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Environmental change provokes rapid macromolecular reallocations within the photosynthetic system in a static population of photobionts in the lichen *Lobaria pulmonaria*

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Abstract: *Lobaria pulmonaria* is an epiphytic lichen that, in south-eastern Canada, inhabits deciduous forests where it must acclimate to large seasonal changes in temperature and in light caused by closing and opening of the leaf canopy. On a seasonal timescale, this acclimation occurs via large shifts in the macromolecular complexes of the photosynthetic system, within a photobiont population that shows no seasonal change in cell numbers. In this study, samples of *L. pulmonaria* were harvested in February and in May from a natural population near Sackville, New Brunswick, and subjected to two simulated intense seasonal changes: (1) early spring warming, simulated by a shift from high light at 5°C to high light at 16°C (February shift), and (2) late spring canopy closure, simulated by a shift from high light at 16°C to low light at 16°C (May shift). Thallus samples were collected daily throughout each week-long shift. There were no significant changes in photobiont cell population size or in the fraction of cells dividing during either shift. During the first day of the February temperature shift, there were, however, large changes in the pools of chlorophyll, the major light capture molecule in the photobionts, the PsbA (D1) core protein of photosystem II whose turnover is highly responsive to changing light and temperature, and the RbcL major subunit of the carbon-fixing RUBISCO enzyme whose levels correlate strongly with achieved photosynthesis in lichens. A static population of photobionts was therefore able to perform large and rapid macromolecular reallocations to cope with rapid environmental change. No significant changes were seen in the chlorophyll, photosystem II or RUBISCO pools across the May light shift, although seasonal-scale macromolecular reallocation does occur in response to decreased light in the summer.

Key words: light acclimation, photosystem II, RUBISCO, seasonal change, temperature acclimation.

Introduction

Lobaria pulmonaria is a lichen with a temperate circumpolar distribution that is subjected to wide shifts in environmental conditions throughout the year. It is a locally common epiphyte in eastern Canada under deciduous forest canopies on the trunks of maple trees (*Acer* spp.) and less commonly on spruce (*Picea* spp.) and other conifers (Jordan 1973; Brodo *et al.* 2001). In deciduous habitats, *L. pulmonaria* is subjected to freezing temperatures and bright light in the open canopy winter months, and warm, wet and dark conditions under the thick leaf canopy in summer months. After water (Jensen & Feige 1991; Lange *et al.* 1999; Nash *et al.* 1990), light is the primary limitation on lichen photosynthesis (Palmqvist 2000). Lichens that grow under the leaf canopy, such as *L. pulmonaria*, are subject to limited irradiance during the summer months (MacKenzie *et al.* 2001) when other conditions, such as water availability and temperature, may be more conducive to photosynthesis and growth.

Photosynthesis and growth of lichens change with seasonally changing environmental conditions (Larson & Kershaw 1975; Kershaw & Webber 1984; Kershaw 1985; Muir *et al.* 1998; Lange *et al.* 1999;

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MacKenzie *et al.* 2001; MacKenzie *et al.* 2002). MacKenzie *et al.* (2001) tracked photosynthetic changes in physiology and macromolecular content in *L. pulmonaria* at 35-day intervals over the period of a year in a deciduous habitat, and showed that *L. pulmonaria* acclimated to changing conditions of temperature and light by changing macromolecular organization of the photosynthetic system in the photobiont and thereby altering the fate of absorbed light energy (MacKenzie *et al.* 2002).

In highly structured lichens such as *Lobaria*, the photobiont cell population is strongly regulated by the mycobiont (Malachowski *et al.* 1980; Hill 1992) with important consequences for acclimation of the photobiont to changing conditions. Photobiont cell division rates coincide with fungal hyphal growth at the margins of the thallus, but in the majority of the thallus, greater than *c.* 2 mm from the growing margins, photobiont cell division is arrested and the cells become outsized and static (Greenhalgh & Anglesea 1979; Hill 1992). We have confirmed a similar pattern of control in the photobiont population of *L. pulmonaria*, and have shown reversible reallocations of macromolecular resources to chlorophyll, RUBISCO (RbcL), photosystem II (PsbA) and other pools that occur to maintain photosynthetic capacity in this photobiont population without significant cell turnover (Schofield *et al.* 2003), when tracked on a seasonal timescale.

In this study, we sought to determine how photosynthetic acclimation to environmental change occurs over short, but ecologically relevant timescales of days in the photobionts of *L. pulmonaria*. Substantial acclimation of photosynthetic gas exchange patterns in similar lichens can occur within two days upon a shift in temperature (Kershaw 1985). We therefore examined *L. pulmonaria* specimens subjected to controlled environmental changes in the laboratory to detect any rapid, day-scale, macromolecular acclimation. We subjected lichens collected in late February to an increase in temperature at constant light intensity, as can occur in the field in early spring before the leaf canopy develops. Lichens collected in May were also subjected to a shift from high light to low light at constant temperature, as can occur in the field as the leaf canopy closes. We applied microscopy and measurements of pool sizes of chlorophyll, photosystem II (PsbA) and RUBISCO (RbcL), macromolecular indicators of capacities for, respectively, light capture, photochemistry and carbon fixation, to confirm whether rapid macromolecular changes can occur *intracellularly* in a static population of photobiont cells.

Materials and Methods

Sample collection and laboratory maintenance

Samples of *Lobaria pulmonaria* (L.) Hoffm. were collected in February and May 2003 from a predominantly maple (*Acer* spp.) dominated deciduous forest site near Walker Road, Sackville, New Brunswick (45°44·22′N, 64°10·01′W). Samples of thallus were removed from tree trunks while dry and metabolically inactive and transported to the laboratory in the dark, dry and over ice, where they were stored for several weeks at −20°C. On the first day of each shift experiment, several thalli were placed in a Conviron controlled environment chamber at the beginning of the day cycle and allowed to equilibrate to the cabinet conditions while dry. Both February and May samples were maintained under 12:12 day:night cycle and misted daily until saturated with *c.* 1 g g thallus $^{-1}$, with Millipore® filtered distilled water *c.* 2 hours after dawn. This hydration scheme allowed maintenance of high efficiency photosynthesis for several hours during the day. The lichens also retained some metabolic activity throughout the day-night cycle because of the high relative humidity of the cabinet. Such continuous activity allowed by humid air is likely to occur for extended times at our field site during the spring, and is a general phenomenon among green-algal lichens in temperate forests (Lange *et al.* 2001). The February samples were held at 5°C for the first day, then shifted to 16°C at the end of the first day period and for the rest of the week-long experiment, under a constant 160 μmol photons m^{-2} s^{-1} during all day periods. The May samples were held under a photosynthetic photon fluence rate (PPFR) of 160 μmol m^{-2} s^{-1} over the waveband 400–700 nm during the first day period, then at a PPFR of 20 μmol m^{-2} s^{-1} in all subsequent day periods, and at a constant 16°C throughout the experiment.

Chlorophyll fluorescence measurement

Computer controlled pulse-amplitude modulated (PAM) chlorophyll fluorometers (FL-2000, Opti-Sciences) monitored photochemical efficiency

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($\varphi_{PSII} = F'_m - F_s/F'_m$) in three arbitrarily selected thalli during each shift experiment, via fibre-optic probes. Fluorescence was excited in the lichen photobionts by a red LED at *c.* 460 nm modulated at 8 Hz, trained continuously on the thallus from several cm distance; thus, the probe did not touch the thallus, nor shade it from overhead illumination. Saturating light pulses were supplied every five minutes to assess photochemical efficiency (φ_{PSII}) of the photobionts, also at *c.* 460 nm and via the same fibre-optic cable. Data for φ_{PSII} was captured and sorted continuously to a computer by a custom software application written by one of the authors (T.D.B.M.)

Pigment extraction and determination

Daily, near the end of the daylight cycle (when the thalli had become relatively dry), six arbitrarily selected lobes (*c.* 10 × 5 mm wide) were removed from the different thalli for pigment and microscopic analyses. Two small (*c.* 2 × 5 mm wide) sections were dissected from the younger marginal end and the older end of each thallus fragment and reserved for later microscopic analysis. The remainder of the tissue was imaged with a Fluor-S Max MultiImager (BioRad, Canada) to determine thallus area, then was extracted with DMSO according to the method of Barnes *et al.* (1992). Spectra of the extract were recorded with a Beckman 640 spectrophotometer (Beckman Coulter, USA) and quantities of chlorophyll pigments (chl *a* chl *b*) were calculated as:

$$\text{chl } a \text{ (mg ml}^{-1}) = 14{\cdot}85\ A_{664{\cdot}8} - 5{\cdot}14\ A_{648{\cdot}2}$$

$$\text{chl } b \text{ (mg ml}^{-1}) = 25{\cdot}48\ A_{648{\cdot}2} - 7{\cdot}36\ A_{664{\cdot}8}$$

after Barnes *et al.* (1992), then were corrected to molar quantities per unit area of thallus.

Protein extraction and determination

Six large (*c.* 3 cm^2) replicate thallus samples were randomly sampled for protein analysis from the stored thalli from the field (hereafter called day 0 thalli) and thalli maintained in the cabinet at the end of days 1 and 6. These times were chosen to assess protein content of the field samples (day 0), after the first day in the controlled environment cabinet at its initial conditions (day 1), and after five full days of acclimation to the new cabinet conditions (day 6). Each sample was imaged using a Fluor-S Max MultiImager (BioRad) and thallus area determined. Before protein extraction, thallus samples were washed with Mg CO_3 buffered 90% acetone to remove any interfering secondary metabolites and surface contaminants. The samples were then dried and their dry weight measured using a Metler AE 166 analytical balance. Dry weight was measured after the acetone wash as it removes substantial non-lichen surface contamination and assists complete dehydration of the thallus. The dried samples were pulverized in liquid nitrogen containing powdered glass (*c.* 0·02 g) with a mortar and pestle. The powder was transferred to a 2 ml microcentrifuge tube on ice. To ensure complete transfer, a small additional amount of powdered glass was then ground with the mortar and pestle to recover any remaining tissue. Pigments were extracted from the powdered sample with 1 ml of Mg CO_3 buffered acetone in the dark on ice for an hour, vortexing every 20 min to remove pigments and more acidic secondary metabolites which may interfere with the protein extraction. The remaining tissue was dried under a stream of nitrogen gas. Protein solubilization buffer (650 µl; 100 mM Tris–HCl pH 9·3, 160 mM sucrose, 1 mM EDTA, 1% sodium dodecyl sulfate, 0·5% DTT added just before use) was added to the dried pellets, frozen in liquid nitrogen, then sonicated using a Branson Digital sonifier (VWR, Canada) until thawed (typically 40 s), then frozen and sonicated again. The samples were heated for 5 min at 95°C and centrifuged for 5 min at × 12 000 g. The supernatants containing solubilized proteins were transferred to clean microcentrifuge tubes and stored at − 80°C until used for immunodetection.

Proteins were separated by SDS-PAGE electrophoresis in pre-cast linear 10% SDS-polyacrylamide NuPAGE Novex Bis-Tris gels (Invitrogen, USA), using an Invitrogen XCell SureLock Mini-Cell electrophoresis unit. Samples were loaded on an equal area per well basis (equivalent to 1·5 mm^2 thallus per well) along with two quantified RbcL standards and one well with 3 µl of a 25:1 mix of Precision Plus Prestained Protein Standard marker (BioRad, USA) and MagicMarkerXP chemiluminescent protein marker (Invitrogen, USA). The gels were electrophoresed in pairs at 200 V for 60 min in NuPAGE MES electrophoresis buffer (Invitrogen, USA). After electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA) at 30 V for 60 to 80 min in transfer buffer (NuPAGE Transfer Buffer, Invitrogen, USA) with an XCell II Blot Module version G (Invitrogen, USA). After transfer the PVDF membranes were washed in 10 ml of 2% ECL Advance blocking agent powder in Tris Buffered Saline Tween-20 (TBS-T; 20 mM Tris–HCl, 137 mM NaCl, 0·1% Tween-20, pH 7·6) and soaked at room temperature on an orbital shaker for 15 min, then stored overnight at 4°C. After blocking, the membranes were incubated with both the anti-PsbA and anti-RbcL primary Global antibodies (Agrisera, Sweden, www.agrisera.se) at 1/50 000 dilution in 10 ml of 2% blocking buffer for 1 h. The membranes were then washed exhaustively with TBS-T 0·1%, before adding the HRP-conjugated rabbit anti-chicken secondary antibody (AbCam, www.abcam.com) at 1/50 000 dilution in 10 ml of 2% blocking buffer for 1 h and again washing exhaustively. Immunoreactive proteins were visualized with ECL Advance (Amersham Bioscience, USA) chemiluminescent reagents and imaged with the Fluor-S Max MultiImager (BioRad, USA). The Quantity One™ (BioRad, USA) software package was used for imaging and analyzing the chemiluminescent band intensities. Intensity measurements of each band were corrected for local background luminescence.

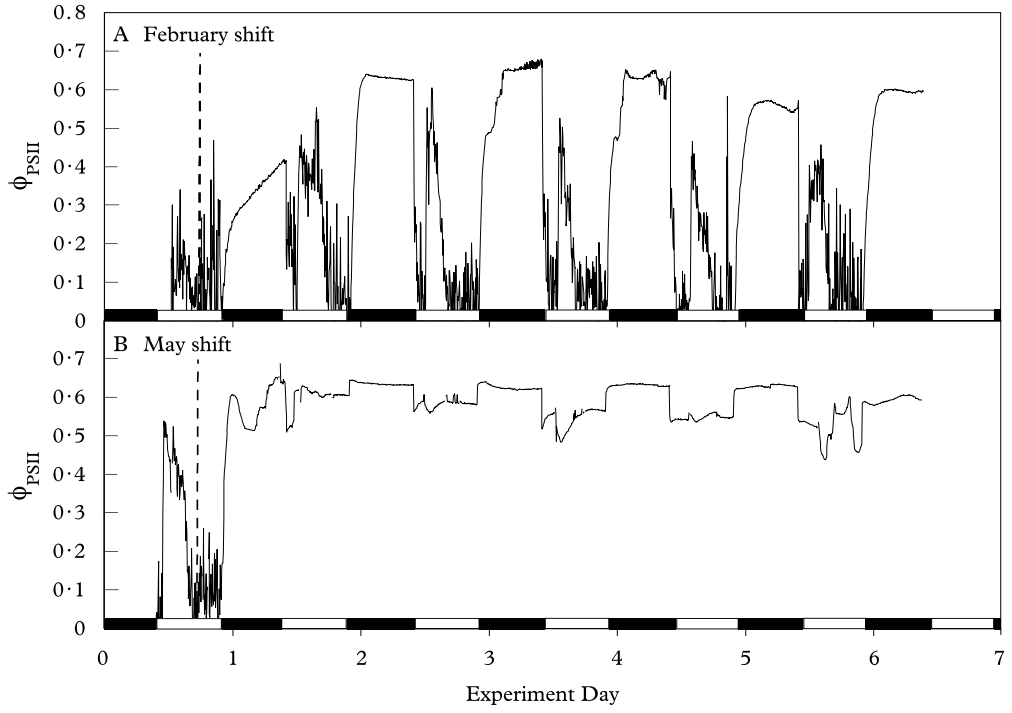

FIG. 1. Continuous φ_{PSII} measurements of *Lobaria pulmonaria*. A, February-collected samples shifted from 5°C to 16°C at a PPFR of 160 μmol m^{-2} s^{-1}; B, May-collected samples shifted from 160 to 20 μmol m^{-2} s^{-1} PPFR at 16°C. Each profile is an average of the simultaneous responses of three individual thalli monitored by separate chlorophyll fluorometers automatically every five minutes during the week-long shift experiment, with the vertical dashed line marking the time of the environmental shift. Samples were maintained in controlled environment chambers under a 12 h light (white bars): 12 h dark (black bars) photoperiod and were sprayed with Millipore® filtered distilled water once per day at approximately 2 h after lights-on.

Photobiont population and division rates

Cross-sections of thallus samples were analyzed to quantify the photobiont cell population and the proportion of dividing photobiont cells. Cross-sections from day 0, 1, 2 and 6 from each shift experiment were fixed using a protocol adapted from Jackson (1989). After the samples were embedded in paraffin, sections were cut to a thickness of 5 μm using an American Optical microtome (Leica KM2135). The thallus cross-sections were examined using a Zeiss (Axioskop 2) microscope and imaged with Axio Vision 3.0 software. The number of cells per image was counted and the area of the image calculated with the NIH Image software package (National Institute of Health, USA) to ultimately calculate cells m^{-2} thallus. Each section was also examined for the number of dividing photobiont cells, which could be distinguished from non-dividing cells by their appearance as small clusters of cells within a common envelope. One-way ANOVA statistical analyses were used to assess changes of photobiont cell population and proportion of dividing photobiont cells through days 0, 1, 2 and 6 during the two shift experiments. We did not use repeated-measures ANOVA, as all microscopic analyses were conducted on separate, independent lobes of sample material. Statistical analyses were performed with Systat 5.2.1 for Macintosh software.

Results

The lichen samples achieved high photosynthetic efficiency (φ_{PSII}) during their week-long incubation in the controlled environment chambers (Fig. 1). The February field samples had a depressed φ_{PSII}, even when wetted, before the shift to higher temperature (Fig. 1A), but then rose rapidly after the shift to increased temperature, to levels similar to the May samples while they were hydrated. The February samples dried sufficiently under the bright light over the day period to limit photosynthetic efficiency, even at relatively high

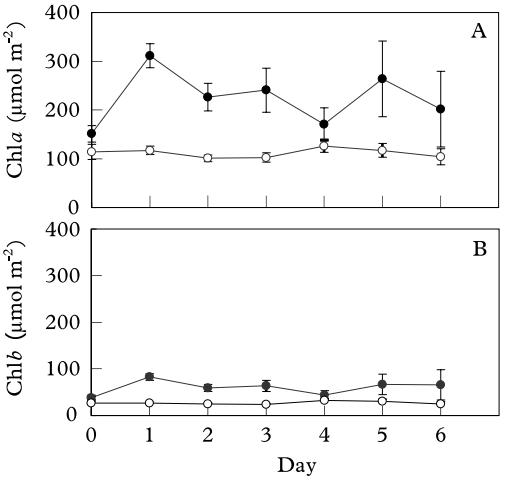

FIG. 2. Chlorophyll content of *Lobaria pulmonaria*. A, chl *a*; B, chl *b*. February shift (closed symbols) and May shift (open symbols). $n=6 \pm$ SEM.

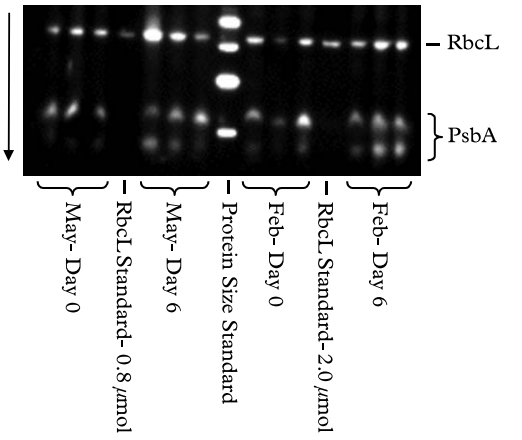

FIG. 3. Representative immunoblot image of RbcL and PsbA proteins in *Lobaria pulmonaria*. PsbA protein was detected as two bands which showed qualitatively similar patterns, therefore the two band intensities were added for subsequent analysis.

temperature. During the night, however, high relative humidity allowed for full activation of PSII (high dark values of φ_{PSII}). The May pre-shift samples had a near maximal φ_{PSII} both before and after the shift to low light (Fig. 1B). As in the February samples, high relative humidity allowed for high φ_{PSII} values at night and during the low daytime light exposure after the environmental shift. Green algal lichens such as *L. pulmonaria* retain photosynthetic efficiency from high humidity, even if liquid water is unavailable (Lange *et al.* 2001).

Pigments and proteins involved in photosynthesis showed large and rapid fluctuations over the February environmental shift, but not over the May shift. Chlorophyll *a* and *b* contents of lichen thalli rose significantly from day 0 to day 1 in the February shift ($P<0{\cdot}001$), although no significant difference was detected between the day 0 and final day 6 values for either pigment (Fig. 2). Thus, the shift from the high-light and subfreezing conditions of the field to one day at moderate light and 5°C caused a significant increase in chlorophyll, while the subsequent rise to 16°C caused little additional effect, with the chlorophyll content actually dropping back toward initial levels. The May shift on the other hand showed no significant change in either Chl *a* or Chl *b* over the course of the shift.

As in chlorophyll, the major protein pools PsbA and RbcL increased sharply in the February shift to higher temperature, but not across the May shift to lower light. Figure 3 shows a representative image of a protein blot treated with antibodies specific for RbcL and PsbA. In the February shift the relative quantity of PsbA, like that of chlorophyll, jumped significantly between day 0 and day 1 ($P=0{\cdot}014$) in the 5°C cabinet, but then returned to levels not significantly different from starting levels by day 6, in the 16°C cabinet (Fig. 4A). May samples on the other hand showed no significant initial or delayed change in PsbA levels. RUBISCO (RbcL) also rose dramatically in the February shift, but not in the May shift (Fig. 4). The rapid rise of RbcL in the February shift was statistically significant ($P=0{\cdot}015$), and was coincident with the rises in chlorophyll and PsbA in the samples (Figs 2A, 4A & B). These macromolecular changes supported the rapid rise in φ_{PSII} in the February samples (Fig. 1A) and suggest a general up-regulation of photosynthesis after the shift to more mild temperature conditions.

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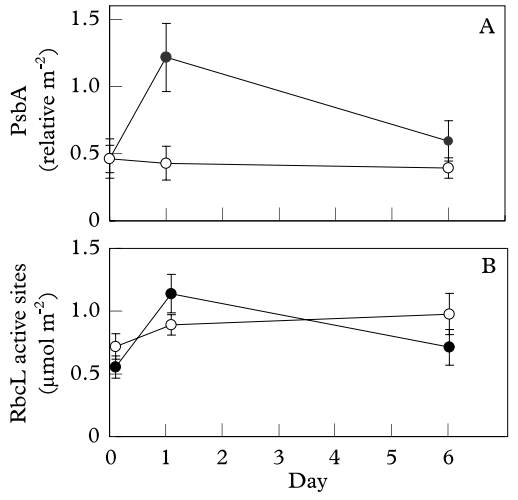

FIG. 4. Photosystem II and RUBISCO content of *Lobaria pulmonaria*. A, relative PsbA; B, absolute RbcL. Content of February shift (closed symbols) and May shift (open symbols). $n=3 \pm$ SEM.

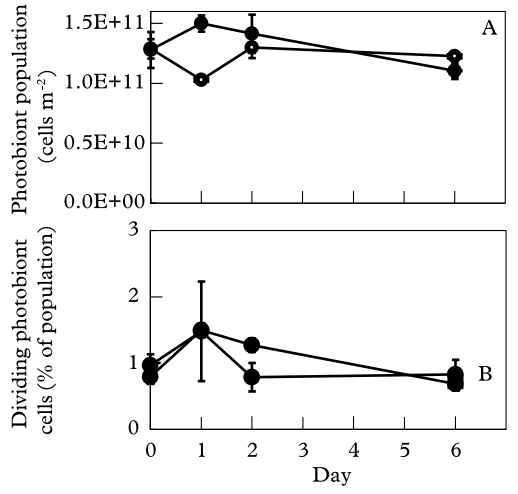

FIG. 5. *Lobaria pulmonaria* photobiont. A, photobiont population; B, proportion of dividing cells. February shift (closed symbols) and May shift (open symbols).

The photobiont populations of *L. pulmonaria* were stable throughout the shift experiments in both the February and May samples. Photobiont population size showed no change over either environmental shift (Fig. 5A), with the slight changes in mean population size over time not statistically significant (one-way ANOVA *P*-values 0·16 for February shift, 0·06 for May shift). Also, the proportion of dividing photobiont cells was very low in the central regions of the thallus sampled for protein and pigment analyses (Fig. 5B). The proportion of dividing photobiont cells in the February samples showed a marginally statistically significant peak (one-way ANOVA *P*-value 0·034), however, the peak was small and transient, and probably had little ecophysiological relevance compared to the much larger changes in macromolecular contents of these cells. The proportion of dividing photobiont cells in the May samples did not change significantly through the shift experiment (one-way ANOVA *P*-value 0·64). These data indicate that cell turnover could not act as a mechanism for any significant physiological or macromolecular acclimation. Our cell counting method was sensitive to differences in the proportion of dividing cells, as we quantified a significantly higher proportion of dividing cells (3–4% of the population) at the thallus margin regions, but even at the thallus margins this proportion of dividing cells did not change over the course of the shift experiments (data not shown).

Discussion

Changes in photosynthetic physiology and related macromolecular pools in *Lobaria pulmonaria* occur over a seasonal timeframe (MacKenzie *et al.* 2001) within an apparently stable photobiont population (Schofield *et al.* 2003) to acclimate their photosynthetic physiology to changing environmental conditions through the year in their deciduous habitat. In this study we have shown that *L. pulmonaria* can perform such photosynthetic and intracellular macromolecular acclimation rapidly in a stable photobiont population exposed to a shift that mimics a quick spring warming change from a cold, high light environment to a warm, high light environment, under an open canopy. No such rapid acclimation was detected in *L. pulmonaria* exposed to a more mild environmental change from warm

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moderate light to warm, low light, which mimics canopy closure in late spring. Schofield *et al.* (2003) and MacKenzie *et al.* (2001) did observe changes in pigments and proteins involved with photosynthesis in response to low summer light over a longer seasonal time scale.

We observed a rapid increase in chlorophyll pigments and PsbA and RbcL proteins in the February samples maintained in relatively warm cabinet conditions. These macromolecular changes were coincident with the large increase in φ_{PSII} in the thallus, indicating a rapid upregulation of efficient photosynthesis. The near- or sub-freezing winter temperatures in bright light cause a near cessation of photosynthesis in *L. pulmonaria* and acclimation of the photosynthetic apparatus into an energy-dissipation state (Schofield *et al.* 2003), with down-regulation of PSII and a large drop in PsbA content. This down-regulation helps the lichens cope with the high excitation pressure imposed on the photosynthetic apparatus by combined high light and the restriction of metabolic energy consumption and protein recycling under low ambient temperature (Huner *et al.* 1998). The increase in temperature relieved this excess excitation pressure by allowing metabolic consumption of energy and protein synthesis to proceed, rendering conditions amenable to the reassembly and return to productive function of the photosynthetic apparatus (e.g. Ensminger *et al.* 2004), indicated by a transient increase in PsbA protein. The increase in temperature and concomitant relief of excitation pressure in PSII would shift the balance between synthesis and photodegradation of PsbA, allowing rapid net accumulation. After several days of acclimation to the new lower-pressure conditions, this overshoot corrected to a lower level, probably through down-regulation of the PsbA synthesis rate (Vasilikiotis & Melis 1994; Huner *et al.* 1998).

In the May high to low light shift there was no severe change in the environment to necessitate a rapid and large change in macromolecular organization. For example, there was no rapid change in PsbA in the May shift samples, probably because the shift from moderate light to low light was not as extreme a change in the excitation pressure and consequent photodegradation rate of PsbA as experienced by the February samples. The May samples had a well-developed photosynthetic apparatus that performed efficiently, with high φ_{PSII} before and after the shift from a moderate light level to a relatively low light level that did not represent removal of a stress factor as occurred in the February shift. We have previously shown that, over *c.* 100 days, slow but substantial changes in chlorophyll and RbcL content do indeed occur to maintain photosynthesis in such low light in the field (MacKenzie *et al.* 2001). Kershaw & Webber (1984) also noted acclimation of CO_2 exchange light-responses due to opening and closing of the deciduous leaf canopy over thalli of *Peltigera*. In controlled environment cabinets, Kershaw (1985) reported that acclimation of CO_2 exchange in *Peltigera* to changes in temperature occurred in 1–2 days, while substantial acclimation to a shift to low light took several weeks. These results on the photosynthetic rate of *Peltigera* are entirely consistent with the results of our physiological and macromolecular investigation of *L. pulmonaria*.

While in this study substantial changes in large macromolecular pools have been noted, they were not accompanied by significant changes in photobiont population or division rates. Our population and division results are consistent with those from our earlier seasonal field study (Schofield *et al.* 2003), and with studies on other lichens (Greenhalgh & Anglesea 1979; Hill 1992; Armstrong & Smith 1998) that have observed a paucity of photobiont turnover in the central regions of lichen thalli, that represent the large majority of the symbiont biomass. The lack of photobiont population turnover in mature thallus regions, now confirmed on a daily and seasonal scale, means that the photobiont of *L. pulmonaria* reallocates photosynthetic macromolecules within existing cells as a mechanism of acclimating to environmental change. Acclimation within existing photosynthetic

cells can only occur to a small degree in annual crop plants and deciduous trees (Huner *et al.* 1998; Savitch *et al.* 2002). Multicellular evergreen plants, such as *Pinus*, do, however, show extensive intracellular reallocations (Ottander *et al.* 1995; Gilmore & Ball 2000; Ivanov *et al.* 2001; Öquist & Huner 2003; Ensminger *et al.* 2004), which are remarkably analogous to the intracellular reallocations we find in the evolutionarily distant, unicellular algal photobionts of lichens.

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