantially affect the reproduction of keystone species such as *Fucus vesiculosus* (Phaeophyceae). In four consecutive benthic mesocosm experiments, we compared the reproductive biology and quantified the temporal development of Baltic Sea *Fucus* fertility under the single and combined impact of elevated seawater temperature and pCO_2 (1100 ppm). In an additional experiment, we investigated the impact of temperature (0–25°C) on the maturation of North Sea *F. vesiculosus* receptacles. A marked seasonal reproductive cycle of *F. vesiculosus* became apparent in the course of 1 year. The first appearance of receptacles on vegetative apices and the further development of immature receptacles of *F. vesiculosus* in autumn were unaffected by warming or elevated pCO_2. During winter, elevated pCO_2 in both ambient and warmed temperatures increased the proportion of mature receptacles significantly. In spring, warming and, to a lesser extent, elevated pCO_2 accelerated the maturation of receptacles and advanced the release of gametes by up to 2 weeks. Likewise, in the laboratory, maturation and gamete release were accelerated at 15–25°C relative to colder temperatures. In summary, elevated pCO_2 and/or warming do not influence receptacle appearance in autumn, but do accelerate the maturation process during spring, resulting in earlier gamete release. Temperature and, to a much lesser extent, pCO_2 affect the temporal development of *Fucus* fertility. Thus, rising temperatures will mainly shift or disturb the phenology of *F. vesiculosus* in spring and summer, which may alter and/or hamper its ecological functions in shallow coastal ecosystems of the Baltic Sea.

Keywords: bladder wrack; mesocosm; multi-factorial change; reproduction; seasonal pattern.

Introduction

In the Baltic Sea, *Fucus vesiculosus* L. is the most common canopy-forming and hence structurally important seaweed dominating the biomass along rocky and stony coasts (Kautsky et al. 1992, Torn et al. 2006, Rönnbäck et al. 2007). *Fucus* communities provide food for numerous organisms, thereby supporting complex trophic interactions (Kautsky et al. 1992, Middelboe et al. 2006, Korpinen et al. 2007) and providing various ecosystem goods and services (Rönnbäck et al. 2007). Vulnerability of *F. vesiculosus* to environmental change is suggested by the sharp decline of Baltic populations over the last five decades (Torn et al. 2006). The severe changes in *Fucus* distribution and biomass have recently been attributed to multifactorial stressors (Wahl et al. 2011), where indirect effects may outweigh direct effects (Wahl et al. 2015a). Crucial points for the persistence of *Fucus* populations have also been identified in its reproductive cycle (Andersson et al. 1994, Serrão et al. 1996a,b, 1999). Hence, it is important to study factors governing the reproduction of this seaweed.

Fucus species generally show a marked seasonality in fertility (Knight and Parke 1950, Carlson 1991, Serrão et al. 1996a, Berger et al. 2001). Reproductive seasonality may be a result of various abiotic factors that are used as environmental cues to induce and synchronize reproduction and/or to meet the physiological requirements for reproduction (Lüning and tom Dieck 1989, reviewed by Brawley and Johnson 1992). In fucoid species, environmental factors not only induce reproduction but also determine the period and duration of reproduction (Mathieson et al. 1976, Terry and Moss 1980, Bäck et al. 1991, Andersson et al. 1994). Photoperiod is the most common factor inducing reproduction in seaweeds (Santelices 1990, Brawley and Johnson 1992). For example, *Fucus distichus* L. and

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Ascophyllum nodosum (L.) Le Jolis formed receptacles at their apices under short-day conditions (SD, e.g. 8 h light:16 h darkness), whereas the apices remained vegetative under long-day conditions (LD, e.g. 16 h light:8 h darkness) in the laboratory (Bird and McLachlan 1976, Terry and Moss 1980). Increase in temperature and/or exceeding temperature thresholds, either alone or in combination with irradiance, stimulate receptacle and gamete maturation in fucoids (Mathieson et al. 1976, Pearson and Brawley 1996, Kraufvelin et al. 2012). Finally, gamete release seems to be governed by endogenous free-running circadian or circalunar cycles (Pearson and Serrão 2006), which may be synchronized by environmental factors such as tidal height, wave action, salinity (Serrão et al. 1999, Ladah et al. 2008), time of day and lunar phase (Andersson et al. 1994). The endogenous cycles, however, have not been unequivocally identified in fucoids.

In the western Baltic Sea, *F. vesiculosus* populations release gametes in early summer (May–July), in autumn (September–October) or in both seasons (Carlson 1991, Berger et al. 2001, Maczassek 2014). Receptacles are induced in summer-reproducing individuals during the preceding autumn under SD conditions, but only mature in the following spring. Possibly low winter temperatures delay the development. Autumn-reproducing individuals initiate receptacles in spring, mature over the summer and release gametes in September and October. Both reproduction types abscise their decaying receptacles after gamete release, and start to develop new vegetative tips before the next reproductive cycle (6–9 months long) starts (Berger et al. 2001). Some populations of *F. vesiculosus* reproduce in both seasons and probably comprise a mixture of both reproduction types (Carlson 1991, Berger et al. 2001). Induction of receptacle initiation by SD has been experimentally proven for summer-reproducing *Fucus* individuals (Berger et al. 2001).

In dioecious *F. vesiculosus*, the reproductive tissue (receptacles) develops at the frond tips and contains the conceptacles with egg-containing oogonia or the antheridia with sperm cells. During maturation, the receptacles grow in volume, the number of conceptacles per receptacle increases, and the egg and sperm cells mature (Bäck et al. 1991, Andersson et al. 1994). Mature conceptacles contain eight eggs per oogonium and 64 spermatozoids per antheridium, although it is not known whether the number of oogonia and antheridia per conceptacle may vary. The number, size or fresh mass of receptacles have been used to calculate reproductive effort *in situ* (Robertson 1987, Carlson 1991, Kalvas and Kautsky 1993, Brenchley et al. 1996, Berger et al. 2001) and as a proxy for *Fucus* fertility (Ruuskanen and Bäck 1999).

As the onset and duration of reproduction in *Fucus* is triggered and regulated by various environmental factors (Bäck et al. 1991, 1993, Andersson et al. 1994, Berger et al. 2001, Kraufvelin et al. 2012), development of reproductive tissue and the corresponding physiological processes may be particularly sensitive to environmental change. Global warming and acidification of the oceans as a consequence of rising atmospheric CO_2 concentrations are the most widespread effects of global change. Mean sea-surface temperatures in the Baltic Sea have increased in all seasons since 1985 (HELCOM 2013). A doubling of atmospheric CO_2 by the year 2100 (Caldeira and Wickett 2005, Orr et al. 2005, Bindoff et al. 2007) is predicted to cause a rise of 3–6°C in surface temperatures of the Baltic Sea by the end of the century (Gräwe et al. 2013, Elken et al. 2015). The increase of the CO_2 partial pressure (pCO_2) in sea-surface waters will cause a pH decrease of 0.1–0.5 units at the ocean surface in the 21st century (Feely et al. 2004, Sabine et al. 2004, Caldeira and Wickett 2005, Orr et al. 2005).

Increasing seawater temperature and ocean acidification are expected to shift synchronously and may singly or interactively affect reproduction of *Fucus*. This impact may vary seasonally according to natural growth and reproduction periods, and because these two factors, together with potentially interacting factors such as photoperiod or nutrient concentration, follow natural fluctuations. Thus, we hypothesized that the influence of warming and increased pCO_2 (singly and combined) on the reproductive biology of *F. vesiculosus* (e.g. receptacle formation, maturation and gamete release) would differ among seasons. We exposed *F. vesiculosus* to the same orthogonally crossed factors of warming and elevated pCO_2 in all four seasons, at levels expected for the shallow western Baltic Sea region within the next 100 years (Gräwe et al. 2013, Elken et al. 2015, Schneider et al. 2015). These near-natural climate-change scenarios, including all natural fluctuations, were simulated using benthic mesocosms (Kiel Outdoor Benthocosms, Wahl et al. 2015b). In an additional experiment we quantified the direct and isolated effect of temperature on the maturation of *F. vesiculosus* receptacles.

Materials and methods

Sampling site

Fucus vesiculosus individuals were collected, still attached to their natural rock substratum, in each season (spring: 2 April 2013; summer: 2 July 2013; autumn: 8 October 2013; winter: 14 January 2014) from a depth of 0.2–1 m in the Kiel

Fjord (Bülk), southwestern Baltic Sea, Germany (54°27′N; 010°12′E). In the atidal Baltic Sea, *F. vesiculosus* occurs between 0.3 and 3 m depth and is adapted to permanent submergence. After collection, the *Fucus* individuals were immediately placed in water-filled buckets and transported to the experimental site at the GEOMAR Helmholtz Centre for Ocean Research. All individuals were tagged for later identification.

Experimental setup and treatments

The experiments were performed in a nearly natural scenario in the Kiel Outdoor Benthocosms (KOB) in the inner Kiel Fjord (54°20′N; 010°09′E), which was described in detail by Wahl et al. (2015b). The KOB system consists of six tanks divided into 12 independent experimental units, each holding 1470 l of water and with gas-tight, translucent covers. The KOB facility is exposed to ambient light (irradiance and photoperiod) conditions year-round. In order to maintain water conditions as close as possible to the actual ambient conditions of the Kiel Fjord, including their environmental fluctuations, the experimental units were supplied with non-filtered seawater pumped from 1 m depth near the KOB facility, with a flow-through of 1800 l per day in each tank. Water inside each tank was circulated by a pump. In each experimental unit, 20 individuals of *Fucus vesiculosus* growing on their rock substrata were established. The rock substratum of each *Fucus* was placed in a small plastic dish (Ø = 14 cm, h = 4 cm) attached to a grating at a water depth of 40 cm in the tank. A diagram of the Benthocosm components and the experimental setup was provided by Wahl et al. (2015b).

The effects of future ocean warming were studied by contrasting ambient temperature of Kiel Fjord water with warmer water (+5°C relative to Fjord water) at two pCO_2 levels, ambient (ca. 400 ppm) vs. ca. 1100 ppm in the headspace above the Benthocosms. Thus, four treatments were tested: (1) ambient temperature with ambient pCO_2 (control), (2) ambient temperature with elevated pCO_2 (+CO_2), (3) elevated temperature with ambient pCO_2 (+Temp), and (4) elevated temperature with elevated pCO_2 (+Temp +CO_2). Each treatment was replicated in three independent experimental units. All treatments were superimposed on the natural fluctuations of all environmental variables. The enhanced levels of both factors were chosen according to climate-change predictions for shallow coastal Baltic habitats over the next 100 years (Gräwe et al. 2013, Elken et al. 2015, Schneider et al. 2015). Before the experiments, the *Fucus* individuals were acclimated to the Benthocosm conditions for 2 days under ambient conditions. The temperature in the warming treatments was elevated by 2°C on the second day and by 3°C on the third day to reach a 5°C higher temperature compared to the natural Kiel Fjord temperature on the fourth day. Under computer control, CO_2 was injected from the second day onwards into the headspace above the acidification tanks in order to maintain the headspace pCO_2 close to 1100 ppm.

The test for seasonal variations of separate and combined effects of simulated ocean warming and acidification was limited by the need to service the technical equipment and sensors every 4 months, and thus the study was divided into four sequential experiments rather than 1 year-round study. The experiments ran from 4 April to 19 June 2013 (spring), from 4 July to 17 September 2013 (summer), from 10 October to 18 December 2013 (autumn) and from 16 January to 1 April 2014 (winter), each lasting for at least 10 weeks.

Monitoring and manipulation of temperature and pCO_2 conditions

Water temperature was continuously monitored by sensors and automatically adjusted by heat exchangers and internal heating elements. For seawater pCO_2 manipulation, the headspace atmosphere was maintained at approximately 1100 ppm CO_2 by computer-controlled injection of pure CO_2 and was continuously logged. The pH in the tanks was continuously logged by sensors (gel-electrolyte filled glass electrode, GHL Advanced Technology, Kaiserslautern, Germany). To permit *post-hoc* correction of sensor drift, the pH was also measured daily using handheld and calibrated sensors (Seven Multi + InLab Expert Pro, Mettler Toledo GmbH, Giessen, Germany). Salinity was continuously logged at the institute pier (<100 m distant) by GEOMAR. The pCO_2 of the water in the four climate combinations was calculated from regular measurements of total alkalinity (TA), dissolved inorganic carbon (DIC), pH, salinity and temperature using the CO2SYS Excel Macro spreadsheet developed by Pierrot et al. (2006). A wave generator regularly induced water motion and thereby promoted diffusion of CO_2 from the headspace into the water column.

Reproductive tissue development in the Benthocosms

For each experiment, *Fucus vesiculosus* individuals 15–25 cm long with 91 ± 30 total apices and apparently

equal vigor were chosen, each individual growing on a stone (10–15 cm in diameter) from a single holdfast. The numbers of vegetative and reproductive apices of three individuals per tank were counted at the beginning of each seasonal experiment (spring: 4 April 2013; summer: 4 July 2013; autumn: 10 October 2013; winter: 16 January 2014) and constituted the "initial reproductive state". At regular intervals during the experiments (spring: 2 and 30 May 2013; summer: 18 July and 15 August 2013; autumn: 7 and 21 November 2013; winter: 13 February and 13 March 2014) and at the end of each experiment (spring: 19 June 2013; summer: 17 September 2013; autumn: 18 December 2013; winter: 1 April 2014) this assessment was repeated. One apex was defined as an incision of the dichotomy larger than 0.5 cm. The temporal development of fertility of *F. vesiculosus* was calculated as the percentage of receptacles vs. vegetative apices per individual. Subsequently, the percentage of receptacles was normalized to the highest single value that developed under control conditions (78%). This enabled better comparison of the chronological development of receptacle formation, as the initial numbers of fertile apices differed among individuals and between successive seasonal experiments. To test for possible temperature or photoperiod effects on receptacle formation and maturation, the normalized number of receptacles was correlated with season and temperature as well as with daylength.

The proportion of reproductive vs. total fresh frond biomass ("reproductive allocation") was calculated according to Robertson (1987) at the end of each experiment. The total fresh frond biomass, including the receptacles originating from one holdfast of each *Fucus* individual per treatment (n = 3), was set as 100%. Total wet mass was determined after removing the macroepiphytes and standardized removal of surface water (gently shaking the plant five times and blotting of surface water between cotton towels; EMB1200-1, Kern, Balingen, Germany). Finally, all receptacles per *Fucus* individual were cut from the fronds and weighed separately for their total wet mass (reproductive biomass). It was not experimentally possible to correct the "reproductive allocation" for the initial reproductive status, because this procedure requires destructive dissection of the receptacles for biomass determination.

The receptacles were classified into four stages of maturity according to Maczassek (2014), and related to the total number of apices. The four stages are: (1) vegetative (apices flat, with or without hair pits), (2) immature (receptacle initials indicated by a slight swelling and thickening of the apices, no conceptacles visible), (3) mature (swollen apices, conceptacles clearly visible

either as dark dots against the light or as dots slightly elevated above the surface), and (4) decayed (gamete release completed and tissue degrading). The different stages of receptacle maturation and abscission as well as the new appearance of receptacles on initially vegetative apices of *F. vesiculosus* in the Benthocosms were recorded.

Laboratory temperature experiment with North Sea *Fucus vesiculosus*

In order to better understand the development and maturation of *Fucus vesiculosus* receptacles along a temperature gradient under otherwise constant conditions, an additional laboratory experiment was conducted. *Fucus* specimens were collected at the island of Sylt (List, Germany) during low tide in April 2013. After sampling, the algal thalli were stored in a refrigerator box with water from the sampling site, transported to Bremerhaven, and stored at 10°C in filtered seawater for 1 day. Experiments were conducted with apical thallus parts comprising 4–6 tips, with at least one tip showing an immature receptacle (maturation stage 2 according to Maczassek 2014). These apical thallus parts were randomly selected and cut from different individuals prior to the experiments. We chose material where onset of fertility obviously had been induced in nature, as we were interested in the effect of temperature on the further receptacle development and not its induction, as these are separate processes. Thus, at the start of the experiment only a few tips per replicate were visually reproductive, and further increase of fertile tips on the experimental thallus parts was considered to be a function of the temperature treatment. The tips were acclimated to laboratory conditions for 3 days in filtered seawater at 10°C before the start of the experiment. Maturation of immature receptacles as well as conceptacle development was quantified over 35 days in a temperature gradient. The temperature gradient was installed in a walk-in constant cooling chamber (0°C) in six water baths (0, 5, 10, 15, 20 and 25°C ± 0.1°C) which were controlled by thermostats (Haake DC3 and DC10, Thermo Fisher Scientific, Inc., Waltham, USA). Each treatment consisted of four 2 l glass beakers (n = 4) filled with four *Fucus* branches each and aerated with artificial air containing 380 ppm CO_2 (gas mixing device; HTK Hamburg GmbH, Hamburg, Germany). *Fucus* individuals were grown in 2-µm-filtered North Sea water enriched with nutrients after Provasoli (1968; 1/10 enrichment) and the medium was exchanged every 3–4 days. The algae

were exposed to 120 μmol photons $m^{-2} s^{-1}$ ±10% (Norka, HQI TS 150W, OSRAM GmbH, Bad Homburg, Germany) measured at the bottom of the beaker under a 14:10 h L:D cycle. Prior to the experiment, the algae were acclimated to final temperatures in steps of 5°C for 2 days each.

Fertility of *F. vesiculosus* was calculated (1) as the percentage of receptacles vs. vegetative apices and (2) as the increase of conceptacles per receptacle. The number of conceptacles on one side of the same randomly chosen receptacle of each thallus (resulting in four observations per beaker; n = 4: mean of four means) was counted weekly under a stereomicroscope at 6.3× magnification. The receptacle was initially marked by a small hole punched with a glass pipette into the thallus tissue below the receptacle. In a pre-experiment, the number of conceptacles per receptacle did not vary significantly between the two sides of one receptacle (data not shown). Thus, data taken from one side were doubled to relate receptacles to the number of conceptacles. At the end of the experiment, the receptacles were cut and the fresh mass was determined after the surface water was removed with paper towels in a standardized way. The fresh mass of each receptacle was then correlated with the number of conceptacles.

Statistical analyses

Benthocosm experiment

Differences in the abundance of receptacles and four maturity stages among the treatments were analysed with repeated-measures analysis of variance (rm ANOVA), with the within-subject factor measurement dates (time) and the between-subject factors pCO_2 and temperature for each experiment separately. If the assumption of sphericity (Mauchly test) was not met, the univariate approach with Greenhouse-Geisser adjusted degrees of freedom and p-values for the F-test was applied. During the summer, autumn and winter experiments, the proportion of decaying receptacles on the *Fucus* individuals was too low for statistical analyses.

In order to assess differences in the relative abundance of receptacles and the allocation of reproductive vs. vegetative fresh tissue mass of *Fucus vesiculosus* among the four different treatments, one-way ANOVAs were applied. The reproductive allocation data were square root-transformed to achieve homogeneity of variances (Underwood 1997). Prior to the use of ANOVAs, data were tested for normality with the Kolmogorov-Smirnov test and for homogeneity of variances with Levene's test.

When the analysis revealed significant differences, pairwise comparisons between means were further explored using a *post-hoc* Tukey's honest significant difference test. Data were analyzed using SPSS Statistics 20 (IBM, Armonk, NY, USA) and the R software (R Development Core Team 2016).

A multifactorial and multivariate analysis was run by relating the presumed drivers "temperature" and "daylength" to the responses "mean % receptacles" and "number of total apices" using the RELATE routine in PRIMER® (Quest Research Limited, Auckland, New Zealand). A partitioning of the variance explanation among the multiple drivers was run using the routine distLM followed by a visualization using dbRDA (both in the PRIMER® package).

Laboratory experiment

In order to analyze the development of receptacles and conceptacles over time, they were normalized to the initial number at the start of the experiment, as this differed among replicates. This enabled us to compare the development independently of the initial size of the measuring parameter. Before statistical analysis, the relative percentage data were logit transformed (Warton and Hui 2011). Differences in the final abundance of receptacles and the relative increase of conceptacles after 5 weeks were investigated with one-way ANOVAs (n = 4, mean of four means, respectively), and pairwise comparisons between means were further explored with *post-hoc* Tukey's tests. Homogeneity of variances was confirmed with the Brown-Forsythe or Bartlett test. Normality was not investigated as the sample size was too small. Data were analyzed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) and STATISTICA 6.0 (StatSoft GmbH Europe, Hamburg, Germany).

Results

Environmental conditions during the study

The water temperature of the Kiel Fjord showed a typical seasonal pattern, with rising mean temperatures in spring and early summer (April–June), reaching maximum values (24.1–24.8°C) in July–August, and declining temperatures during autumn and early winter (September–December). In January a minimum water temperature of 4.2°C was reached and afterwards the temperature increased again

from February to March (Figure 1, Table S1). Daylength was calculated for the location of the Kiel Benthocosms with the Online-Photoperiod Calculator V 1.94 L by L. Lammi (http://www.sci.fi/~benefon/sol.html). During the course of the spring experiment, daylength increased from 14 h to 17 h and in the summer experiment it decreased from 17 h to 13 h. In the autumn experiment, daylength decreased further from 10 h to 8 h and during the course of the winter experiment increased again from 8 h to 12 h (Figure 1). In the spring and autumn experiments, water temperature was positively correlated with daylength (spring: $R^2 = 0.95$; autumn: $R^2 = 0.99$), in contrast to the summer and winter experiments (summer: $R^2 = 0.21$; winter: $R^2 = 0.04$).

The pH of the Kiel Fjord surface water was high (8.5) in spring (April–June) and low (7.7) in autumn (October–November). The pH, pCO_2, TA and DIC differed among the treatments and among seasons (Table S2). The overall mean effect of head-space enrichment with CO_2 from ambient (380–450 ppm) to 1050–1100 ppm was to reduce the tank water pH by 0.18 ± 0.08 pH units (Figure S1). The mean difference between the ambient and increased CO_2 treatments was 340 ppm CO_2 at ambient temperature and 460 ppm CO_2 at elevated temperature (M. Böttcher and V. Winde, pers. comm.). However, the seasonal amplitude of pH and pCO_2 in the water of the Benthocosms was larger than the treatment size. In addition, the *in-situ* seasonal fluctuations were altered by the diurnal metabolic activity of *Fucus*. The original raw data for the key environmental parameters of each Benthocosm are available at the PANGAEA® data platform (http://doi.pangaea.de/10.1594/PANGAEA.842739).
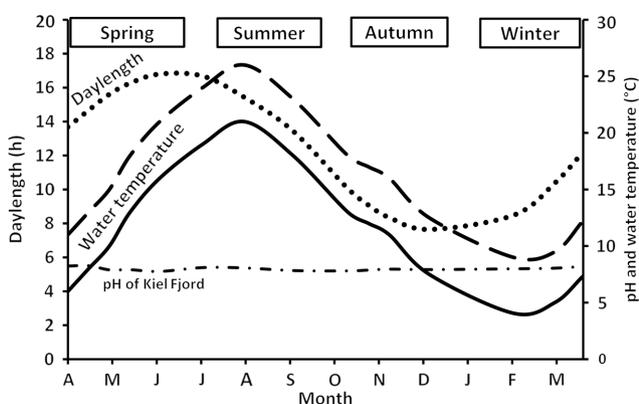

Figure 1: Mean water temperatures (solid line: temperature of the Kiel Fjord; broken line: temperature of the Kiel Fjord +5°C) and daylength (dotted line) in the four experiments.
Mean pH in the Kiel Fjord (dash-dot line) during 1 year. Experiments: spring: 4 April–19 June 2013; summer: 4 July–17 September 2013; autumn: 10 October–18 December 2013; winter: 16 January–1 April 2014.

Development of Baltic Sea *Fucus vesiculosus* fertility in the Benthocosms

The overall temporal development of *Fucus vesiculosus* fertility followed a complex pattern, varying over the seasons. In total, temperature effects were much more pronounced than CO_2 effects, which are described in detail in the following sections.

Receptacle development in relation to temperature and daylength

In *Fucus* individuals grown in the Benthocosms, the receptacles started to develop in early April and >60% of the apices were receptacles in early May (Figure 2). After gamete release and abscission of the decaying reproductive tissue in late May to early June, almost no receptacles were present from July through September (Figure 2). In the autumn experiment, new receptacles appeared in November and December and, from January to April, 60–100% of all apices were receptacles. This general receptacle development pattern of *Fucus vesiculosus* growing in ambient environmental conditions of temperature and pCO_2 (control) in the Benthocosms was also apparent in all other treatments (Figure 2).

During the spring experiment, the receptacles started to grow, ripened, released their gametes, and finally decayed. This sequence of events in the spring experiment resulted in a significant receptacle decrease of $37 \pm 17\%$ (mean ± standard deviation) of total apex numbers under all treatments (time, $F = 36.41$, $df = 1.32$, $p < 0.001$). Warming under ambient and enhanced pCO_2 accelerated the maturation of receptacles and advanced the release of gametes by up to 2 weeks (pers. obs.). The subsequent receptacle abscission under warming led to significantly fewer receptacles (May: $26 \pm 18\%$, June: $11 \pm 7\%$) compared to ambient conditions (May: $84 \pm 26\%$, June: $61 \pm 29\%$; one-way ANOVA, 30 May: $F = 4.53$ and 19 June: $F = 4.34$, $df = 3$, $p < 0.05$; Figure 2). The impact of abiotic factors on receptacle development in the spring experiment is reflected in a significant interaction between the factors warming and CO_2 (Temp $\times$ CO_2, $F = 7.3$, $df = 1$, $p < 0.05$; Table 1).

In the summer experiment, 0–30% of apices were receptacles (Figure 2), but this proportion differed significantly among the measurement dates (time, $F = 6.06$, $df = 1.16$, $p < 0.05$; Table 1). Warming reduced the abundance of receptacles significantly, to 0–3% in summer (temperature, $F = 7.4$, $df = 1$, $p < 0.05$; Table 1). During an unexpected natural heat-wave in the Fjord, experimental
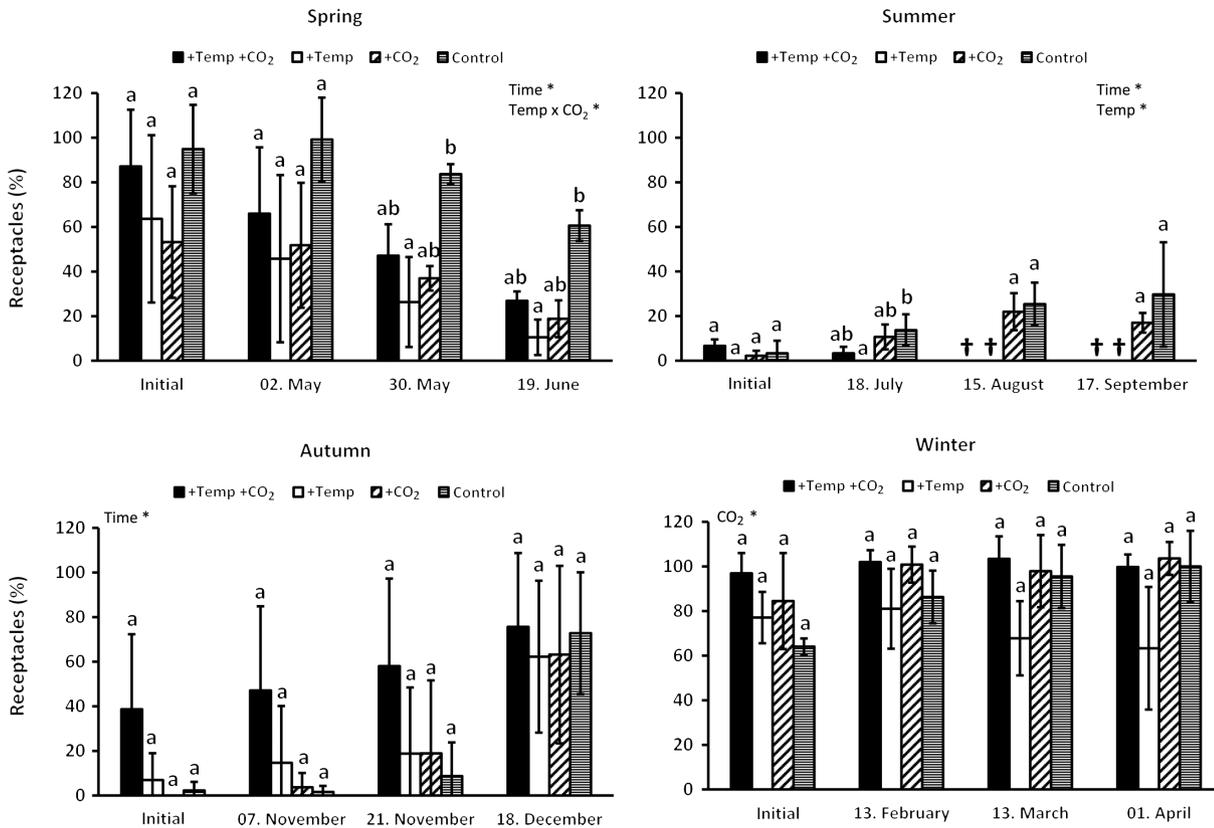
Figure 2: Initial receptacle status and receptacle development (% of total number of apices) of *Fucus vesiculosus* during experiments in the Benthocosms with various temperature and pCO$_2$ conditions over different seasons.

Seasons: spring: 4 April–19 June 2013; summer: 4 July–17 September 2013; autumn: 10 October–18 December 2013; winter: 16 January–1 April 2014. Temperature and pCO$_2$ conditions: +Temp + CO$_2$: elevated temperature and pCO$_2$, +Temp: elevated temperature and ambient pCO$_2$, +CO$_2$: ambient temperature and elevated pCO$_2$, control: ambient temperature and pCO$_2$. The means and SDs of the highest percentage of receptacles of *F. vesiculosus* grown under control conditions (78%) were normalized to 100% (n = 3). Factors listed with an asterisk for each season are those shown by repeated-measures ANOVA to have significant effects. Different letters above the bars indicate significant differences (p-value < 0.05) between treatment means within a sampling date (Tukey's test). Cross (†) indicates dieback of *F. vesiculosus* in the summer experiment under warming.

warming produced peak temperatures of 27–30°C over a period of 30 days (Table S3). This period of high water temperatures resulted in a dieback of the *Fucus* assemblage in the warmed treatments. Under ambient temperature conditions, enhanced CO$_2$ did not increase the abundance of receptacles in the course of the summer experiment (CO$_2$, F = 0.06, df = 1, p = 0.812; Table 1).

During the autumn experiment, new receptacles were formed and reproductive tissue developed on most of the frond tips (Figure 2). Thus, the abundance of *Fucus* receptacles differed significantly among the measurement dates and increased from 0%–40% to 60%–80% of total apex numbers under all treatments until December (time, F = 28.47, df = 1.85, p < 0.001, Table 1). No significant effects of warming or increased CO$_2$ were apparent (Figure 2). During the winter experiment, the abundance of receptacles did not differ among measurement dates,

and 60–100% of apices were receptacles (time, F = 1.04, df = 1.18, p = 0.35, Table 1). This indicates a retardation of receptacle maturation from January to early April.

Concerning the different treatments, receptacle abundance of *Fucus* was inversely related to water temperature (Figure 3). Under LD (>12 h) in the spring and summer experiments, the number of receptacles decreased or remained low, respectively, while under SD (<12 h) in autumn and winter, the number of receptacles increased (Figure 3). The formation of new receptacles of the *Fucus* individuals in the autumn experiment was not altered or accelerated under warming and/or increased CO$_2$ (Figures 2 and 3). However, under warmed conditions, receptacle maturation was enhanced in the autumn, winter and spring experiments, which resulted in a slightly faster increase but also an earlier decrease of receptacle numbers depending on season. This altered receptacle

Table 1: Results of repeated-measures ANOVA for effects of temperature, CO_2 and time during each experiment on receptacle abundance (%) in *Fucus vesiculosus* in different seasons.

Source of variation	df	F-value	p-value
(a) Spring			
Temperature	1	1.731	0.225
CO_2	1	1.047	0.336
Time	1.32	36.406	**<0.001**
Temp×CO_2	1	7.296	**0.027**
Temp×Time	1.32	2.992	0.106
CO_2×Time	1.32	0.110	0.814
Temp×CO_2×Time	1.32	0.107	0.817
(b) Summer			
Temperature	1	7.400	**0.026**
CO_2	1	0.060	0.812
Time	1.16	6.061	**0.042**
Temp×CO_2	1	0.521	0.491
Temp×Time	1.16	3.914	0.075
CO_2×Time	1.16	0.419	0.562
Temp×CO_2×Time	1.16	0.110	0.783
(c) Autumn			
Temperature	1	1.808	0.216
CO_2	1	1.105	0.324
Time	1.849	28.473	**<0.001**
Temp×CO_2	1	1.093	0.326
Temp×Time	1.849	1.665	0.223
CO_2×Time	1.849	0.974	0.394
Temp×CO_2×Time	1.849	0.057	0.934
(d) Winter			
Temperature	1	0.590	0.464
CO_2	1	7.936	**0.023**
Time	1.179	1.044	0.347
Temp×CO_2	1	1.832	0.213
Temp×Time	1.179	1.685	0.229
CO_2×Time	1.179	0.012	0.941
Temp×CO_2×Time	1.179	0.666	0.459

Seasons: spring: 4 April–19 June 2013; summer: 4 July–17 September 2013; autumn: 10 October–18 December 2013; winter: 16 January–1 April 2014. Bold type indicates p-values < 0.05.

maturation under warmed conditions was mitigated by increased CO_2 as indicated by the continually high presence of receptacles in the course of the winter experiment. However, this mitigating effect of increased CO_2 on receptacle development was nullified when an upper temperature threshold of 24°C was exceeded in summer.

Integrated over all seasons, the fertility of *F. vesiculosus* was strongly influenced by water temperature and daylength. Indeed, across all sampling dates, the resemblances based on temperature and daylength and the resemblances based on mean % receptacles and number of total apices were correlated (RELATE rho: 0.257, p < 0.05). However, temperature alone explained most of the variance in the pattern of receptacles and apices (AIC

26), while the inclusion of daylength added only slightly to this explanatory power (AIC 22). Mean % receptacles was correlated negatively with temperature and positively with daylength, while the total number of apices showed the inverse relationships (Figure 4).

Allocation of biomass to reproduction

Allocation of reproductive vs. vegetative fresh biomass of *Fucus vesiculosus* showed a clear seasonal pattern, with higher allocation to reproduction than to vegetative biomass in spring and winter (30%) compared to summer and autumn (7%; one-way ANOVA, F = 71.6, df = 3, p < 0.001, Figure 5). In spring, warming decreased the allocation of biomass to reproduction by 60% at the end of the experiment in June compared to ambient temperatures (one-way ANOVA, F = 7.64, df = 3, p < 0.001). At the end of the autumn experiment in December, reproductive allocation was also decreased by 60% under warming, and this was even more pronounced under CO_2-enriched conditions (one-way ANOVA, F = 4.91, df = 3, p < 0.05). In the subsequent winter experiment, allocation of biomass to reproduction was 25%–30% in *F. vesiculosus* under all treatments (Figure 5). The pattern of allocation of reproductive biomass to vegetative tissue was comparable to the pattern observed for receptacle development (receptacle %) in spring, summer and winter. In autumn, however, warming decreased the relative reproductive biomass allocation significantly, which was not obvious from the numbers of receptacles (Figures 2 and 5).

Reproductive maturation

In contrast to the total receptacle development, the proportion of apices with mature receptacles under enhanced CO_2 (ambient and warmed treatment) increased by 30% in the winter experiment (CO_2, mature receptacles: F = 6.9, df = 1; p < 0.05, Table S4; Figures 2 and 6). During the spring experiment, the four apex maturation stages differed among measurement dates (time, vegetative apices: F = 38.25, df = 3; immature receptacles: F = 9.12, df = 1.11; mature receptacles: F = 11.86, df = 3; decayed receptacles: F = 18.52, df = 1.33; p < 0.05, Tables S4–S7), because this was the main reproductive phase of *Fucus vesiculosus* with strong alteration of apex maturation stages compared to the other seasons (Figure 6). In spring, under warming, enhanced CO_2 increased the proportion of vegetative apices by 20% and decreased the proportion of apices with mature receptacles by 15% (Temp × CO_2, vegetative
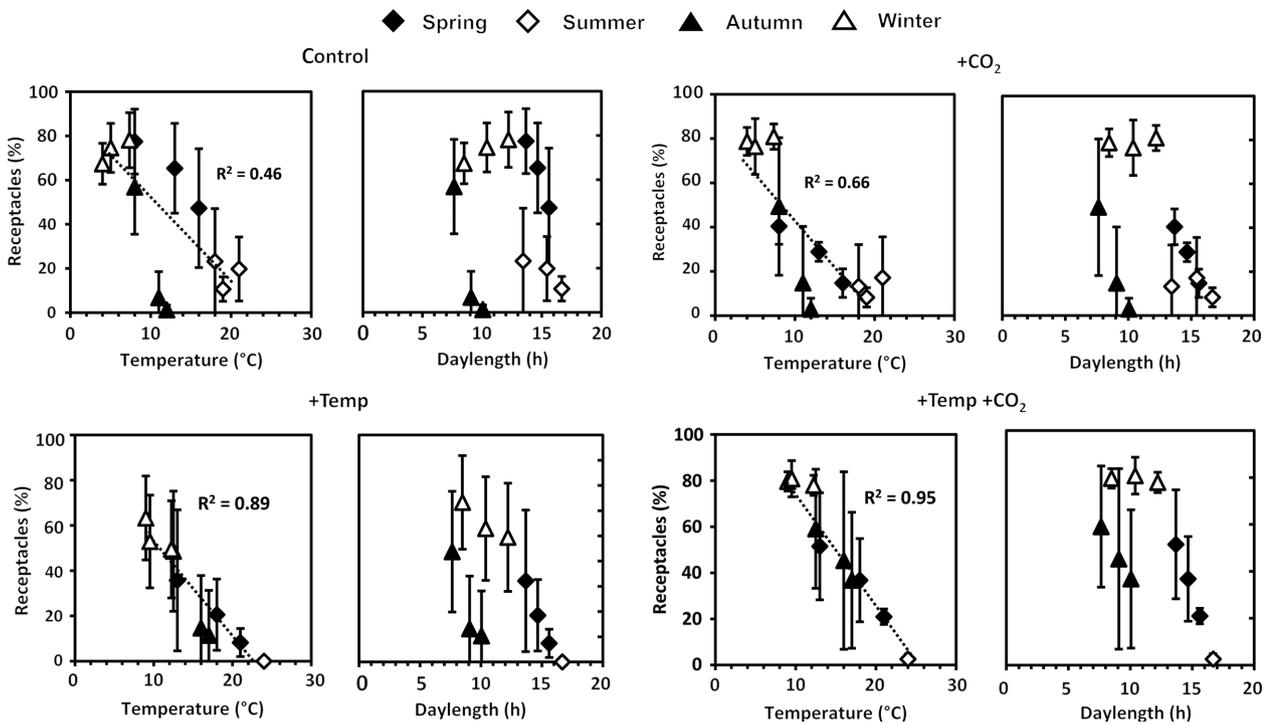
Figure 3: Receptacle development (% of total number of apices) as a function of mean water temperature and mean daylength for *Fucus vesiculosus* grown under different conditions in the Benthocosms (see Figure 2 for details).
Mean values $\pm$ SD (n = 3); trend lines and coefficients of determination (R^2) are shown for receptacles as a function of temperature.
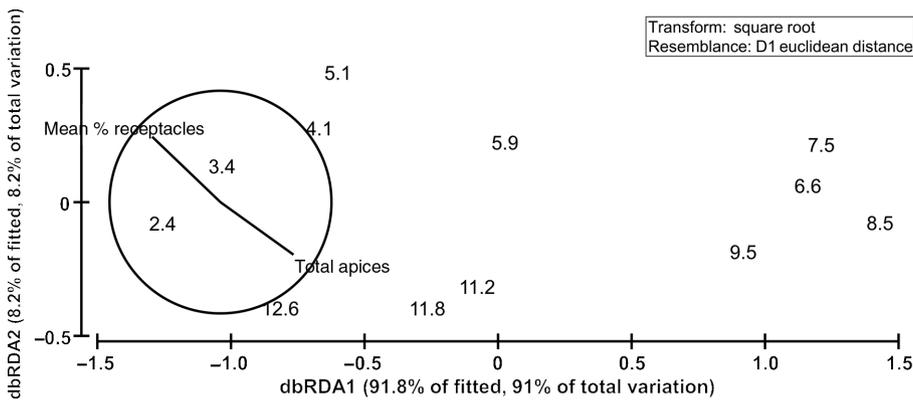

Figure 4: Multiple regression representation between the presumed drivers temperature and daylength and the mean % receptacles and number of total apices.
Numbers represent sampling events as "months" (e.g. 11.2 = early November and 11.8 = late November). Sampling dates are distributed along axis 1, which correlates strongly with temperature ($r = -0.93$) and weakly with daylength ($r = 0.37$), and axis 2, which correlates strongly with daylength ($r = -0.93$) and weakly with temperature ($r = 0.37$). Distance between samplings represents their dissimilarity. Projected across sampling date matrix are vectors for receptacle percentage and number of apices. The receptacle percentage correlates positively with daylength and negatively with temperature.

apices: F = 12.27, df = 1, mature receptacles: F = 11.74, df = 1; p < 0.05, Tables S4 and S5). The proportion of decaying receptacles in spring was enhanced by warming, CO_2 and time (Temp $\times$ CO_2 $\times$ time, F = 6.47, df = 1.33, p < 0.05, Table S7). In summer, the percentage of vegetative apices was constantly above 80% under all treatments. During the autumn experiment, the proportion of vegetative apices to apices with immature receptacles differed among measurement dates under all treatments (time, vegetative apices: F = 28.79, df = 1.55; immature receptacles: F = 33.3, df = 3; p < 0.001, Tables S5 and S6). The proportion of vegetative apices decreased by 47% and apices with immature
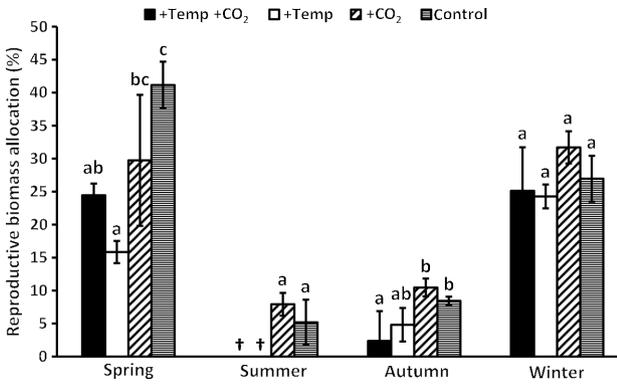
Figure 5: Reproductive biomass allocation of *Fucus vesiculosus* experimental individuals at the end of the experiments in the Benthocosms, with various temperature and pCO$_2$ conditions over different seasons (see Figure 2 for details).
Reproductive biomass is expressed as percentage of total frond biomass. Mean values ± SD (n = 3). Different letters above the bars indicate significant differences (p-value < 0.05) between treatment means within a sampling date (Tukey's test). Cross (†) indicates dieback of *F. vesiculosus* in the summer experiment under warming.

Reproductive maturation of North Sea *Fucus vesiculosus* receptacles over a temperature gradient

Temperature affected the formation of new receptacles and ripening of immature receptacles over 5 weeks (ANOVA, F = 11.76, df = 5, p < 0.001). Between 5 and 25°C the response was quite uniform: after 5 weeks, 84%–94% of *Fucus* tips had formed receptacles irrespective of temperature, and fertility was significantly lower (58%) only at 0°C (Figure 7A). The relative increase of conceptacles after 5 weeks also showed a significant temperature response (Figure 7B; ANOVA, F = 6.80, df = 5, p = 0.001). At 10°C, conceptacles increased by 70%, significantly more than at 0, 20 and 25°C (20, 24, 31%, respectively). At 5°C the conceptacle increase was 61% and thus similar to the proportion at 10°C, but was not significantly different from 15 to 25°C (Figure 7B). Earliest gamete release from receptacles occurred after 3 weeks at 15–25°C, while it was observed only after 4 weeks at 5 and 10°C and after 5 weeks at 0°C (Figure 7B). Correlation of receptacle fresh weight with the number of conceptacles per receptacle after 5 weeks showed a weak but significant relationship (Figure 8; R^2 = 0.476, p < 0.05). The heavier the receptacle, the more conceptacles were present.

receptacles increased by 50% (Figure 6), indicating that vegetative apices developed into immature receptacles under all treatments.
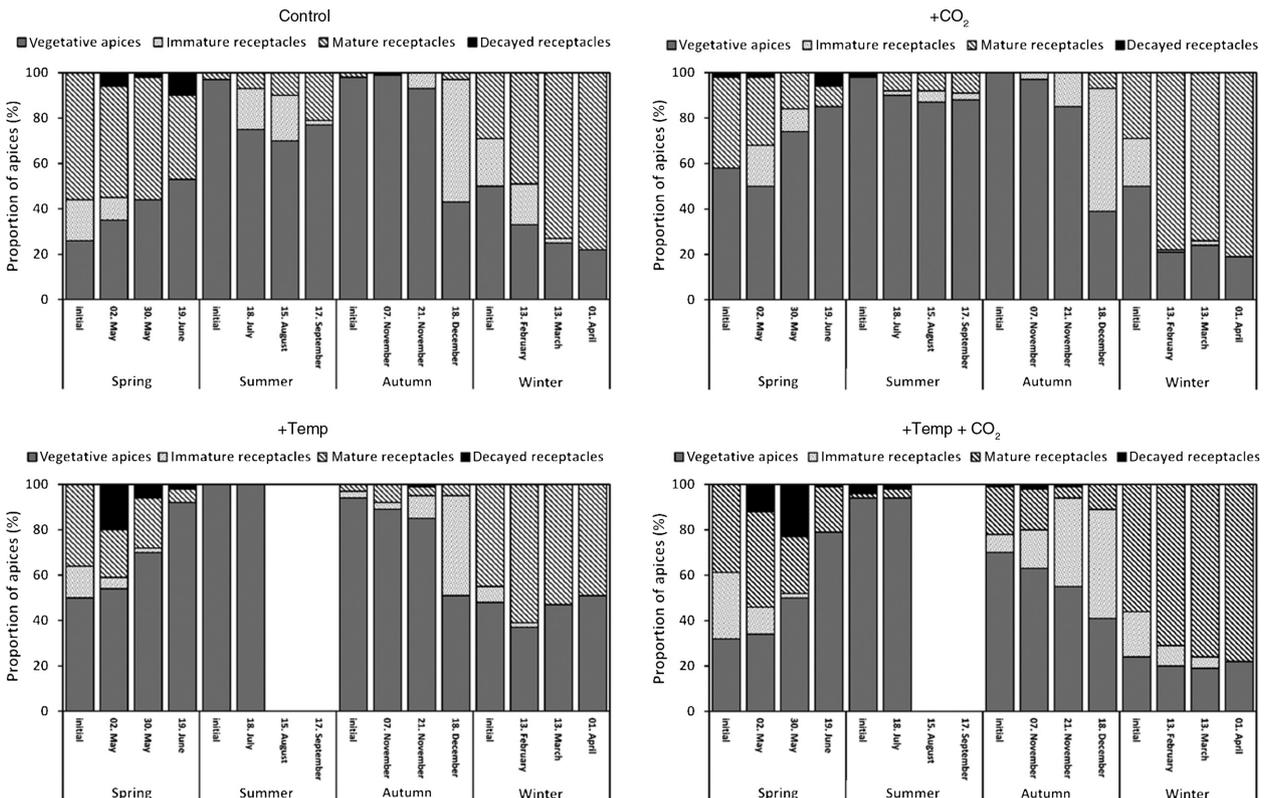
Figure 6: Initial reproductive status and reproductive maturation of *Fucus vesiculosus* apices during experiments in the Benthocosms, with various temperature and pCO$_2$ conditions over different seasons (see Figure 2 for details).
Maturity levels were classified according to Maczassek (2014). Mean values (n = 3).
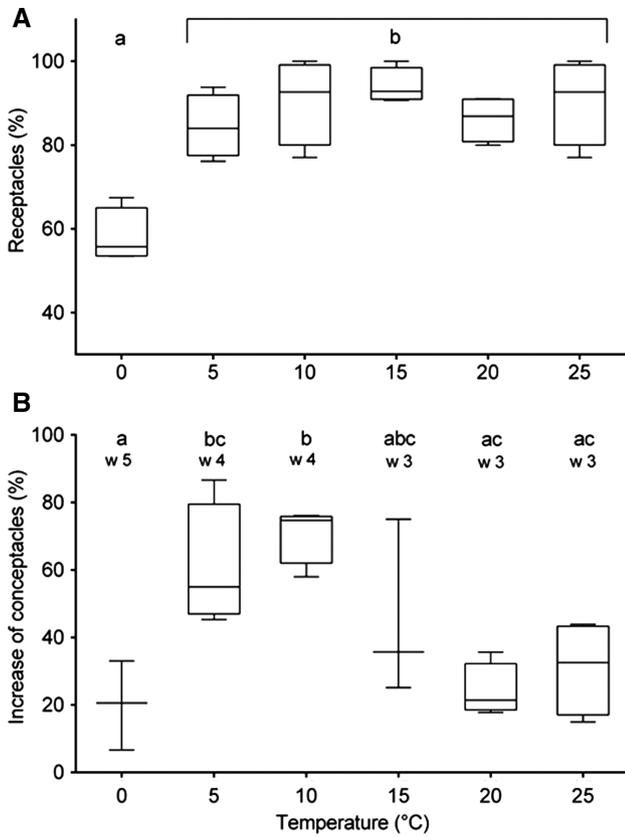
Figure 7: Receptacle and conceptacle ripening of fertile tips of *Fucus vesiculosus* (North Sea) along a temperature gradient after 5 weeks in the laboratory.

(A) Proportion of tips with fertile receptacles (n = 4; median of means ± SD and min/max values). (B) Relative increase of conceptacles (n = 4; 0 and 15°C n = 3; median of means ± SD and min/max values) and indication of earliest gamete release (w = week). Different letters above box-plots indicate significant differences (p-value < 0.05) between treatment means (Tukey's test).
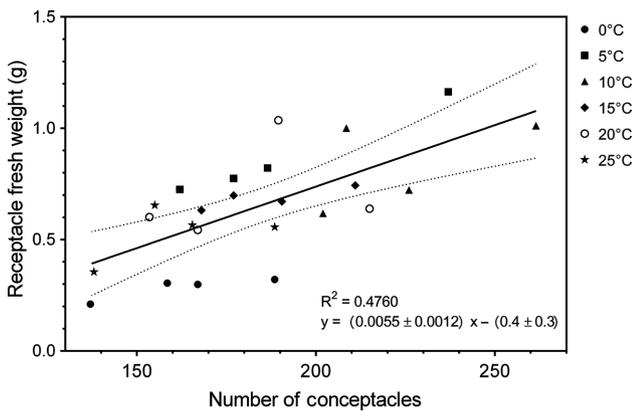

Figure 8: Correlation between fresh weight of receptacles and number of conceptacles in *Fucus vesiculosus* (North Sea) after 5 weeks of exposure to a temperature gradient in the laboratory. Black and dotted lines: linear regression ± confidence interval.

Discussion

A marked seasonal reproductive cycle with a defined sequence of events became apparent under all treatment conditions, and is shown schematically in Figure 9. New receptacles appeared in November under short-day conditions (<10 h), a process that was not influenced by elevated pCO_2 and/or temperature. During the winter experiment, elevated pCO_2 in ambient and warmed conditions increased the proportion of mature receptacles significantly and mitigated the negative effect of warming. In spring, warming and elevated pCO_2 accelerated maturation of receptacles, resulting in release of gametes as
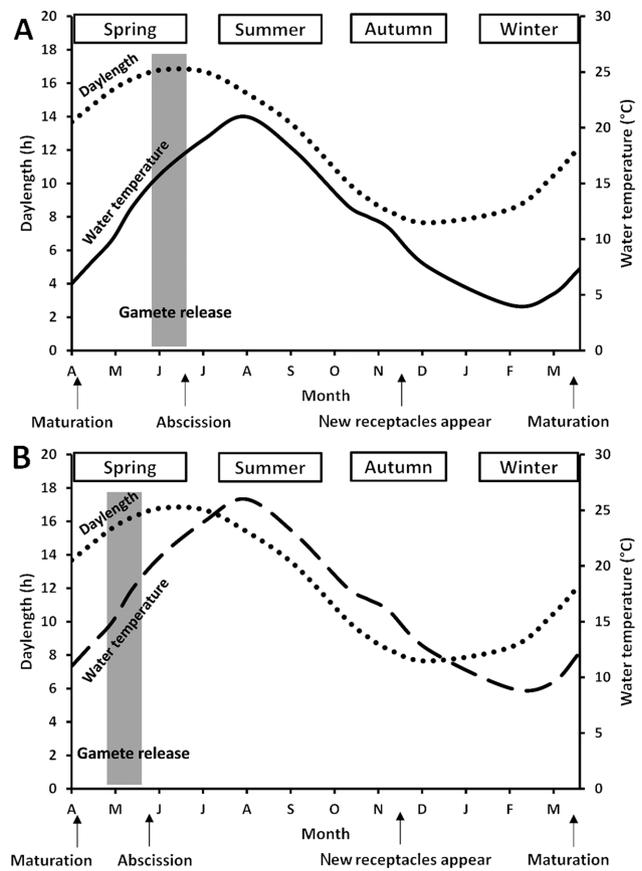

Figure 9: Conceptual sketch based on observations of the annual cycle of receptacle formation, maturation, gamete release and receptacle decay in *Fucus vesiculosus* in the Benthocosms under (A) temperature of the Kiel Fjord (solid line) and ambient pCO_2, or (B) temperature of the Kiel Fjord + 5°C (broken line) and enhanced pCO_2 (1100 ppm).

The times of maturation and abscission of receptacles, as well as of the formation of new receptacles from vegetative apices and development of immature receptacles on *F. vesiculosus* in the Benthocosms are indicated with arrows. Seasons: spring: 4 April–19 June 2013; summer: 4 July–17 September 2013; autumn: 10 October–18 December 2013; winter: 16 January–1 April 2014.

much as 2 weeks earlier. This agrees with the results of the unifactorial laboratory approach, where receptacle abundance was similarly high at all temperatures between 5 and 25°C, but conceptacles matured 2–3 times faster at 5 and 10°C than at lower or higher temperatures. Although our results demonstrated that, depending on the season, elevated pCO_2 affected the development and maturation of *Fucus vesiculosus* receptacles alone or interactively with warming, temperature had a stronger effect on reproduction. In general, reproduction of *F. vesiculosus* was directly controlled by fluctuating environmental parameters, especially photoperiod and temperature, differently influencing the different phases of its reproductive cycle. Thus, it is crucial to consider the whole annual cycle of an alga or at least its different seasonal reproductive states, in order to assess the full ecological implications of future climate-change scenarios.

Seasonal reproduction and reproductive biomass allocation

Fucus species generally show marked seasonal variation in growth and reproduction in temperate regions (e.g. Berger et al. 2001), growing most rapidly in late spring and early summer (Lehvo et al. 2001, Graiff et al. 2015a) and reproducing mostly in restricted periods, in summer and/or autumn (Berger et al. 2001). The *Fucus* population in the Kiel Fjord reproduces in both seasons (Maczassek, pers. comm.), as was partly confirmed by our results. However, more *Fucus* individuals released gametes in early summer (May–June) during the spring experiment, while no gametes were released during the autumn (pers. obs.). Apparently, we collected mostly summer-reproducing *Fucus* individuals according to the definition by Berger et al. (2001). In our study, fertility obviously had been induced in nature before the experiments started. Thus, the preceding environmental conditions that influenced the receptacle initiation must be considered in order to correctly interpret the results.

In *Fucus vesiculosus* there is a trade-off between production of receptacles and of vegetative tips (Kautsky et al. 1992). Thus, the relationship between the numbers of receptacles and vegetative tips indicates the reproductive allocation (Bäck et al. 1991, Kautsky et al. 1992). The cost of gamete production, measured as biomass, is small in fucoids (Vernet and Harper 1980), but the thallus structures supporting reproduction are considered to be relatively costly (Mathieson and Guo 1992). Schiel (1985) suggested that the true cost of reproduction in fucoids must also take into account the vegetative structures that support the fertile material, and are produced and shed annually. Therefore, the reproductive allocation is commonly determined as the ratio between total frond biomass and receptacle biomass (Cousens 1986). Reproductive biomass allocation in *Fucus* grown in the Benthocosms under ambient conditions was high at the end of the spring and winter experiments (27%–40%), which is in agreement with the reproductive biomass allocation of 23%–25% observed for *Fucus* individuals on the east coast of Sweden and in the northern Baltic Sea in the same seasons (Bäck et al. 1993, Berger et al. 2001). This indicates that, in winter and spring, *Fucus* invests highly in newly produced biomass for reproduction, compared to vegetative biomass. In autumn, reproductive allocation was lower (5%–8%) in Baltic *F. vesiculosus* compared to *Fucus spiralis* L. (12%–25%) (Robertson 1987). Although the results show a clear pattern, it should be considered that the water content in the receptacles might increase during maturation, leading to a proportionally lower carbon content compared to the vegetative thalli. Because reproductive and vegetative tissues are both photosynthetically active, reproductive allocation based on biomass alone probably overestimates the full cost of reproduction as well as the costs of the reproductive effort and success.

Reproduction under ocean warming and acidification

The ongoing and expected future warming of the Baltic Sea may have consequences for the seasonality of *Fucus* reproduction, as the reproductive cycle seems to be especially sensitive to this and other environmental factors (Kraufvelin et al. 2012). The first signs of receptacle growth of *Fucus vesiculosus* occurred 5–6 weeks earlier and receptacles matured 2–3 weeks earlier during warm than during cold springs in the field (Kraufvelin et al. 2012). This observation agrees with our laboratory and Benthocosm results, where warming accelerated maturation. Receptacle development was rather rapid over a wide range of temperatures, between 5 and 25°C, in the laboratory. The number of conceptacles per receptacle was highest in the 5–10°C temperature range, but was only weakly related to receptacle fresh mass, which also changed with temperature and season in the Benthocosm. A positive relationship between conceptacle number and receptacle size was previously observed for *Fucus distichus* (Ang 1991). From these data it is assumed that reproductive output, which has not been quantified either in the Benthocosm or in the laboratory study, will be highest during periods of continuous low to moderate temperatures that normally occur

in spring to early summer or late autumn. This ideally matches the temperature relationship of the next life-cycle stage, namely the germination capacity of zygotes (Maczassek 2014) and of adult *F. vesiculosus,* which grow best at 15–20°C (Graiff et al. 2015b), corresponding to late spring to summer in the field. Another issue influencing reproductive capacity, which is still unknown for *F. vesiculosus*, is whether each conceptacle in *F. vesiculosus* always contains the same number of gametes, or whether this number is also dependent on temperature or other environmental factors. The only study quantifying the number of eggs per conceptacle in Fucales dealt with *Ascophyllum nodosum* (Åberg and Pavia 1997). In this species, the number of eggs per conceptacle did not vary among individuals, although the authors considered only one environmental setting. Furthermore, simulated warming in the spring experiment accelerated maturation of receptacles, with an earlier release of gametes and subsequent receptacle abscission compared to control conditions in the Benthocosms. Under simulated ocean warming, temperature maxima exceeded 15°C for longer periods in spring, which probably accelerated receptacle maturation, leading to earlier maturation of the receptacles. However, in the laboratory and Benthocosm experiments, we did not differentiate between female and male individuals of this dioecious *Fucus* species, and cannot exclude the possibility that receptacles of females and males may mature at different rates. Also, sperm and egg cells were released 1–2 weeks earlier under higher temperatures (15–25°C) compared to cooler temperatures (0–10°C) in the laboratory experiment. Similarly, in the Benthocosms, warmed conditions resulted in release of gametes as much as 2 weeks earlier and subsequent receptacle abscission. Thus, global warming will mainly influence the reproductive period of *F. vesiculosus* in spring, thereby perhaps leading to a shift between the timing of *F. vesiculosus* zygote settlement and the cover of competing filamentous algae (Kiirikki and Lehvo 1997, Berger et al. 2004, Råberg et al. 2005, Kraufvelin et al. 2007). These possible changes in species interactions require further investigation in order to better understand their possible consequences.

High CO_2 and/or dissolved inorganic carbon (DIC) conditions stimulate growth and photosynthesis of different non-calcifying seaweeds (Gordillo et al. 2001, Nygård and Dring 2008, Wu et al. 2008, Olischläger et al. 2012, Saderne 2012, Koch et al. 2013). In addition to the effect of enhanced CO_2 on growth and photosynthesis of seaweeds, reproduction may be impacted by the DIC concentration in seawater. Gamete release by *F. distichus* is triggered and synchronized by sensing low DIC concentrations, probably via the HCO_3^- concentration in the seawater

(Pearson et al. 1998). Low DIC concentrations characteristically indicate low water movement (and sharp gradients at small scales), which ensures high fertilization success for species with external fertilization (Pearson and Serrão 2006). In the present study, in spring, *F. vesiculosus* developed fewer receptacles under acidification, but the receptacles matured slightly faster and released their gametes earlier compared to control conditions in the Benthocosms. However, in the course of the winter experiment, elevated CO_2 significantly enhanced the biomass of *F. vesiculosus* (Graiff et al. 2015a) and also increased the formation of mature receptacles. During the winter experiment, most of the vegetative apices developed into receptacles, which comprised a considerable proportion of the biomass at the end of the experiment. The increased formation of mature receptacles might have been due to reduced energy investment in the operation and/or maintenance of active carbon concentration mechanisms (CCMs) at higher pCO_2, resulting in surplus energy for reproduction or vegetative growth.

In conclusion, the interactive effects of ocean warming and acidification on the reproductive biology of *F. vesiculosus* (e.g. receptacle formation, maturation and gamete release) have not been studied before. These effects are, however, important for predicting the impact of global change on reproductive periodicity and population dynamics. For example, critical daylengths for photoperiodic responses are shorter at lower temperatures in some seaweeds (reviewed by Dring 1974), and photoperiodic responses are restricted to certain temperatures in others (Maggs and Guiry 1987, Hales and Fletcher 1990). Such interactions precisely synchronize the timing of reproduction (Brawley and Johnson 1992). In the present study, the onset of receptacle formation in autumn was not altered by warming and elevated CO_2, either singly or in combination. Probably, photoperiod acted as a trigger and induced fertility before the *Fucus* individuals were transferred to the Benthocosms. Thus, new receptacles appeared and developed under SD conditions in the autumn experiment, irrespective of the subsequent environmental conditions. In winter, however, elevated pCO_2 enhanced the proportion of mature receptacles under the warming scenario. Apparently the indicated negative effect of warmed winter temperatures on the receptacles was mitigated by increased CO_2 resulting in slightly higher reproductive biomass allocation in the spring and winter experiments compared to warming alone. The supply of DIC may have increased – antagonistically to warming – the performance of *F. vesiculosus*. This finding is consistent with those of Olischläger and Wiencke (2013) and Sarker et al. (2013), who examined the combined effects

of CO_2 and temperature on *Neosiphonia harveyi* (Bailey) M.-S. Kim, H.-G. Choi, Guiry & G.W. Saunders and *Chondrus crispus* Stackh., respectively. Both studies described a mitigation of the temperature stress response due to increased CO_2. Here, we also found that temperature had a stronger effect than pCO_2, but pCO_2 did not mitigate the effect of stressful high summer temperatures, as the tolerance threshold of western Baltic Sea *F. vesiculosus* had been exceeded. In addition, the observed phenology shift of *F. vesiculosus* under warming was neither increased nor mitigated by enhanced pCO_2.

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Bionotes

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tems and their acclimation and adaption capacities.

Graphical abstract

Angelika Graiff, Marie Dankworth, Martin Wahl, Ulf Karsten and Inka Bartsch

Seasonal variations of *Fucus vesiculosus* fertility under ocean acidification and warming in the western Baltic Sea

Research article: Global warming will have the potential to alter the phenology of *Fucus vesiculosus* in the Baltic Sea as simulated warming in spring induced faster maturation of receptacles with release of gametes up to 2 weeks earlier compared to ambient conditions.

Keywords: bladder wrack; mesocosm; multi-factorial change; reproduction; seasonal pattern.