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Intrinsic and extrinsic control of reproduction in the green tide-forming alga, *Ulva rigida*

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Abstract

The green seaweed *Ulva* is the causative genus behind nuisance green tides, but also has uses in the food and feed industries. Growing interest in *Ulva* cultivation has highlighted knowledge gaps in the mechanisms that regulate maturation and reproduction, particularly interacting intrinsic and extrinsic factors. In this study, the effects of temperature shock, dehydration, culture temperature, nitrate concentration, and thallus fragmentation were investigated on blade and basal tissue from *U. rigida* thalli of varying ages. A 20-minute temperature shock induced a mean reproductive response of 94.7% in blade tissue by day five. The reproductive rate of blade tissues increased with the degree of fragmentation and with growth media renewal. Combining temperature shock with fragmentation triggered 97.3% of blade tissues to reproduce by day three. In contrast, dehydration reduced reproduction. A temperature of 18°C in combination with a nitrate concentration of 100 µmol L^{-1} halved the maturation period (28.4 days) compared to cultivation under lower temperature and nitrate conditions (62.1 days). Reproduction in blade tissues increased with plant age but basal tissues remained in the vegetative state even after temperature shock and fragmentation. Furthermore, the presence of basal tissues suppressed reproduction of blade tissues. These findings indicate that extrinsic factors such as temperature shock and fragmentation induce reproduction in blade but not basal tissues, which appears to be under the control of intrinsic factors such as sporulation inhibitors. The

differentiation of *Ulva* cells could support the rapid growth of *Ulva* when environmental conditions are favourable and also facilitate survival under unfavourable conditions.

Keywords: dehydration; differentiation; fragmentation; reproduction; temperature; *Ulva*

1. Introduction

Ulva is a cosmopolitan genus of green seaweed that can be found from tropical to polar climates and from fresh to fully saline environments (Rautenberger and Bischof, 2006; Shimada et al., 2008; Kirkendale et al., 2013). Robust capacities for nutrient uptake and growth have contributed to certain *Ulva* species becoming the problematic agents behind worldwide green tide events (Smetacek and Zingone, 2013). On the other hand, *Ulva* species are important in the food and feed industries and are gaining increasing recognition as potential biofuel feedstock and as nutrient scrubbers for bioremediation applications (Bikker et al., 2016). *Ulva* cultivation is widely practiced (Lehahn et al., 2016), yet there remain gaps in our knowledge on key facets of *Ulva*'s biology, including its reproductive biology. Reproduction not only directly affects productivity but can also be used to supply gametes for seedlings and should eventually lead to full domestication.

Ulva species tend to have complicated life cycles, generally involving an alternation of isomorphic diploid sporophyte and haploid gametophyte phases. In

addition, parthenogenetic development of gametes has also been found (Kapraun, 1970). In all cases, reproductive cells are directly transformed from vegetative cells (Hiraoka and Enomoto, 1998). The transition is initiated by extrinsic factors, with environmental temperature known to be important. For example, *U. lactuca* from Groton, USA, only reproduce in warmer months (Niesenbaum, 1988), whereas North Sea populations of *U. pseudocurvata* have approximate weekly reproductive peaks during the summer and biweekly peaks during colder seasons (Lüning et al., 2008). Kalita and Titlyanov (2011) shed further light on temperature regulation of reproductive rhythmicity by showing that the reproductive period of *U. fenestrata* decreased from 30 to five days when temperature increased from 10 to 20°C, and ceased at 5°C. Furthermore, rapid temperature changes have been found to induce gamete release over periods of hours to days (Niesenbaum, 1988; Carl et al., 2014a).

The effect of dehydration on gamete release is less clear. For example, Smith (1947) and Corradi et al. (2006) found that most *Ulva* blades discharged gametes five to 10 minutes after a dehydration period of one hour or less when followed by rehydration, whereas Carl et al. (2014a) found dehydration to be ineffective to increase sporulation.

Tissue fragmentation is considered a powerful factor inducing reproduction (Nordby and Hoxmark, 1972; Nordby, 1977; Hiraoka and Enomoto, 1998; Dan et al., 2002; Gao et al., 2010). Fragmentation dramatically improved the sporulation rate of

U. mutabilis from 15.8 to 80.0% (Nordby, 1977). Hiraoka and Enomoto (1998) reported that reproduction of *U. pertusa* was induced two to three days after fragmentation, with the reproductive rate increasing when fragment diameter decreased from 10 to 0.9 mm. Gao et al. (2010) demonstrated that *U. prolifera* fragments of 0.5 mm diameter were almost entirely converted to sporangia whereas in larger diameter fragments the sporangia only formed from the marginal and submarginal cells.

Nutrient availability also plays a role in reproduction of *Ulva* species. Mohsen et al. (1974) showed that nitrogen enrichment induced rapid sporogenesis and sporulation, whereas depleted nitrogen led to zygospore formation in *U. fasciata*. In addition, nitrate enrichment could significantly promote reproduction in *U. rigida* over a 12-day cultivation (Gao et al., 2017).

Intrinsic regulatory factors also contribute to reproductive regulation. Föyn (1959) and Thiadens and Zeuthen (1966) reported that growth medium renewal induced *U. mutabilis* to reproduce, inferring that substances in the fresh medium induced sporulation rather than suppressing factors in the spent medium. However, Nilsen and Nordby (1975) demonstrated that *U. mutabilis* reproduction was blocked by high molecular weight carbohydrates extracted from living thallus fragments. Further, Stratmann et al. (1996) identified two regulatory factors that maintain *U. mutabilis* in the vegetative state: a glycoprotein termed sporulation inhibitor-1 (SI-1)

and a non-proteinaceous compound termed sporulation inhibitor-2 (SI-2). SI-1 is a cell wall component, the excretion of which decreases with thallus maturation, while SI-2 occurs in the space between the two blade cell layers. The overall concentration of SI-2 in the thallus remains constant throughout the life cycle. Each sporulation inhibitor can inhibit gametogenesis. Furthermore, gamete release after gametogenesis in species of *Ulva* could be controlled by a third substance, termed swarming inhibitor (Wichard and Oertel, 2010; Vesty et al., 2015).

Although the *Ulva* thallus is organized simply with little functional differentiation within the thallus, it does consist of at least two cell types: rhizoidal cells in the basal parts and blade cells in the marginal parts of the thallus. Different degrees of reproduction have been demonstrated between these thallus regions. More than 90% of excised discs from the upper parts of the thallus in *U. pertusa* sporulated while almost all discs from the basal parts did not mature three days after excision (Hiraoka and Enomoto, 1998). A similar trend was found in *U. pseudocurvata*, in which the degree of fertility increased from the basal to apical part of the thallus (Lüning et al., 2008). Different reproductive performances across the *Ulva* thallus might be due to the uneven distribution of sporulation inhibitors within the thallus, with the highest concentration near the holdfast (Stratmann et al., 1996).

The studies aforementioned used mature *Ulva* plants, so little is known about the effects of intrinsic and extrinsic factors on reproduction of the blade and basal

parts of *Ulva* at varying ages. Accordingly, the precise role and interplay of intrinsic and extrinsic factors remains unclear, particularly in relation to plant age. The responses of *Ulva* at varying ages to environmental factors could be differential. In our previous study, young *U. rigida* (less than 60 days post germination) did not show any reproduction events during 60 days of culture, while the adult plants (more than 60 days post germination) grown at higher temperature and nutrient replete conditions (nitrate) had a reproduction rate of 64 ± 5% by day 12 (Gao et al., 2017). Therefore, young *Ulva* is deemed to be favourable for commericial cultivation compared to adult *Ulva* as it could avoid periodic reproduction and thus growth fluctuation (Hiraoka and Oka, 2008). Based on the previous studies, we hypothesised that the interplay of intrinsic and extrinsic factors on reproduction of blade and basal tissues in *U. rigida* would differ with plant age. We therefore investigated the role of temperature shock, fragmentation and (assumed) sporulation inhibitors in controlling reproduction in blade and basal tissues of *U. rigida* of different ages. This research provides important insights into the regulation of reproduction in a commercially and ecologically important green alga.

2. Materials and methods

2.1. Seaweed collection

Vegetative *U. rigida* plants of 12–15 cm in length were collected from the low intertidal reaches of Cullercoats Bay, UK (55.03° N, 1.43° W). Reproduction and

swarmer release from temperate *Ulva* species usually occur during spring tides (Lüning et al., 2008); therefore, plants were collected two days after a spring tide to ensure that the plants were at an early stage of a lunar reproduction cycle and thereby minimizing any effect of spring tides. The fronds were placed in a plastic bag and transported to the laboratory within one hour and gently rinsed in 1 μm filtered seawater to remove any sediment, epiphytes or small grazers. The *Ulva* species used in this study was identified by DNA barcoding at the Institute of Oceanology, Chinese Academy of Sciences. It was found that the sequence, excluding the primers at both ends, fully matched (100%) to *U. rigida* SSBO0102 isolated from Skara Brae, Orkney, Scotland (Gao, 2016). The plants were identified as gametophytes by microscopically observing the released swarmers that had two flagella and exhibited positive phototaxis.

2.2. Culture conditions and determination of reproduction

Ulva discs were excised from the vegetative thalli using a stainless steel punch and rinsed with autoclaved seawater. After various treatments stated in the following subsections, *Ulva* discs were cultured in 500-mL conical flasks filled with 300 mL autoclaved seawater at 18°C with a 16: 8 (L: D) photoperiod and light intensity of 80 μmol photons m^{-2} s^{-1}. The flasks were aerated with a flow rate of 100 L min^{-1}. The nitrate and phosphate concentrations in the seawater were 100 and 5 μmol L^{-1}

140 respectively, and the media was renewed daily. The temperature and photoperiod
141 were chosen as they are the ambient conditions at the sampling site during summer.

142 Reproductive *Ulva* discs were recognized by a colour change and was verified by
143 microscopy (Fig. 1). Vegetative cells were green in colour, containing several
144 granular chloroplasts (Fig. 1A). Thalli became pale green when becoming fertile due
145 to a change in the chloroplast position (Fig. 1B). Approximately 24 hours later,
146 pyriform gametes were formed within the sporangia (Fig. 1C) causing the thalli to
147 develop a yellowish appearance; gametes were subsequently liberated from the
148 sporangia (Fig. 1D). The cells become empty after discharging all gametes (Fig. 1C).
149 Sporulation in more than half of the disc area was considered as equivalent to
150 complete sporulation; the samples were checked daily. The reproduction rate was
151 recorded from the day when reproduction occurred to the end of the experiments
152 when the *Ulva* tissue samples yielded a reproductive success of over 90%. Different
153 time periods were used in the following experiments as the effectiveness of different
154 induction methods varied. Triplicates were used in all experiments. The number of
155 discs used per replicate is stated within each experimental section.

156 *2.2.1. Temperature shock I*

157 Excised discs, with a diameter of 20 mm, were placed in 500 mL flasks
158 containing 300 mL seawater at 4°C for 0, 10, 20, 30, 60, 120, 180, or 360 minutes,
159 within a refrigerator. These flasks were then transferred to an 18°C incubator and the

temperature of seawater in the flasks rose to 18°C within two hours. The flasks were cultured as described above (see 2.2) for five days. The lower temperature and time treatments were based on Carl et al. (2014a). The reproduction rates on days three, four and five were recorded. Reproductive rate was determined as the ratio of reproductive discs to all discs within a treatment.

2.2.2. Temperature shock II

To further understand the role of temperature shock, additional discs were treated in five conditions. One group was kept at 18°C for two weeks with no temperature shock (Zero); another was transferred from 18 to 4°C for two hours and returned to 18°C and then cultured at 18°C for two weeks; the remaining three groups were transferred from 18 to 4°C and then cultured at 4°C for one, two, and three weeks, respectively. In both temperature shock experiments each group had 25 discs per replicate flask.*2.2.3. Dehydration*

The effect of dehydration was investigated by air-drying blade discs with a diameter of 20 mm at room temperature (20°C) for 0, 15, 30, 60, 120 and 180 minutes, then rehydrating in seawater and culturing them at 18°C as described above for two weeks. These dehydration periods were chosen according to the previous studies on *Ulva* species (Smith, 1947; Corradi et al., 2006; Carl et al., 2014a). The reproduction rates on days 10, 12, and 14 were recorded. Twenty five discs were placed in each flask.

2.2.4. Fragmentation and media

The upper regions of thalli were punched into discs with diameters of 2.5, 4, 6, 8, and 10 mm. Disc sizes were based on Hiraoka and Enomoto (1998) and Gao et al. (2010). To test the hypothesis that fragmentation can stimulate reproduction via releasing sporulation inhibitors into the media (Hiraoka and Enomoto, 1998), half of the discs were cultured in media without renewal while the other half was cultured in media that was renewed daily for seven days under culture conditions as described in section 2.2. The consumed nitrate and phosphate were replenished daily for those media without renewal to avoid any effect of nutrient limitation. The reproduction rates on days five, six, and seven were recorded. The numbers of discs with diameters of 2.5, 4, 6, 8, and 10 mm were 320, 125, 56, 31, and 20, respectively, to guarantee equal densities across treatments.

2.2.5 Combination of temperature shock, dehydration, and fragmentation

Discs (fragments: FR) with diameters of 2.5, 4, and 6 mm were punched from the upper thalli regions (FR) and subjected to either one hour of dehydration (FR+DE), six hours of temperature shock (FR+TS), or six hours of temperature shock plus one hour of dehydration (FR+TS+DE). The disc size, temperature shock, and dehydration times were selected due to their effects on reproductive induction in the experiments in sections 2.3, 2.4, and 2.6. The number of discs with diameters of 2.5, 4, and 6 mm

were 320, 125, and 56, respectively, to ensure equal densities across treatments. The reproduction rates on days two, three, and four were recorded.

2.2.6. Reproduction of blade and basal parts of Ulva *thalli with age*

The effects of intrinsic and extrinsic factors throughout development were investigated using 2.5 mm diameter discs excised from the blade and basal regions of thalli of differing ages (20, 30, 40, 50, and 60 days). We define basal regions as those within a 15 mm distance from basal end and blade regions as those within 105-150 mm distances from basal end (Hiraoka and Enomoto, 1998; Lüning et al., 2008). Twenty five discs were placed in each of three flasks per treatment. Every flask experienced six hours of temperature shock (4^{o}C) before being cultured for 14 days as described above. The reproduction rates on days seven, 10, and 14 were recorded. The period was selected as it was sufficient for reproduction of the blade parts based on the experience of normal laboratory culture. Different ages of *Ulva* thalli were obtained from the development of gametes. Discharged gametes were attracted to a point-source light using a fibre optic illuminator, collected with a pipette, and transferred to sterile seawater. Afterwards, 20 mL aliquots of gamete suspension with a concentration of 1.0×10^5 gametes mL^{-1} were placed in Petri dishes and kept in darkness for 24 hours to ensure even settlement. The culture conditions for settled gametes were the same as for the disc cultures.

2.2.7. Inhibitory effects of basal cells

Discs from blade (20 mm diameter discs) and basal (5 mm diameter discs) parts of the thalli were used to study the potential mechanisms that basal cells use to maintain vegetative status across the whole life history. Different sized discs were used to easily distinguish between tissue types during reproduction examination. Four ratios of blade to basal discs were set up in increments of 20 discs: (1: 0, i.e. 20 blade: 0 basal discs), blade: basal (1: 1, i.e. 20: 20), blade: basal (1: 2, i.e. 20: 40) and blade: basal (1: 3, i.e. 20: 60). These ratios were based on a preliminary experiment showing that a ratio of 1: 3 could completely inhibit reproduction in blade discs. The experiment was conducted for 15 days and in triplicate. The reproduction rates on days 13, 14, and 15 were recorded.

2.3. Effects of temperature and nitrate on maturation of U. rigida

The gametes used in this experiment were collected and settled as described in section 2.2.6. To investigate the effects of extrinsic factors on maturation, settled gametes were cultured at two fully crossed temperature (14, 18°C) and nitrate (6, 100 μmol L^{-1}) conditions, giving four treatments: 1) low temperature (14°C) and nitrate (6 μM N), 2) low temperature (14°C) and high nitrate (100 μM N), 3) high temperature (18°C) and low nitrate (6 μM N), and 4) high temperature (18°C) and nitrate (100 μM N). The phosphate concentration in the seawater was set at 5 μmol L^{-1} for all treatments. The lower temperature and nitrate levels reflected those at the sampling

site (Gao et al., 2017). The higher temperature is the projected value by the end of this century and the higher nitrate level is indicative of coastal eutrophication (Gao et al., 2017). Germlings were cultured as described above and manually detached when they reached 1 mm in length, and ten individuals from each treatment were randomly selected for further culture. Each individual was grown in a 500 mL conical flask. The culture conditions were the same as aforementioned. Germling length was measured every three or four days and the time taken to reach 1.5 cm was recorded (see supplementary Fig. S1).

2.4. Statistical analysis

Results were expressed as means of replicates ± standard deviation. Statistical analysis was carried out with SPSS v21. The data from every treatment conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and had equal variances (Levene's test, $P > 0.05$). A repeated measures ANOVA (RM-ANOVA) was conducted to assess the effect of culture time on reproduction in each experiment. One-way multivariate ANOVAs (MANOVAs) were used to analyse the effects of temperature shock, dehydration, age, or basal cells on reproduction of blade cells on different days. A two-way MANOVA was conducted to assess the effects of fragmentation and media on blade cell reproduction on different days. A three-way MANOVA was conducted to assess the effects of temperature shock, dehydration, and fragmentation on different days. A two-way ANOVA was conducted to assess the effects of temperature and

nitrate on maturation. Tukey's honest significant difference (Tukey HSD) tests were conducted for *post hoc* investigation. A confidence interval of 95% was set for all tests.

3. Results

3.1. Temperature shock

There was a significant interaction between culture time and temperature shock time (RM-ANOVA, $F_{(14, 32)} = 104.530$, $P < 0.001$). *Ulva* discs without temperature shock did not show any reproduction during five days of culture while those experiencing temperature shock became reproductive within five days (Fig. 2). There were statistically significant differences in reproduction rates between temperature shock times on days three, four, and five (MANOVA, $F_{(7, 16)} > 133.029$, $P < 0.001$; Fig. 2). On day three, a 20-minute temperature shock was insufficient to trigger reproduction, while a 30-minute shock caused a reproduction rate of 24.0 ± 4.0%, increasing to 36.0 ± 4.0% with a 60-minute shock (Tukey HSD, $P < 0.05$). Further increases to shock duration were ineffective until 360 minutes whereupon reproduction increased to 48.0 ± 4.0%. Reproduction rates on day four follow a similar trend as on day three—a slight increase for extended shock periods with the highest rate at the longest temperature shock time. By day five, a 10-minute shock triggered 36.0 ± 4.0%, increasing to 94.7 ± 2.3% after a 20-minute shock. Further shock period extensions had no significant impact. It is interesting to note that *Ulva*

discs did not sporulate if they experienced a low temperature of 4°C for one, two, or three weeks without returning to the high temperature of 18°C (Fig. 3).

3.2. Dehydration

There was a significant interaction between time of culture and dehydration (RM-ANOVA, $F_{(10, 24)} = 28.887$, $P < 0.001$). In general, reproduction rate increased over time and decreased with increasing dehydration times (Fig. 4). This trend was consistent over time, and dehydration significantly reduced reproduction compared to the control (ANOVA, $F_{(5, 12)} > 140.800$, $P < 0.001$). Notably, there was no reproduction in discs dehydrated for 180 minutes within 14 days of culture.

3.3. Fragmentation and media

There was a significant interaction between culture time and fragmentation (RM-ANOVA, $F_{(8, 40)} = 19.058$, $P < 0.001$) or media (RM-ANOVA, F = 7.777, df = 2, 40, $P = 0.001$), which suggests that the changes of reproduction rates with time were different between fragmentation or media treatments. On day five, fragmentation and media interacted significantly (MANOVA, $F_{(4, 20)} = 74.902$, $P < 0.001$; Fig. 5). The influence of the media treatments was reduced as disc size increased (Fig. 5). For instance, the reproduction of 2.5 mm discs was 75.5 ± 2.4% in renewed media and 39.1 ± 1.6% in non-renewed media, whereas for 4 mm discs it was 24.3 ± 2% and 18.1 ± 2% in renewed and non-renewed media, respectively. This trend was similar on day six and seven (Fig. 5). The mean reproduction rate of discs grown in renewed

298 media (56.2 ± 26.0%) was higher than that in non-renewed media (39.0 ± 22.1%) on
299 day seven and this trend was consistent from five days onwards. The reproduction rate
300 decreased with increasing disc size with a minimum of 5.0 ± 5.0% (non-renewed
301 media) and 20.0 ± 5.0% (renewed media) for 10 mm discs and a maximum of 64.7 ±
302 2.7% (non-renewed media) and 92.2 ± 5.6% (renewed media) for 2.5 mm discs on
303 day seven.

304 *3.4. Combination of temperature shock, dehydration, and fragmentation*

305 The combined effects of temperature shock, dehydration, and fragmentation on
306 reproduction, over four days of culture were investigated (Fig. 6). Culture time had an
307 interactive effect with fragmentation (RM-ANOVA, $F = 3.725$, df = 4, 48, $P = 0.010$)
308 and temperature shock (RM-ANOVA, $F = 403.774$, df = 2, 48, $P < 0.001$). The
309 maximum reproduction rate of small discs was reached after three days while medium
310 and large discs reached a maximum on day four (Fig. 6). Discs without temperature
311 shock did not show any reproduction during five days of culture while those with
312 temperature shock demonstrated an increased reproduction rate with time (Fig. 6). On
313 day two, temperature shock interacted with fragmentation (MANOVA, $F_{(2, 24)} =$
314 94.920, $P < 0.001$) and dehydration (MANOVA, $F_{(1, 24)} = 146.463$, $P < 0.001$) (Fig.
315 6A). The reproduction rates of three sizes of discs with temperature shock (FR+TS)
316 were 64.0 ± 3.9% (small discs), 48.0 ± 4.0% (medium discs), and 34.5 ± 2.1% (large
317 discs) respectively, with none reproducing without a temperature shock (FR).

Similarly, discs experiencing dehydration (FR+DE) did not reproduce, while 44.0 ± 3.9% (small discs), 24.0 ± 4.0% (medium discs), and 6.5 ± 6.3% (large discs), respectively, were reproductive when temperature shocked (FR+DE+TS). On day three (Fig. 6B), any combination of two of the three factors interacted to promote reproduction (MANOVA, $F > 7.958$, $P < 0.01$). Discs of all sizes did not reproduce without a temperature shock. Small discs demonstrated the biggest decline in reproduction rate when dehydration was added. The highest reproduction (97.3 ± 2.4%) was in the smallest discs in combination with temperature shock. The pattern on day four (Fig. 6C) was similar to day three. On all three days, fragmentation significantly affected reproduction (MANOVA, $F_{(2, 24)} > 65.976$, $P < 0.001$) as reproduction decreased with increasing disc size.

3.5. Reproduction of blade and basal U. rigida *tissues with age*

There was a significant interaction between culture time and disc age (RM-ANOVA, $F_{(8, 20)} = 10.162$, $P < 0.001$), suggesting the changes of reproduction of blade discs at different ages with culture time were not the same. For example, the reproduction rates of 20-day-old discs were 1.3 ± 2.3% on day seven, 1.3 ± 2.3% on day 10, 2.7 ± 2.3% on day 14, while they were 50.7 ± 4.6% on day 7, 84.0 ± 4.0% on day 10 and 92.0 ± 4.0% on day 14 for 40-day-old discs (Fig. 7). There were statistically significant differences in reproduction rates of blade discs of differing ages on days seven, 10, and 14 (ANOVA, $F_{(4, 10)} > 115.578$, $P < 0.001$; Fig. 7). On

day seven (Fig. 7A), 20-day-old discs showed little reproduction, with reproduction rate increasing with age (from 30 to 60 days), reaching 84.0 ± 4.0% in 60-day-old discs. On day 10 (Fig. 7B), the reproduction rate increased by more than five times when disc age changed from 20 to 40 days (Tukey HSD, $P < 0.05$). The difference in reproduction between 40- and 50-day-old or 50- and 60-day-old discs was not statistically significant. Sixty-day-old discs, however, had a higher reproduction rate (97.3 ± 2.3%) than 40-day-old discs. The pattern on day 14 (Fig. 7C) was the same as day 10. No reproduction was found in basal discs regardless of age or culture time (Fig. 7).

3.6. Inhibitory effects of basal cells

There was a significant interaction between the ratios of blade to basal discs and culture time (RM-ANOVA, $F_{(6, 16)} = 10.162$, $P = 0.001$), suggesting that the changes of reproduction with varying ratios of blade to basal discs with time were different. For example, reproduction rates of blade discs with 60 basal discs (1:3) were 0.0 ± 0. 0% (day 13), 3.3 ± 2.9% (day 14), and 8.3 ± 2.9% (day 15) respectively; while they were respectively 25.0 ± 5.0% (day 13), 53.3 ± 5.8% (day 14), and 70.0 ± 5.0% (day 15) for blade discs with 20 basal discs (1:1) (Fig. 8). There were significant differences in reproduction rates of blade discs with the addition of basal discs (MANOVA, $F_{(3, 8)} > 122.769$, $P < 0.001$; Fig. 8). On day 13, blade discs alone (1:0) had 60.0 ± 5.0% reproduction, which declined to 25.0 ± 5.0% when 20 basal discs

were added (1:1), and further to 8.3 ± 2.9% with 40 basal discs (1:2) (Tukey HSD, $P < 0.05$). Blade discs mixed with 60 basal discs (1:3) did not become reproductive. Similar pattern were detected on days 14 and 15 except that the treatment with 60 basal discs (1:3) did show some reproduction, albeit at very low levels (3.3 ± 2.9% on day 14 and 8.3 ± 2.9% on day 15).

3.7. Effects of temperature and nitrate on maturity of U. rigida

Temperature and nitrate had a significant interaction on maturity time of *U. rigida* (ANOVA, $F_{(1, 36)} = 33.085$, $P < 0.001$) and each of them had a main effect (ANOVA, $F_{(1, 36)} = 461.693$, $P < 0.001$ for temperature; ANOVA, F (1, 36) = 532.399, $P < 0.001$ for nitrate). Maturation took 62.1 ± 2.8 days when plants were grown under conditions of low temperature and low nitrate (Fig. 9). High temperature (18°C and 6 μM N) and high nitrate (14°C and 100 μM N) shortened the time to 40.3 ± 2.2 and 41.5 ± 2.5 days respectively. The high temperature and high nitrate condition (18°C and 100 μM N) promoted the shortest maturation period of 28.4 ± 2.0 days.

4. Discussion

The intrinsic and extrinsic factors regulating the reproductive biology of the problematic green tide-forming green algae, *Ulva rigida*, were investigated in this

study. The intention was to better inform the management of green tide events through a more in-depth understanding of reproductive cues − an important consideration given the predicted response of green tides to climate change (Gao et al. 2017) − and to support the development of hatchery systems for sustainable *Ulva* cultivation.

4.1. Effects of temperature shock

Temperature shock is an established method to induce gamete release, but the efficacy of the approach varies with species and ecotype. In the present study, a minimum of a 10-minute temperature shock was necessary to trigger reproduction (36 ± 4%) after culturing for five days (Fig. 2). Carl et al. (2014a), using the tropical *Ulva* sp. 3 (subsequently named as *U. tepida*; Masakiyo and Shimada, 2014), reported 10 minutes to be the saturation time for reproductive induction, with extended shock duration failing to enhance zooid formation. Our data, however, show that 20 minutes is required for saturation in this temperate *U. rigida* strain. In terms of release period, it took five days for discs experiencing 10-minute temperature shock to become reproductive (36.0 ± 4.0%) in the current study, yet Carl et al. (2014a) achieved a similar sporulaton rate after two days using the same temperature shock time. Furthermore, Niesenbaum (1988) reported that *U. lactuca* thalli became reproductive 18 hours after a 2°C wash.

Ulva from the tropics should theoretically be more sensitive to cold temperature stimulation. However, Carl et al. (2014a) switched between four and 25°C compared with four and 18°C in the present study; the 7°C difference equates to a more definitive temperature shock that may also have contributed towards the saturation time differences. Assuming that the degree of deviation from the environmental norm is a telling factor in the effectiveness of temperature shock, then by extrapolation, *Ulva* from more polar environments would be expected to experience even lower reproductive rates as a drop to 4°C would equate to the weakest temperature shock relative to its ecological norm. This, however, assumes equal sensitivity to temperature across geographically- and ecologically-distant strains. As far as the authors are aware, this latitudinal relationship between temperature shock and reproduction in *Ulva* has not been investigated.

The shorter time required for fertility of *Ulva* species experiencing temperature shock reported in previous studies (Niesenbaum, 1988; Carl et al., 2014a) could be down to the pre-fertile status of the plants, as gamete discharge was also found in the control group; temperature shock merely accelerated the release process. It is suggested that a minimun of two days is required for the transition from vegetative to reproductive status (Wichard and Oertel, 2010). The enhanced reproduction under temperature shock may be a survival strategy under unfavourable conditions (Li and Brawley, 2004).

Whether temperature shock stopped the excretion of sporulation inhibitors or triggered another reproductive pathway remains to be determined. The process appears to involve a two-step mechanism. The first step is from high temperature to low temperature and the second is returning to a high temperature. Neither is dispensable, as continuous low temperature induction for three weeks, without returning to high temperature, did not trigger reproduction. This finding is supported by previous studies wherein it was shown that *U. fenestrata* cannot form reproductive cells at 5°C (Kalita and Tytilanov, 2011) and gamete release in *U. mutabilis* only occurs within a narrow temperature range between 15 and 25^{o}C (Wichard and Oetel 2010).

4.2. Effects of dehydration

Dehydration did not stimulate reproduction of *U. rigida* despite the use of longer dehydration times (up to 180 minutes) than previously reported (maximum 60 minutes). On the contrary, reproduction decreased as dehydration time extended. This agrees with Carl et al. (2014a) but contrasts with Corradi et al. (2006) in which thalli subjected to a 10- or 20-minute dehydration released gametes within three days. Furthermore, Smith (1947) reported that *Ulva* blades dried for one hour liberated gametes five to 10 minutes after reimmersion. Those thalli should be already reproductive and dehydration merely stimulated gamete release as no transformation from vegetative to reproductive status could happen within such a short time

436 (Wichard and Oertel, 2010). Dehydration, unlike temperature shock, may not serve as
437 an effective environmental stimulus as *Ulva* growing intertidally will experience two
438 emersion periods per lunar day. Tidal emersion does not convey seasonal cues, unlike
439 temperature and photoperiod for instance.

440 *4.3. Effects of temperature and nitrate on maturation*

441 Extrinsic factors that are more stable and persistent than temperature shock can
442 also accelerate *Ulva* maturation and reproduction. Culturing at elevated temperature
443 and/ or nitrate conditions shortened the time to maturity in this study by over half.
444 Likewise, the reproductive rhythm of *U. fenestrata* decreased from 30 to five days
445 when temperature was increased from 10 to 20°C (Kalita and Titlyanov, 2011) and
446 higher temperature interacting with nitrate also induced more reproduction in *U.*
447 *rigida* (Gao et al., 2017). Moderate temperatures can accelerate growth and
448 reproduction by increasing enzyme activity, and increased nitrogen availability can
449 support an accelerated synthesis of nucleotides and proteins (Iken, 2012). For
450 example, up to 32% of genes in *Saccharina latissima* had altered expression profiles
451 in respose to changes of temperature and light, with the highest transcription rates at
452 the high temperature and light treatments (Heinrich et al., 2012). In terms of the time
453 to maturity, thalli of *U. mutabilis* became fertile at an age between 18 and 24 (± 2)
454 days (Stratmann et al., 1996), which was earlier than the *U. rigida* (28 ± 2 days) even

455 under optimal conditions in this study. This can mainly be ascribed to species
456 difference since the *U. mutabilis* used was a fast-growing mutant.

457 *4.4. Effects of fragmentation*

458 *Ulva* species grow along with *Porphyra yezoensis* by attaching to the net curtain
459 and rafts used for *Porphyra* aquaculture in China. The thalli fragments of *Ulva*
460 produced during cleaning of ropes, rafts and other attachments after *P. yezoensis* is
461 harvested are deemed to be the sources of green tides in China (Liu et al., 2009). In
462 addition, fragmentation of *Ulva* thalli commonly occurs in the sea due to the action of
463 grazers, waves, and propellers, and is suggested as one of the main factors hastening
464 the occurrence and spread of green tides by inducing vast sporulation events (Gao et
465 al., 2010). For *U. rigida*, smaller tissue discs resulted in greater reproduction which is
466 consistent with *U. pertusa* (Hiraoka and Enomoto, 1998) and *U. prolifera* (Gao et al.,
467 2010). These findings indicate that an increase in reproduction with decreasing
468 fragment size may commonly exist in *Ulva* species.

469 There are two hypotheses relating to fragmentation-induced reproduction. The
470 first is that wounding triggers the expression of genes coding for sex-inducing
471 pheromones, such as in the green alga *Volvox* (Amon et al., 1998). Smaller discs have
472 a higher ratio of wounded cells to total cells, which would translate into a stronger
473 reproductive stimulation. This hypothesis could now be tested in *U. linza* as a baseline
474 set of transcripts is available (Zhang et al., 2012), but currently the molecular tools are

475 unavailable for *U. rigida*. The second hypothesis is that *Ulva* blade cells produce two
476 reproduction suppressors; one (SI-1) is excreted into cell walls and the other (SI-2) is
477 located in the inner space between the two cell layers (Stratmann et al., 1996). Cutting
478 breaks the cell wall and extracelluar matrix structure making it easier for small discs
479 to leach inhibitors, thereby removing the regulatory block to reproduction (Hiraoka
480 and Enomoto, 1998). This hypothesis was supported by differences in reproduction
481 between *Ulva* grown in old and renewed media. More wounding sites could have
482 resulted in greater release of inhibitors into the media which were removed following
483 media exchange. Testing of this hypothesis would require a detailed metabolomics
484 study, such as advocated by Simpson et al. (2011), Goulitquer et al. (2012) and Gupta
485 et al. (2014). To our knowledge, such a focused metabolomics study has yet to be
486 been done on *Ulva* in relation to growth and reproduction inhibitors.

487 *4.5. Combined effects of extrinsic and intrinsic factors*

488 As mentioned above, dehydration did not stimulate reproduction of *U. rigida*
489 during 14-day culture, while temperature shock and fragmentation could induce
490 reproduction occurrence by days three and five, respectively. This suggests that
491 temperature shock has more power to induce reproduction in *U. rigida* compared to
492 dehydration and fragmentation. Meanwhile, a combination of fragmentation and
493 temperature shock shortened the time to zooid formation and discharge compared to
494 each factor singularly. This indicates that the regulation of *Ulva* reproduction is a

comprehensive and interactive process of intrinsic and extrinsic factors. Fragmentation would have freed the tissue from regulatory sporulation inhibition (Hiraoka and Enomoto, 1998), thus initiating the transition from vegetative to reproductive status, while temperature shock will have accelerated the process. This promoting effect was moderated somewhat by dehyration, providing futher evidence that dehydration is not a fertility stimulator (Carl et al., 2014a).

4.6. Reproduction of blade and basal cells with age

The reproduction rate of blade cells increased with age and indicates that blade cells of older *Ulva* are more sensitive to stimulatory triggers. This changing sensitivity might be due to differing regulatory factor excretion capabilities in blade cells with age. Stratmann et al. (1996) reported that the excretion of sporulation inhibitor SI-1 in blade cells decreased with maturation of *U. mutabilis*. That is why young *Ulva* seldom become reproductive whereas old *Ulva* are liable to release swarmers (Gao et al., 2017). When the amount of excreted sporulation inhibitor is not enough to control the induction of enviromental factors, reproduction occurs. The finding that reproduction is highly age-depedent has also been noted in *U. tepida* that became reproductive after five days of nursery and 13 days of outdoor cultivation (Carl et al., 2014b).

On the other hand, no reproduction occured in basal cells from either young or mature thalli that experienced temperature shock and fragment, which suggests the

basal cells can maintain vegetative status over the whole life span. This could be due to their lifetime excretion of sporulation inhibitors. The sporulation inhibitor produced by basal cells can be released into the medium (Stratmann et al., 1996), and was observed to proportionately inhibit reproduction in blade discs. Furthermore, the excretion of sporulation inhibitors in basal cells was not affected by environmental stresses, such as temperature shock and fragmentation. The robust excretion of sporulation inhibitors from basal cells − thus inhibiting the reproduction of blade cells − may potentially explain the phenomenon that swarmer release of *Ulva* species usually happens during spring tides (Smith, 1947; Lüning et al., 2008). Consider *Ulva* species living in closed ditches or tide pools, or within the upper intertidal zone; the reproduction of blade cells is inhibited by basal cell sporulation inhibitors . Seawater can reach the highest level of the intertidal zone and may dilute the sporulation inhibitors during spring tides. Consequently, swarmers are induced and released without the direct control of sporulation inhibitors (Smith, 1947; Stratmann et al., 1996; Lüning et al., 2008). It is worth noting that the dilution effect should not be the only link between swarmer release and spring tides. The moonlight could also contribute to the periodic reproduction of *Ulva* species in the field (Lüning et al., 2008). The synchronous swarming would result in a considerable enhancement of mating success and support the optimal distribution of swarmers and offspring within the habitat (Stratmann et al., 1996).

The different inhibitor(s) excretion patterns in basal (consistant excretion) and blade cells (decreasing excretion) with age may have led to the functional differentiation between the cell types. This differentiation could benefit *Ulva*'s response to environment signals. Blade cells are more sensitive to environmental change. High productivity and quick growth of the blade allows *Ulva* to rapidly invade primary substrata as an opportunistic species when conditions are favourble. Meanwhile, under unfavourable conditions the blade can quickly transform from vegetative to reproductive states, discharge swarmers and finally die off, while basal tissue persists from which new thalli arise during each subsequent growing season.

5. Conclusions

The interactions of extrinsic and intrinsic factors regulating the reproduction of differently aged *U. rigida* thalli were investigated for the first time. Blade tissues of mature thalli responded strongly to both temperature shock and fragmentation whereas dehydration countered maturation and gamete release. The combination of temperature shock and fragmentation was the most powerful tool to induce reproduction in blade tissues. The response of blade tissues increased with age whereas basal tissues remained non-reproductive. Basal tissues suppressed the reproductive response of blade tissues when they were co-cultured, indicating that intrinsic signalling factors, such as sporulation inhibitors, dominate reproductive regulation. The differentiation of *Ulva* cells with time leads to contrasting

reproductive performance in both regions of the plant, which not only supports the rapid growth of *Ulva* when environmental conditions are favourable but also aids survival during unfavourable conditions. These findings provide important information that furthers our understanding of the ecological success of *Ulva*, particularly in green tide situations. This will become increasingly important as climate change and human land use practices continue to create conditions favourable for green tide formation. In addition, the findings in the present study also make a useful contribution to the development of hatchery systems for sustainable *Ulva* cultivation.

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Figure legends

Fig. 1. Light micrographs of *Ulva rigida* in the process of reproduction. (A) vegetative cells, (B) reproductive cells before discharging gametes (red arrow), (C) reproductive cells discharging gametes (blue arrow), and (D) discharged swarmers. The scale bars represent 10 μm.

Fig. 2. The reproduction rates of *Ulva rigida* treated with increasing temperature shock periods (in minutes). The reproduction rates were assessed three, four, and five days post temperature shock treatment. Data are means ± SD (n = 3) with each replicate including 25 discs.

Fig. 3. Effects of two-step temperature shock on the reproduction rate of *Ulva rigida*. The results were obtained on day five for the group of two hours. Zero represents discs kept at 18 °C for two weeks without experiencing a temperature shock, and two hours represents discs experiencing two hours of a 4 °C treatment before returning to 18 °C. One week, two weeks and three weeks indicate discs transferred from 18 °C to 4 °C and kept at 4 °C for one week, two weeks, and three weeks respectively. Data are means ± SD (n = 3) with each replicate including 25 discs.

Fig. 4. The reproduction rates of *Ulva rigida* treated by increasing dehydration periods (in minutes). The reproduction rates were assessed 10, 12, and 14 days post dehydration treatment. Data are means ± SD (n = 3) with each replicate including 25 discs.

Fig. 5. Effects of tissue fragment size and media condition on the reproduction rates of *Ulva rigida* on days five, six, and seven. Closed squares represent non-renewed media treatment and open squares represent renewed media treatment. Data are means ± SD (n = 3). Each replicate in the 2.5 mm diameter treatment includes 320 discs, each replicate in the 4 mm diameter treatment includes 125 discs, each replicate in the 6 mm diameter treatment includes 56 discs, each replicate in the 8 mm diameter treatment includes 31 discs, and each replicate in the 10 mm diameter treatment includes 20 discs.

Fig. 6. Combined effects of temperature shock, dehydration and fragmention on reproduction of *Ulva rigida* on days two (A), three (B), and four (C). Small, Medium, and Large are discs with diameters of 2.5, 4, and 6 mm respectively. FR, FR+DE, FR+TS, and FR+TS+DE represent discs without temperature shock or dehydration treatments, discs with a one hour dehydration treatment, discs with a six hour temperature shock treatment, and discs with a six hour temperature shock and a one hour dehydration treatment, respectively. Data are means ± SD (n = 3).

Fig. 7. Reproduction rates of blade and basal tissues of different ages observed seven (A), 10 (B), and 14 (C) days after fragmentation and temperature shock treatments. Data are the means ± SD (n = 3).

Fig. 8. Inhibitory effects of basal tissues on the reproduction rates of blade tissues on days 13, 14, and 15. Data are means ± SD (n = 3).

719 **Fig. 9.** Effects of temperature and nitrate on maturation of *Ulva rigida*. Data are

720 means ± SD (n = 10).

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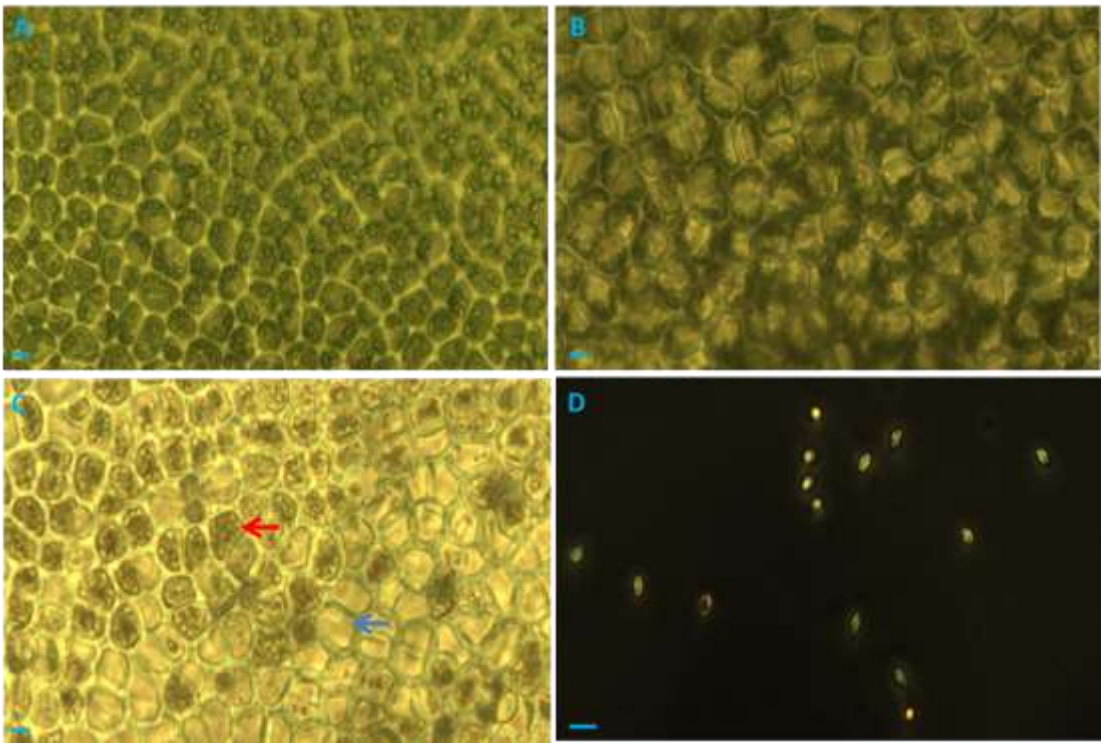

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724 **Fig. 1.**

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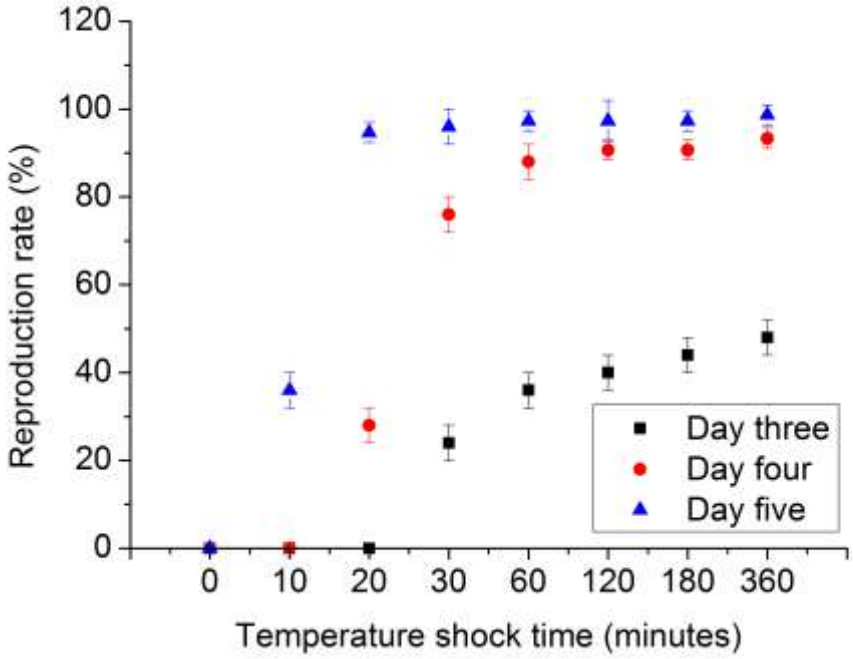

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728 **Fig. 2.**

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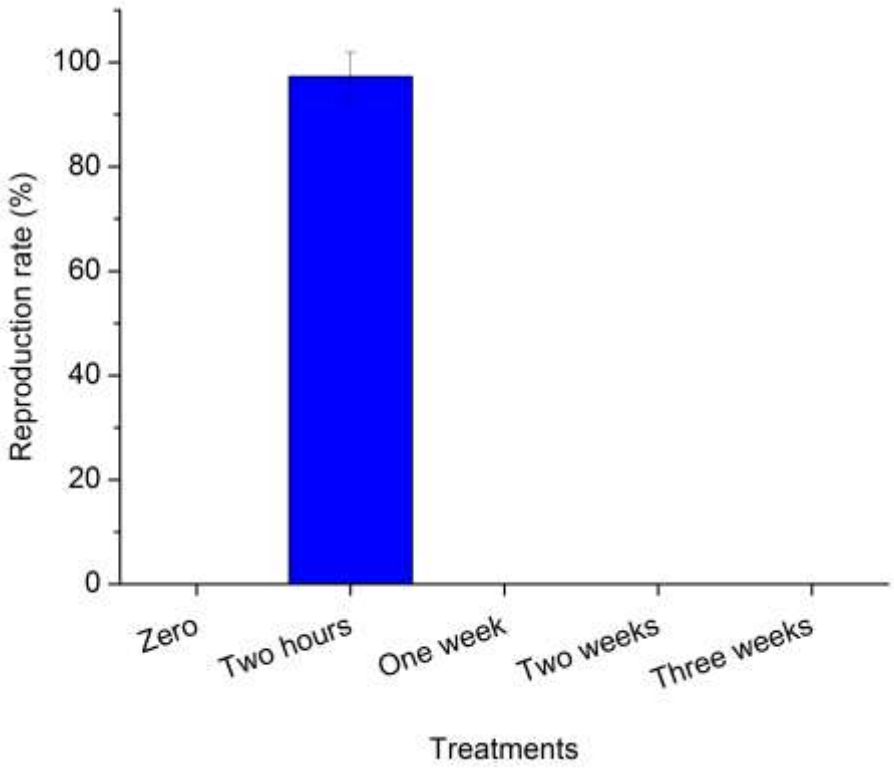

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732 **Fig. 3.**

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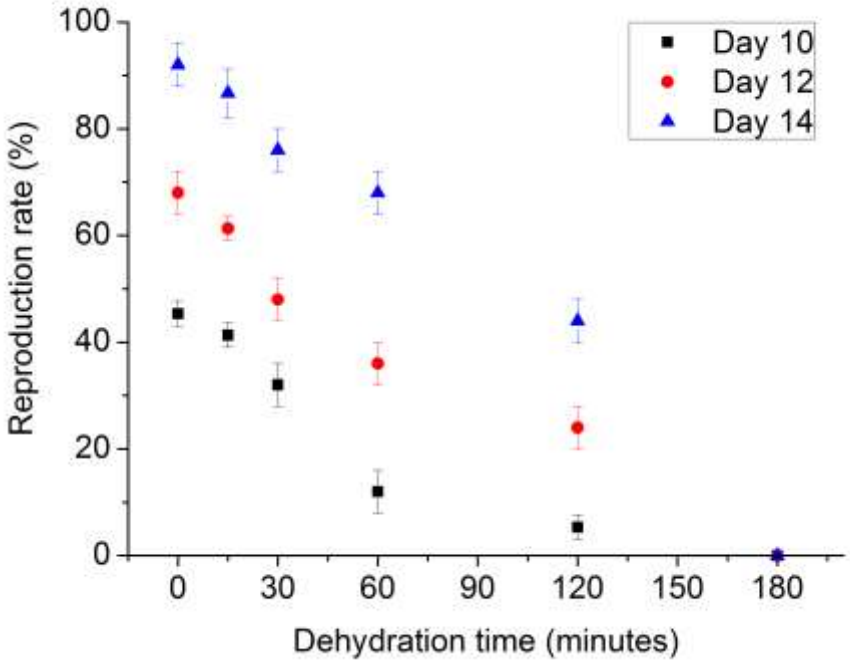

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737 **Fig. 4.**

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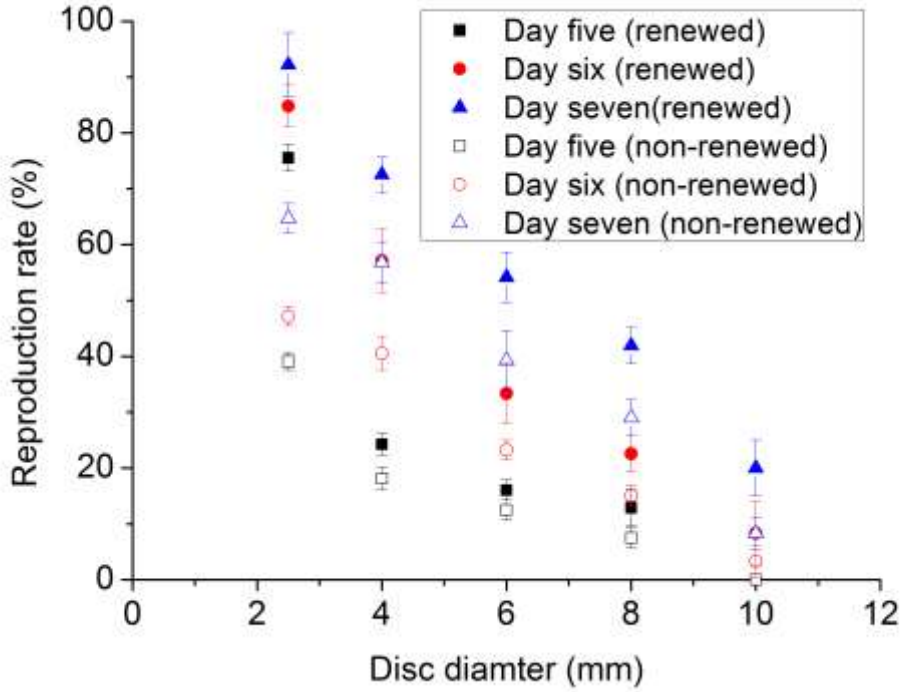

740

741 **Fig. 5.**

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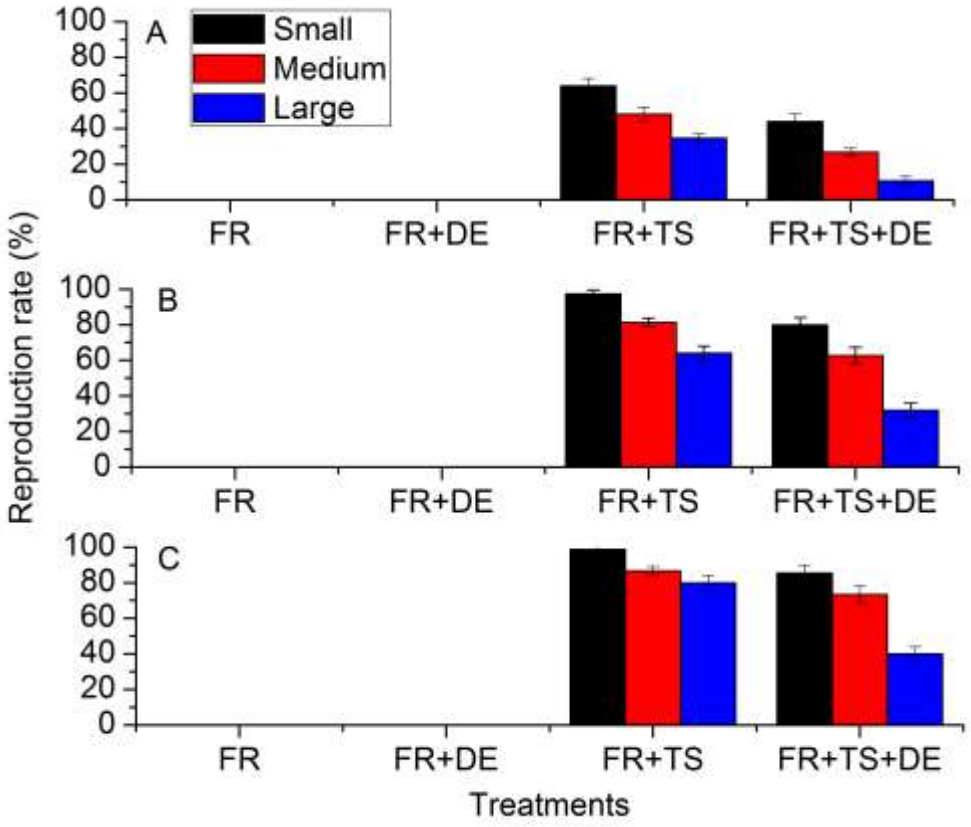

743

744 **Fig. 6.**

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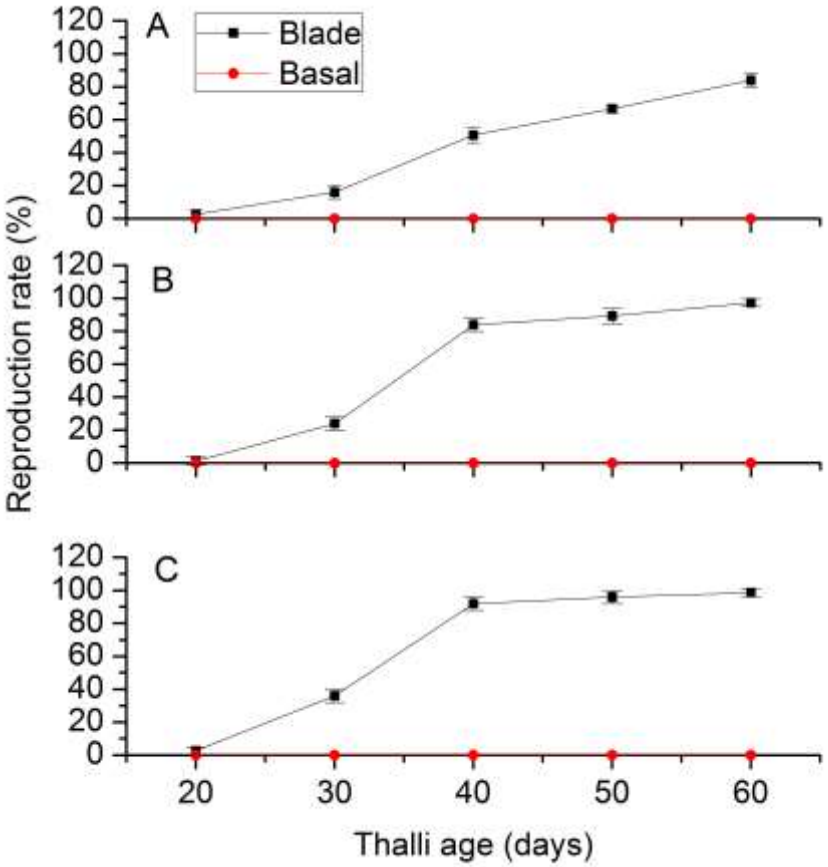

748

749 **Fig. 7.**

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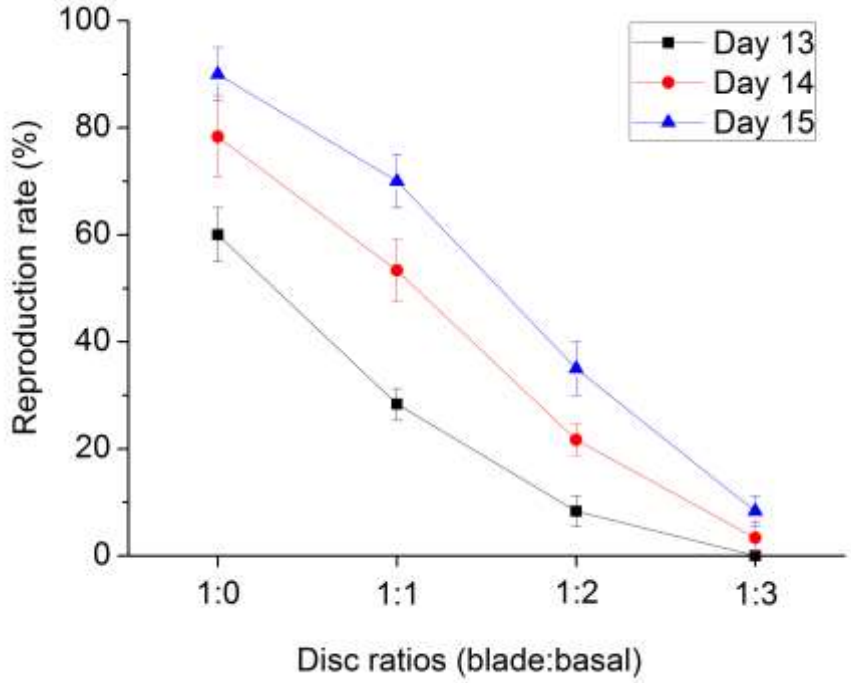
Day 13
Day 14
Day 15
Reproduction rate (%)
100
80
60
40
20
0
1:0
1:1
1:2
1:3
Disc ratios (blade:basal)

Fig. 8.

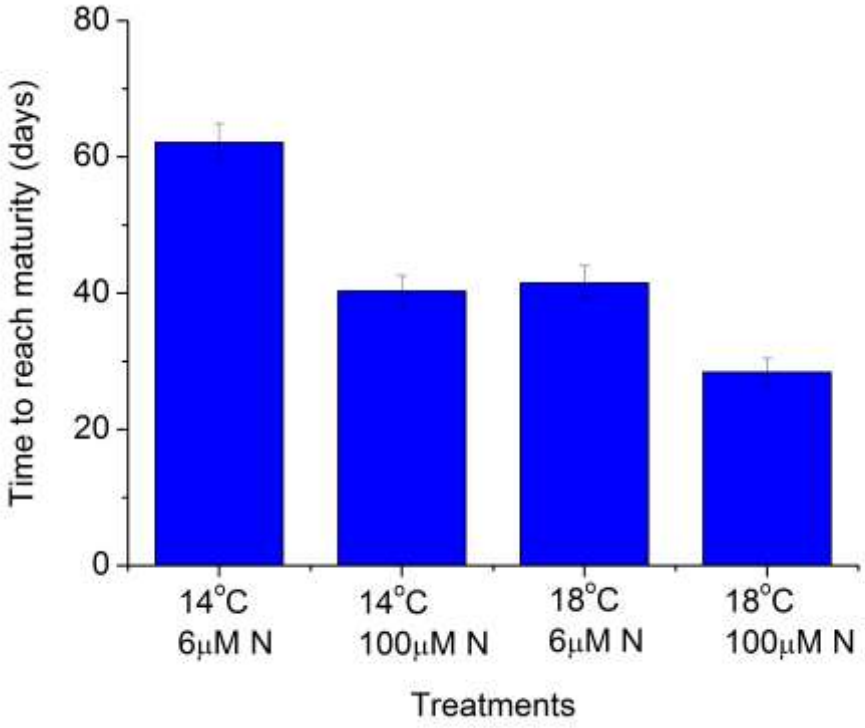
Time to reach maturity (days)
80
60
40
20
0
14°C
6μM N
14°C
100μM N
18°C
6μM N
18°C
100μM N
Treatments

Fig. 9.