

Fluorescence and photosynthetic competency in single eggs and embryos of *Ascophyllum nodosum* (Phaeophyceae)

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Pulse amplitude modulation (PAM) fluorometry was used in 2004 and 2005 to measure chlorophyll *a* fluorescence associated with photosystem II in eggs and embryos of *Ascophyllum nodosum* (Linnaeus) Le Jolis from the Atlantic coast of Nova Scotia. Fucoid eggs and embryos were maintained in the laboratory for 8 days over which fluorescence measurements were taken. Newly released eggs saturated at about 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with maximum electron transport rate (rETR_{max}) declining to <3 after 3 days. Embryos saturated at about 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and rETR_{max} increased to >11 by day 3. Although some egg senescence was apparent by day 3, some eggs continued to generate a strong photosynthetic signal throughout the 8-day experimental period, even though rETR_{max} was reduced. Differences in photosynthetic responses associated with fertilisation were apparent in F_v/F_m , within 24 h, and in rETR_{max} after 2 days. Eggs showed relatively stable or declining responses, whereas embryos showed stable or increasing responses. By day 8, quantum yield of embryos was similar to values reported elsewhere for mature fucoids.

KEY WORDS: *Ascophyllum*, chlorophyll *a* fluorescence, development, eggs, embryos, Fucaceae, microscopy-PAM, quantum yield

INTRODUCTION

Pulse amplitude modulation (PAM) fluorometry uses chlorophyll *a* fluorescence to characterise the photosynthetic process. Chlorophyll *a* fluorescence analysis has become one of the most powerful approaches for measuring photosynthetic activity of algae *in vivo* (Schreiber *et al.* 1994). It is particularly useful for measuring the impact of environmental stress on the physiological state of photosynthesis. Due to recent technical progress in optoelectronics and photonics, instruments with exceptional sensitivity and selectivity have been developed that can be applied in practically all situations where photosynthesis takes place *in vivo*. Microscopy-PAM (Walz GmbH) allows characterisation of photosynthetic parameters on very small spatial scales or in single cells (Schreiber 1998). This is especially useful where small specimens are available or where processes may differ in adjacent cells or tissues. Previous applications of the microscopy-PAM fluorometry in algal research include examination of expelled zooxanthellae in the corals *Cyphastrea serailia* (Forskål) and *Pocillopora damicornis* (Linnaeus) (Ralph *et al.* 2001) and diurnal changes of the diatom *Ethmodiscus* (Villareal 2004).

PAM fluorometry has been used previously for studies of fucoid photosynthesis. Early applications by Raven & Samuelsson (1988) and Huppertz *et al.* (1990) examined photoinhibition in *Fucus* species. More recent studies examined photosynthetic physiology of embryos of *Fucus* species (Major & Davison 1998; Lamote *et al.* 2003) in bulk culture. Here we use microscopy-PAM to examine persistence of photosynthetic fluorescence parameters in unfertilised eggs and their

development in embryos of *Ascophyllum nodosum* (Linnaeus) Le Jolis.

Early development of zygotes and embryos in *A. nodosum* has been followed with studies focusing on morphology, eco-physiology, hybridisation and symbiosis (e.g. Moss 1975; Shearer & Moss 1975; Garbary & MacDonald 1995; Kim *et al.* 1997). Fucoid embryos in general have become a model system for studies of a wide range of cellular phenomena (e.g. Bourget *et al.* 2001; Lamote *et al.* 2003). Photosynthesis in eggs and embryos was examined in *Fucus serratus* Linnaeus (McLachlan & Bidwell 1978) with radioactive carbon tracers, and this provided some insight into photosynthetic competency and carbon metabolism. Lamote *et al.* (2003) recently examined the assembly of the photosynthetic apparatus in *F. serratus* in early embryogenesis. The objective in this paper is to examine developmental changes in unfertilised eggs, and to compare these with embryos of *A. nodosum* to evaluate stage-specific photosynthetic competency.

MATERIAL AND METHODS

Isolation and cultivation

Ten to 20 fertile fronds of *A. nodosum* were collected in the mid-intertidal zone from Drumhead, Nova Scotia (45.14°N, 61.59°W) in early June 2004, and from Tor Bay, Nova Scotia (45.19°N, 61.34°W) in early June 2005. Both sites are fully marine and vary from fully wave exposed to slightly wave protected. Thalli were sexed based on microscopic examination, and excised receptacles with male or female conceptacles were placed on moistened tissue paper in separate plastic Petri dishes and refrigerated overnight at 4°C. Receptacles onto

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which eggs or sperm had exuded were placed in seawater in different dishes. Gentle swirling of the dishes separated the gametes from the conceptacle surface. The receptacles were removed, and eggs were rinsed in several changes of seawater and divided into two groups. To one group of eggs, 1 ml of the sperm/seawater was added to induce fertilisation. Eggs and embryos were dispersed with Pasteur pipettes into five 90- by 20-mm plastic Petri dishes. Eggs and zygotes were maintained at 15°C under continuous illumination provided by Philips Econ-o-watt 25-Watt bulbs at an irradiance of 35–50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (2 π collector, Li-Cor, Lincoln, NB, USA). Ultraviolet-treated seawater was replenished every 48 h.

Eggs and embryos were removed from their dishes with a pipette and placed on a well slide in seawater at room temperature. At each time interval (0, 0.5, 1, 2, 5, 8 days after fertilisation) a minimum of five eggs and five zygotes were individually examined for photosynthetic parameters. Experimental protocol differed slightly in 2004 and 2005. In 2005, at each time interval, five eggs or embryos were assayed from each of five dishes, and a single mean value was calculated for each dish. To avoid pseudoreplication, statistical significance was calculated using $n = 5$. In 2004, all of the eggs and embryos were left in single large dishes, and material was removed for assay at the appropriate times. Temperature was not controlled on the microscope stage. Initial experiments showed that photosynthetic parameters of eggs and zygotes were not affected by heat from the microscope or by the 1 h that cultures were at room temperature (*c.* 22°C) at each sampling time. Eggs or zygotes associated with diatoms were not used for fluorescence analysis.

Fluorescence measurement

Fluorescence measurements on single eggs and embryos of *A. nodosum* were performed using a microscopy-PAM facility (Walz GmbH, Effeltrich, Germany). This consists of a modified epifluorescence microscope, the PAM control unit and a notebook computer with dedicated Windows-software (WinControl) for system operation and fluorescence analysis. Using visual inspection and an iris diaphragm, the active field is narrowed so that the fluorescence characteristics of a small area (*i.e.* cell or tissue) can be assessed. The microscopy-PAM apparatus is equipped with a pin-hole micro quantum sensor (MC-MQS) to assess quantum flux density of the blue excitation light in the object plane (Schreiber 1998).

Microscopy-PAM was used to assess the maximum quantum yield of charge separation in PSII, F_v/F_m , effective quantum yield of charge separation in PSII, $\Phi_{\text{PSII}} = (F_m' - F)/F_m'$, and non-photochemical quenching (NPQ), $(F_m - F_m')/F_m'$ (Genty *et al.* 1989). Constant settings of the PAM fluorometer were used for the duration of the experiment. Accordingly, the measuring light frequency (MF) was set at 8; the saturating light intensity (SI) was set at 12; the saturating light width (SW) was set at 0.8 and the photomultiplier gain (PG) was set at 11. First, minimum fluorescence, F_o , was induced by low irradiation pulses in 10–15-min dark-adapted samples. Following a single saturating flash, maximal fluorescence, F_m , was detected. Variable fluorescence, F_v , was calculated as the difference between F_o and F_m . When the sample is preirradiated, F_m usually decreases, and is then called F_m' , while F_o , then referred to as F , either increased or decreased.

Since no widely accepted absorption coefficients have yet been developed for fucoids, the relative electron transport rate (rETR) between PSII and PSI is calculated as $\text{rETR} = \Phi_{\text{PSII}} \times \text{PFD} \times 0.5$, where PFD is photosynthetic photon flux density of photosynthetically active radiation (PAR 400–700 nm). This equation assumes that PSII absorbs half (0.5) the quanta of available light (Schreiber *et al.* 1994). Rapid light curves (RLC) were obtained from nine measurements after 10-s sample illumination with increasing levels of actinic light from 0 to 130 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The RLCs showed no photo-inhibition at an irradiance up to 130 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Consequently, RLCs of photosynthesis (P) can be expressed as rETR where the P-E (photosynthesis-irradiance) curves were fitted using the following function from Webb *et al.* (1974):

$$P = P_m[1 - \exp(-\alpha E/P_m)],$$

where P_m is the maximal rate in the absence of photoinhibition and α is the initial slope of the RLC. The non-photochemical quenching [$\text{NPQ} = (F_m - F_m')/F_m'$] was determined as the highest NPQ value on the RLC.

Data analysis

Statistical analysis was carried using one or two-way ANOVAs to determine if significant differences occurred among times for F_o , Φ_{PSII} , rETR_{max} and α^{ETR} , and between eggs and embryos. All data were tested for normality and homogeneity of variance using the Kolmogorov–Smirnov and Levene tests, respectively. Where a significant difference occurred, a Duncan *post hoc* comparison was used to determine which times were different. All statistics were calculated using SPSS 12.0KO for Windows (SPSS Inc., Chicago, IL). Values are all expressed as mean and standard error (*i.e.* mean $\pm s_{\bar{x}}$).

RESULTS

Results varied somewhat from 2004 and 2005 (Table 1) with eggs from 2005 providing higher values for photosynthetic fluorescence parameters. We attribute this to differences in timing of gamete maturation and release. In 2004, gamete release was well advanced at Drumhead when the collections were made, and this likely contributed to the more rapid decline of the eggs in 2004. In 2005, a delayed spring with cooler air temperatures likely slowed maturation and resulted in eggs with greater viability. Despite this variation, the general pattern from both years was that photosynthetic fluorescence parameters declined with time in eggs and were constant or increased with time in embryos. Consequently, we present the detailed results for 2005 (Figs 1–6) and a comparison of the 2004 and 2005 results for day 2 and day 5 for primary parameters (Table 1). The experiments described here were carried out over 8 days. By the eighth day few unfertilised eggs remained intact in 2004 whereas in 2005 most eggs had significant, if reduced, photosynthetic capacity. In addition, after 8 days the non-rhizoidal portions of embryos had undergone many rounds of cell division and were expanding beyond the initial size of the fertilised egg.

An hour after fertilisation there were no apparent differences in photosynthetic physiology. Minimum fluorescence

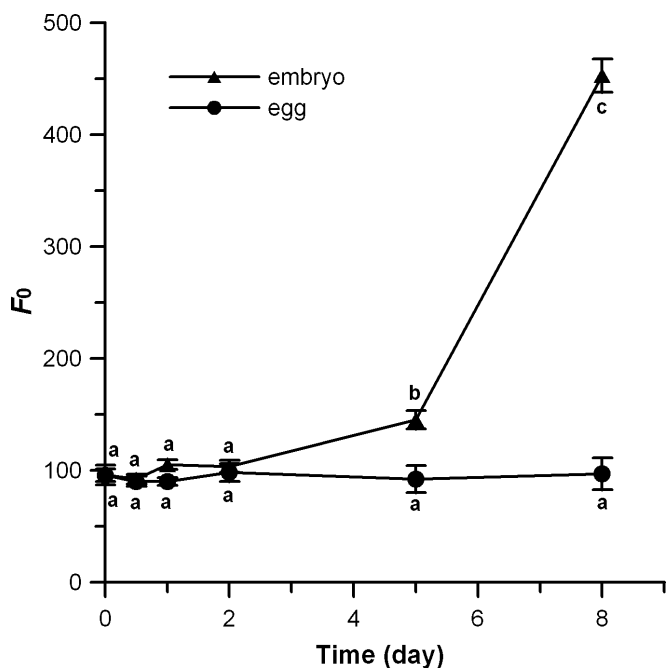
Table 1. Comparisons of photosynthetic parameters, given as a mean ($\pm s_x$) ($n = 5$). ANOVAs were performed to test for differences in the means of the 2 consecutive years.

	Day 2			Day 5		
	2004	2005	<i>P</i>	2004	2005	<i>P</i>
Eggs						
F_v/F_m	0.57 (0.02)	0.59 (0.01)	0.52	0.42 (0.03)	0.57 (0.03)	0.01
rETR _{max}	2.56 (0.24)	4.79 (0.52)	0.01	1.98 (0.26)	3.39 (0.54)	0.05
α^{ETR}	0.16 (0.01)	0.17 (0.02)	0.96	0.12 (0.22)	0.17 (0.01)	0.06
NPQ	9.02 (1.31)	10.51 (1.85)	0.53	2.62 (0.54)	4.58 (0.66)	0.06
Embryos						
F_v/F_m	0.61 (0.02)	0.67 (0.01)	0.02	0.66 (0.01)	0.67 (0.01)	0.48
rETR _{max}	3.70 (0.46)	6.79 (0.26)	0.00	8.42 (0.78)	7.57 (1.28)	0.59
α^{ETR}	0.24 (0.02)	0.28 (0.01)	0.10	0.23 (0.02)	0.26 (0.01)	0.16
NPQ	11.92 (2.18)	10.44 (0.85)	0.63	3.64 (0.83)	3.14 (0.51)	0.63

(F_o) (Fig. 1) provides a measure of relative photosynthetic biomass (i.e. chlorophyll content). In 2005, F_o remained stable over 8 days in the eggs. In the embryos, F_o increased slightly at day 5, and then dramatically by day 8. By contrast, in 2004, F_o remained stable in the eggs for only 2 days and then declined (data not shown). Thus, significant differences in F_o were apparent between eggs and embryos in 2004 within 12 h of fertilisation; this required 5 days in 2005.

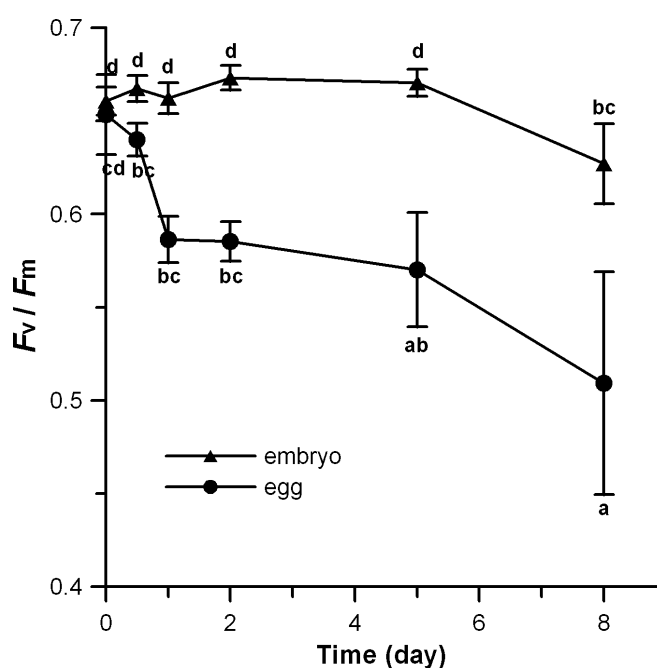
Maximum quantum yield (F_v/F_m) (Fig. 2) was initially about 0.66 for both eggs and embryos; however, by 12 h they were significantly different, and the values for eggs had declined. In 2004, F_v/F_m in embryos increased, whereas in 2005, it remained stable (Table 1). In both years, eggs showed reasonable values (>0.57) for at least 2 days; however, by day 5, F_v/F_m declined to 0.40–0.45 in 2004 while remaining at 0.57 in 2005 (Table 1).

Eggs and embryos had different optimum light intensity

**Fig. 1.** Changes in minimum fluorescence (F_o) in developing eggs (●) and embryos (▲) of *A. nodosum* (mean $\pm s_x$, $n = 5$). Bars with the differing letters are significantly different as determined by Duncan *post hoc* comparison.

(also called the light saturation parameter, $I_k = \text{rETR}_{\text{max}}/\alpha^{\text{ETR}}$) that changed over time (Figs 3, 4). Newly released eggs saturated an irradiance of about $30 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ with rETR_{max} of over 6. At day 8, light saturation of eggs was at $16 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, and rETR_{max} had declined to <3 . By day 8, saturation of embryos was almost $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, and rETR_{max} was always above 11. Results from both years were similar except that differences in embryos were more apparent earlier in development in 2004.

Maximum electron transport rate (rETR_{max}) ran in parallel for eggs and embryos for 2 day, and then rapidly diverged (Fig. 5). This function increased by over 80% in embryos and declined by over 50% in eggs. Differences in rETR_{max} between eggs and embryos were much more apparent in 2004 than in 2005 (Table 1), although the pattern of declining values in eggs and increasing values for embryos were consistent in both years.

**Fig. 2.** Changes in maximum quantum yield (F_v/F_m) in developing eggs (●) and embryos (▲) of *A. nodosum* (mean $\pm s_x$, $n = 5$). Bars with the differing letters are significantly different as determined by Duncan *post hoc* comparison.

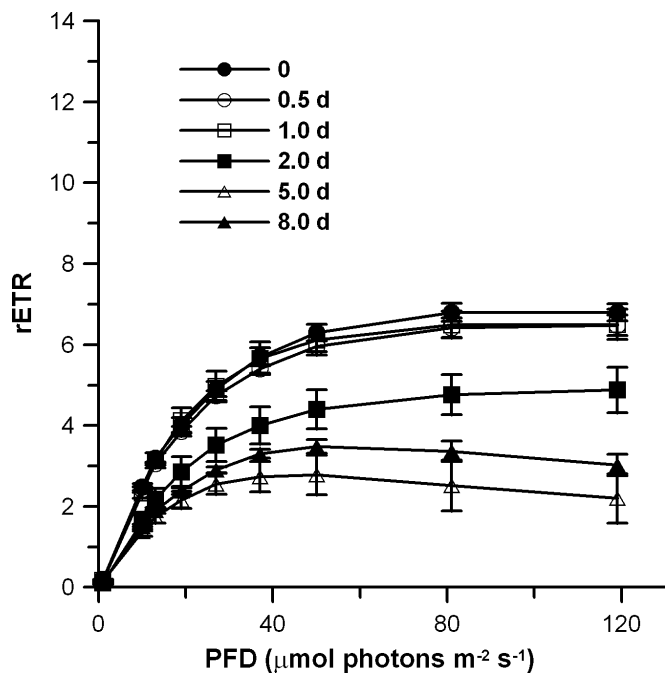


Fig. 3. Relative electron transport rate (rETR) in relation to photon flux density (PFD) in senescing eggs of *A. nodosum* ($n = 5$, figures indicate mean $\pm s_x$).

Photosynthetic efficiency (α^{ETR}) fluctuated in the first day in both eggs and embryos. Embryos subsequently stabilised between 0.25 and 0.30 whereas eggs dropped dramatically at day 2 and retained a constant level between 0.15 and 0.18 (Fig. 6). The differences between eggs and embryos after day 2 are highly significant.

Non-photochemical quenching (NPQ) (Table 1) was highly

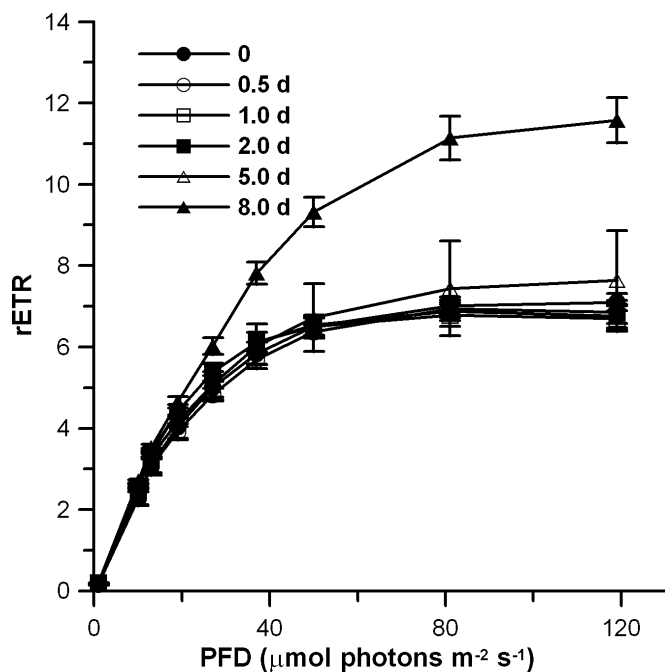


Fig. 4. Relative electron transport rate (rETR) in relation to photon flux density (PFD) in embryos of *A. nodosum* during early development ($n = 5$, figures indicate mean $\pm s_x$).

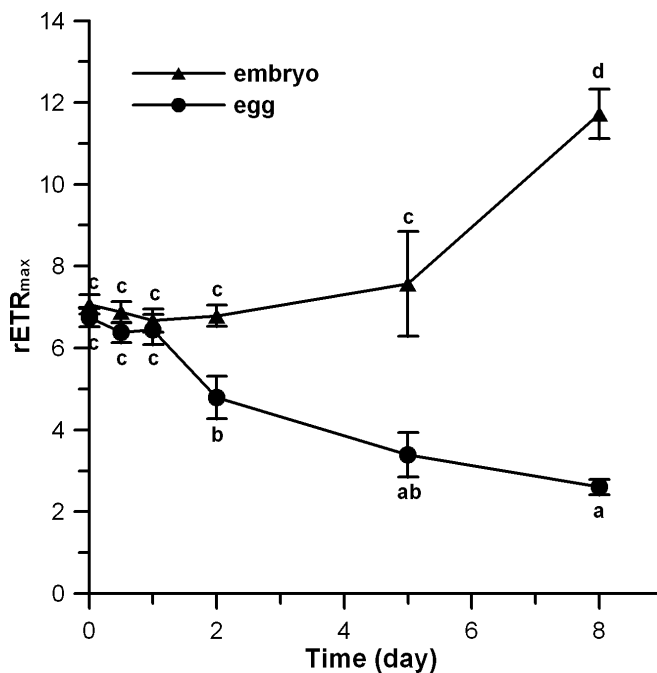


Fig. 5. Changes in maximum electron transport rate ($rETR_{max}$) in eggs (●) and embryos (▲) of *A. nodosum* (mean $\pm s_x$, $n = 5$). Bars with the differing letters are significantly different as determined by Duncan *post hoc* comparison.

variable in both eggs and zygotes; however, consistent patterns of change occurred. In both 2004 and 2005, NPQ was initially (day 2) high in both eggs and embryos, and then declined (day 5). These declines were 30–60% of day 2 values. The healthier eggs in 2005 retained high levels of NPQ relative to the 2004 eggs, which had much lower $rETR_{max}$.

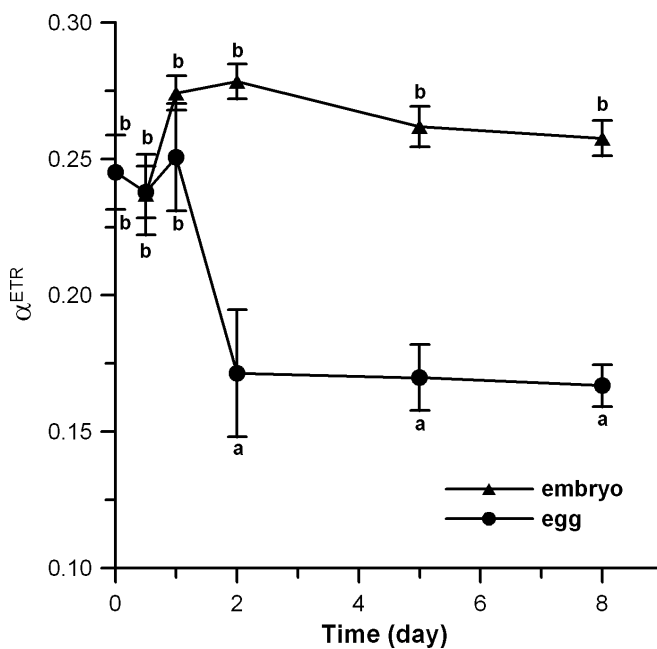


Fig. 6. Changes in photosynthetic efficiency (α^{ETR}) in eggs (●) and embryos (▲) of *A. nodosum* (mean $\pm s_x$, $n = 5$). Bars with the differing letters are significantly different as determined by Duncan *post hoc* comparison.

DISCUSSION

The two most important observations from this work are that: (1) the photosynthetic machinery of unfertilised eggs can be maintained for up to at least a week; and (2) unfertilised eggs and embryos have different photosynthetic characteristics that become apparent soon after fertilisation. Based on ^{14}C studies, McLachlan & Bidwell (1978) previously pointed out that the eggs, sperm and zygotes of *F. serratus* were photosynthetically competent. They further determined that carbon fixation of eggs declined between initial release and 48 h. Over 3 days, carbon fixation of the embryos tripled. Although *F. serratus* and *A. nodosum* in Nova Scotia grow at different tidal heights (*F. serratus* is typically lower on the shore or in pools), they belong to closely related genera, and similar physiology might be expected. Our results are consistent with those of McLachlan & Bidwell (1978).

Although electron transport rate (ETR) is recognised as an imperfect proxy for photosynthesis (Barranguet & Kromkamp 2000), fluorescence rapid light curves (RLCs) have been readily adopted as proxies for traditional P versus E curves to characterise the photoacclimation status of diverse marine algae (e.g. Morris & Kromkamp 2003). Thus despite limitations in interpretation, we can compare our RLCs with previous results of Stengel & Dring (1998), who used oxygen evolution as their measure of photosynthesis in *A. nodosum*, and provided P-E curves for different aged tissues of mature thalli. Older thallus parts were saturated at lower irradiances (150–250 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) compared to younger, actively-growing thallus parts (saturation at 400–500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). This contrasts with low light-adapted eggs and embryos in the laboratory in which the light-saturation of photosynthesis occurred between 30 and 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ thalli.

Photoinhibition, however, does not explain the extremely low recruitment of *A. nodosum* based on zygote settlement and growth (see Chapman 1995 for review). Some eggs survived for 1 week, and some surviving eggs had a quantum yield as high as mature fucoids (Raven & Samuelsson 1988). In theory, these long-lived eggs should enhance recruitment. It remains to be determined, however, if these eggs can still be fertilised and then develop. Garbary & MacDonald (1995) showed that embryos of *A. nodosum* had more rapid growth when infected with the symbiont, *Mycophycias ascophylli* (Cotton) Kohlmeyer & Volkmann-Kohlmeyer. It would be interesting to know the impact of this symbiosis on photosynthetic fluorescence parameters.

Maximum quantum yield (F_v/F_m) reflects the maximum efficiency of PSII photochemistry (Anderson *et al.* 1997). When values decline, this can reflect the development of mechanisms either for photoprotection or photodamage. Since the light levels used in the culture were at least one third of what would be required for photoinhibition, neither of these mechanisms apply. The decline in F_o and rETR in eggs suggests a major decline in chlorophyll content associated with senescence. When values are reduced, it suggests a greater proportion of inactive PSII reaction centres based on degradation of D1 proteins (Anderson *et al.* 1997). This is apparent in our data in the collapsed values in eggs for F_v/F_m .

In other experiments (Kim & Garbary, unpublished data using imaging-PAM), we examined maximum quantum yield

(F_v/F_m) in mature fronds of *A. nodosum*. These fronds were variable based on age of tissue and tissue type. Regardless of this variation, we obtained means of 0.72 to 0.66 in 1- to 4-year-old branch segments, respectively. The maximum quantum yield in our 9-day-old embryos was about 0.65, suggesting the photosynthetic machinery was close to fully functional relative to mature fronds. These values for (F_v/F_m) are comparable to those obtained by Raven & Samuelsson (1988) and Huperz *et al.* (1990) for *Fucus* species.

Lamote *et al.* (2003) showed changes in relative pigment composition in *F. serratus* within 8 h of fertilisation that was attributed to pigment-protein complex CP43 of PSII. This is consistent with the increase in F_o that we observed after 12 h. There is a major discrepancy between results obtained by Lamote *et al.* (2003) and those obtained by McLachlan & Bidwell (1978) and our study. The former show no variable fluorescence ($F_v/F_m = 0$) for the first 24 h after fertilisation, and they concluded that the photosynthetic apparatus was not functioning in this period because PSII assembly was not complete. McLachlan & Bidwell (1978), however, showed significant CO_2 uptake in both eggs and embryos in this period in *F. serratus*, and this is consistent with high levels of photosynthetic parameters that we found in eggs and embryos of *A. nodosum* for at least the first 48 h. Thus the observation and the explanation for the absence of variable fluorescence in the first 24 h of development in *F. serratus* by Lamote *et al.* (2003) must be questioned. The final values obtained by these authors after 8 days are consistent with those in *Fucus evanescens* C. Agardh (Major & Davison 1998) and those in *A. nodosum* obtained here after a comparable growth period. Major & Davison (1998) suggested an uncoupling of growth and photosynthesis during early embryogenesis in *F. evanescens*. This is particularly apparent in *A. nodosum*, where healthy values (even if declining) for photosynthetic parameters can remain for 2–5 days in eggs that are clearly not growing. In addition, substantial growth of the rhizoids and cell divisions are occurring in the embryo during the first days when many photosynthetic parameters are stable. Both of these phenomena may be explained by the concentration of storage materials in fucoid eggs (McLachlan & Bidwell 1978).

In general, reduction in NPQ results from an exposure to environmental stress and involves photoprotective down-regulation of PSII efficiency (e.g. Gévaert *et al.* 2002, 2003). The numbers given for NPQ in Table 1 were calculated based on the highest NPQ value on the RLC. Consequently, our samples were adapted to 35–50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ when we determined NPQ. In addition, the short time over which the RLCs were acquired (about 2 min) does not allow for the down regulation of photosynthesis and the xanthophyll cycle to become activated (Ralph *et al.* 1999). Thus differences in NPQ between 2-day-old and 5-day-old embryos are real, and not a consequence of photoacclimation.

In our results, changes in NPQ between 2-day-old and 5-day-old eggs and embryos likely reflects two different consequences. On the one hand, NPQ would be expected to decline in senescing eggs. Senescence was much more advanced in 2004 by day 5, and this explains the much greater reduction relative to 2005. On the other hand, developing embryos would be expected to reduce NPQ because of their much higher electron transport rate and the need for increasing photosynthetic carbon assimilation. Our observations are consistent

with increasing metabolic requirements in rapidly developing embryos. Further studies on photoacclimation of developing embryos are warranted.

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