



# *Ascophyllum nodosum* and its symbionts: XI. The epiphyte *Vertebrata lanosa* performs better photosynthetically when attached to *Ascophyllum* than when alone

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*Vertebrata lanosa* is an abundant and obligate red algal epiphyte of *Ascophyllum nodosum* that forms part of a complex and highly integrated symbiotic system that includes the ascomycete, *Mycophycias ascophylli*. As part of ongoing studies to resolve interactions among species in the symbiosis, we used pulse amplitude modulation fluorimetry of chlorophyll *a* fluorescence, from photosystem II (PSII), to measure the maximum quantum yield ( $F_v / F_m$ ) of PSII [QY(II)<sub>max</sub>] and relative photosynthetic electron transport rates (rETR), as a function of light intensity, in order to evaluate the photosynthetic capacity of the two algal symbionts in the field and in the laboratory under different treatments. Our primary question was 'Is the ecological integration of these species reflected in a corresponding physiological integration involving photosynthetic process?' In the laboratory we measured changes in QY(II)<sub>max</sub> in thalli of *V. lanosa* and *A. nodosum* over one week periods when maintained together in either attached or detached treatments or when maintained separated from each other. While the QY(II)<sub>max</sub> of PSII of *A. nodosum* remained high and showed no significant variation among treatments, *V. lanosa* showed decreasing performance in the following conditions: *V. lanosa* attached to *A. nodosum*, *V. lanosa* in the same culture, but not attached to *A. nodosum*, and *V. lanosa* alone. These results are consistent with observations in which rETR was reduced in *V. lanosa* maintained alone versus attached to *A. nodosum*. Values for QY(II)<sub>max</sub> in *V. lanosa* measured in the field in fully submerged thalli were similar to those measured in the laboratory when *V. lanosa* was attached to its obligate host *A. nodosum*. Our results provide evidence of a physiological association of the epiphyte and its host that reflects the known ecology.

**Key Words:** *Ascophyllum nodosum*; epiphytism; photosynthesis; quantum yield; symbiosis; *Vertebrata lanosa*

## INTRODUCTION

*Ascophyllum nodosum* (L.) Le Jolis is the host of a complex symbiotic system that includes an obligate endophytic ascomycete, *Mycophycias ascophylli* (Cotton) Kohlmeyer & Volkmann-Kohlmeyer, and the obligate, epiphytic red alga, *Vertebrata lanosa* (L.) Christensen (Garbary and Deckert 2001). *V. lanosa* is, in turn, associated with the host-specific, red algal parasite *Choreocolax polysiphoniae* Reinsch (Irvine 1983). These are the

four core members of a symbiosis that also involves the obligate, but not host-specific, epiphyte, *Elachista fucicola* (Velley) Areschoug (also on *Fucus* spp.), and the chironomid insect, *Halocladus variabilis* (Staiger), which is typically associated with *E. fucicola* (Garbary et al. 2009a, Tarakhovskaya and Garbary 2009, Brown et al. 2013). Considerable progress has been made in understanding the geographic and ecological distributions of these sym-



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bioses across the North Atlantic Ocean, as well as the anatomical and ultrastructural interactions of these associations (e.g., Lining and Garbary 1992, Deckert and Garbary 2005a, 2005b, Garbary et al. 2005b, Longtin and Scrosati 2009, Longtin et al. 2009, Toxopeus et al. 2011, Garbary and Tarakhovskaya 2013).

From a physiological perspective, the interactions of *A. nodosum* and *V. lanosa* (hereafter referred to as *Ascophyllum* and *Vertebrata*, respectively) have been the most intensively studied. Given the host specificity, it was initially assumed that host specificity was based on a biochemical dependency, and there was considerable interest in the possible transfer of materials between *Vertebrata* and *Ascophyllum*. A variety of studies showed photosynthate and inorganic nutrient transfer between the symbionts (Citharel 1972, Harlin 1973, Penot 1974, Harlin and Craigie 1975, Turner and Evans 1977, Penot et al. 1993, Ciciotte and Thomas 1997). None of these studies, however, provided a strong physiological explanation for the association, in that movement of materials was in both directions, and rarely in excess of what was considered simple diffusion. Indeed, Pearson and Evans (1990, 1991), Garbary et al. (1991), and Longtin and Scrosati (2009) suggested that the association had a strong ecological basis in recruitment limitation provided by host surface features. These included the number of settlement sites on *Ascophyllum* associated with scars from receptacle abscission and herbivore surface wounds, as well as epidermal shedding (Garbary et al. 2009b).

In this study, we used pulse amplitude modulation (PAM) of chlorophyll *a* fluorescence to measure photosynthetic parameters of *Vertebrata* and *Ascophyllum* when isolated from each other, or when in combination, where the two species were either attached or not attached to each other. We evaluated a series of hypotheses: 1) that *Vertebrata* is parasitic on *Ascophyllum* and will have a negative impact on the photosynthetic performance of the host; 2) that the survival of *Vertebrata* is based on exudates from *Ascophyllum*; and 3) that maintenance of photosynthesis in *Vertebrata* requires it to be in direct contact (i.e., rhizoid penetration) with *Ascophyllum* so that transfer of materials can be facilitated from host to epiphyte.

## MATERIALS AND METHODS

### Study site and source of algal material

All algal material came from Gullivers Cove in the Bay

of Fundy of Nova Scotia, Canada (44°36.85' N, 65°55.56' W) (Fig. 1A), and experiments were conducted during November-December 2012. Gullivers Cove is an undeveloped area that was previously studied for algal diversity (Edelstein et al. 1970), and is currently used for commercial harvest of *Palmaria palmata* (L.) Weber & Mohr (Garbary et al. 2012a). *Ascophyllum* grew on the basalt bedrock characteristic of Nova Scotian shores facing the lower Bay of Fundy (Garbary et al. 2012a), and where fronds typically reach 1 m long (Fig. 1B). The site has a continuous bed of *Ascophyllum* through the mid intertidal zone from ca. 1.5 to 5 m elevation, and *Vertebrata* (Fig. 1C-E) was abundant at all but the extreme upper and lower portions of the *Ascophyllum* distribution.

### Photosynthetic fluorescence parameters

We examined photosynthetic performance of *Ascophyllum* and *Vertebrata* by measuring the maximum quantum yield of photosystem II (PSII) [ $QY(II)_{max}$ ], a measure of the photochemical efficiency of PSII, and the relative rates of photosynthetic electron transport (rETR) at various light intensities. Both photosynthetic parameters were measured as changes in the PSII chlorophyll *a* fluorescence emission using a portable, PAM fluorometer (Diving-PAM; Heinz Walz, Effeltrich, Germany). The  $QY(II)_{max}$  was estimated as:

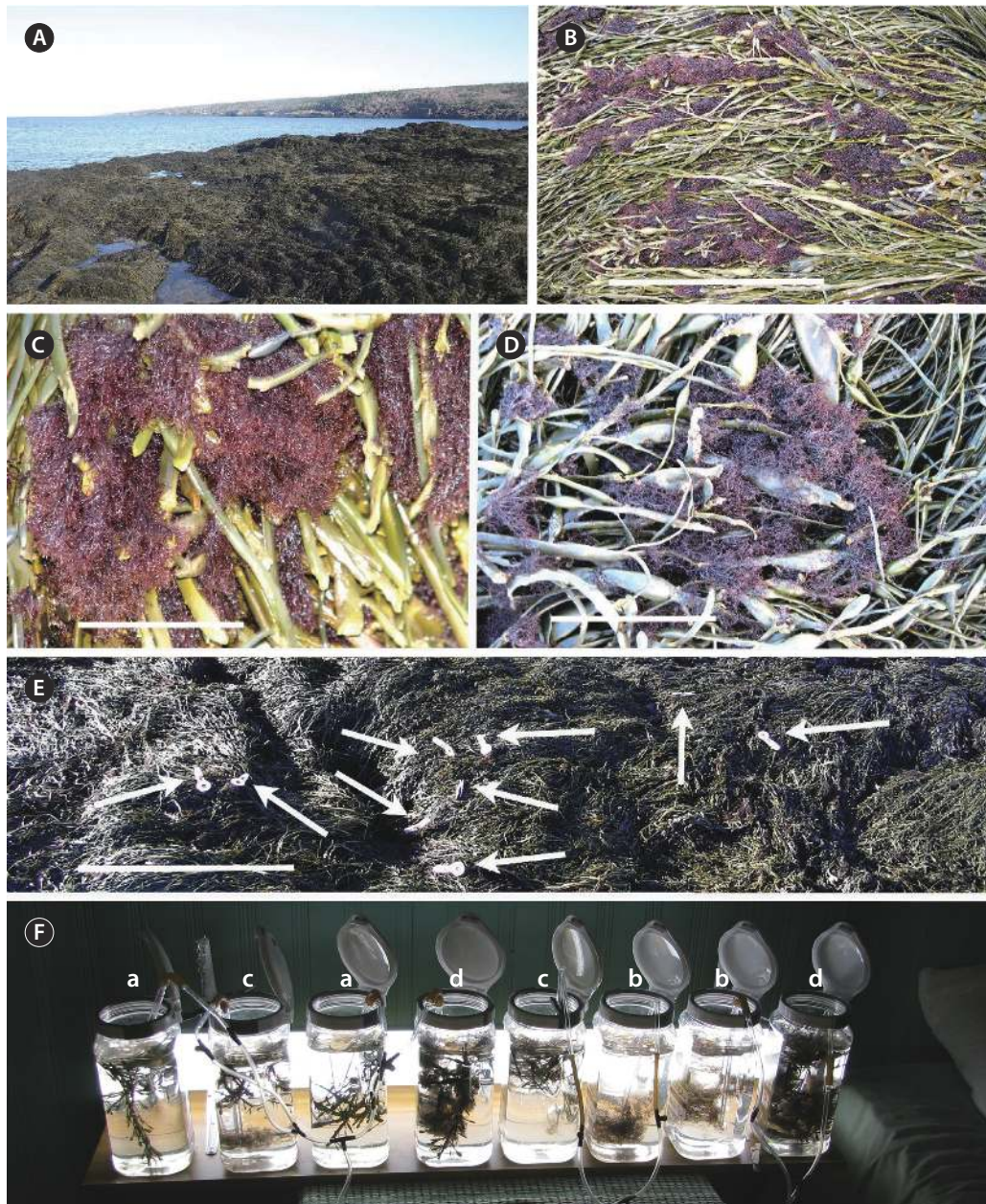
$$QY(II)_{max} = (F_m - F_o) / F_m = F_v / F_m$$

, where  $F_o$  is the minimal value of chlorophyll *a* fluorescence from PSII in dark-adapted thallus (10-15 min) when both photochemical quenching (qP) is at a maximum (i.e., at 100%) and non-photochemical quenching (NPQ) is at a minimum (i.e., at 0).  $F_m$  is the maximum value for chlorophyll *a* fluorescence from PSII measured from dark-adapted thalli during a non-modulated flash of light that fully reduces PSII and is called a saturating flash (SF).  $F_v$ , the difference between  $F_m - F_o$ , is called the variable fluorescence (Papageorgiou and Govindjee 2004). To obtain thalli in the dark-adapted state samples were placed in the 'dark leaf clips' (DIVING-LC; Heinz Walz) and dark-adapted for 10-15 min prior to taking the readings. Gain settings on the Diving-PAM were set at 1 and 4 for *Ascophyllum* and *Vertebrata*, respectively. Chlorophyll *a* fluorescence nomenclature follows Kromkamp and Forster (2003).

The intensity of the monitoring beam of the PAM fluorometer was 0.04  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  and the intensity of the SF was 10,000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ .

The  $QY(II)_{max}$  measurements give information only





**Fig. 1.** (A) Field site at Gullivers Cove, Nova Scotia with mid-intertidal zone dominated by *Ascophyllum nodosum*. (B) Abundant thalli of *Vertebrata lanosa* on *A. nodosum* in situ. (C) Clumps of fully moistened *V. lanosa* exposed on surface of *A. nodosum*. (D) Partially dried thalli of *V. lanosa* after several hours of desiccation on exposed surface of *A. nodosum*. (E) 'Leaf clips' (arrows) attached to *V. lanosa* during dark adaptation prior to fluorescence measurement. (F) Apparatus for laboratory experiment with replicated containers of *A. nodosum* alone (a); *V. lanosa* alone (b); both species with *V. lanosa* detached (c); both species with *V. lanosa* attached (d). Scale bars represent: B & E, 50 cm; C & D, 5 cm.

about the photochemical efficiency of PSII. In order to investigate the effect of treatments on the photosynthetic electron transport capacity downstream from PSII we measured rETR at various light intensities using the rapid light curve (RLC) option of the Diving-PAM fluorometer. The RLC protocol used began with dark-adapted thalli and the thallus was exposed to eight sequential light

periods (10 s each) of increasing light intensity (24 to 1,341  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). The rETR was determined using the following equation:

$$\text{rETR} = \text{QY(II)}_{\text{eff}} \times \text{PAR} \times 0.5$$

, where  $\text{QY(II)}_{\text{eff}}$  (also called  $\Phi_{\text{PSII}}$ ) is the effective (i.e., actual) quantum yield of PSII at given intensity of photosyn-

thetically active radiation (PAR).  $QY(II)_{\text{eff}}$  is dimensionless while the intensity of PAR has units of  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ .  $QY(II)_{\text{eff}}$  is calculated, by the Diving-PAM, using the following equation from Genty et al. (1989):

$$QY(II)_{\text{eff}} = (F_m' - F_t) / F_m'$$

, where  $F_m'$  is the maximum chlorophyll *a* fluorescence, during a SF, measured during exposure of the thallus to the given PAR intensity and  $F_t$  is the steady-state level of fluorescence under the given PAR intensity at time *t*.  $QY(II)_{\text{eff}}$  is lower than  $QY(II)_{\text{max}}$  because of the presence of qP and NPQ under continuous PAR. The factor 0.5 in the equation is used to account for the assumption that photons absorbed by the thallus are partitioned equally between PSII and PSI (see Genty et al. 1989 for derivation of the equation). To calculate the absolute ETR, rather than rETR, requires information on the fraction of photons incident upon the thallus that are actually absorbed by the photosystems.

The degree of qP, due to increasing reduction of the first stable electron acceptor in PSII, Quinone A ( $Q_A$ ), with increasing PAR intensity is calculated by the Diving-PAM (Walz 1998) by the equation:

$$qP = (F_m' - F_o) / (F_m' - F_o')$$

and the degree of NPQ using the equation:

$$NPQ = (F_m - F_m') / F_m'$$

### Photosynthetic parameters in the field

To establish baseline values for the photosynthetic parameters of *Vertebrata* growing under natural conditions, we measured  $F_o$ ,  $F_m$ , and  $QY(II)_{\text{max}}$  (measured as  $F_v / F_m$ ) *in situ* at low tide on November 23, when air temperature was about 13°C and there was intermittent cloud cover. To establish a range of natural values in the intact symbiosis, and to ensure that the immersed condition was optimal for our experiments, we compared thalli of *Vertebrata* on the surface of *Ascophyllum* fronds that were partially desiccated (max of 3 h of exposure) (Fig. 1E) with thalli following re-immersion in seawater. Re-immersion was done by draping long *Ascophyllum* fronds with their associated *Vertebrata* into plastic dishpans filled with seawater collected from the water's edge (9°C). The Diving-PAM "leaf clips" were attached to hydrated *Vertebrata* within one minute, and thalli were dark adapted for 10-15 min prior to taking measurements. Eight to ten values of photosynthetic parameters were taken at 15 min intervals. These data were obtained over about 2 h when incident light was measured with a meter (LI-250;

LI-COR, Lincoln, NE, USA) with a cosine corrected detector. Irradiance varied from  $615 \pm 11.5$  to  $76.1 \pm 0.6$   $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  (mean  $\pm$  standard deviation [SD], with six replicate readings taken within 30 s). Light was measured at nine, 10-15 min intervals from 1:30 PM to 3:20 PM. The extensive variation in light was based on changes in cloud cover, and the declining angle of the sun, which eventually set below the horizon of an adjacent cliff. An initial 30 chlorophyll *a* fluorescence readings of  $QY(II)_{\text{max}}$  were made of exposed thalli, and then all subsequent readings ( $n = 69$ ) were done with thalli that were immersed in seawater.

### Laboratory experiments

In an initial experiment, freshly collected fronds of *Ascophyllum* with and without epiphytic thalli of *Vertebrata* from the mid portion of the *Ascophyllum* zone (ca. 2.5 m elevation) were dissected to produce segments of *Ascophyllum*  $14 \pm 2$  cm long that included an air bladder at either end, with or without epiphytic *Vertebrata* (A), and clumps of *Vertebrata* 2-4 cm long (B). Ten, 2-4-year-old segments of *Ascophyllum* (with or without *Vertebrata*), and ca. 25 clumps of *Vertebrata* (with or without *Ascophyllum*) were placed in each culture vessel. Duplicate 3.0 L plastic containers had one of four algal treatments: 1) *Ascophyllum* alone, 2) *Vertebrata* alone, 3) *Vertebrata* attached to *Ascophyllum*, and 4) *Vertebrata* not attached to *Ascophyllum* (Fig. 1F). Culture medium was seawater from Gullivers Cove. Seawater was collected every one-two days in 20 L plastic containers from the water's edge, and used following a maximum of 48 h after collection. There was a 50% replacement of the unfiltered seawater each day to avoid potential nutrient depletion. Culture vessels were numbered and assigned algal condition using random numbers. Each day, culture vessels were rotated one position along the single culture shelf to minimize potential effects of position. Cultures were maintained at  $14 \pm 2^\circ\text{C}$  by opening and closing windows in the culture room. Cultures were continuously aerated, and illuminated with F32W T8 fluorescent bulbs with an irradiance of 40-90  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ . This was supplemented during daylight hours with indirect natural light through the room windows (max. 10  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ). Photosynthetic measurements were done from 8-11 AM each day with monitoring done on days 1, 3, 5, and 7. Data from day 5 was subsequently omitted because an extended power outage on day 4 produced aberrant values in all conditions on day 5, with subsequent recovery by day 7.



In a subsequent experiment (experiment 2), we omitted the condition, *Ascophyllum* alone, and monitored  $F_o$ ,  $F_m$ , and  $QY(II)_{max}$  daily for six days ( $n = 10$  for each condition) in each of the triplicate culture vessels, but only for *Vertebrata*. On day 7 we also measured rETR and recorded associated quenching parameters (qP and NPQ) by carrying out RLCs on four samples from each culture vessel (total of  $n = 12$  for each condition).

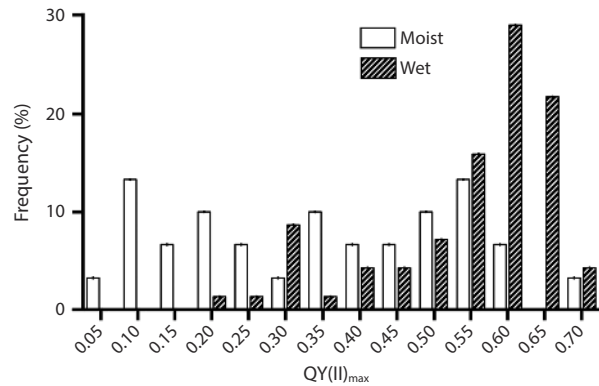
## RESULTS

### In situ measurements

A wide range in quantum yield  $QY(II)_{max}$  was measured *in situ* for *Vertebrata* in the intertidal zone of Gullivers Cove. Initially we evaluated naturally desiccated thalli on the surface of the *Ascophyllum* bed at low tide (Fig. 1B-E), and compared these to thalli immediately after re-immersion in seawater. Values of  $QY(II)_{max}$  for partially desiccated thalli ranged from 0.121 to 0.718 (mean of  $0.321 \pm 0.177$ ,  $n = 20$ ). This was significantly lower ( $p < 0.05$ , Student's t-test) than the quantum yield of thalli following re-immersion in seawater ( $0.587 \pm 0.109$ ,  $n = 69$ ). Even here there was a wide range of values (0.210 to 0.710) and a clear peak between 0.601 and 0.650 (Fig. 2).

### Laboratory experiment 1

This experiment showed several clear photosynthetic responses on the part of *Vertebrata* with respect to the occurrence of *Ascophyllum*; however, there was no evidence for the converse. Accordingly, there was no significant difference in  $QY(II)_{max}$  of *Ascophyllum*, regardless of whether *Vertebrata* was attached or not, or *Vertebrata* was present or absent in the same culture vessel (Table 1). The variation in  $QY(II)_{max}$  for *Ascophyllum* in the three conditions



**Fig. 2.** Percent frequency distribution of maximum quantum yield [ $QY(II)_{max}$ ] of *Vertebrata lanosa* measured *in situ* at low tide in partially desiccated ( $n = 30$ ) thalli and following re-immersion in seawater ( $n = 69$ ).

after seven days was as follows:  $0.758 \pm 0.032$  (*Ascophyllum* alone),  $0.771 \pm 0.021$  (*Ascophyllum* with *Vertebrata* attached), and  $0.767 \pm 0.022$  (*Ascophyllum* with detached *Vertebrata*; all values mean  $\pm$  SD). The largest difference in  $QY(II)_{max}$  between these means and the means after 24 h was 0.005, suggesting that there had been no deterioration in photosynthetic capacity of *Ascophyllum* over the seven day culture period.

In the same experiment there were major differences in  $QY(II)_{max}$  of *Vertebrata* in the three treatments, i.e., *Vertebrata* attached to *Ascophyllum*, *Vertebrata* with *Ascophyllum*, but not attached, and *Vertebrata* alone. Differences in the three conditions were apparent after 24 h and remained for the duration of the experiment (Table 1). A 2-way ANOVA evaluating  $QY(II)_{max}$  of *Vertebrata* at days 1, 3, and 7 and three treatments (attached to *Ascophyllum*, detached with *Ascophyllum*, and alone) was significant (F ratio = 52.13;  $p < 0.001$ ), with condition, and the interaction between condition and day significant at  $p < 0.05$ . Thus after 24 h attached *Vertebrata* had the highest maximum quantum yield ( $0.621 \pm 0.047$ ), and *Vertebrata* in the

**Table 1.** Results of laboratory experiment 1 showing maximum quantum yield,  $QY(II)_{max}$ , in *Ascophyllum* and *Vertebrata* on days 1, 3, and 7 when growing alone (alone), detached, but in same culture vessel, and attached to its symbiont

Species and condition	Day 1	Day 3	Day 7
<i>Ascophyllum</i> (attached)	$0.770 \pm 0.194$	$0.746 \pm 0.068$	$0.762 \pm 0.016$
<i>Ascophyllum</i> (not attached)	$0.766 \pm 0.026$	$0.753 \pm 0.021$	$0.765 \pm 0.023$
<i>Ascophyllum</i> alone	$0.763 \pm 0.025$	$0.745 \pm 0.032$	$0.752 \pm 0.022$
<i>Vertebrata</i> (attached)	$0.621 \pm 0.051^{abc}$	$0.659 \pm 0.033^a$	$0.654 \pm 0.052^{ab}$
<i>Vertebrata</i> (not attached)	$0.589 \pm 0.051^{cde}$	$0.603 \pm 0.035^{cd}$	$0.605 \pm 0.066^{bcd}$
<i>Vertebrata</i> alone	$0.571 \pm 0.049^{de}$	$0.544 \pm 0.043^e$	$0.543 \pm 0.058^e$

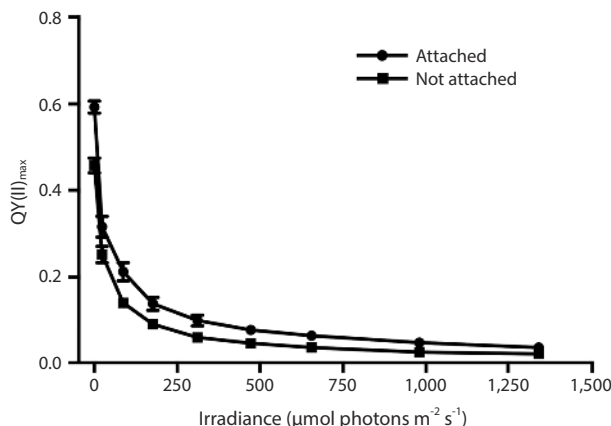
Values are presented as mean  $\pm$  standard deviation ( $n = 20$ ); different letter exponents for *Vertebrata* show significant difference at  $p < 0.05$  based on Tukey *post-hoc* tests in separate two-way ANOVAs for each species.

absence of *Ascophyllum* had the lowest  $QY(II)_{max}$  ( $0.571 \pm 0.049$ ), with detached *Vertebrata* with *Ascophyllum* being intermediate between the other values ( $0.589 \pm 0.051$ ). Over the seven day experimental period there was no significant decline in  $QY(II)_{max}$  of *Vertebrata* when attached to *Ascophyllum* ( $p = 0.616$ ). Tukey post hoc tests showed that  $QY(II)_{max}$  of *Vertebrata* attached to *Ascophyllum* was always significantly higher than *Vertebrata* alone, with the detached thalli (but in the same culture vessel) being intermediate between the other conditions, with considerable overlap in values (Table 1).

### Laboratory experiment 2

Experiment 2 evaluated only the performance of *Vertebrata* in the three treatments. The same primary result was obtained in this repeated experiment with respect to the  $QY(II)_{max}$  of *Vertebrata* when alone, or either attached or not to *Ascophyllum*, but present in the same culture vessel as *Ascophyllum*. After seven days (Table 2), *Vertebrata* alone had the lowest  $QY(II)_{max}$  ( $0.455 \pm 0.040$ ), *Vertebrata* attached to *Ascophyllum* had the highest  $QY(II)_{max}$  ( $0.620 \pm 0.061$ ), and *Vertebrata* when separated (but in the same culture vessel as *Ascophyllum*) was intermediate ( $0.513 \pm 0.100$ ). The one-way ANOVA was highly significant (Table 2). The chlorophyll *a* fluorescence parameters of  $F_0$  and  $F_m$  showed the same trends as  $QY(II)_{max}$  although the mean of  $F_0$  for *Vertebrata* ( $520 \pm 105$ ) when not attached (but in the same culture vessel) were intermediate but not significantly different from the other culture conditions (Table 2).

The values for  $QY(II)_{max}$  between day 1 and day 7 for attached *Vertebrata* ( $0.631 \pm 0.053$  and  $0.633 \pm 0.062$ , respectively) were not significantly different (Student's *t*-test,  $p = 0.89$ ) despite significant declines in both  $F_0$  and  $F_m$  over the same time period in this condition ( $0.727 \pm 0.152$ ,  $0.555 \pm 0.107$  and  $2,023 \pm 529$ ,  $1,496 \pm 368$  for  $F_0$  and



**Fig. 3.** Changes in  $QY(II)_{eff}$  of *Vertebrata lanosa* during rapid light curves for thalli attached to *Ascophyllum nodosum* or when grown alone. Figures show mean  $\pm$  standard error ( $n = 12$ ).

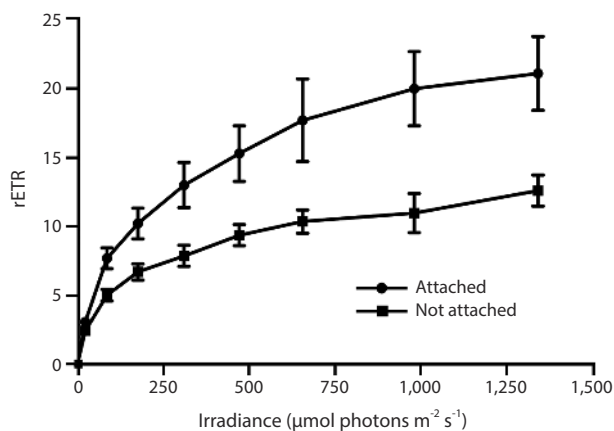
$F_m$ , respectively, Student's *t*-test  $p < 0.001$  for both). These declines (0, 23, and 26% for  $QY(II)_{max}$ ,  $F_0$ , and  $F_m$ , respectively) were generally lower than the equivalent reductions for *Vertebrata* alone (21, 33, and 49%, respectively) and *Vertebrata* with *Ascophyllum*, but detached (23, 20, and 46%). All of these declines from day 1 to 7 in  $F_0$ ,  $F_m$ , and  $QY(II)_{max}$  in *Vertebrata* alone and *Vertebrata* detached were highly significant (Student's *t*-test,  $p < 0.001$ ). The declines in these parameters may be based on a reduction in pigmentation (not measured), in that *Vertebrata* thalli in all conditions appeared to be a lighter red.

RLC for the determination of rETR at various intensities of PAR were only done with *Vertebrata*, either when alone or attached to *Ascophyllum*. For each RLC the first Diving-PAM measurement provides the value  $F_v / F_m$ , the estimate of  $QY(II)_{max}$ , because the thalli were dark-adapted. Subsequent Diving-PAM measurements provide values of  $QY(II)_{eff}$ , because the eight measurement subsequent to the first are obtained with the thallus now under illumination, from 24 to  $1,341 \mu\text{mol photons m}^{-2}$

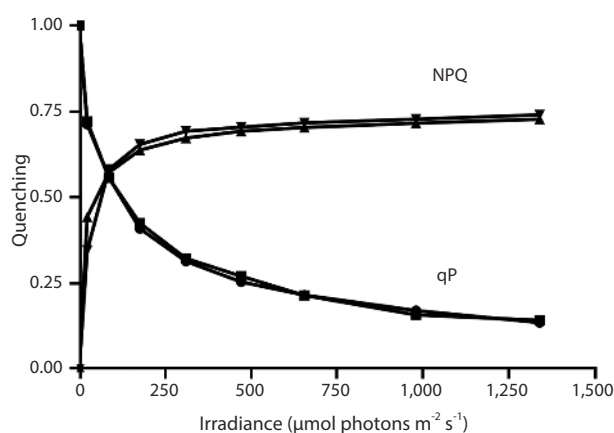
**Table 2.** Results of fluorescence parameters in *Vertebrata lanosa* in experiment 2 on day 7 showing  $F_0$ ,  $F_{max}$ , and  $QY(II)_{max}$

Experimental condition	Fluorescence parameter		
	$F_0$	$F_{max}$	$QY(II)_{max}$
<i>Vertebrata</i> (attached)	$555 \pm 107^b$	$1,496 \pm 368^c$	$0.620 \pm 0.061^c$
<i>Vertebrata</i> (not attached)	$520 \pm 105^{a,b}$	$1,081 \pm 317^b$	$0.513 \pm 0.101^b$
<i>Vertebrata</i> alone	$470 \pm 75^a$	$721 \pm 234^a$	$0.455 \pm 0.040^a$
F ratio	5.807	46.578	40.749
p-value	0.004	<0.0001	<0.0001

Values are presented as mean  $\pm$  standard deviation ( $n = 30$ ); different letter exponents show significant difference at  $p < 0.05$  based on Tukey *post-hoc* test in separate one-way ANOVAs for each photosynthetic parameter.



**Fig. 4.** Changes in relative photosynthetic electron transport rate (rETR) of *Vertebrata lanosa* during rapid light curves for thalli attached to *Ascophyllum nodosum* or when not attached (i.e., grown alone). Figure shows mean  $\pm$  standard error (n = 12).



**Fig. 5.** Changes in photochemical quenching (qP) and non-photochemical quenching (NPQ) of *Vertebrata lanosa* during rapid light curves for thalli attached to *Ascophyllum nodosum* or when grown alone. Note strong overlap in curves for the two *Vertebrata* conditions. Figure shows mean  $\pm$  standard error (n = 12).

$s^{-1}$  (Fig. 3). With each subsequent light period during performance of the RLC the value of  $QY(II)_{\text{eff}}$  decreases (Fig. 3) because of the increasing degree of quenching by qP and NPQ. The values for attached *Vertebrata* for  $QY(II)_{\text{eff}}$  were significantly higher at each irradiance than those *Vertebrata* grown alone (Student's t-test,  $p < 0.05$ ) (Fig. 3). The difference of in  $QY(II)_{\text{eff}}$  values for the two treatments, while of smaller absolute values with increasing irradiance, showed an increase in percent difference from ca. 20% at low irradiances to ca. 40% at high irradiances. Over the range of PAR intensities evaluated, rETR reached maxima of  $21.1 \pm 9.2$  and  $12.6 \pm 3.9$  (mean  $\pm$  SD) at  $1,341 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for *Vertebrata* attached and *Vertebrata* grown alone, respectively (Fig. 4). Mean values of rETR in attached thalli were 21% to 45% higher in *Vertebrata* when attached to its host and this was significant at almost all irradiances ( $p < 0.05$  Student's t-test, except at  $24 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  where  $p = 0.052$ ).

The degree of NPQ was the same whether *Vertebrata* was grown alone or grown attached to its host *Ascophyllum*, and this was the case for all PAR intensities (Fig. 5). There was no significant difference in mean values at any irradiance. qP for the two conditions was also similar, and the curves were strongly overlapping through the range of PAR intensities (Fig. 5).

## DISCUSSION

Photosynthetic parameters, determined by analysis of changes in chlorophyll *a* fluorescence emission from

PSII have provided useful data on the relationship between *Ascophyllum* and *Vertebrata*. For the first time we have demonstrated a conclusive physiological basis for this symbiotic association. With regard to our initial hypotheses we have demonstrated that: 1) photochemistry of PSII [reflected in the value of  $QY(II)_{\text{max}}$ ] of *Ascophyllum* is not negatively affected by the presence of *Vertebrata* (Table 1), and thus there is no evidence of a parasitic interaction with respect to this important aspect of photosynthesis; 2) photosynthetic parameters are partially maintained when *Vertebrata* is separated from the host, but in the same culture, thus suggesting a role for diffusible substance from the host (Tables 1 & 2); and 3) long term maintenance of photosynthetic processes requires permanent attachment to *Ascophyllum* (Figs 3 & 4).

The values for  $QY(II)_{\text{max}}$  we obtained for *Ascophyllum* (i.e., means of 0.74 to 0.77) (Table 1) are among the best for brown algae (cf. Garbary and Kim 2005, Kim et al. 2006, Kim and Garbary 2009). The maintenance of high values for maximum quantum yield in *Vertebrata* in control conditions (i.e., attached to *Ascophyllum*) in the laboratory (i.e., laboratory experiment 1,  $0.608 \pm 0.075$ ; laboratory experiment 2,  $0.632 \pm 0.060$ ; mean  $\pm$  SD) were clearly higher than the attached, immersed samples measured *in situ* (i.e.,  $0.569 \pm 0.123$ ). These values for both *Ascophyllum* and *Vertebrata* confirm that the laboratory measures for quantum yield were not depressed based on limitations of our culture conditions.

Laboratory results from the RLCs of *Vertebrata* (Figs 3 & 4) gave very different results for rETR when it was attached to *Ascophyllum* or alone in a culture. We therefore

expected to observe differences in qP and NQP quenching characteristics for thalli in the two conditions (Fig. 5). This anomalous result may be explained by the apparent loss of pigmentation in *Vertebrata* when cultured alone. Unfortunately, this loss of pigmentation was a subjective determination that could not be quantified because of loss of samples during a subsequent power outage.

The virtually obligate association between *Vertebrata lanosa* and *Ascophyllum nodosum* would have been observed by Linnaeus (1767) (as *Fucus lanosus* Linnaeus, not seen, cited from AlgaeBase) (Guiry et al. 2014). Despite extensive ecological studies of the interactions (e.g., Garbary et al. 1991, Levin and Mathieson 1991, Cardinal and Lesage 1992, Garbary and Deckert 2001, Scrosati and Longtin 2010), this is the first time that a physiological basis for the dependency of *Vertebrata* on *Ascophyllum* has been clearly demonstrated. While we cannot demonstrate an underlying mechanism for the photosynthetic interactions, our results show that photosynthetic activity of *Vertebrata* declines when it is not attached to its host (Tables 1 & 2, Figs 3 & 4). The extent of this reduction in terms of  $QY(II)_{max}$  and rETR suggest that *Vertebrata* requires its host for long-term maintenance of photosynthesis.

We have experimentally examined only one set of interactions, i.e., between *Ascophyllum* and *Vertebrata*; however, we cannot separate the potential interactions of *Vertebrata* and the mutualistic fungal endophyte *M. ascophylli* (hereafter *Mycophycias*). Garbary et al. (2005a) showed that fungal hyphae of *Mycophycias* regularly colonize the endophytic rhizoids of *Vertebrata* that penetrate into the cortical tissue of the host. Consequently, distinguishing between possible transport of materials between *Ascophyllum* and *Vertebrata*, and between *Mycophycias* and *Vertebrata*, is difficult. Garbary et al. (1991) suggested that host specificity of *Vertebrata* was based primarily on ecological factors associated with the production and number of suitable settlement sites of the host (e.g., receptacle dehiscence scars) and the timing of spore production by *Vertebrata* (Garbary et al. 1991, Kaczmarek and Dowe 1997). Colonization sites also are produced by surface wounds (Longtin and Scrosati 2009, Scrosati and Longtin 2010).

*Mycophycias* is always present in *Ascophyllum* (Kohlmeyer and Kohlmeyer 1972, Garbary and Deckert 2001, Deckert and Garbary 2005a, Garbary 2009), and the fungal hyphae are dispersed throughout the host from holdfasts through to branch apices and receptacles (Garbary and Gautam 1989, Deckert and Garbary 2005a, 2005b, Xu et al. 2008). In all frond portions, hyphae form complex

networks in which rings of hyphae surround host cells, particularly in the cortex. Garbary and MacDonald (1995) showed that zygotes of *Ascophyllum* grew faster and with a different morphology following colonization by *Mycophycias*. Subsequent experiments showed that zygotes of *Ascophyllum* were more tolerant of desiccation following infection by *Mycophycias* (Garbary and London 1995). The abundant penetration of *Vertebrata* rhizoids by *Mycophycias* suggests that analogous physiological performance of *Vertebrata* may be partly based on colonization of the red algal rhizoids by the fungus. An alternative explanation for the decline in photosynthetic performance of *Vertebrata* when separated from its host is that it is based on phenolic (or other exudates) from *Ascophyllum*. Pearson and Evans (1991) showed stimulation in rhizoid production of *Vertebrata* based on exudates from tissues of a variety of brown algae. While this might partially explain our photosynthetic results, it is not consistent with the host specificity of *Vertebrata*.

On the one hand, we have little evidence for a mechanism to explain the causal basis for the reduction of quantum yield and electron transport rates in *Vertebrata* following detachment from *Ascophyllum*. On the other hand, the latter is a known source of phytohormones (e.g., Tarakhovskaya et al. 2007), and is harvested and used in the manufacture of commercial extracts that have a variety of applications in animal husbandry and agriculture (review by Craigie 2011). Externally applied phytohormones to embryos of *Fucus vesiculosus* modify photosynthetic processes (Tarakhovskaya et al. 2013), and this might also be the case for *Vertebrata*. The commercial extract from *Ascophyllum* can regulate phytohormone biosynthesis and accumulation in *Arabidopsis* (Wally et al. 2012). We speculate that one or more of the secondary metabolites (e.g., phytohormones or phenolics) from *Ascophyllum* may be responsible for the maintenance of photosynthetic function when *Vertebrata* is attached to its host. However, we cannot rule out the possibility that the interaction is based on a chemical interaction with *Mycophycias* via direct connections with the rhizoids of *Vertebrata* (Garbary et al. 2005a). The intermediate response of quantum yield of *Vertebrata* when detached from *Ascophyllum*, but maintained in the same culture vessel as *Ascophyllum*, is strong support for some diffusible substance produced by *Ascophyllum* as being required for maximizing photosynthetic processes of *Vertebrata*. The production of secondary rhizoids by *Vertebrata* when exposed to fucoid extracts (Pearson and Evans 1991) demonstrates that *Vertebrata* has complex metabolic interactions with brown algae, and equivalent



interactions may be part of the photosynthetic responses.

What is the nature of the *Vertebrata-Ascophyllum* interaction? Rawlence (1972) showed that cell death occurred during penetration of the *V. lanosa* rhizoids into the host, and Garbary et al. (2005a) demonstrated a hypersensitive reaction of some host cells adjacent to the red algal rhizoids. These interactions suggest a parasitic interaction between epiphyte and host. However, our photosynthetic data show no decline in photosynthetic performance when *Ascophyllum* has abundant *Vertebrata* compared to when it is absent. Indeed, Gauna et al. (2011) suggested that, both in terms of growth and initiation of receptacles, that there was no reduction in performance of the *Ascophyllum* when *Vertebrata* was present, and that host growth may even be stimulated by the epiphyte. The limited cell death induced by the epiphyte is minor compared to the massive sloughing associated with host epidermal shedding that occurs at regular intervals (Garbary et al. 2009b, 2012b, Halat et al. 2013). Thus the interactions between these species would appear to be at least commensal in nature, and may even be mutualistic.

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