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MELANISATION OF LICHENS: THE COMPOSITION OF MELANIN AND THE ROLE OF ULTRAVIOLET LIGHT (UV) IN PELTIGERALEAN AND NON-PELTIGERALEAN LICHENS

By

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PREFACE

The experimental work and thesis preparation described in this thesis was carried out in the School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg under the supervision of Professor Richard P. Beckett, from February 2016 to November 2017.

These studies present original work by the author and have not been otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of other, it is duly acknowledged in the text.

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PUBLICATIONS DECLARATION

CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication;

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ABSTRACT

Lichens are unique organisms widely known for their ability to tolerate extreme environmental conditions due to the symbiotic relationship between a fungus and algae or cyanobacteria. This includes the ability to synthesise melanins to protect themselves from ultraviolet radiation and high light. Melanins are found in different forms, the eumelanins (DOPA), often synthesised by Peltigeralean lichens while the non-Peltigeralean produce dark pigments that appear not to be DOPA melanins. Increased levels of UV and high light affect the physiology of many organisms, as a result this study investigates the effects of the photoprotective pigment, melanin on the photosynthetic apparatus of both chlorophycean and cyanobacterial bionts. The first aim of this thesis was to study the effect of using different light regimes to induce melanins. The second aim was to compare the properties of melanin between different lichens with those from free-living fungi. Lastly, the effect of melanisation on the photosynthesis was investigated. Results presented here suggest that melanins are insoluble in organic solvents, except DMSO and strongly absorb in the UVB and UVA wavelengths. The induction of melanin was slow and was better induced beneath the screens that transmitted UV. Transplanting nonmelanised *Lobaria pulmonaria*, to an open site for four weeks induced melanic pigments. Melanised thalli had normal chlorophyll contents and normal maximum rates of photosynthesis. Chlorophyll fluorescence analysis showed that the maximum quantum yield and relative electron transfer rates were similar to those of non-melanised thalli. However, at light levels lower than 100 µmol photons m^{-2} s^{-1} melanisation of the upper cortex of the lichen reduced rates of CO_2 fixation by more than 40%. Melanic thalli also had a higher chlorophyll a/b ratio and more xanthophyll cycle pigments. In *Lobaria retigera*, the +UV screen decreased the photosynthetic rate more than other light treatments. Photoinhibition of wet thalli was rapid compared to dry, though complete recovery was reached after a day. From these results, it can be confirmed that melanisation has a protective action against high light as melanised thalli were more tolerant to excess light. While protecting photobionts from high light, melanisation reduced photosynthetic efficiency and protects lichens from photoinhibition in both the wet and dry states.

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LIST OF ABBREVIATIONS

BRI	browning reflectance index
CBSC	carbon based secondary compounds
Chl	chlorophyll fluorescence
CHL	chlorophyll content
CRISPR	clustered regularly interspaced short palindromic repeats
DHN	1, 8-Dihydroxynaphthalene
DM	dry mass
DNA	deoxyribonucleic acid
DOPA	3, 4-Dihydroxyphenylalanine
DW	dry weight
ETR	electron transport rate
Fm	maximum fluorescence
Fv/Fm	maximum fluorescence
GHB	glutaminyl-4-hydroxybenzene
IPD	immediate pigment darkening
IRGA	infrared gas analyzer
MAA	mycosporine-like amino acids
NPQ	non-photochemical quenching
PAR	photosynthetically active light
PKS	pentaketide pathway
pksP	polyketide synthase gene
PS I	photosystem I
PSII	photo system II
QY	quantum yield
Qy	quantum yield
RH	relative humidity
ROS	reactive oxygen species
RWC	relative water content
SDS	sodium dodecyl sulphate
UV	ultraviolet light

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CHAPTER 1: LITERATURE REVIEW

Lichens

Lichens are comprised of fungal, algal and yeast partners. For about 140 years, lichens were defined as an association of a mycobiont (95%) and a photobiont (5%) (Molnár and Farkas, 2010; Solhaug and Gauslaa, 2012) but more recently it was realized that many species contain an additional layer of a basidiomycete yeast (Spribille et al., 2016). The significance of this second mycobiont is not well-known, but it may serve a pathogen defense function. There are more than 17 000 species of lichens which includes 16 750 of lichenized Ascomycetes, 200 Deuteromycetes and *c.* 50 Basidiomycetes. Bacteria often found in lichens are mainly from genera such as Actinobacteria, Verrucomicrobia, Acidobacteria, Firmicutes, Proteobacteria and Planctomycetes (Bates et al., 2011). Lichens can either have a "blue-green" or cyanobacterial component or a green alga. The green algal partners are mostly from the Chlorococcales (Friedl, 1995), and the most cyanobacterial photobionts come from the genera *Nostoc*, *Gloeocapsa*, *Scytonema*, or *Calothrix* (Ahmadjian, 1993; Huneck, 1999). This association improves the longevity of the individual bionts (Kranner et al., 2005). An important difference between green and cyanobacterial photobionts is that cyanobacteria lack the xanthophyll cycle. There are four morphological forms of lichens namely crustose, fruticose, foliose and gelatinous (Watson, 1929).

Lichens can either be broadly taxonomically grouped as Peltigeralean or non-Peltigeralean species. The Peltigeralean lichens are often differentiated from non-Peltigeralean species by having unstalked apothecia (Miadlikowska and Lutzoni, 2000). They often grow on trees, although a few species are terricolous or muscicolous. Many Peltigeralean lichens have cyanobacterial photobionts (Miadlikowska and Lutzoni, 2000; Hodkinson et al., 2014) and can therefore fix nitrogen (Hodkinson et al., 2014). Non-Peltigeralean lichens generally have chlorophycean photobionts (Hale, 1967). Some Peltigerales are defined by the absence of the lower cortex e.g. *Leptogium* and *Peltigera* (Miadlikowska and Lutzoni, 2000). Peltigeralean lichens tend to display higher activities of redox enzymes such as tyrosinase and laccase (Laufer et al., 2006).

Lichen distribution

Lichens dominate on about 10% of the terrestrial biosphere (Ahmadjian, 1993), often in areas characterized by high stress e.g. high light/radiation, temperature extremes and low water availability. They have a remarkable ability to adapt to extreme environments and conditions (Kappen, 1973; Rothschild and Mancinelli, 2001; Sapmak et al., 2015), and commonly found growing on soil, tree trunks, and rock substrata (Cannon and Kirk, 2007). Lichens grow well in both shady and light-exposed habitats.

Synthesis of lichen substances

The review of Huneck and Yoshimura (1996) indicates that about 700 lichen substances have been isolated and identified. They belong to different groups which are further differentiated based on the color, solubility and whether they are an acid or phenolic. These physico-chemical properties were described by Shibata (1963). Common examples of lichen substances include, usnic acid, parietin, atranorin, chloroatranorin, salazinic acid, lecanoric acid, 7-chloroemodin (Huneck and Yoshimura, 1996), perlatolic acid and physodic acid (Reddy et al., 2016).

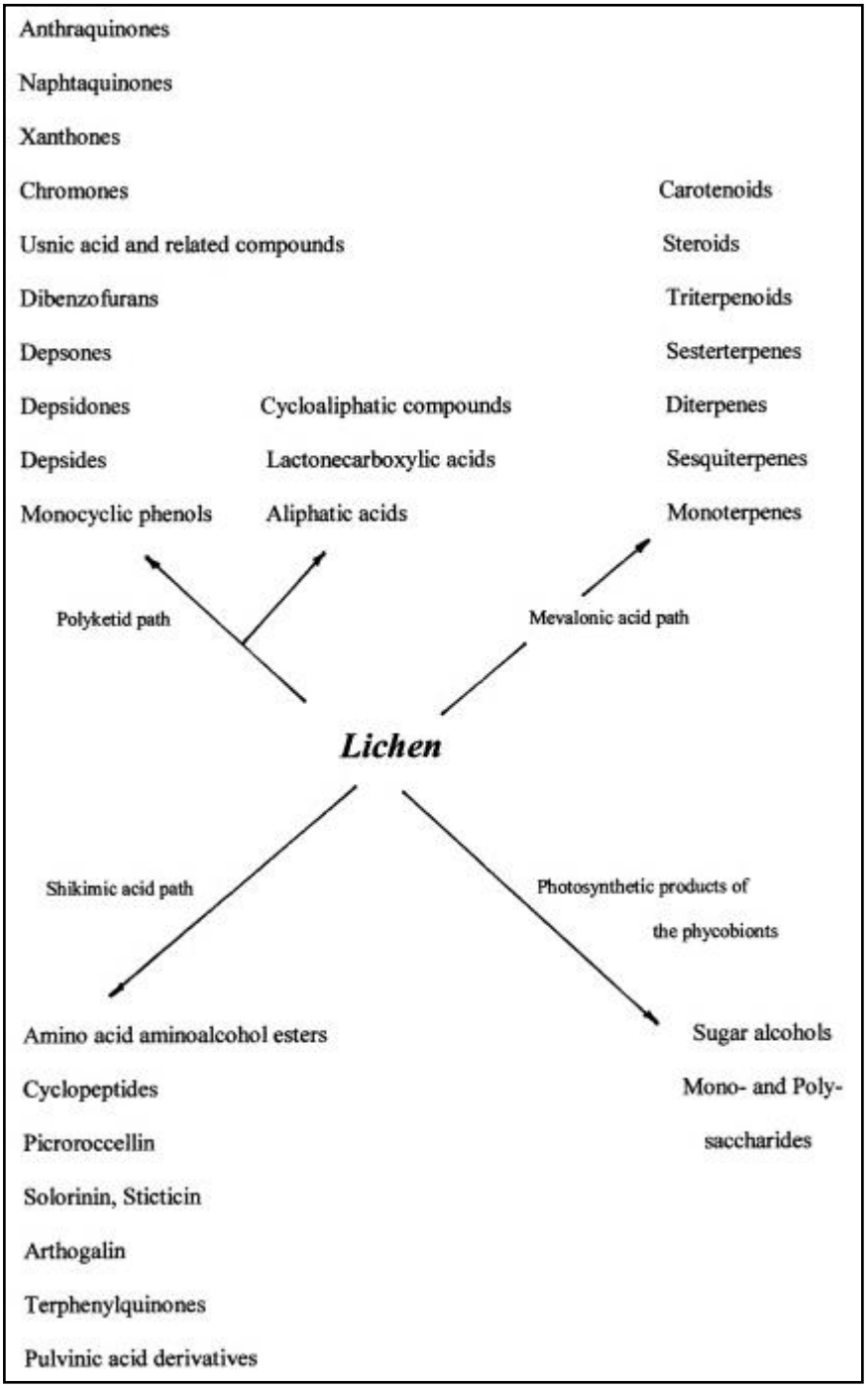

Figure 1.1: Classes of lichen substances and their reaction pathways (Huneck, 1999).

Screening roles of secondary metabolites

Cockell and Knowland (1999) list four criteria that should be fulfilled to confirm a specific light screening role to pigments such as secondary metabolites and melanins, and these criteria can be generalized to include other roles of pigments in stress tolerance. The criteria are:

1. The compound must be shown in the laboratory to have the properties that protect against the stress;

2. The compound should be induced or boosted by the stress;

3. A role in stress tolerance should be demonstrated in vivo e.g. by comparing the stress tolerance of melanised and pale individuals;

4. The compound should protect against ecologically relevant stress levels.

As it will become clear in the remaining sections of this review, the only reasonably safe deductions on the role of melanin and other secondary metabolites in the biology of at least some lichens are that first, they protect the mycobiont from high UV, and second, they protect the photobiont against high PAR. While it seems likely that compounds play other roles in the tolerance of lichens to a diversity of biotic and abiotic stresses, this needs to be confirmed by more work.

Screening the mycobiont from UV

A recent short-term experiment using growth chambers with and without UVB radiation showed that while UVB did not affect pure photobiont responses, it substantially reduced growth driven by both symbionts together (Chowdhury et al., 2016). The implication is that the mycobiont is more sensitive to UVB than the photobiont. As discussed in more detail below, lichens produce a range of secondary metabolites that also effectively screen both symbionts from UV (Solhaug and Gauslaa, 2012; Nguyen et al., 2013). However, a key role of these compounds appears to be to protect the fungus rather than the photobiont from the harmful effects of UV radiation.

Protection of the alga from UV and high PAR

Protection from UV

In contrast to the fungus, apparently photobionts do not need secondary metabolites to protect them from UV radiation. For example, in *Lobaria pulmonaria*, even an intact unmelanised upper cortex protects the underlying algal layer from UV-induced photoinhibition (Gauslaa et al., 2017). While removing the upper cortex of this lichen (exposing the algal-layer) renders the photobionts sensitive to UV, if the upper cortex is left

intact even high doses of UV have no effect on photosynthesis. The implication is that a normal upper cortex can protect the photobiont against UV.

Protection from PAR

High PAR probably has little effect on the mycobiont, except possibly indirectly by causing heat stress. By contrast, in the photobiont high PAR may cause oxidative stress and temporary or permanent photoinhibition. Photoinhibition is caused, directly or indirectly, by the stimulation of the production of reactive oxygen species (ROS) that occurs when photosystems cannot use the light energy they are absorbing, and this energy rather activates oxygen (Gururani et al., 2015; Pospíšil, 2016). The D1 protein, a key component of photosystem II, is believed to be most sensitive to damage during photoinhibition. Some damage appears to occur even in under moderate light intensities, and photosynthesizing organisms must therefore, continuously repair the damage to this protein. The "PSII repair cycle", occurring in chloroplasts and in cyanobacteria, involves degrading and synthesis of the D1 protein, followed by activation of the reaction centre. Recent studies of photoinhibition have suggested that light may directly damage PSII, and the resulting ROS that are produced inhibit D1 protein repair (Nishiyama and Murata, 2014). Convincing evidence exists that photoinhibition regularly occurs in lichens in field situations (Leisner et al., 1997; Jairus et al., 2009), and will reduce the ability of lichen photobionts to photosynthesize.

Plants have several mechanisms to reduce photoinhibition. For example, ROS formation can be reduced by converting the excess light energy into thermal energy, a process termed non-photochemical quenching (NPQ). NPQ occurs by a variety of mechanisms such as the xanthophyll cycle, and the transfer of energized electrons to O_2 which is then used in photorespiration and the Mehler peroxidase reaction (Nishiyama and Murata, 2014; Duffy and Ruban, 2015; Gururani et al., 2015). Lichens, in addition to these mechanisms, reduce photoinhibition by synthesizing cortical compounds (Solhaug and Gauslaa, 2012). Apart from melanins, the most widespread cortical pigments are the classic lichen substances such as usnic acid, atranorin and parietin (Solhaug and Gauslaa, 2012); the synthesis of these compounds is also induced by UV light. In addition to reducing UV, although most of these compounds are only marginally pigmented, their crystalline structure reflects PAR, significantly reducing light intensities in algal layer (Solhaug et al., 2010).

Melanisation is a highly effective way of protecting lichen photobionts again high PAR. For example, in a field study, McEvoy et al. (2007) transplanted *Lobaria pulmonaria* to three sites with low, medium and high light levels for 100 d. Lichens at the site with highest light level became significantly melanised. Thalli from each of these sites were then transplanted to a highly-exposed area for 12 d. The melanised thalli displayed a much smaller reduction in Fv / Fm than the material that had been in the more shaded sites. While other mechanisms may have been involved, results strongly suggest that melanins are involved in photoprotection. Further suggestion that melanins play protective roles came from the study of Färber et al. (2014) on pendulous lichens that dominate canopies of boreal forests. Typically, dark *Bryoria* species grow in the upper canopy, and pale *Alectoria* and *Usnea* species in lower canopy. Exposing these lichens under controlled conditions to a light level of 400 µmol m^{-2} s^{-1} for 7 d caused much less photoinhibition in the melanised *Bryoria* species than the non-melanised *Alectoria* and *Usnea*.

Another class of a sun screening pigment was discovered in the genera *Collema*, *Gonohymenia* and *Peltula* (Büdel et al., 1997b), from which they were isolated from the bacterium *Nostoc commune* in the fungi (Böhm et al., 1995), namely scytonemin (Garcia-Pichel and Castenholz, 1991). Based on phylogenetic evidence, scytonemin is the oldest UV related pigment dating from the Precambrian period common in extracellular cyanobacterial sheaths. It was found in heterocystous and unicellular *Gloeothece* groups. During the Precambrian period UVC was an important environmental stress factor. UVC at 250 nm caused direct damage of cellular contents (Proteau et al., 1993; Dillon and Castenholz, 1999). The synthesis of this pigment is induced in organisms that inhabit high latitudes with increased photosynthetic photon flux density and UV. Scytonemin absorbs UVA and UVB wavelengths. It directly prevents about 85 to 90% of incident UVA radiation from penetrating the cells (Garcia-Pichel and Castenholz, 1991; Garcia-Pichel et al., 1992; Proteau et al., 1993). Evidently, at blue-UVA scytonemin functions as a passive sunscreen though radiation may cause mutations or inhibitions of growth and enzyme activity. A study supporting UV sun screening in a cyanobacterium *Chlorogloeopsis spp*. Strain O-89-Cgs (1) suggests that UVB absorption assists in protecting cells (Garcia-Pichel et al., 1992). Here, scytonemin will be quantified to investigate whether it plays a role in photoprotection in lichens.

Melanin

The word “melanin” comes from the ancient Greek, melanos, meaning “dark,” and in a general sense refers to dark pigments that occur throughout all domains of life (Solano, 2014). They are found within all phyla of the fungi. Melanins are difficult to define due to their structural complexity, but in fungi mostly belong to either the eumelanin or allomelanin groups. Shared properties include broadband absorption spectra, paramagnetism, charge transport and remarkable structural stability. It is therefore perhaps not surprising that melanins have been suggested to have many roles in fungal biology (Cordero and Casadevall, 2017). The biosynthesis of fungal melanins was recently reviewed by (Belozerskaya et al., 2015). Briefly, most fungal melanins are generated from the polymerization of either tyrosine (DOPA or eumelanins) using tyrosinase or in some case laccases, or 1, 8-dihydroxynaphthalene (DHN or allomelanins) using polyketide synthases. During melanin synthesis, the phenolic precursors undergo multiple oxidation and reduction steps, which can occur enzymatically or passively by spontaneous polymerizations. Melanins appear to aggregate to form spherical to rather disordered particles which have been termed “melanin granules”. These granules may be contained at the cell surface or released into the extracellular space. The exact location of melanin granules at the cell surface varies between fungal species. Granules can occur between the plasma membrane and the innermost part of the cell wall or within or at the surface of the cell wall matrix. Some fungal species are constitutively melanised while others melanise facultatively only under specific developmental phases (e.g. conidia, yeast filamentous growth), in response to environmental queues, and/or in the presence of phenolic melanin precursors. Perhaps surprising, there appears to have been no attempt to review the occurrence and roles of melanic pigments in lichenized ascomycetes.

Occurrence of melanins in lichens

There has been no attempt to review the taxonomic distribution of melanins in lichens, although they appear to occur in all lichenized orders. Gostincar et al. (2012) suggest that the earliest lichens were derived from oligotrophic black fungi growing on surfaces e.g. bare rock with almost no usable organic-carbon. Part of the lichen colonized by black fungi benefits

from the aromatic polyketide secondary substances that are probably involved directly or indirectly in the synthesis of melanin. Such fungi inevitably attempted to improve their carbon supply by attaching to microscopic algae. Rock-inhabiting fungi tend to develop into "lichenoid structures" within months when co-cultured with lichen algae (Gorbushina et al., 2005; Brunauer et al., 2007). Recently, Siletti et al. (2017) reviewed the phylogenetic distribution of fungal melanin concentrations across fungal genomes. These concentrations are correlated with melanin protein precursors involved in melanin biosynthesis. He further predicted that, the production of melanin not be correlated with the species growth. Because increased growth rates are possible when melanin is inhibited (Fernandez and Koide, 2014). This inverse relationship increases the role of soil C storage. Also, Gorbushina and Broughton (2009) interpreted the rock surface as a kind of "symbiotic playground," where competitive interactions between species are probably rare. It seems likely that the transition from a rock-inhabiting to a lichenized lifestyle in early ascomycetous evolution was driven by the high stress of their habitat. Desiccation and high levels of UV and PAR increase the formation of reactive oxygen species in both fungi and algae, but the lichen symbiosis increases the efficiency of the protective mechanisms compared to isolated symbiotic partners (Kranner et al., 2005). These mechanisms involve the glutathione redox system, which is also known from black fungi (Jurgensen et al., 2001). In addition, small protective molecules that accumulate in black fungi as stress-responsive osmolytes could be involved in the transition from rock-inhabiting to the lichen life style. Polyols such as ribitol, sorbitol, and erythritol, as well as glucose, provided by algae and cyanobacteria, respectively, are taken up by lichen fungi as food molecules and transformed to mannitol (Friedl and Budel, 2008). Efficient osmolyte metabolism, as found in oligotrophic black fungi, might serve as a pre-adaptation to facilitate the transition to a lichen symbiotic life style. A close relationship between lichens and black fungi is also suggested by molecular phylogenetic studies. Rock-inhabitants are basal to the large lichenized lineages of Arthoniomycetes and Verrucariales (Gueidan et al., 2008; Ruibal et al., 2009).

In extant lichens, black pigments may be synthesized constitutively as part of development, e.g. the cilia of *Heterodermia*, in the lower cortex of genera such as *Parmelia*, or throughout the whole thallus in genera such as *Bryoria*. In other cases, melanins are environmentally induced, for example by UV in the upper cortex of genera such as *Lobaria* (Solhaug and

Gauslaa, 2012). Melanins are now common in many species of lichens that live in a variety of habitats.

Structure of lichen melanins

The chemical nature of lichen melanins remains complex and unclear as this pigment generally consists of aromatic rings and several carboxylic groups (Fernandez and Koide, 2014). However, the melanins of *Lobaria pulmonaria* (Matee et al., 2016) behave as classic DOPA melanins; they are extracted with NaOH, and precipitated with HCl. The resulting black precipitate readily dissolves in DMSO, and has an absorption spectrum resembling that of commercially available melanins. The low C: N ratio of these melanins (10:1) indicates that, as in humans, they were almost certainly synthesized from L-DOPA rather than hydroxylated naphthalene-derived molecules that produce "allomelanins" with a C: N ratio close to 100:1 (Loganathan and Kalyanasundaram, 1999; Solano, 2014). Matee et al. (2016) suggest that this is a consequence of *L. pulmonaria* containing N_2-fixing cephalodia that supply thalli with N. Interestingly, melanisation was accompanied by an increase in laccase rather than tyrosinase activity, suggesting that in *L. pulmonaria* DOPA may be polymerized by laccase, but more work is needed to confirm the melanin biosynthetic pathway in the species.

The nature of melanins in non-Peltigeralean lichens remain uncertain, particularly in species that appear to synthesize constitutively high concentrations of melanins. While the pigments from some species for example, the very dark genus *Bryoria*, can readily be extracted by NaOH, they are not precipitated by acidification, making their chemistry difficult to study. Addition of $FeCl_3$ to acidic solutions does however induce precipitation, and the C: N ratio of these precipitates is typically around 100:1, typical for allomelanins. This is consistent with these species containing green algae rather than N_2 fixing cyanobacterial photobionts, and therefore having less available nitrogen to synthesize protective molecules. While similar soluble melanins are known from bacteria (Aghajanyan et al., 2005; Aghajanyan et al., 2011), it is premature to speculate on the nature of the darkly coloured pigments that occur in non-Peltigeralean lichens. It is possible that they may differ significantly in chemical structure from other recognized fungal melanins.

Melanins compared with other light screening pigments

The roles of secondary metabolites in light-screening was discussed above. Melanisation is particularly common in lichens growing in high stress environments (Gostincar et al., 2012), and therefore intuitively, it seems likely that melanins will play a role in tolerance to high light. However, anecdotal observations suggest that lichens may use classic lichen substances such as usnic acid, atranorin and parietin more frequently than melanins to protect themselves against the effects of high light (Solhaug and Gauslaa, 2012). Interestingly, lichens with lichen substances or melanic compounds often coexist in open habitats, and some lichens with atranorin or usnic acid such as *Cetraria* and *Cladonia* additionally produce melanic compounds under very sun-exposed conditions (Solhaug and Gauslaa, 2012). The more widespread occurrence of secondary metabolites may be because they can play other roles in lichen biology e.g. as a result of their antibiotic properties (Molnár and Farkas, 2010). Another reason for the more widespread occurrence of secondary metabolites rather than melanins may be that melanisation can increase thalli temperature by up to 3 °C (McEvoy et al., 2007), possibly inducing heat stress. Secondary metabolites reflect rather than absorb PAR, and therefore have less effect on the heat balance of lichens. However, more work is needed to understand the relative advantages and disadvantages of melanins and secondary metabolites as sun screens in lichens.

Lichens and thermoregulation

There is some evidence that in free-living fungi melanins may protect lichens against heat stress. Examples include melanin-deficient mutants of *Monilinia fructicola* producing conidia that are more susceptible to high temperatures (Rehnstrom and Free, 1996) and in *Cryptococcus neoformans*, melanisation increasing tolerance to heat and cold stress (Rosas and Casadevall, 1997). Exactly why melanin reduces temperature stress is unknown, although it has been suggested that it reduces stress-induced ROS formation (Cordero and Casadevall, 2017). It is currently unknown whether melanins increase the heat tolerance of lichens.

It is important to distinguish the effects of temperature stress, and the effect of high light levels on thallus temperature. As discussed above, melanins are very effective at absorbing solar radiation and dissipating it “radiationlessly” in the form of heat. While this may reduce

ROS formation in the fungus, and shade the algae from high levels of PAR, a potential disadvantage of melanisation is that it increases thalli temperature

Oxidative Stress

A common property of all biological pigments is their ability to accept and neutralize exogenous free radicals (McGraw, 2005). Melanins are powerful antioxidants. While no data is available on lichens, in free-living fungi they contribute to virulence by interfering with host defence factors, e.g. by neutralizing the oxidative burst of phagocytic cells (Schnitzler et al., 1999). Fungal melanins can also protect from hypochlorite, permanganate and hydrogen peroxide (Jacobson et al., 1995). However, it is perhaps unlikely that mycobiont melanins can scavenge ROS from a photobiont. In future experiments, the effects of ROS generating agents on mycobiont and photobiont health in melanised and non-melanised thalli should be tested.

Protection against desiccation

Arguably, what makes lichens special, and what separates them from most other eukaryotic organisms, is their ability to tolerate desiccation, and then rapidly recover activity when rewetted (Beckett et al., 2008). Surprisingly, there has been no attempt to determine whether melanins contribute to desiccation tolerance in lichens. In the free-living fungus *Cenococcum geophilum*, inhibiting melanin synthesis increases susceptibility to osmotic stress and desiccation (Fernandez and Koide, 2014). Presumably, melanins increase desiccation tolerance by scavenging desiccation-induced ROS as discussed above, toughening cell walls, or by reducing the rate of drying by decreasing cell wall porosity. In lichens, melanins probably have trivial effect on desiccation tolerance in the photobiont. However, given our knowledge of free-living fungi, and the correlation of melanisation in fungi and lichens growing in extreme habitats (Gostincar et al., 2012), future studies should test the effect of melanisation on desiccation tolerance of the mycobiont.

Protection against grazing

A wide range of invertebrates are known to feed on lichens (Gerson and Seaward, 1977). It is often considered that the main herbivore deterrents in lichens are the classic lichen substances such as usnic acid and atranorin (Solhaug and Gauslaa, 2012). However, in free-living fungi, melanins can deter insect grazing by grazing. Scheu and Simmerling (2004) fed melanised (wild type) and pale mycelia of the ascomycete *Aspergillus fumigatus* to Collembola ("Springtails"). Growth and reproduction of the Collembola were low when feeding on the wild type but high when feeding on the melanin-deficient form, showing that melanin synthesis strongly affects the food quality of fungi for fungal feeding invertebrates. Collembola are also well known to graze lichens (Hale, 1972), and although not tested it seems likely that melanised lichen thallus will also be of lower quality than unmelanised. However, it remains unknown whether melanisation has any effect on the lichen grazing preference of lichens, or whether grazing stimulates melanin synthesis in lichens. More work is needed to assess the importance of melanisation in grazing resistance in lichens.

Protection against pathogen attack

The importance for melanisation in the resistance of lichens to attack by microbial pathogens is unknown. Recent research has shown that lichens are far more than a single fungus and a single cyanobacterium or green algae living together. Rather, it is better to view lichens as a micro-ecosystem inhabited by a variety of microorganisms in addition to the main photo- or mycobionts (Grube and Wedin, 2016). For example, Grube et al. (2015) reported that the lichen *Lobaria pulmonaria* contains 800 species of bacteria. Some bacteria and fungi that are regularly found on lichens appear to be beneficial, while others are harmful. There appear to have been no comparisons between the microbial flora of melanised and pale lichens. Also, it is unknown whether melanisation has any effect on the susceptibility of lichens to fungal or bacterial attack, or whether attack by pathogens stimulates melanin synthesis in lichens. However, studies carried out with free-living fungi suggests that melanisation may increase the resistance of lichen fungi. For example, melanisation increases the resistance of cell walls to hydrolytic enzymes capable of digesting the cell wall component (Potgieter and Alexander, 1966; Bloomfield and Alexander, 1967; Kuo and Alexander, 1967). This implication is that if potentially harmful microorganisms are secreting hydrolytic enzymes, then melanised hyphae will be more resistant. Melanisation is also known to increase mechanical strength of fungal

cells walls (Nosanchuk et al., 2015), and will therefore increase the resistance of hyphae to e.g. physical attack by penetration pegs from pathogenic fungi. Clearly, more work is needed to assess the importance of melanisation in pathogen resistance in lichens.

Tolerance to heavy metals

Given the aromatic composition of fungal melanins, it is not surprising that they can form molecular interactions with many metals (Cordero and Casadevall, 2017). In free-living fungi, it has therefore been suggested that melanins are involved in heavy metal tolerance. Melanins exhibit both high binding affinity and capacity to many different metal ions. Metal binding involves interactions with carboxyl, amine, and hydroxyl functional groups of the pigment. Mg, Ca, and Zn, are coordinated preferentially by carboxyl, Cu by hydroxyl groups and iron by hydroxyl, amine, imine and acetate groups. It is likely that melanins are responsible for binding metals in lichens. For example, Williamson et al. (2004) used X-ray element mapping across a lichen-rock interface to show that in the lichen *Trapelia involuta* melanins are the likely molecules responsible for binding U, Fe, and Cu. However, there are no studies that show melanised lichens are more common on metal rich sites, or that metals can induce melanin biosynthesis. While Spagnuolo et al. (2011) found that lichens suspended in a metal polluted area became melanised, this was more likely caused by exposure to high light. In addition, Siletti et al. (2017) showed that melanins tend to prevent fungal growth by scavenging heavy metals. Therefore, the significance for melanins in heavy metal tolerance requires further investigation.

Introduction to the study

The aims of the present study were as follows. First, an attempt was made to determine which kinds of melanins lichens contain. It was hypothesised that Peltigeralean lichens may synthesize high N-containing eumelanins because their photobionts are cyanobacterial, and they therefore having an excess of nitrogen from atmospheric nitrogen fixation. Other species may need to synthesize low N-containing allomelanin-like pigments. The second aim was to confirm that melanins do protect lichens from the high light stress. A range of lichens were

surveyed, comparing the sensitivity to high light of pale and melanised thalli from the same species growing close to each other. It was predicted that melanic thalli should be much more resistant to short term photoinhibition than pale thalli.

Finally, we tested the "cost" of melanisation in *Lobaria*. While melanisation screen light, offering photoprotection, it was hypothesised that the efficiency of photosynthesis in melanised thalli could be reduced. The implication of this would be that melanised lichens would be at a significantly disadvantage if light levels return to lower values, more typical for those habitats in which this shade adapted lichen is most abundant. The overall aim of the work described here was to increase our understanding of the role of melanins in lichen biology.

CHAPTER 2: GENERAL MATERIALS AND METHODS

The general methods and techniques used for the collection and preparation of lichens, measuring of photosynthetic performance, and quantification of scytonemin are detailed below. Specific details on experiments carried out are given in the relevant Chapters.

2.1 Lichen species and collection

Lichen material was collected at various locations between June 2015 and May 2017. Only healthy thalli were selected for all experiments. Material of *Lobaria pulmonaria* was stored at -18 ℃ for *c.* 4 months before use. All the other lichen material was kept at – 20 ℃, typically for up to two weeks, until needed. Table 2.1 illustrates the description of study species and collection locality.

Table 2.1: Location and brief description of study lichens. SA-South Africa.

Taxonomic Order	**Species**	**Collection locality**
Lecanorales	*Cetraria islandica* (L.) Willd	Langangen, Norway
Lecanorales	*Parmelia cetrarioides*	Fort Nottingham reserve, SA
Peltigerales	*Leptogium furfuraceum* (Harm) Sierk.	Monks Cowl, Fort Nottingham reserve, SA
Peltigerales	*Lobaria pulmonaria* (L.) Hoffm.	Langangen, Norway
Peltigerales	*Lobaria retigera* (Bory) Trevis	Monks Cowl, SA
Peltigerales	*Peltigera aphthosa (L.) Ach*	Ås, Norway
Peltigerales	*Peltigera membranaceae* (Ach) Nyl	Ås, Norway
Peltigerales	*Pseudocyphellaria aurata* (Ach) Vein.	Monks Cowl, Fort Nottingham reserve, SA
Peltigerales	*Pseudocyphellaria gilva* (Ach) Malme	Monks Cowl, Fort Nottingham reserve, SA
Peltigerales	*Sticta sublimbata* (J. Steiner) Swinscow and Krog	Fort Nottingham reserve, SA
Teloschistales	*Heterodermia speciosa* (Wulfen) Trevis.	Fort Nottingham reserve, SA

Figure 2.1a: Species used in this study. (A) *Cetraria islandica* (B) *Heterodermia speciosa* (C) *Leptogium furfuraceum* (D) *Lobaria pulmonaria* (E) *Lobaria retigera* and F) *Parmelia cetrarioides* collected from various locations. Copyright reserved (Kok van Herk, Adrea Aptroot, Mike, Papp Beata, Lokos Laszlo and Stephen Sharnoff).

Figure 2.1b: Species used in this study (continued). (G) *Peltigera aphthosa* (H) *Peltigera membranaceae* (I) *Pseudocyphellaria aurata* (J) *Pseudocyphellaria gilva* and (K) *Sticta sublimbata.* Copyright reserved (Kok van Herk, Adrea Aptroot and Stephen Sharnoff).

2.2 Induction of melanin

Induction of melanin was attempted at two localities. The first locality was the Botanical Gardens, UKZN, Pietermaritzburg for a period of 3 to 6 weeks, as shown in Fig. 2.2. Initially, to acclimate lichens to high light, thalli (2-8 g) were exposed to 50% of ambient light for two weeks and then moved to 70% ambient light. In total, 120 thalli were used; 15 thalli were attached in each frame and each treatment consisted of two frames; in addition, thalli were kept in the freezer as a control. In a second experiment, thalli were treated with different light intensities in an open site where they were exposed to full sunlight, with no filter, placed beneath a neutral acrylic screen to test for the effect of the physical presence of the screen (+UV, 1.82 x 0.72 m, 3 mm thick), and beneath a polycarbonate screen (-UV, 1 x 1 m, 3 mm thick). In both experiments, the screens were placed 5 cm above the wooden frames where thalli were attached. The lichens were attached to a mesh net on the frames (47.5 x 54.5 cm) with black cotton thread.

At the second locality, thalli of *Lobaria pulmonaria* were placed in an open site in Ås, Norway for four weeks to induce melanic pigments. Whole thalli (N=15) for both melanic, (exposed to open site) and non-melanic thalli (kept in the freezer, control) were attached with a cotton thread to wooden frames (15.9 × 15.9 cm^2) covered with nylon mesh netting. In experiments conducted in Pietermaritzburg, thalli were hydrated daily with distilled water before sunrise and after sunset. In Ås, thalli were sprayed with deionized water every morning at about 9:00 a.m. and evening after sunset. Thalli were not watered on rainy days. In all experiments, replicate thalli were randomly attached in each frame and the treatments were randomized. The thalli were covered with a thin mesh wire to deter birds. The control treatment was kept in the freezer.

Figure 2.2: Experiment setup beneath normal light (control, no screen put above material), a +UV acrylic screen and a -UV polycarbonate screen to induce melanins.

2.3 Quantification of lichen substances

2.3.1 Melanins

Thalli (1 g) were cleaned and placed in 25 ml Beckman centrifuge tubes. Exactly 20 ml 2 M NaOH was added, tubes left at room temperature overnight and then centrifuged twice for 20 min at 5000 x *g* (Beckman Coulter, Avanti® J. E, centrifuge) and the pellet was discarded. The pH of the supernatant was reduced to pH 1 using 5 M HCl and melanins left to precipitate overnight. When acid precipitation was unsuccessful, melanins were precipitated using 0.1% $FeCl_3$. In both cases, tubes were centrifuged at 5000 x *g* for 20 min and the supernatant discarded. The pellets were then washed successively with 5 ml of distilled water, chloroform, ethyl acetate and acetone. The pellets were dried at 60 °C and then for the melanins that did not require $FeCl_3$, 15 - 25 mg re-dissolved in 1 ml DMSO. The method was conducted according to Ellis and Griffiths (1974) with modifications.

2.3.2 Quantification of scytonemin using HPLC

Scytonemin was quantified on a 1200 Series HPLC with a 1040 M diode-array detector and a fraction collector G1364C (Agilent Technologies, Waldbronn, Germany) according to Garcia-Pichel et al. (1992) and Büdel et al. (1997a) with modifications . Approximately 10 - 20 mg of fine powder of *L. retigera* was ground with a ball mill (Retsch model MM400, Retsch GmbH Hann, Germany), and then transferred to an Eppendorf tube with 1.5 ml acetone. Subsequently, the samples were sonicated (VWR, ultrasonic cleaner) for 30 min at 30 ℃, centrifuged (Epperndorf, Centrifuge 5417 C) at 5000 x *g* for 3 min and 1 ml of supernatant collected. Then, 1.5 ml acetone added to the supernatant, sonicated for 15 min and centrifuged. The extracts were left to dry in a vacuum (Eppendorf, Concentrator plus) for 45 min at 30 ℃, re-dissolved with 1 ml acetone, sonicated for 3 min, and then filtered using a syringe filter (13 mm, VWR International, (w/0.45 µm) PTFE membrane) with a 2 ml syringe (BD Plastipak™) into 1.5 ml pill vials. Samples (20 µl) were injected into the HPLC. Scytonemin was eluted by a binary gradient system of degassed solvents and was observed at 385 nm. Separation was achieved on an ODS Hypersil 4.6250 mm column (Agilent Technologies). Absorption spectra were recorded between 350 and 600 nm on the HPLC-separated peaks. Solvent A was distilled water, whereas solvent B consisted of 75% acetonitrile, 15% methanol and 10% tetrahydrofuran. The run started with 70% B for 11 min before increasing solvent B to 100% for 15 min. At the end of the run, solvent B was reduced to 85% within 1 min and the column flushed with 15% B for 5 min before the next run. A standard scytonemin solution collected from *Collema* was prepared using the fraction collector to collect the scytonemin peak from five HPLC runs with 50 µl injected each time. The scytonemin concentration was measured with a Shimadzu UV2001 PC spectrophotometer using the extinction coefficient 112.6 L g^{-1} cm^{-1} at 384 nm.

2.4 Determination of absorption spectra and nitrogen content

Melanins from Peltigeralean and non-Peltigeralean species (*Lobaria retigera*, *Pseudocyphellaria aurata*, *Heterodermia speciosa* and *Parmelia cetraioides*) were prepared as per section 2.3.1. To determine the absorption spectra for melanins from Peltigeralean lichens, 15- 25 mg pellets were re-dissolved in 1 ml DMSO. The absorbance was read over 250 and 700 nm (Cary 60 UV-Vis, Agilent Technologies). The measured spectra was compared to that of standard melanin published by Meeßen et al. (2013). To determine the C: N ratio of

the samples, three replicates of 0.6 - 1 mg were then packed in a foil and analyzed using the vario MICRO cube Elementar.

2.5 Measurements of Photosynthesis

Comparisons were made between pale (non-melanic) and melanic thalli at different light intensities. A few randomly selected thalli are shown below.

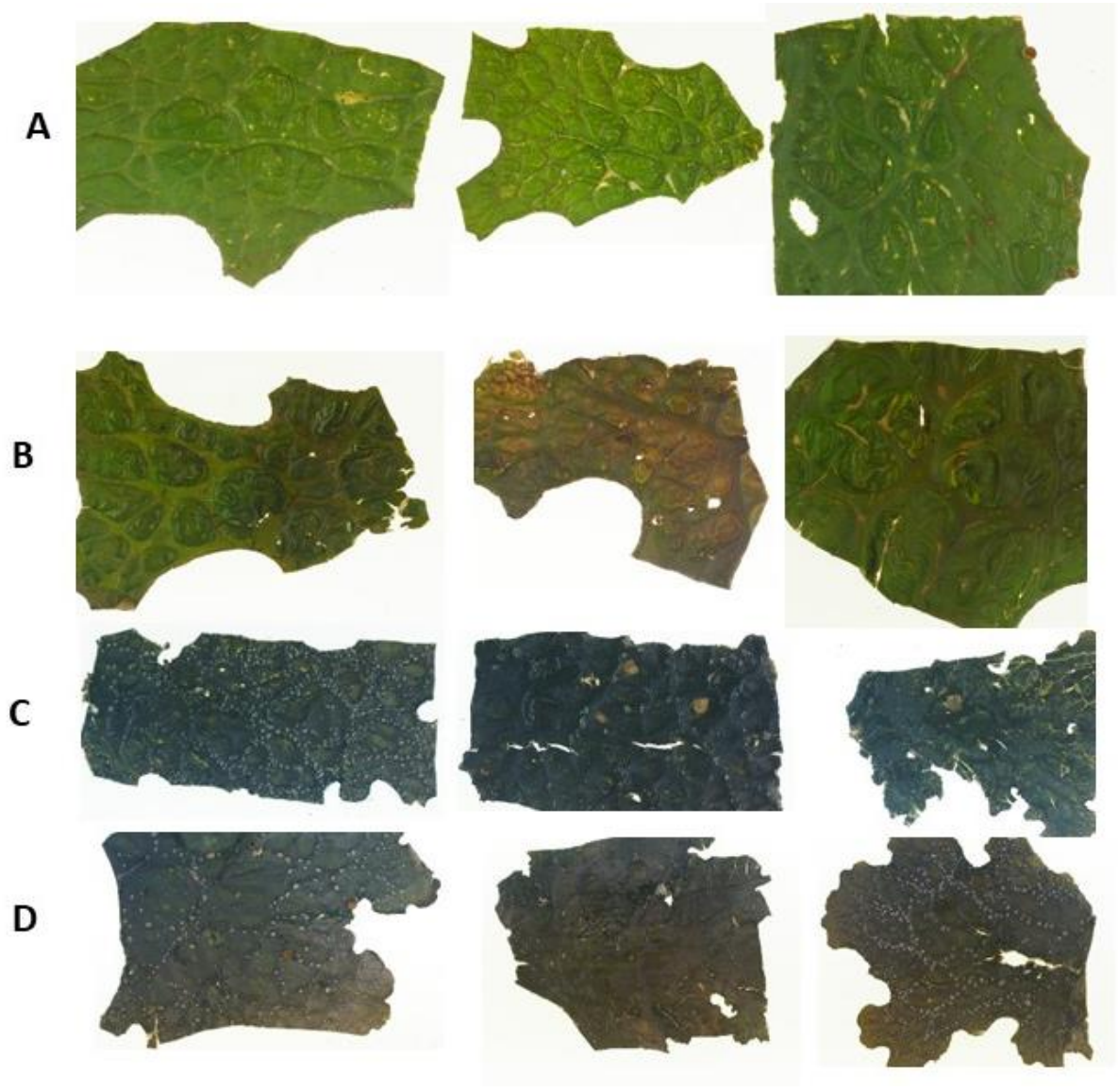

Figure 2.3: Differences in non-melanic (row A, C) and melanic (row B, D) thalli of *Lobaria pulmonaria* (A, B) and *Lobaria retigera* (C, D).

2.5.1 Gas-exchange measurements

Before all photosynthetic measurements, thalli were moistened with distilled water and acclimated at 15 °C under low light (*c.* 10 µmol m^{-2} s^{-1}) until the next day. Photosynthetic O_2 evolution was measured using an O_2 electrode chamber (model LD2, Hansatech, King's Lynn, Norfolk, UK) with a red LED light source (model LH36/2R, Hansatech) at 20 °C and 5% CO_2. Photosynthetic CO_2 uptake was measured with a portable infrared gas-analyzer (LI-6400XT, LiCor, Lincoln, NE, USA) with a LiCor 6400-24 bryophyte chamber and a LI-6400-18 RGB LED light source. In all gas-exchange measurements white light, derived from a mixture of red, green and blue LEDs was used. Before measurement, thalli were acclimated for 10 min at room temperature at 200 µmol m^{-2} s^{-1} from an LED light-source panel (Model SL-3500, Photon System Instruments, Brno, Czech Republic) with equal irradiances from blue, green and red light. They were then fully hydrated by spraying with distilled water and blotted with filter paper to remove superficial water. CO_2 uptake was measured at 200 µmol m^{-2} s^{-1} until stable and optimal thallus water contents were reached (assessed by typical maximal rates of photosynthesis). Unless indicated otherwise, measurements were made at a flow rate of 200 ml min^{-1} of 400 ppm CO_2 at a light level of 200 photons m^{-2} s^{-1}.

2.5.2 The effect of temperature and thallus water content on photosynthetic CO_2 uptake

Thalli were equilibrated for 10 min after each change in temperature before measurements were taken. Rates of photosynthesis at 15, 20, 25 and 30 °C were 2.11 ± 0.75, 1.68 ± 0.54, 1.63 ± 0.50 and 1.46 ± 0.45 µmol CO_2 m^{-2} s^{-1} (n = 5). While the optimum temperature for carbon fixation was thus 15 °C, the temperature inside the IRGA cuvettes was much easier to control at 20 °C and rates were only slightly lower than those at 15 °C. Furthermore, occasional condensation occurred in the leaf chamber at 15 °C. Therefore, all subsequent carbon fixation measurements were carried out at 20 °C. To measure the effect of thallus water content on photosynthesis, thoroughly wetted thalli (*c*. 4 cm^2) were blotted to remove excess water, weighed to obtain the maximum water content, and then put into the IRGA cuvette. After stabilization, three measurements were made at 10 s intervals, and then thalli removed from the cuvette and again weighed. This was repeated until the thalli displayed no net photosynthesis, at which point they were dried overnight at 70 °C and weighed. The

relative water contents (RWC) of the thalli at each sampling interval were calculated as described by Beckett (1995).

2.5.3 Determination of the light response curves of photosynthetic CO_2 uptake

For O_2 production, each thallus was exposed to irregular increasing irradiances in steps of 1 min from 0 to 500 µmol photons m^{-2} s^{-1}, and O_2 production measured during the last 30 s of each step. For CO_2 uptake, thalli were placed into the cuvette, and when net photosynthesis (A) was stable at 200 µmol photons m^{-2} s^{-1}, they were exposed to light levels of 0, 25, 50, 75, 100, 200, 400 and 600 µmol photons m^{-2} s^{-1}. CO_2 uptake was logged when A was stable, typically about 2 - 3 min after changing the light level. Quantum yield (QY) of CO_2 uptake was estimated with the Photosynthesis Assistant (Dundee Scientific Ltd., Scotland, UK) software.

2.5.4 Chlorophyll fluorescence measurements (for comparison of pale and melanised thalli)

Chlorophyll fluorescence was measured using the red version of the Imaging PAM fluorimeter (Walz, Effeltrich, Germany). To determine the effect of light level on ETR, six thalli, three controls and three melanised, were measured simultaneously. Thalli were hydrated for *c*. 24 h in dim light (10 µmol photons m^{-2} s^{-1}) and then acclimated for 5 to 10 min at 200 µmol photons m^{-2} s^{-1} red light from an LED panel (Model SL-3500, Photon System Instruments, Brno, Czech Republic). Rapid light response curves of ETR were then measured by increasing the actinic light in 14 small steps over 7 min from 0 to 400 µmol photons m^{-2} s^{-1} with saturating flashes at the end of exposure to each light level. ETR was calculated as:

$$ETR = \emptyset \text{ PSII} \times \text{light intensity} \times 0.5$$

To determine maximal efficiency of PSII, (Fv/Fm), and for quenching analysis, thalli were initially dark-adapted at room temperature for 10 min. At the start of a run, a saturating flash was applied to determine Fv/Fm. An actinic light of 186 µmol photons m^{-2} s^{-1} was then turned on, and saturating flashes was applied every 30 s for 12 min until fluorescence yields had

stabilized. The actinic light was then turned off, and relaxation measured for 15 min with saturating flashes given at increasing intervals. NPQ was calculated after 12 min using the formula suggested by Bilger et al. (1995):

NPQ = (Fm - F'm)/F'm

2.5.5 Determination of chlorophyll content

Chlorophylls were extracted as described by Palmqvist et al. (1998). All steps were done under dim light. Dry thalli (10 - 20 mg) were homogenized in a ball mill (Retsch model MM400, Retsch GmbH Hann, Germany). Weighed samples were extracted with 1.5 ml DMSO in an Eppendorf tube. The Eppendorf tube was then sonicated in a VWR Ultrasonic water bath (Ultrasonic cleaner, USC 200TH) at 60 ℃ for 40 min and then centrifuged at 16 400 x *g* for 3 min. The absorbance of the supernatant was then measured. The chlorophyll content was determined using the formulae of Wellburn (1994).

2.5.6 Determination of xanthophyll cycle (VAZ) pigments

Thalli were stored at -80 ℃ until extraction, and then 50 - 100 mg put into 1.5 ml of DMSO in Eppendorf tubes and extracted for 48 h in darkness. The solution was filtered and analyzed by HPLC (Agilent 1100 HPLC system, Diode array detector) with an ODS Hypersil 250 x 4.6 mm column according to the method of Niinemets et al. (1998). Standards were made by separating chloroplast pigments on TLC. Violaxanthin was scraped off the TLC plate, dissolved in ethanol, and the concentration of measured spectrophotometrically as described by (Hager and Meyer-Bertenrath, 1966) This pure violaxanthin extract was used as a standard in the HPLC for the other xanthophylls because violaxanthin, antheraxanthin and zeaxanthin have very similar extinction coefficients (Hager and Meyer-Bertenrath, 1966).

2.6 Measurement of the photoinhibitory response

2.6.1 Sample preparation

A day before each experiment, 2 cm disks were cut, or for small thalli, thallus fragments selected. Lichens were stored overnight on wet filter paper in petri dishes under dim lighting (5 µmoles m^{-2} s^{-1}) at a cool temperature (15 °C, thermostatically controlled room). Before high light treatment, chlorophyll fluorescence parameters were measured, and then material either slowly dried for 4 h, or used immediately.

2.6.2 Chlorophyll fluorescence measurements (for assessing effects of photoinhibition)

Chlorophyll fluorescence was measured using the red version of the PAM 2000 fluorometer (Walz, Effeltrich, Germany) in Norway and the FMS2 chlorophyll fluorometer in Pietermaritzburg (Hansatech instruments, King's Lynn, England). To determine maximal efficiency of PSII, (Fv/Fm) and ϕPSII or ETR, thalli were initially dark-adapted at room temperature for 10 min. At the start of a run, a saturating flash was applied to determine Fv/Fm. Thalli were then exposed to PAR at 30 µmoles m^{-2} s^{-1} for 10 min, and then another saturating flash applied to estimate ϕPSII.

2.6.3 High light stress

High light stress was given using a white LED light-source panel (Model SL-3500, Photon System Instruments, Brno, Czech Republic). The light was given at 800 - 1000 µmoles m^{-2} s^{-1} until the maximum quantum yield of PSII photochemistry (Fv / Fm) was reduced from values of around 0.5 - 0.7 to 0.2 - 0.4, with each species needing different times.

Chlorophyll fluorescence measurements were made in moist material before high light treatment ("time zero"), at the end of the high light treatment, and then at intervals for up to 24 h. Thalli exposed to high light in the dry state were rapidly hydrated following exposure by placing them on moist filter paper and spraying with distilled water. During recovery, lichens were maintained at room temperature (20 °C) and dim lighting (5 µmoles m^{-2} s^{-1}) as recommended by Gauslaa et al. (2017).

2.7 Reflectance measurements to estimate melanin content

Reflectance spectra of the upper cortex were recorded on dry thalli as described by Solhaug et al. (2003). Briefly, an integrating sphere (model ISP-50-REFL OceanOptics, NL-6961 LL Eerbeek, The Netherlands) was pressed against the thalli, and the thalli then illuminated by a halogen lamp (model DH2000, OceanOptics) through a 600 µm optical fibre (thick) connected to the input port of the integrating sphere. Reflectance (400 - 1050 nm) was measured with a spectrometer (model SD2000, OceanOptics) connected to the output port of the sphere with a 400 µm fibre. Reflection was calculated relative to a reference spectrum derived from a white reference tile (WS-2, OceanOptics). BRI, calculated as (1/R550 - 1/R700)/R750) (Chivkunova et al., 2001), was used as a quantitative estimate of melanic compounds.

2.8 Statistical analysis

The best-fit curve for the effect of relative water content on photosynthesis were determined using SigmaPlot version 11. Quantum yields of photosynthetic CO_2 uptake and O_2 evolution were estimated with the software Photosyn Assistant version 1.1, Dundee Scientific. Light response curves for O_2 evolution were also fitted with Photosyn Assistant. All contrasts between melanised and control thalli were tested with ANOVA. The rest of the experiments were statistically analysed using regression analysis and t-tests. The statistics were performed using IBM SPSS statistic v24.

CHAPTER 3: THE EFFECT OF THE VARIOUS LIGHT REGIMES ON THE INDUCTION OF MELANINS AND THE STUDY OF MELANIN PROPERTIES

3.1 Introduction

The structure and roles of fungal melanins were reviewed in Chapter 1. Perhaps the only specifically lichen melanin to be investigated in detail is that of *Lobaria pulmonaria* (Matee et al., 2016). As discussed in Chapter 1, *L. pulmonaria* possesses a chlorophycean photobiont with cyanobacterial cephalodia, and melanin is synthesized by the mycobiont, mainly in response to UVB. Chemical analysis of purified melanin from the species strongly indicated that it was "eumelanin". The aim of the experiments described in this chapter was to test if cyanobacterial and green algal lichens possess different types of melanins, specifically eumelanins (derived from 3, 4- dihydroxyphenylalanine) and allomelanins (derived from 1, 8-dihydroxynaphthalene) respectively. The properties of lichen melanins were compared to those from free-living fungi. The ability of various light regimes to induce melanins in several lichens were also tested. Lastly, the presence of scytonemin, a compound that screens UV, was tested (Büdel et al., 1997b).

3.2 Methods

3.2.1 Sample preparation to determine melanin properties

Melanised material of *Leptogium furfuraceum*, *Pseudocyphellaria gilva*, *Heterodermia speciosa* and *Parmelia cetrarioides* were collected directly from their natural habitat at Fort Nottingham and Monks Cowl, KwaZulu Natal. A fresh mass of 1 g was extracted for melanins as described by Ellis and Griffiths (1974) with slight modifications as described in Chapter 2. In *Lobaria retigera* and *Pseudocyphellaria aurata*, melanins were induced in a transplant

experiment as described below. The nitrogen content was determined after the melanins were extracted, using three replicates of 0.6-1 mg in the vario MICRO cube Elementar. Furthermore, samples were dissolved in DMSO and the absorption spectrum measured according to Meeßen et al. (2013).

3.2.2 Transplant experiment to induce melanin

The aim of the experiment was to acclimatize the lichens *Pseudocyphellaria aurata* and *Lobaria retigera* to high light conditions to induce melanin as described in Chapter 2, section 2.2. Initially, 20 whole thalli were randomly selected. The thalli were fastened with cotton to wooden frames (two replicate frames for each treatment) covered with a nylon mesh net. Each frame (47.5 x 54.5 cm) had 10 thalli attached. The thalli were initially exposed to 50% ambient light and then 70% ambient light under shade cloth. For both *P. aurata* and *L. retigera*, thalli were exposed for three weeks to 50% ambient light and for six weeks to 70% ambient light. Before moving the material to 70% ambient light, chlorophyll fluorescence was measured. In a second experiment, new thalli of *Lobaria retigera* were prepared as described above. The thalli were exposed to full sunlight (normal ambient light), beneath a +UV acrylic screen and beneath a -UV polycarbonate screen for three weeks. In both experiments, the control treatment were thalli kept in a freezer at -20 ℃. Lichens were sprayed daily in the mornings and evenings with distilled water, except on rainy days. Measurements of chlorophyll fluorescence parameters were taken at the start and at the end of the experiments. Thereafter, scytonemin was quantified as explained in Chapter 2, 2.4.2.

3.3 Results

3.3.1 Properties of melanin

The properties of melanins from the lichens screened are given in Table 3.1. The melanins in Peltigeralean lichens were synthesized along the thalli margins and lichen body while in non-Peltigeralean lichens, the lower cortex and cilia were pigmented. In Peltigeralean lichens, melanins were precipitated by the addition of HCl, while *Heterodermia speciosa* and *Parmelia cetrarioides* precipitation required $FeCl_3$. Only melanins of the Peltigeralean group could be re-dissolved in DMSO. The melanins from *Leptogium furfuraceum* and *Pseudocyphellaria aurata* absorbed light strongly from 280 to 380 nm (Figure 3.1).

Species	Pigmentation Location	Precipitate solution	Solubility (d.H_2O, chloroform, ethyl acetate acetone)	Pigment colour
Peltigerales				
Leptogium furfuraceum	lichen body	HCl	insoluble	pale green
Lobaria retigera	Marginal	HCl	insoluble	dark green
Pseudocyphellaria aurata	Marginal	HCl	insoluble	green
Pseudocyphellaria gilva	lichen body	HCl	insoluble	brown
Non-Peltigerales				
Heterodermia speciosa	Cilia	$FeCl_3$	insoluble	brown/ black
Parmelia cetrarioides	lower cortex	$FeCl_3$	insoluble	brown/ black

Table 3.1: General properties of melanised Peltigeralean and non-Peltigeralean lichens.

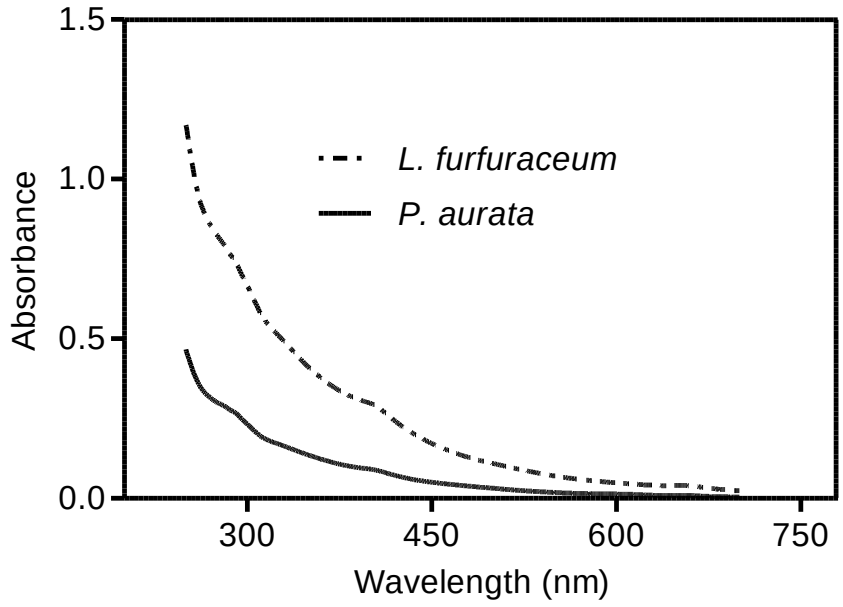

Figure 3.1: Absorption spectra (250 nm – 700 nm) of melanins extracted from *Pseudocyphellaria aurata* and *Leptogium furfuraceum*.

3.3.2 The induction of melanin

Table 3.2 gives the visual observations recorded during the experiment. Thalli of *L. retigera* melanised slowly, although some thalli seemed to experience photooxidation as they melanised. Increasing the light intensity to 70% of ambient light induced melanisation in *P. aurata* after four weeks.

Table 3.2: Morphological changes observed in *Pseudocyphellaria aurata* and *Lobaria retigera* during exposure to different light intensities. Light treatments were: 50% ambient light, 70% ambient light, full sunlight (normal ambient light), -UV (lichens placed below a polycarbonate screen) and a +UV (lichens placed beneath an acrylic screen).

Species	**Light treatment**	**Period of exposure (weeks)**	**Observation**
P. aurata			
Experiment 1	50%	0 - 3	No changes
	70%	0 - 4	Slow browning of the thalli margins, thalli appeared healthy
		5 - 6	Thalli started to bleach
L. retigera			
Experiment 1	50%	0 - 3	Slow browning observed at centre of thalli body
	70%	0 - 5	Browning of the thalli margins, after fifth week 50 % of the thalli appeared damaged.
		6	80 % of the thalli damaged
Experiment 2			
	Full sun	0 - 3	60 % of the thalli appeared brown, 40 % bleached
	+UV	0 - 3	Rapid browning of the thalli margins, thalli appeared healthy
	-UV	0 - 3	Browning started after the second week

Exposure to 70% ambient light reduced ETR in *P. aurata*, while NPQ increased (Figure. 3.2a). In *L. retigera,* thalli exposed to 50% and 70% ambient light behaved similarly, however, at *c.* 320 µmol m^{-2} s^{-1}, ETR in material exposed to 70% ambient light increased (Figure 3.2b).

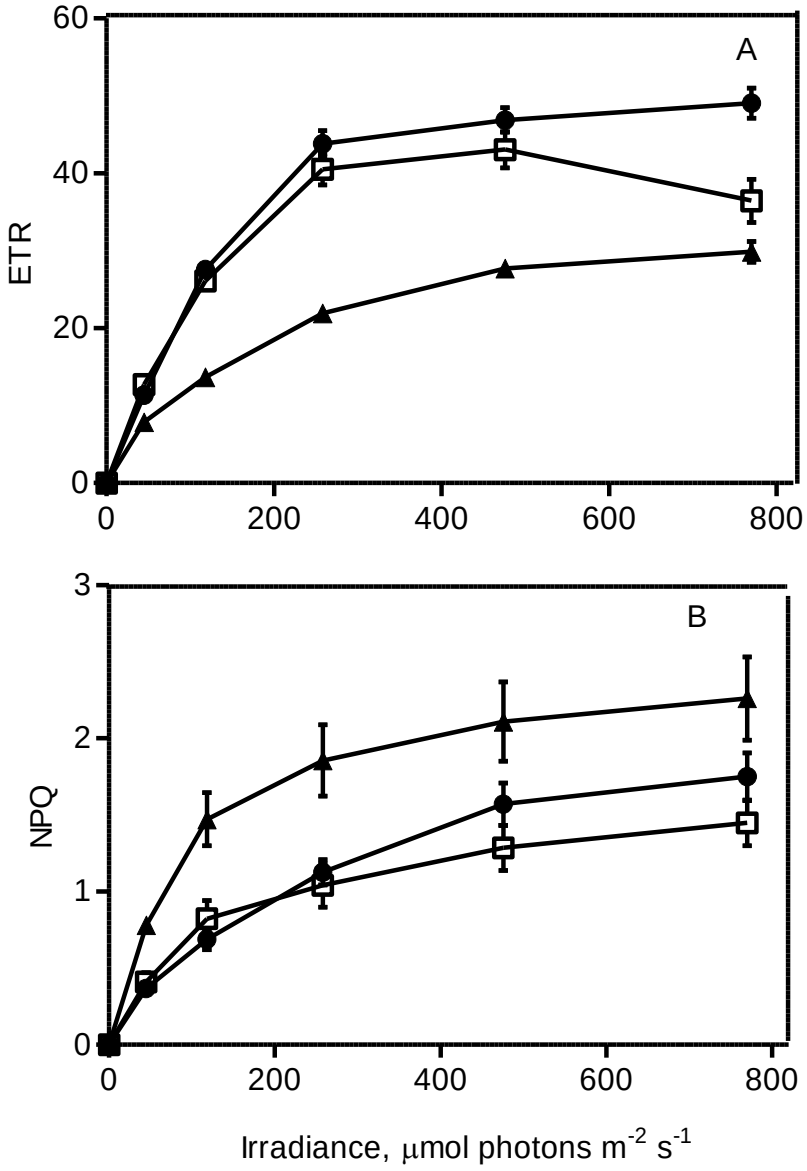

Figure 3.2a: The ETR and non-photochemical quenching (NPQ) of *Pseudocyphellaria aurata.* Values are given ±S.E., n =15. Treatments were the control (closed circle), 50% ambient light (open square) and 70% ambient light (closed triangle).

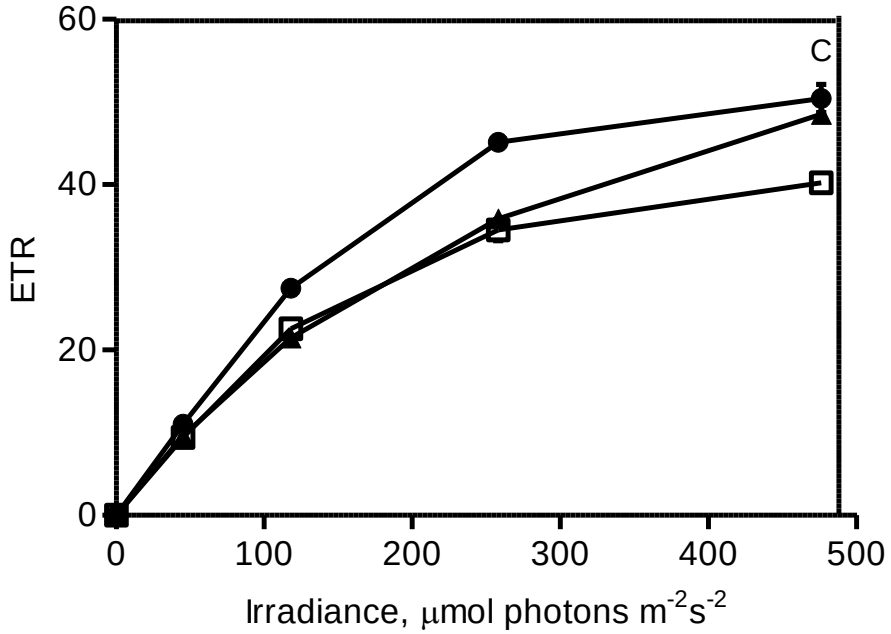

Figure 3.2b: The electron transport rate of *Lobaria retigera* at different light regimes. Values are given ±S.E., n =15. Treatments were the control (closed circle), 50% ambient light (open square) and 70% ambient light (closed triangle).

In the second experiment, exposure of *L. retigera* to different light regimes was studied. All treatments sharply reduced the ETR, however values for thalli beneath +UV ETR were slightly higher than -UV, Figure 3.3.

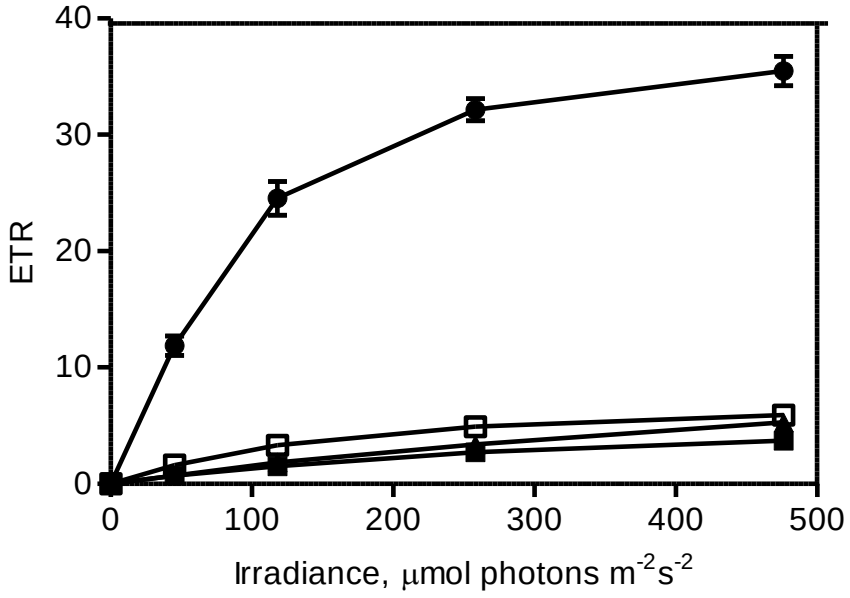

Figure 3.3: Electron transport rate of *Lobaria retigera* acclimatised at different light regimes. Values are given ±S.E., n =15. Treatments were the control with untreated thalli (closed circle), full sun (open square), beneath a screen that allowed +UV light through (closed triangle), and beneath a screen that blocked -UV light (closed square).

3.3.3 Determination of nitrogen content in lichen melanins

The melanins from Peltigeralean lichens had much higher nitrogen contents than those from non-Peltigeralean lichens (Table 3.3).

Table 3.3: Comparison of carbon to nitrogen content in the melanins from Peltigeralean and non-Peltigeralean lichens.

Species	C: N
Peltigerales	
Lobaria retigera	6.80
Pseudocyphellaria aurata	10.69
Non-Peltigerales	
Parmelia cetraioides	222.62
Heterodermia speciosa	155.30

3.3.4 Quantification of scytonemin in *Lobaria retigera*

Scytonemin was present in very low amounts in *L. retigera*. The HPLC only showed traces of scytonemin compared with extracts from the lichen *Collema*. During the test runs, there appeared copious amounts of chlorophyll and a few unknown lichen substances. The amount of scytonemin under a -UV polycarbonate screen was increased compared to when exposed to full sun. It was lowest when exposed to +UV acrylic screen.

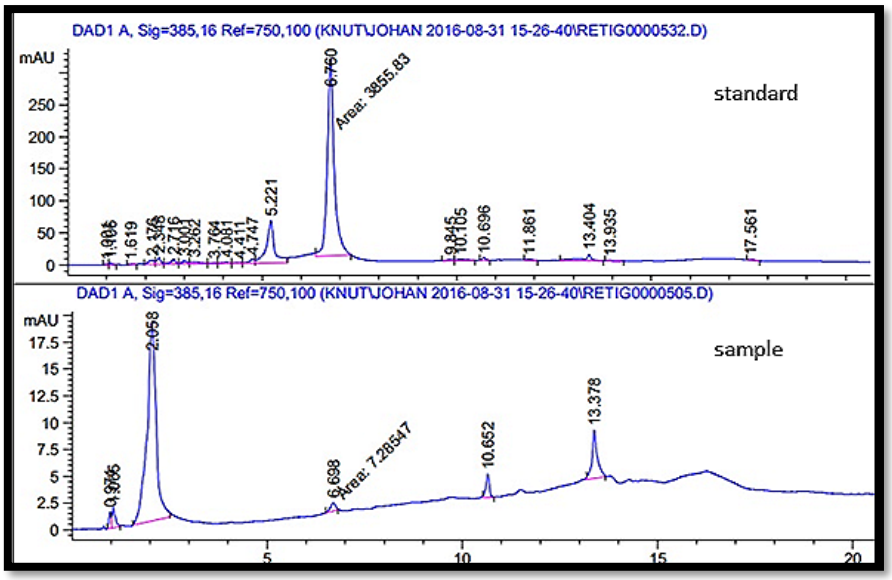

Figure 3.4: HPLC illustration of scytonemin content extracted from *Lobaria retigera* with a retention time of ± 6 min. The standard was prepared from a *Collema spp.* at 385 nm.

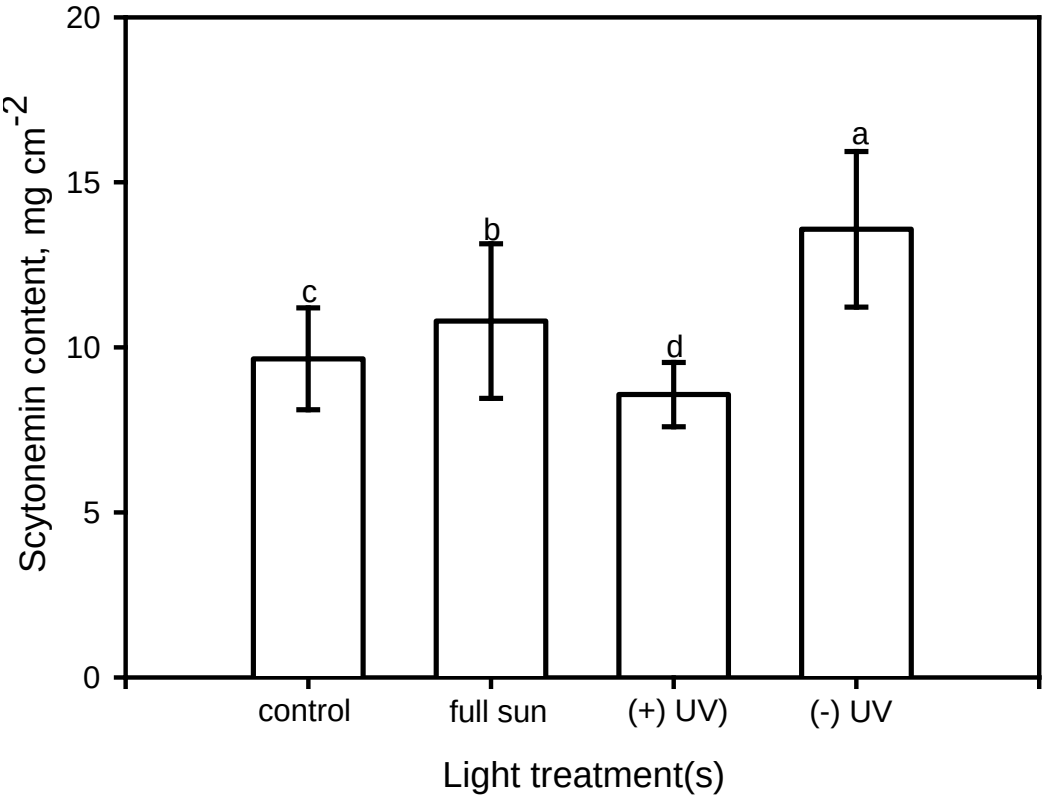

Figure 3.5: Scytonemin content in *Lobaria retigera.* Values are given ±S.E., n = 15. Treatments were the control (kept in the freezer), full sunlight (normal ambient light), a -UV polycarbonate screen and a +UV acrylic screen.

3.4 Discussion

Melanins prepared here were insoluble in organic solvents, except melanins from Peltigeralean lichens which solubilized only in DMSO (Table 3.1). The absorption spectra of these melanins showed that they absorbed light strongly from 280 to 380 nm, corresponding to the UVB and a portion of UVA wavelengths. The solubility and spectral properties of melanin are similar to those reported by Meeßen et al. (2013) from *Buellia frigida* and *Rhizocarpon geographicum*.

The ETR data from experiment 1 (Figure 3.2a, b) show that thalli were damaged by exposure to 50% and then 70% light, although for *P. aurata* NPQ did increase, possibly due to the synthesis of xanthophyll cycle pigments. The pool size of xanthophyll cycle pigments is often positively correlated with NPQ (Baker, 2008). Exposure of *L. retigera* to 70% light slightly reduced ETR (Figure 3.3). At this light intensity, *L. retigera* melanised more strongly than following exposure to 50% light. Success in inducing melanins here was probably a consequence of hydration in the morning and evening following the recommendations of Gauslaa and Solhaug (2001). In experiment 2, exposure of *L. retigera* to various light regimes all greatly reduced ETR (Figure 3.3). This is probably due to damage to the photosystem II, and suggests that alternative ways need to be found of studying the induction and effects of melanisation in this species.

The low C: N ratio of the melanins from the two Peltigeralean species studied here (*c.* 10:1) (Table 3.3) indicates that, as in humans, they were almost certainly synthesized from L-DOPA rather than hydroxylated naphthalene-derived molecules that produce “allomelanins” with a C: N ratio close to 100:1 (Loganathan and Kalyanasundaram, 1999; Solano, 2014). Results were consistent with those obtained by Matee et al. (2016) with *L. pulmonaria.* Matee et al. (2016) suggest that ability of Peltigeralean lichens to synthesise eumelanins is a result of their containing N_2-fixing cyanobacteria to boost the supply of N to the thalli. The much lower N contents of the melanins from the non-Peltigeralean species suggests that they were probably allomelanins, although more work is required to confirm this.

Only very small quantities of scytonemin were found in *L. retigera*, and it is clear that this lichen substance is not the main sunscreen (Figures 3.4 and 3.5). In some cyanobacteria, scytonemin does play a photoprotective role (Büdel et al., 1997b; Rastogi et al., 2013). Rather, it seems likely that melanin is the main photoprotectant, although to confirm this more work is needed. Interestingly, *L. retigera* has been reported to synthesize the secondary metabolite, thelephoric acid (Shukla et al., 2014), although its photoprotective role in lichens is not known.

3.5 Conclusion

It can be concluded that in South Africa the transplant method used was not a successful way of studying melanin induction. Chlorophyll fluorescence measurements showed that transplanting lichens often caused severe stress. Results contrasted with the similar experiment of Matee et al. (2016) and Chapter 4, where melanins were successfully induced in *Lobaria pulmonaria* in Norway, presumably because conditions were less harsh. However, in the South African studies some melanins synthesis was induced, and it was possible to study their properties. In the Peltigeralean species, melanins were extracted by alkaline solutions and precipitated by HCl, consistent with them being DOPA melanins. This was also supported by their high N content. The type of melanins possessed by non-Peltigeralean lichens remains unclear, although they could be DHN melanins.

CHAPTER 4: MELANISATION IN THE OLD FOREST LICHEN *LOBARIA PULMONARIA* (L) HOFFM. REDUCES THE EFFICIENCY OF PHOTOSYNTHESIS

4.1 Introduction

Lichens with classic lichen substances or melanic compounds often coexist in open habitats, and some lichens with atranorin or usnic acid such as *Cetraria* and *Cladonia* additionally produce melanic compounds under very sun-exposed conditions (Solhaug and Gauslaa, 2012). While lichens appear able to effectively protect themselves against high light, very little information is available on the resulting metabolic cost. There is some evidence that synthesis of classic lichen substances may reduce the efficiency of photosynthesis. In *X. parietina*, the quantum yield of photosynthetic O_2 evolution in blue light was lower in control thalli than in parietin-free thalli, although under red light the quantum yield did not differ between control and parietin-free thalli (Solhaug and Gauslaa, 1996). However, the cost to lichens of synthesising cortical melanins is unknown.

The aim of the work presented here was to study the cost of photo-protection by melanic pigments in the lichen *Lobaria pulmonaria*. The species has a wide distribution in Europe, Asia, North America and Africa, preferring damp habitats with high rainfall, especially in coastal areas (McCune and Geiser, 1997). *L. pulmonaria* normally grows in relatively shaded habitats. The average light level throughout the whole year for the habit where the *L. pulmonaria* grows can be as low as 14 µmol m^{-2} s^{-1}, although it is ten times higher on the exposed side of tree trunks (Gauslaa and Solhaug, 2000). However, at times lichens may be exposed to higher light levels, for example following leaf fall in autumn. Lichens will receive more light when openings in forests occur, for example because of tree fall, or where local conditions such as avalanches, poor soils, or fire damage create semi-permanent clearings. While *L. pulmonaria* upregulates NPQ and xanthophyll cycle pigments in response to increased exposure to PAR and UV (MacKenzie et al., 2002), the most important adaptation is probably the synthesis of cortical melanin pigments (Solhaug et al., 2003) Here, a variety of techniques were used to compare photosynthetic performance in melanised and nonmelanised thalli. We were particularly interested in studying the effect of melanisation on the efficiency of photosynthesis at lower light levels i.e. the “quantum yield.”

4.2 Materials and Methods

4.2.1 Lichen Material

L. pulmonaria was collected from the trunks of oak trees *c.* 5 m above the ground in an old oak forest at Langangen, Norway (59° 06 43 N, 9° 50 05 E, 140 m above sea level) in June 2015 and April 2016. After storage at -18 ℃ for *c.* 4 months, half of the thalli were placed in an open site in Ås for four weeks to induce melanic pigments. They were sprayed with distilled water on days without rain. The other half of the thalli remained in the freezer as controls. Non-melanic (control) and melanic thalli with ten replicates each, were randomized within the two groups before start of experiments.

4.2.2 Comparison of melanised and non-melanised thalli

Light response curves were measured with infra-red gas analysis, the O_2 electrode chamber, and chlorophyll fluorescence as described in Chapter 2. Initially, the effect of temperature and thallus water content on photosynthetic CO_2 uptake was determined to ensure optimal conditions were used. Reflectance spectra of the upper cortex (dry thalli), the chlorophyll content, and the concentrations of VAZ pigments were measured in melanised and non-melanised thalli as described in Chapter 2.

4.2.3 Statistical analysis

Best-fit curve for the effect of relative water content on photosynthesis were determined using SigmaPlot version 11. Quantum yields of photosynthetic CO_2 uptake and O_2 evolution were estimated with the software Photosyn Assistant version 1.1, Dundee Scientific. Light response curves for O_2 evolution were also fitted with Photosyn Assistant. All contrasts between melanised and control thalli were tested with t-tests carried out with the “R” open source programming language

4.3 Results

4.3.1 Measure of photosynthetic efficiency using Infra-red gas analysis

The optimum RWC for carbon fixation was between 30 and 60% (Figure 4.1). Carbon fixation in both non-melanised and melanised thalli increased linearly from 0 to 75 μmol photons $m^{-2} s^{-1}$ (Figure 4.2). The quantum yield of photosynthetic CO_2 uptake, assessed as the initial slope

of the graph, was more than 40% lower in melanised thalli (Table 4.1). However, maximum rates (at *c.* 400 µmol photons m^{-2} s^{-1}) did not differ significantly between non-melanised and melanised thalli.

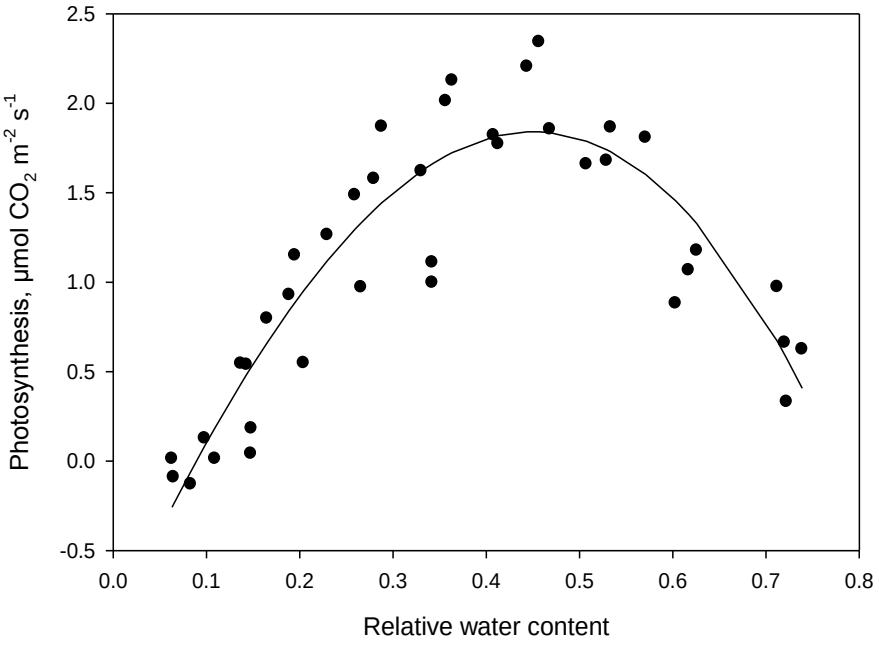

Figure 4.1: Photosynthetic CO_2 uptake at 200 µmol photons m^{-2} s^{-1} as a function of relative water contents in four thalli of *Lobaria pulmonaria* during dehydration.

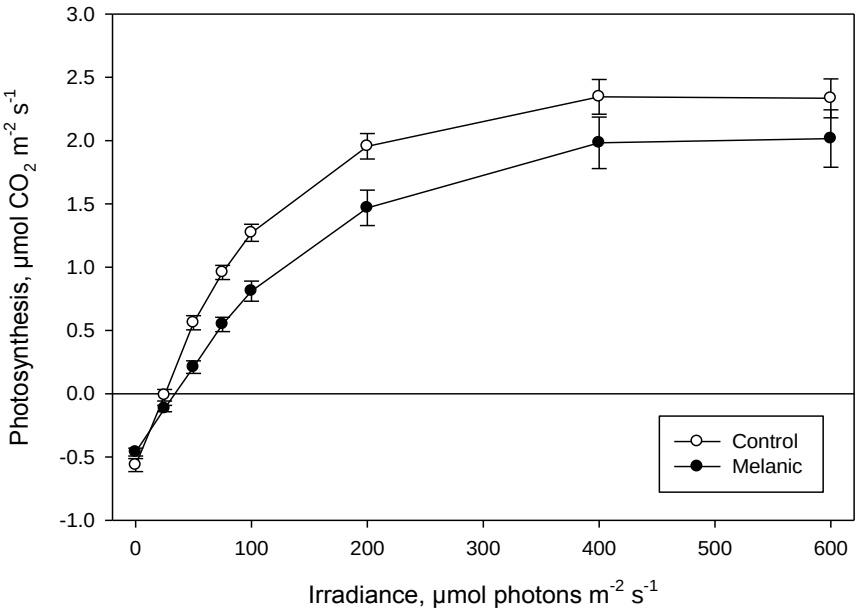

Figure 4.2: Light response curves for photosynthetic CO_2 uptake for melanic (closed symbols) and control (open symbols) thalli of *Lobaria pulmonaria*. Each curve is the mean of light response curves for 15 thalli and error bars show ±SE.

4.3.2 Photosynthetic O_2 evolution

The quantum yield of photosynthetic O_2 evolution was more than 40% lower in melanised thalli (Table 4.1), whereas maximum rates of O_2 uptake were not significantly different (Figure 4.3).

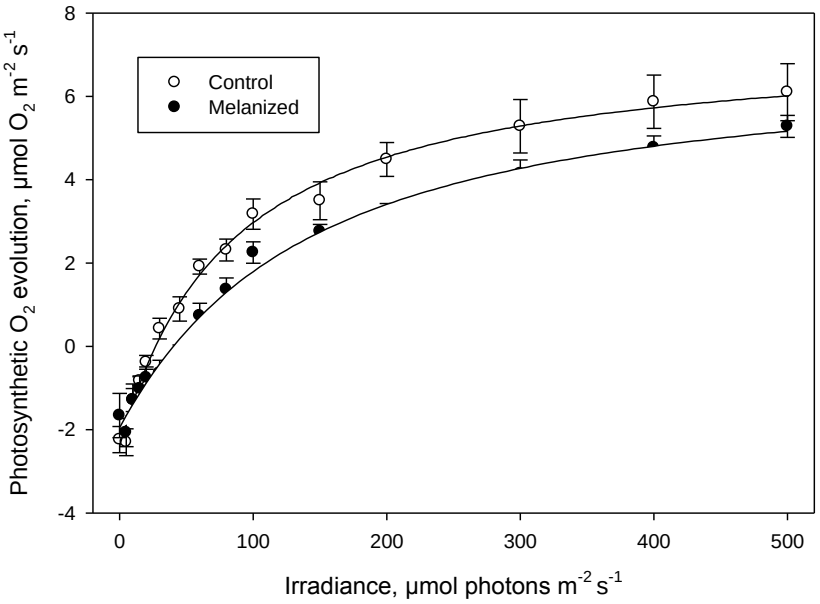

Figure 4.3: Light response curves for photosynthetic O_2 evolution for melanic (closed symbols) and control (open symbols) thalli of *Lobaria pulmonaria*. Each curve is the mean of light response curves for six thalli and error bars show ±SE.

4.3.3 Chlorophyll Fluorescence measurements

Fv/Fm was similar in both non-melanised and melanised thalli, and NPQ was about 20% lower in melanised thalli (Table 4.1). The relative ETR of non-melanised and melanised increased with irradiance in a same way; at irradiances higher than 200 µmol photons m^{-2} s^{-1} relative ETR started to decline (Figure 4.4).

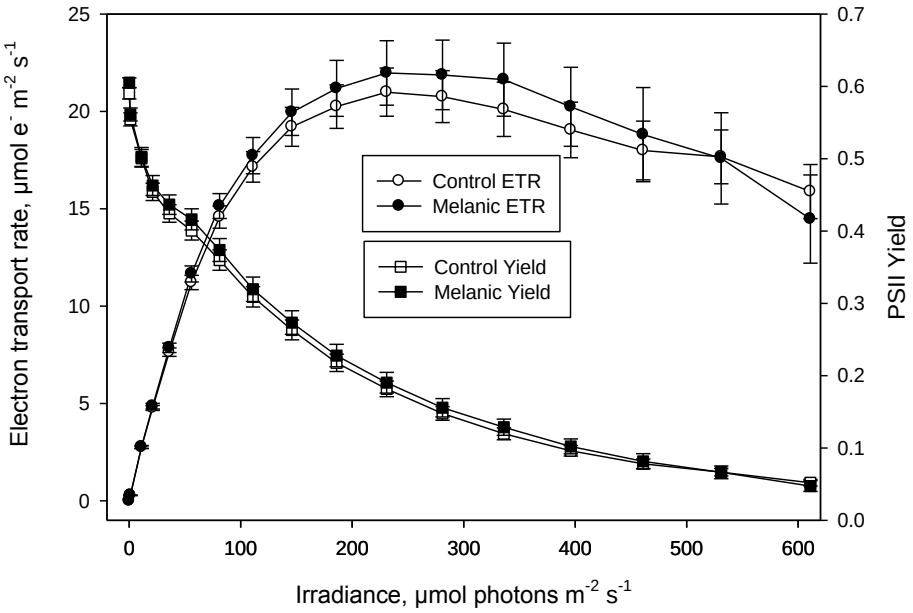

Figure 4.4: Light response curves of electron transport rate (circles) and PSII yield (squares) for melanic (closed symbols) and control (open symbols) thalli of *Lobaria pulmonaria*. Each curve is the mean of light response curves for 15 thalli and error bars show ±SE.

4.3.4 Reflectance measurements

Melanic thalli had lower reflectance compared with the non-melanic thalli across most wavelengths, particularly in the red and infra-red portions of the spectrum (Figure 4.5, Table 4.1). Overall, thalli used in 2015 were more melanised than those used in 2016.

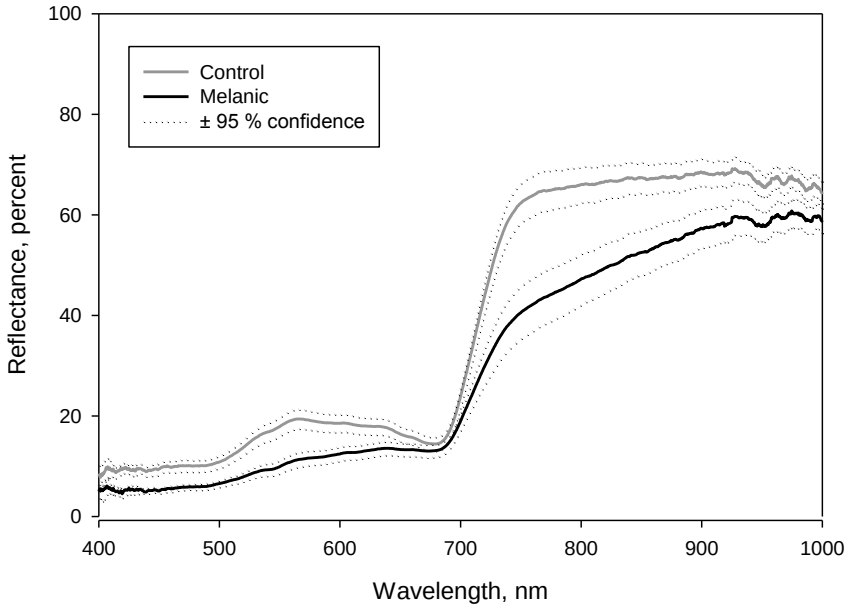

Figure 4.5: Percent reflectance from melanic (black lines) and pale control (grey lines) thalli of *Lobaria pulmonaria*. Each line is the mean of 15 thalli and the dotted lines show ±95% confidence intervals.

4.3.5 Chlorophylls and VAZ pigments

There was a tendency for higher contents of the pool size of VAZ pigments in melanised thalli (P=0.078, t-test). The chlorophyll a/b ratio was higher in melanised thalli than in pale control thalli (Table 4.1), whereas there were no differences in the concentrations of chlorophyll.

Table 4.1: Effect of melanisation by high light exposure on various parameters in *Lobaria pulmonaria*. Values shown are mean values ±SE and n=6 to n=15. Different letters on control and melanic mean values indicate significant differences analysed by t-test (P > 0.05).

	2015 experiment		**2016 experiment**	
	Control	**Melanic**	**Control**	**Melanic**
QY CO_2 uptake			0.025 ± 0.001[a]	0.014 ± 0.001[b]
QY O_2 evolution	0.071±0.07[a]	0.041±0.05[b]		
Chl a+b mg g^{-1}			1.76 ± 0.05[a]	1.54 ± 0.04[b]
Chl a+b mg m^{-2}	82.30 ± 4.52[a]	84.46 ± 5.56[a]		
Chl a/b	2.90 ± 0.06[a]	3.06 ± 0.05[b]	3.07 ± 0.02[a]	3.16 ± 0.03[b]
VAZ mg g^{-1}	0.19 ± 0.02[a]	0.25 ± 0.03[a]		
Fv/Fm			0.672 ± 0.003[a]	0.661 ± 0.005[a]
NPQ			4.26± 0.15[a]	3.47 ± 0.18[b]
BRI			2.91 ± 0.68[a]	15.55 ± 2.50[b]
Reflectance VIS			14.54 ± 0.65[a]	9.75 ± 0.46[b]
Reflectance NIR			63.53 ± 1.31[a]	50.06 ± 1.83[b]

4.4 Discussion

The lichen *Lobaria pulmonaria* typically grows in rather wet habitats, often in coastal areas, and on the more shaded sides of trees (McCune and Geiser, 1997). As discussed earlier, when exposed to high light, *L. pulmonaria* is well known to protect itself by synthesising cortical melanins (Matee et al., 2016). Various parameters indicate that the photobionts of melanised thalli are healthy. Chlorophyll contents and maximum rates of photosynthesis are high (Table 4.1, Figure 4.2), while chlorophyll fluorescence analysis showed that the maximum quantum yield and relative electron transfer rates are similar in non-melanised and melanised thalli (Table 4.1, Figure 4.3). However, at light levels lower than 100 µmol photons m^{-2} s^{-1}, melanisation reduces CO_2 fixation by more than 40% (Table 4.1, Figures 4.2, 4.3). The main conclusion of the data presented in this chapter is that although melanisation effectively reduces high-light stress in lichen photobionts, should light availability fall e.g. by seasonal changes, canopy closure or re-growth of plants surrounding the host tree, it is highly likely that growth will be reduced.

Optimal conditions for photosynthesis

Preliminary experiments were carried out to determine the optimum conditions for CO_2 fixation. For the material used in the present experiment, the optimal RWCs were between 30 and 60% (Figure 4.1). This is very similar to the optimum absolute water content of 100% reported by Scheidegger et al. (1995), the only other published study on the effect of water content on photosynthesis in *L. pulmonaria*. At higher water contents photosynthesis was depressed, presumably because "oversaturation" reduces the rate that CO_2 can diffuse to the photobionts (Cowan et al., 1992). The temperature optimum for photosynthesis in a given lichen is remarkably plastic (Domaschke et al., 2013), but for the present material was 15^{o}C. However, as discussed in Materials and Methods (Chapter 2), photosynthesis at 20^{o}C was only slightly lower, and for practical considerations, this temperature was used in subsequent experiments.

Melanisation in L. pulmonaria

Cortical melanins in *L. pulmonaria* are produced following exposure to high light, and here were quantified by spectral reflectance in the 400 – 1050 nm region (Figure 4.5, Table 4.1). The "browning index" or BI increased by more than five times in melanic thalli. Recently, Matee et al. (2016) identified the melanins in *L. pulmonaria* as being of the DOPA melanin type. In Chapter 3, DOPA melanins were identified in *L. retigera*, based on the C: N ratio, suggesting that DOPA melanins are common in this genus.

Effect of melanisation on photobiont health

A variety of measurements indicated that photobiont health is normal in melanised thalli. Chlorophyll fluorescence analyses showed that the maximum efficiencies of PS II (Fv/Fm) are similar in non-melanised and melanised thalli (Table 4.1). Total chlorophylls were slightly lower in melanised thalli, while the ratio of Chl-a/b showed a small but significant increase (Table 4.1). These changes are typical responses of photobionts to high light; similar results have been obtained from comparisons shade and sun (but not melanised) thalli of *L. pulmonaria* (Pannewitz et al., 2002). Taken together, results suggest that melanins effectively protected the photobionts against the effects of high light.

Effect of melanisation on photobiont carbon fixation

While the photobiont cells showed no evidence of damage, measurements made with IRGA and the O_2 electrode showed that at light levels up to 100 µmol m^{-2} s^{-1} melanisation reduced the quantum yield of photosynthesis by more than 40% (Figures 4.2 and 4.3). While ETR estimates derived by chlorophyll fluorescence suggest that the quantum yields of non-melanised and melanised thalli are the same (Table 4.1), this is an artefact of chlorophyll fluorimetry. ETR estimates from chlorophyll fluorescence are calculated as the product of light intensity and the quantum yield of PSII during exposure to light (ΦPSII), with ΦPSII being the ratio $(Fm' - F_t)/Fm'$. A true estimate of ETR by chlorophyll fluorescence would need to consider the reduced level light reaching the photobiont in melanised thalli. Without knowing how much light is actually absorbed by the photobionts, calculation of "ETRabs" in lichens is not possible (Solhaug et al., 2010). While the ETR estimates confirm that the photobionts are in good health, more direct measurements of photosynthetic activity by IRGA and the O_2 electrode clearly show that when light levels are below about 100 µmol m^{-2} s^{-1} melanised thalli will display significantly lower rates of carbon fixation.

Effect of melanisation on NPQ and xanthophyll cycle pigments

Apparent higher NPQ in non-melanised thalli than in melanised thalli (Table 4.1) is probably caused by less light reaching the photobiont in melanised thalli with measurements at the same level of incident light. Therefore, the same type of artefact is present for NPQ as for a true estimate of ETR. However, the tendency for melanised thalli to have a higher pool size of xanthophyll cycle pigments in (Table 4.1) indicates that high light adapted thalli actually have a higher capacity for NPQ, because the xanthophyll cycle is probably the main component of NPQ (Baker, 2008).

The "clearcut anomaly"

As discussed in the Introduction, melanisation will be of particular benefit to a lichen if there is a sudden increase in light level. An example of a situation when this may occur is following the clearcutting of the forest surrounding the tree on which a lichen is growing. A clearcut is created when many trees in an area are uniformly cut down, for example to promote even age stands. *Lobaria pulmonaria* it normally considered a shade-adapted lichen with no or little melanin (Gauslaa and Solhaug, 2000). Rather surprisingly, Gauslaa et al. (2006) showed that *L. pulmonaria* growing in areas subject to clearcutting synthesize melanins and may have

higher growth rates than specimens from normal shaded habitats. A logical deduction from these results is that the realized niche *L. pulmonaria* should extend into microhabitats with higher light levels than those in which this species is normally found, giving rise to the "clearcut anomaly". However, most likely reason that *L. pulmonaria* is quite rare or absent in exposed, high light sites is that such habitats experience occasional, variable extreme weather conditions that damage the thalli. The transplantation experiment of Gauslaa et al. (2006) was carried out in southern Norway from July to October, and growth rates were probably high because rain was quite frequent during this particular period. Under such conditions, with both frequent rain and light above saturation level, first, melanins will reduce high light stress and second, damage caused by photoinhibition can be readily repaired when thalli are moist and metabolically active. However, there would be no possibility to repair photo-inhibitory damage if lichens remain for long periods in the dry state exposed to high light (Gauslaa and Solhaug, 2000; Gauslaa et al., 2012). Although a sudden increase in light availability of *L. pulmonaria* may increase growth rates, eventually stressful conditions are likely to occur, resulting in lethal stress, and the normal absence of this species in such sites. Thus, while melanisation may allow survival for a short period, lichens are only likely to survive if light levels are soon reduced, but then melanisation will reduce photosynthetic rates.

4.5 Conclusion

Melanisation, at least in the short term, is an effective strategy to protect lichen photobionts from high light. However, the main conclusion of the work presented here is that melanised thalli will be disadvantaged should light levels fall below about 100 µmol m^{-2} s^{-1}. Falling light levels may occur for several reasons e.g. reductions in light availability during autumn, development of leaves in branches above lichens or by the growth of plants surrounding the host tree. The latter is realistic, as *Lobaria* species can live over 20 y (Rhoades, 1983; Larsson et al., 2009; Gustafsson et al., 2013). Furthermore, there is no evidence that melanins can be broken down. Under shaded conditions, melanisation will reduce carbon fixation by up to 40%, which will result in a decline in growth rate. A further potential disadvantage of melanisation will be that it will increase thalli temperature by up to 3 °C (McEvoy et al., 2007), possibly inducing heat stress. Increases in thallus temperature may explain why it appears that more lichens use classic secondary metabolites as sun shields rather than melanins.

Secondary metabolites appear as effective as melanins in reducing the transmission of PAR (Solhaug et al., 2010), but reflect rather than absorb PAR, and therefore have less effect on the heat balance of lichens. However, more work is clearly needed to understand the relative advantages and disadvantages of melanins and secondary metabolites as sun screens in lichens.

CHAPTER 5: THE EFFECT OF HIGH LIGHT ON THE PHOTOSYNTHETIC PERFORMANCE IN *LOBARIA RETIGERA*

5.1 Introduction

The aim of the work presented here was to complement that of Chapter 4, and to study the cost of photo-protection by melanic pigments in the cyanolichen *Lobaria retigera.* Anecdotal observations suggest that *L. retigera* prefers even damper, wetter habitats than *Lobaria pulmonaria.* Like *L. pulmonaria*, *L. retigera* can at times be exposed to higher light levels, for example following leaf fall in autumn, or when light when openings in forests occur, for example, because of tree fall. Little is known about the biochemical adaptations of *L. retigera* to high light stress, but like *L. pulmonaria* the most important adaptation is probably the synthesis of cortical melanin pigments (Solhaug et al., 2003), particularly as cyanobacteria lack the xanthophyll cycle found in green algal lichens (Campbell et al., 1998). Unfortunately, the treatments designed to induce melanisation proved too stressful for the lichens (Chapter 3), and resulted in severe damage to the photosynthetic apparatus. As a result, more work is required to establish whether melanisation reduces efficiency of photosynthesis in cyanobacterial lichens.

5.2 Methods

Exposure to ambient light

In the first experiment, the effect of different types of light on the photosynthetic performance of *L. retigera* was tested. Fifteen thalli each treatment was treated with normal ambient light (full sun), an acrylic screen (+UV, to test for the physical presence of the screen), and a polycarbonate screen (-UV, removes UV). Control thalli were kept in the freezer at - 20 °C. Each treatment had two wooden frames (47.5 x 54.5 cm) with shade cloth mesh netting to attach thalli with a total fresh mass of about 4 g. The lichen material was collected from trees at in Monks Cowl, KwaZulu Natal. Of the two frames, one frame had 7 thalli attached while the other had 8 thalli. The frames were randomly placed in the exposure site (Botanical Gardens, UKZN, Pietermaritzburg) and left for three weeks. The treatments were watered

daily with distilled water before sunrise and at sunset, except on rainy days to keep them photosynthetically active. After three weeks, thalli were harvested for analyses of photosynthetic activities in Norway.

In the second experiment, photoinhibition was measured in nonmelanised and melanised lichen thalli collected directly from their natural habitats. Thalli were collected from their host tree, *Leucosidea sericea*, and cleaned. The treatments were wet and dry thalli that were nonmelanised and melanised. Ten thalli for each treatment were cut into 2 cm discs and hydrated for 24h to 72 h to measure time zero (initial) Fv/Fm and PS II yield. Thereafter, wet thalli were given 1000 µmol m^{-2} s^{-1} for 1.5 h. The thalli for the dry treatment were left to dehydrate in a bench at room temperature. The thalli were given 800 µmol m^{-2} s^{-1} for 10 h. Recovery was recorded at time intervals, 1, 2, 3, 4 ,5 and 24 h.

5.3 Results

5.3.1 Measurement of photosynthetic performance

Placing lichens into the field reduced the photosynthetic quantum yield of CO_2 uptake to approximately one third, Table 5.1. However, the lichens under the polycarbonate filter had slight higher yields that the other two treatments. The BRI of thalli in full sun was greatest, although those under the acrylic filter (which transmits UV) were significantly greater than the controls, and the lichens under the polycarbonate filter which blocks UV.

Table 5.1: The effect of exposure to high light on the quantum yield of carbon fixation and the browning reflectance in *Lobaria retigera*. The control represents thalli kept in the freezer. Values are given ±S.E., n =15 and within each column the values with the same letter do not differ significantly (P > 0.05).

Treatment	**Quantum yield of CO_2 uptake**	**Browning Reflectance Index**
Control	0.108 ± 0.017^{a}	0.002 ± 0.02^{d}
Full sun	0.032 ± 0.007^{c}	0.583 ± 1.68^{a}
- UV, polycarbonate screen	0.037 ± 0.006^{b}	0.004 ± 0.03^{c}
+ UV, acrylic screen	0.030 ± 0.009^{d}	0.048 ± 0.04^{b}

5.3.2 Chlorophyll (CHL) content

Exposure to light significantly reduced the chlorophyll content (Figure 5.1).

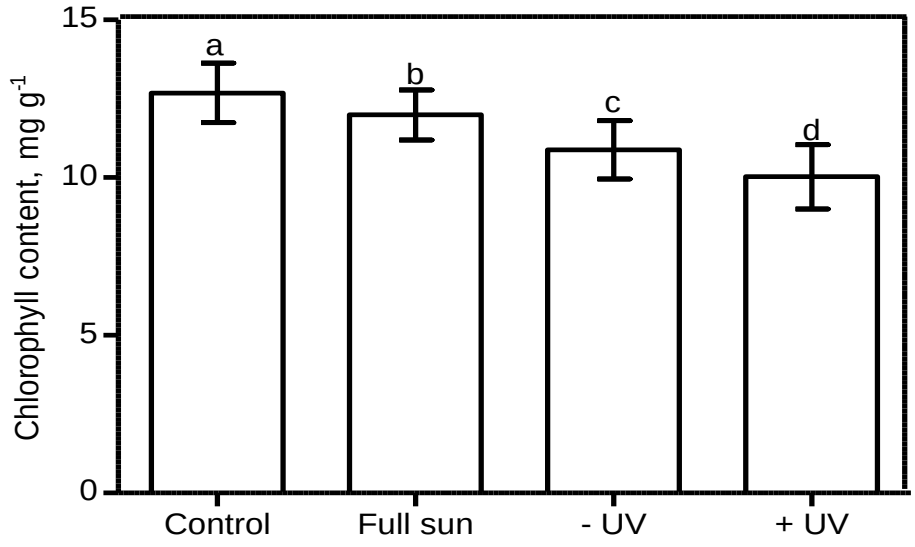

Figure 5.1: The effect of high light on the chlorophyll content of *Lobaria retigera*. Values are given ± S.E., n = 15, and columns with the same number on top do not differ significantly (P > 0.05).

5.3.3 Measurement of the state of the photosynthetic apparatus

Exposure to all combinations of light in the field significantly decreased the rate of photosynthesis compared with the control, although lichens grown without UV light had slightly higher rates of photosynthesis than those grown with UV (Figure 5.2).

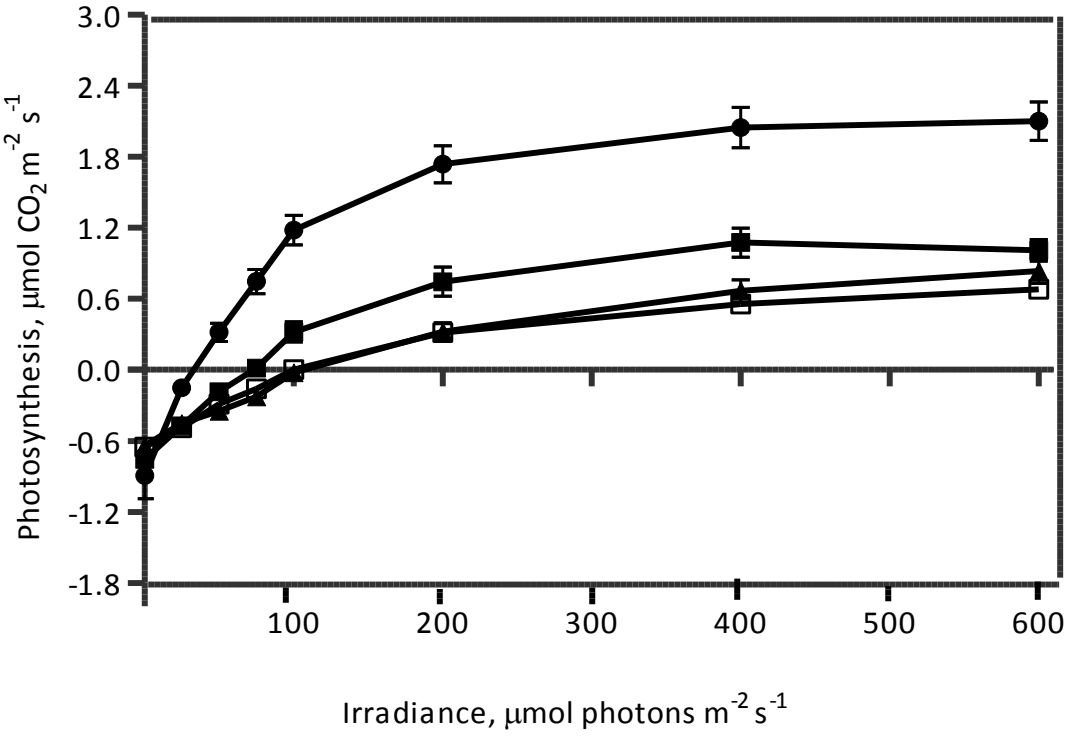

Figure 5.2: Photosynthetic CO_2 uptake of *Lobaria retigera* at varying irradiances. Values are given ±S.E., n = 15. Treatments were the control (thalli not light treated, closed circle), full sunlight (open square), + UV (closed triangle) and -UV (closed square).

Initial values of Fv / Fm and ϕPSII were similar in melanised and unmelanised thalli (Figure 5.3). In the hydrated state, melanisation greatly reduced the effects of an exposure to a photoinhibitory light intensity. After 24 h, although Fv / Fm measurements suggested that PSII had fully recovered in both melanised and nonmelanised thalli, estimates of ϕPSII indicated that recovery was not complete in pale thalli. In dry thalli, melanisation had little effect of the resistance of thalli to photoinhibition, although the reduction Fv / Fm was initially slightly lower in melanised than pale thalli.

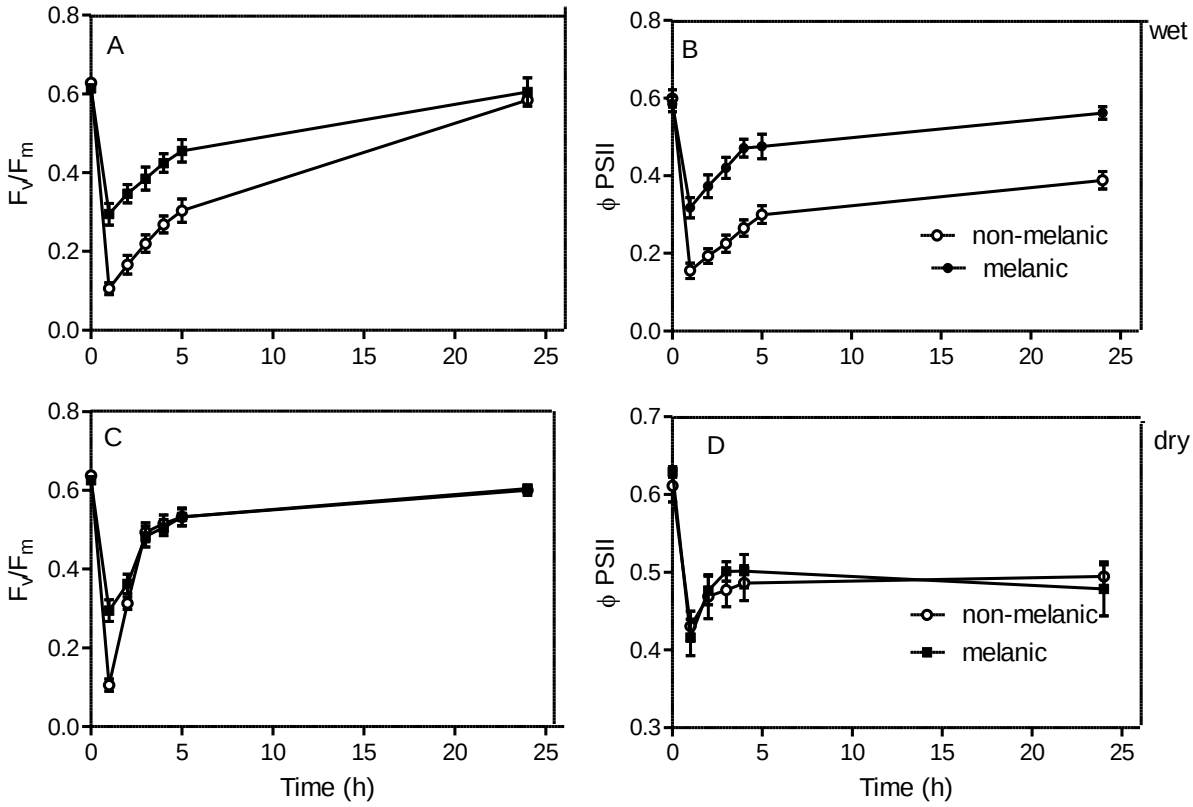

Figure 5.3: The effect of a photoinhibitory exposure to light on Fv / Fm and ϕPSII in *Lobaria retigera.* The wet thalli were given 800 µmol m^{-2} s^{-1} for 10 h and dry thalli exposed to 1000 µmol m^{-2} s^{-1} for 1.5 h. Values are given ±S.E., n =10. The non-melanised thalli are represented by open symbols, while melanised thalli have closed symbols.

5.4 Discussion

The effect of exposure to different types of light on the photosynthetic performance of L. retigera

Placing the shade-adapted lichen *L. retigera* into the sun in the open or under the various frames caused severe stress, as indicated by the photosynthetic light response curves of material from different treatments in Figure 5.2 and their quantum yields in Table 5.1. Results differed from those obtained with *L. pulmonaria* (Matee et al., 2016), which appeared to suffer little stress following transplantation as discussed in Chapter 3. This was probably because the experiment with *L. retigera* was carried out in South Africa, while the experiments with *L. pulmonaria* were carried out in Norway. Presumably, the much higher ambient light levels in South Africa compared to Norway were too stressful for the lichen, although possibly *L. retigera* is inherently less tolerant to high light than *L. pulmonaria*. Only the thalli exposed to full sunlight (without any filters) significantly melanised, suggesting that unlike *L. pulmonaria*, UV alone is insufficient to cause melanisation. Exposing thalli to full sunlight apparently induces a signal that initiates melanisation that is absent in thalli under filters, even those that transmit UV. The nature of this signal remains unclear. The chlorophyll contents of sun exposed thalli were reduced (Figure 5.1), and because of the stress caused by the high light treatments (Figure 5.2), it was not possible to deduce whether melanisation reduced the photosynthetic efficiency of *L. retigera* in the same way that it does for *L. pulmonaria*.

Melanin provides protection to hydrated thalli exposed to high light. Lange et al. (1999) shows a similar trend for a dry cyanobacteria due to lack of the xanthophyll cycle to dissipate energy in wet thalli as these are very sensitive. The dry thalli are more resistant to high light than wet for reasons discussed in Chapter 6. When thalli are dry they are inactive, and any light-induced production of reactive oxygen species will be reduced; however, photoinhibition eventually occurs. For reasons that remain unclear, melanins are less rather than more effective at protecting dry thalli as compared to the results presented for species discussed in Chapter 6, where melanins protect both dry and wet thalli.

5.5 Conclusion

Much information is available about melanisation in the green algal lichen *Lobaria pulmonaria* Matee et al. (2016), but relatively little is known about this process in cyanobacterial species

in general. Therefore, *L. retigera* was chosen for further study because it is closely related to *L. pulmonaria*, but has a cyanobacterial photobiont. Unfortunately, probably because experiments were carried out in Pietermaritzburg rather than Norway, our treatments designed to induce melanisation resulted in severe damage to the photosynthetic apparatus. Therefore, whether melanisation reduces the quantum yield of photosynthesis as with *L. pulmonaria* (Chapter 4) could not be fully investigated. It is recommended that the experiments described here be repeated in a place with lower light levels, for example, Norway. However, results from naturally melanised *L. retigera* (collected from its natural habitat) showed that, at least in the moist state, melanised thalli are most resistant to photoinhibition than pale thalli.

CHAPTER 6: MELANINS PROTECT HYDRATED AND DESICCATED LICHENS FROM PHOTOINHIBITION

6.1 Introduction

The main of the aim of the work presented in this Chapter was to systematically test under controlled conditions whether melanin synthesis increases the tolerance to photoinhibition in *Lobaria pulmonaria* and a range of other lichens, species with chlorophycean and cyanobacterial species, and that grow in shaded or more exposed habitats. A further aim of the present study was to test whether melanisation is more effective in protecting dry or wet thalli. It is well known that lichens suffer light stress even when desiccated (Kershaw and Macfarlane, 1980). The reasons for this remain unclear, as it is difficult to image how photoinhibition occurs in dry thalli. It may be relevant that changes in gene expression and/or protein synthesis have been observed in dry seeds of *Nicotiana* (Bove et al., 2005; Leubner-Metzger, 2005) and *Arabidopsis (*Cadman et al., 2006; Chibani et al., 2006). These results have been interpreted as indicating the existence of hydrated pockets in which ROS formation could occur, and possibly these pockets occur in lichens. Even if only small amounts of ROS are produced, when thalli are desiccated normal mechanisms that repair DNA and other biomolecules do not take place (Buffoni Hall et al., 2003). In *L. pulmonaria* cortical transmittance to PAR is typically between one third and one half lower in the air-dry compared with the hydrated state (Gauslaa and Solhaug, 2001). Melanisation significantly reduces transmittance, and interestingly the transmittance of the upper cortex of melanised thalli to PAR is about 40% lower than pale thalli when the thalli are dry, but only about 30% lower when the thalli are wet. We therefore hypothesized that melanisation may give greater photoprotection when the thalli are air-dry.

6.2 Methods

6.2.1 Plant Material

Lichen material was collected from the localities indicated in Table (6. 1). For each species, melanised and pale thalli were selected from exposed and shaded individuals from the same population, never more than 5 m apart. If collected wet, material was slowly dried at room

temperature. Material was stored at -20 ^{o}C until ready for experimentation for a maximum of two weeks. One day before each experiment, 2 cm disks were cut, or for small thalli, thallus fragments selected. Lichens were stored overnight on wet filter paper in petri dishes under dim lighting (5 µmoles m^{-2} s^{-1}) at a cool temperature (15 ^{o}C, thermostatically controlled room). Before high light treatment, chlorophyll fluorescence parameters were measured, and then material either slowly dried for 4 h, or used immediately.

Table 6. 1: Expanded details on species from Table 2.1 used in this Chapter, their photobiont types and an indication of light availability in their typical habitat.

Species	**Collection Locality**	**Photobiont**	**Shade / Sun species**
Cetraria islandica (L.) Ach.	Soil, Langangen, Norway	Chlorophycean	Sun
Lobaria pulmonaria (L.) Hoffm.	Oak trees, Langangen, Norway	Chlorophycean	Shade
Peltigera aphthosa (L.) Willd.	Soil, outskirts of Ås, Norway	Chlorophycean	Sun
Peltigera membranacea (Ach.) Nyl.	Soil, outskirts of Ås, Norway	Cyanobacterial	Sun
Pseudocyphellaria gilva (Ach.) Malme	*Leucosidea sericea* trees, Fort Nottingham, RSA	Cyanobacterial	Shade
Sticta sublimbata (J. Steiner) Swinscow & Krog	*Leucosidea sericea* trees, Fort Nottingham, RSA	Cyanobacterial	Shade

6.2.2 Chlorophyll fluorescence

Chlorophyll fluorescence for some species was measured using the red version of the PAM 2000 fluorimeter while for other the Hansatech FMS 2 was used. The maximal efficiency of PSII, (Fv/Fm) and the operating efficiency of PSII (ØPSII) were measured. Thalli were initially dark-adapted at room temperature for 10 min. At the start of a run, a saturating flash was applied to determine Fv/Fm. Thalli were then exposed to PAR at 30 µmoles m^{-2} s^{-1} for 10 min, after which fluorescence had reached a stable value. Another saturating flash was then applied, and ØPSII was calculated as:

$$ØPSII = (Fm' - F_t)/Fm'$$

where F_t is the stable fluorescence signal in the light, and Fm' is the maximum fluorescence when a saturating pulse is given in the light.

6.2.3 High light stress

High light stress was given using a white LED light-source panel (Model SL-3500, Photon System Instruments, Brno, Czech Republic). Light was given at 800 or 1500 µmoles m^{-2} s^{-1} until the maximum quantum yield of PSII photochemistry (F_v / F_m) was reduced from values of around 0.7 to 0.3 - 0.4, with each species needing different times as indicated in Table (6. 2).

Table 6.2: Light intensity and time of exposure used to induce photoinhibition.

Species	**Dry**		**Wet**	
	Time (h)	**Light Intensity (µmol m^{-2} s^{-1})**	**Time (h)**	**Light Intensity (µmol m^{-2} s^{-1})**
Cetraria islandica	36	800	3	1500
Lobaria pulmonaria	12	800	8	800
Peltigera aphthosa	12	800	5	1500
Peltigera membranacea	12	800	12	800
Pseudocyphellaria gilva	6	800	5	800
Sticta sublimbata	4	800	2	800

Chlorophyll fluorescence measurements were made in moist material before high light treatment at “time zero” as indicated above, at the end of the high light treatment, and then at intervals for up to 40 h. Photoinhibition in thalli exposed to high light in the dry state was measured at intervals following the hydration of replicate samples. When sufficient inhibition had occurred, they were immediately hydrated following exposure by being placed on moist filter paper and then sprayed with additional distilled water. During recovery, lichens were maintained at room temperature (20 ^{o}C) and dim lighting (5 µmoles m^{-2} s^{-1}) as recommended by Solhaug (2017). Reflectance spectra of the upper cortex were recorded on dry thalli as described in Chapter 2. The results were analysed using a two-way ANOVA, with melanisation and time as the two factors.

6.3 Results

Table (6.2) presents the times and light levels needed to photoinhibit the lichens. Lichens were more tolerant to high light when dry; much longer exposures were needed to create similar photoinhibition than for wet thalli. In general, lichens collected from open habitats required longer times or high light levels to create similar levels of photoinhibition compared with species from more shaded localities.

Measurement of thallus reflection indicated that the extent of melanisation varied greatly between different lichen species (Table 6. 3). For example, in *Pseudocyphellaria gilva* the BRI of melanised thalli was only 20% higher than pale thalli, while in *Cetraria islandica* melanisation the BRI was more than one hundred times greater (Table 6. 3).

Table 6. 3: Browning reflectance index (BRI) of the lichens used in this study. Figures are given ±1 s.d., n = 12. BRI was calculated as described by Solovchenko et al. (2001).

Species	**BRI Non-melanised thalli**	**BRI Melanised thalli**	**Increase in BRI in melanised thalli**
Pseudocyphellaria gilva	7.5 ± 1.9	9.3 ± 1.9	1.2
Sticta sublimbata	8.2 ± 2.6	24.8 ± 4.0	3.0
Lobaria pulmonaria	4.4 ± 1.1	48.4 ± 42.4	11.0
Peltigera membranacea	2.8 ± 0.5	31.6 ± 4.1	11.3
Peltigera apthosa	2.1 ± 2.1	24.8 ± 15.6	11.8
Cetraria islandica	0.6 ± 0.8	87.9 ± 27.1	146.5

Figures 6.1, 6.2 and 6.3 show the effects on Fv/Fm and ØPSII of a photoinhibitory exposure of light to wet and dry thalli of six lichen species. Initial values of Fv / Fm and ØPSII were very similar in melanised and pale thalli. In most cases, 24 h after the photoinhibitory stress almost all lichens had fully recovered, although the recovery was not complete in some cases e.g. unmelanised *C. islandica* (Figure 6.1B, D) and wet *Peltigera membranacea* (Figure 6.2E, F) and *Pseudocyphellaria gilva* (Figure 6.3B). In all cases, melanisation significantly reduced photoinhibition in both wet and dry thalli (ANOVA analyses not shown). The protective effect of melanisation was correlated with the relative increase in BRI between pale and melanised thalli. For example, the greatest protective effect of melanisation occurred in *C. islandica* (Figure 6.1A, B, C, D), the species that showed the greatest increase in BRI (Table 6.3). By contrast, melanised thalli of *Pseudocyphellaria* and *Sticta* had BRI values only slightly greater than those of pale thalli, and in these two species melanins conferred only slight (but significant) photoprotection (Figure 6.3). In general, ØPSII appeared to be a more sensitive indicator of recovery from photoinhibition than Fv/Fm. For example, in *C. islandica* Fv/Fm indicated that after 24 h pale thalli had completely recovered from photoinhibition (Figure 6.1A, C), while ØPSII measurements showed that recovery was incomplete (Figure 6.1B, D).

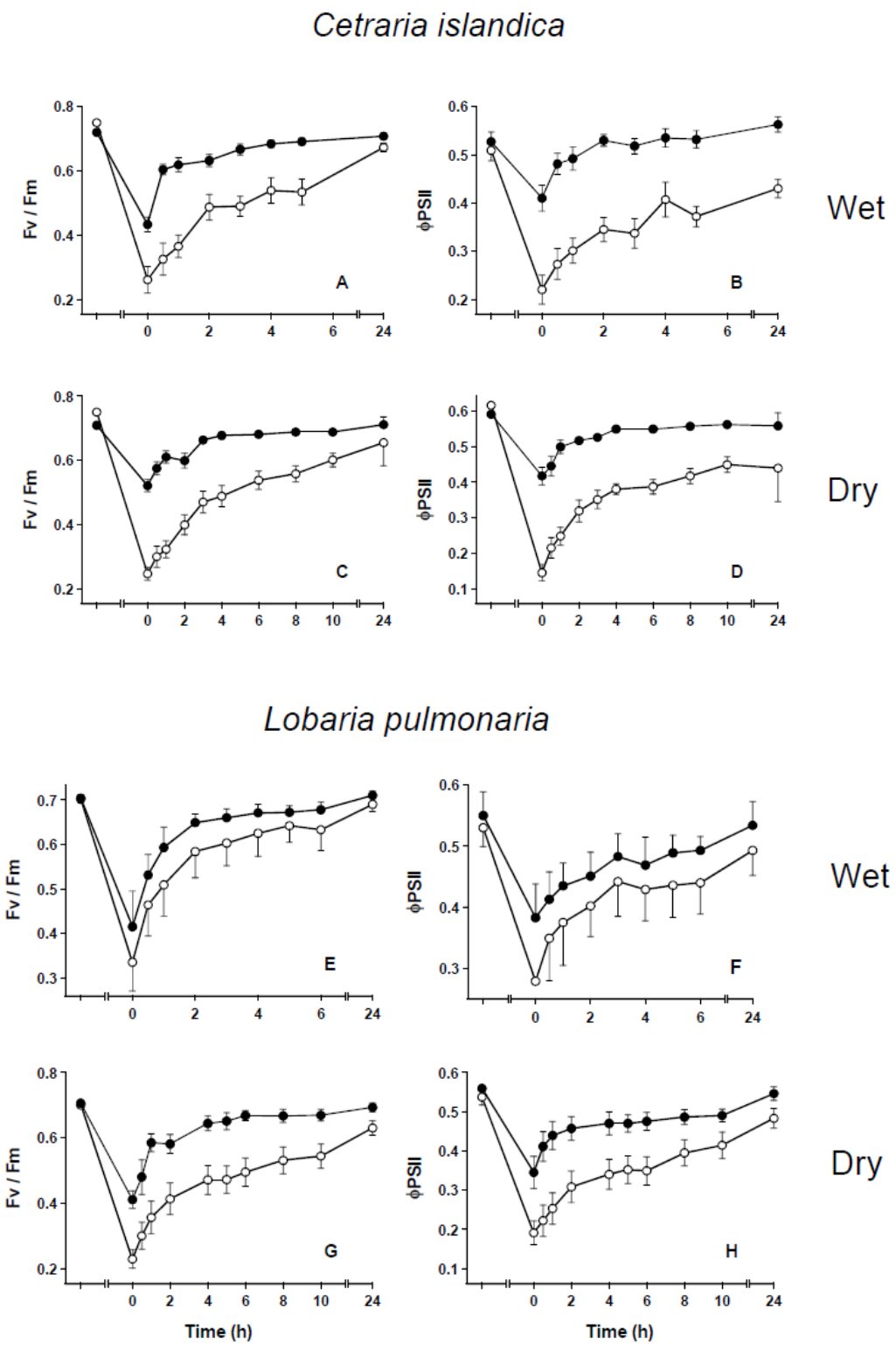

Figure 6.1: Photoinhibitory response of wet and dry nonmelanised (open symbols) and melanised (closed symbols) thalli of *Cetraria islandica* (A, B, C, D) and *Lobaria pulmonaria* (E, F, G, H). In this Figure 6.1, 6.2 and Figure 6.3, values are given ±SE of the mean, and n = 10 to 15.

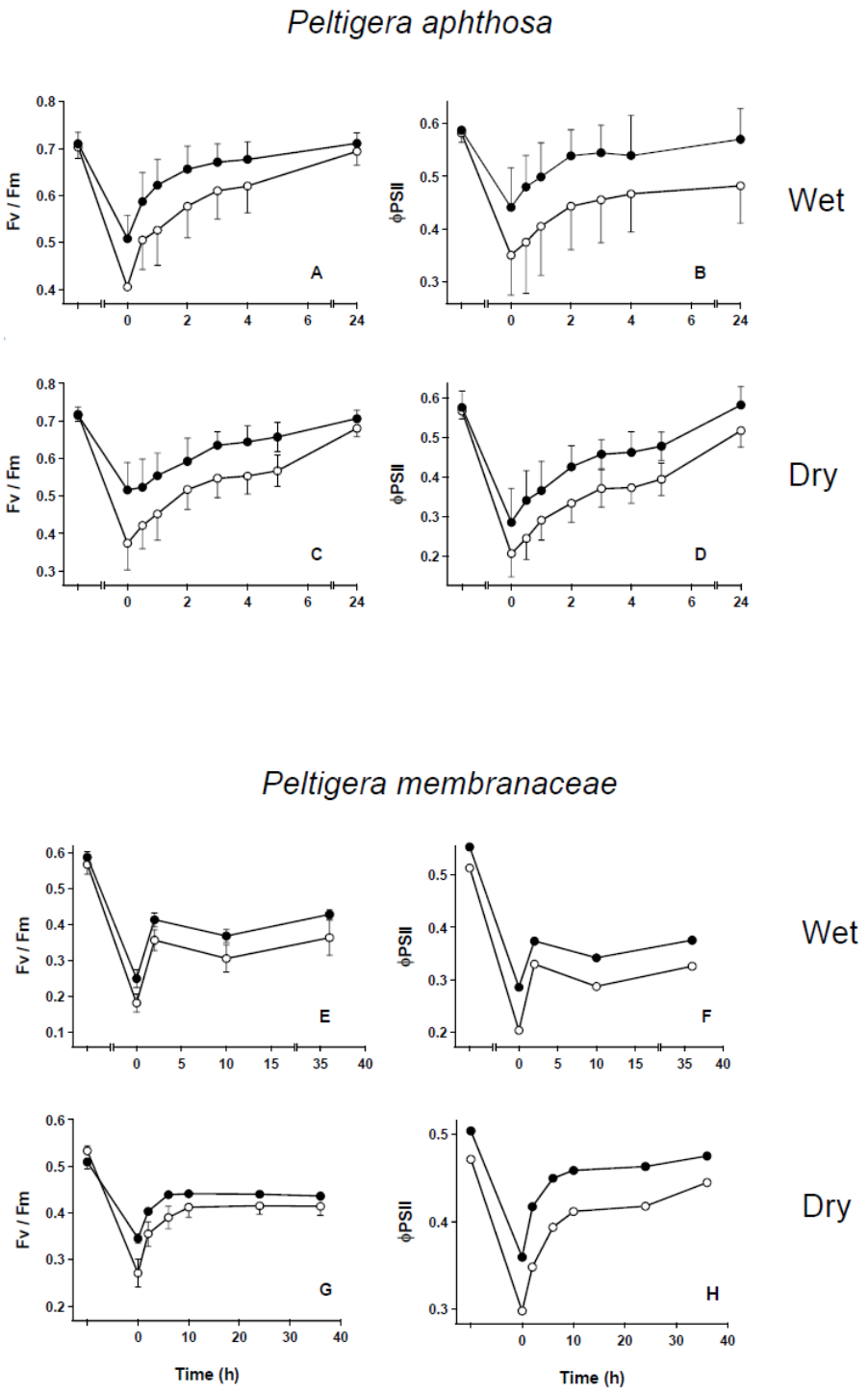

Figure 6.2: Photoinhibitory response of wet and dry nonmelanised (open symbols) and melanised (closed symbols) thalli of *Peltigera aphthosa* (A, B, C, D) and *Peltigera membranaceae* (E, F, G, H).

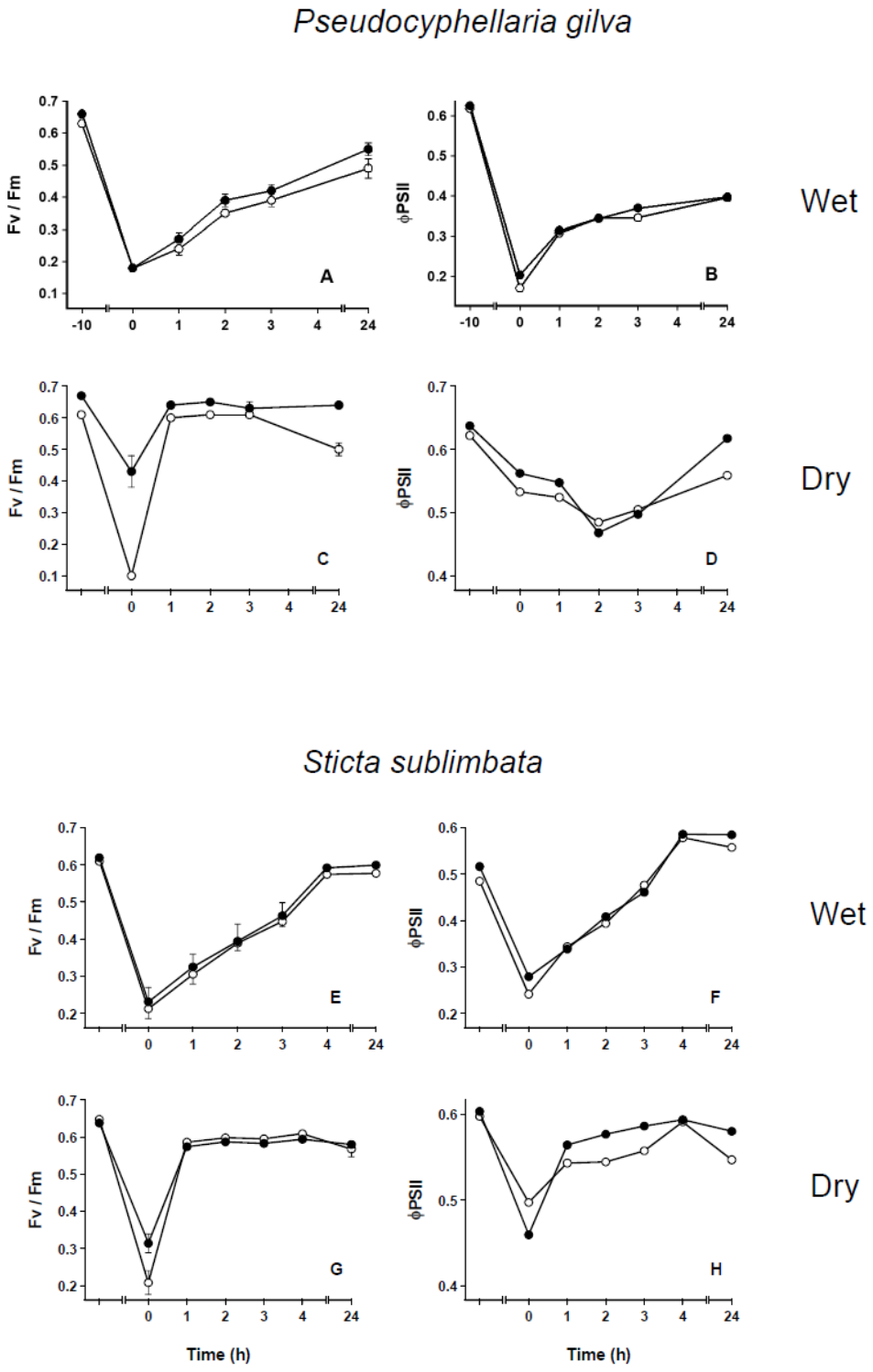

Figure 6.3: Photoinhibitory response of wet and dry nonmelanised (open symbols) and melanised (closed symbols) thalli of *Pseudocyphellaria gilva* (A, B, C, D) and *Sticta sublimbata* (E, F, G, H).

6.4 Discussion

The work presented in this Chapter indicate that melanisation effectively reduces photoinhibition in lichens, and can protect both desiccated and hydrated lichens. Perhaps surprisingly, apart from the field experiment of McEvoy et al. (2007) with just one species (*Lobaria pulmonaria*), the present study is the first to show directly that melanisation increases tolerance to photoinhibition in lichen photobionts. In general, the effectiveness of melanisation is greatest in those species with the largest difference in BRI between melanised and pale thalli. For example, melanins provide the greatest protection in *C. islandica* (Figure 6.1A, B, C, D), the species with largest difference in BRI between pale and melanised thalli (Table 6.3). By contrast, for species in which differences in BRI are less e.g. *Pseudocyphellaria gilva* and *Sticta sublimbata* (Table 6.3) melanins are much less effective in providing photoprotection (Figure 6.3). Collectively, results show that melanins provide photoprotection in species that grow in both high and more shaded habitats, and that possess either green or cyanobacterial photobionts.

Roles of melanins in lichens

Melanins probably play different roles in the mycobiont and the photobionts. The fungal symbiont is responsible for melanin synthesis, and the trigger for synthesis is UV light (Matee et al., 2016). As discussed in Chapter 1, fungi apparently need more protection from UV that the photobionts, as elevated UV affects overall lichen growth more than purely photobiont responses such as chlorophyll content and the quantum yield of photosynthesis (Chowdhury et al., 2016). Interestingly, photobionts may be tolerant to UV levels that greatly exceed those currently being experienced by lichens in the field, as even in un-melanised thalli the cortex effectively blocks transmittance of light with wavelengths less than 325 nm (Gauslaa et al., 2017). However, while not needing UV protection, results presented here show that melanisation increases photobiont tolerance to photoinhibition by high PAR. Therefore, the although the mycobiont benefits directly from melanin synthesis by having increased UV protection, protecting the photobionts from high PAR indirectly benefits the mycobiont by increasing the supply of photosynthates to the whole thallus.

Melanin effectively protects dry lichens from photoinhibition

Melanisation effectively protects both wet and dry thalli from photoinhibition. Lichens are more tolerant of light stress when dry, but eventually become photoinhibited when exposed to even moderate light intensities (Table 6.2). This is perhaps surprising, as at low thallus water contents metabolism is almost inactive, presumably reducing light-induced ROS formation and the resulting photoinhibition. Furthermore, the cortical transmittance to PAR is much lower (between one third and one half lower) in air-dry compared with hydrated thalli (Gauslaa and Solhaug, 2001). While more research is needed to understand the mechanism of photoinhibition in dry lichens, recovery may take many hours (Figures 6.1, 6.2 and 6.3), reducing carbon fixation for the symbiosis. Interestingly, comparing wet and dry lichens, melanisation is sometimes more effectively at preventing photoinhibition when thalli are dry (for example, compared Figure 6.1F with 6.1G, Figure 6.3A with 6.3C and Figure 6.3E with 6.3G). One explanation for this result is that melanisation has different effects on cortical transmittance in wet and dry lichens. While in wet thalli the transmittance of the upper cortex of melanised thalli of *L. pulmonaria* to PAR is about 30% lower than pale thalli, in dry thalli the reduction is about 40% (Gauslaa and Solhaug, 2001). Assuming a similar difference occurs in other lichens, a greater reduction in cortical transmittance when lichens are dry thalli may explain why melanisation can be more effective at reducing photoinhibition in these thalli.

ØPSII can be a more sensitive indicator of photoinhibition than Fv/Fm

For some lichens, measuring the operating efficiency of PSII (ØPSII) can give a more sensitive measure of the effects of high light stress than simply measuring maximum quantum yield of PSII photochemistry (Fv / Fm). Fv / Fm can be quickly and easily measured, and is therefore commonly used in photoinhibition studies (Míguez et al., 2017; Solhaug, 2017). However, ØPSII can be also readily derived from chlorophyll fluorescence measurements. Measurement of ØPSII takes longer than Fv / Fm, as an actinic light must be switched on and fluorescence must reach a stable value (Ft) before a saturating pulse is given and Fm' measured. ØPSII is calculated as the ratio (Fm' - Ft)/Fm', and then the rate of electron transport between PSII and PSI (ETR) can be calculated as follows:

$$ETR = ØPSII \times PAR \times Abs \times 0.5$$

where PAR is incident irradiance and Abs is the fraction of incident irradiance absorbed by the photobiont. A strong linear relationship often exists between ETR and carbon fixation, although discrepancies may occur under certain stress conditions (Fryer et al., 1998). In this Chapter, recovery from photoinhibition is presented using ØPSII rather than ETR, as melanisation decreases Abs by an unknown amount making it difficult to compare ETR in melanised and pale thalli (see Chapter 4 for more discussion on this point). However, in some cases measuring ØPSII can give a better indication of the effects of stress on the photosynthetic apparatus than Fv / Fm. For example, in both wet and dry unmelanised *C. islandica* (Figure 6.1) and wet *Pseudocyphellaria gilva* (Figure 6.3) simply measuring Fv / Fm would indicate that lichens had almost completely recovered from photoinhibition (Figure 6.1A, C and Figure 6.3A), while ØPSII shows that some residual effects remain (Figure 6.1B, C and Figure 6.3B). In general, for all species tested, ØPSII measurements show that melanisation facilitates a faster and more complete recovery from photoinhibition than Fv / Fm, which underestimates the protective effect of melanisation. Therefore, although more time consuming to measure, ØPSII can be a more sensitive indicator of high light stress.

In the present study, recovery from photoinhibition was measured under low light (30 µmoles m^{-2} s^{-1}). In the field, lichens may need to recover under higher light levels. If we had used higher light levels during recovery here, photosynthesis would have been saturated. As a result, any reduction in "Abs" caused by melanisation would have had little effect on ETR. By comparison, under low light pale the photobiont absorbs a higher fraction of incident light thalli, and therefore will have relatively higher rates of ETR and photosynthesis. It seems likely that, providing light levels are not high enough to cause further photoinhibition, melanisation will be even more effective in facilitating recovery from photoinhibition when light levels are high.

6.5 Conclusions

Results presented in this Chapter clearly show that melanisation is an effective strategy to protect lichen photobionts from high light. As discussed in Chapter 1, melanisation is not the only mechanism of photoprotection, and more work is needed to compare the importance and efficiency of melanisation with, for example, other lichen secondary metabolites and non-photochemical quenching. Melanisation may not invariably be beneficial for lichens. As

discussed in Chapter 4, melanisation reduces the efficiency of photosynthesis at low light levels, and can increase thalli temperatures possibly inducing heat stress. However, should light levels remain high, melanisation is likely to significantly reduce the time that lichens are photoinhibited in the field.

CHAPTER 7: GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

This study examined the effects of melanisation induced by high light on the photosynthetic apparatus of green-algal and cyanobacterial lichens. Based on these findings, the following conclusions can be drawn.

Climate change increases solar radiation (Caldwell et al., 2007) which in turn affects lichen physiology. Over the past years, some studies have shown that lichens have started to develop signs of sensitivity, such as damage or death of thalli, a decrease or inhibition of metabolic activities (Bjerke et al., 2003). Some organisms are unable to withstand the detrimental effects of increased radiation (Caldwell et al., 2007). However, lichens, alpine and desert species are amongst the unique organisms as they have developed strategies to minimise or tolerate high light stress (Gauslaa and Solhaug, 1999; Korner, 2003). One of the strategies employed to resist or protect from high light is the synthesis of melanins in the upper cortex or in some species, in the lower cortex and cilia. In this study, results suggest that melanins are of different forms between lichen groups. Tentatively, results suggest that Peltigeralean lichens make eumelanin (DOPA) while non-Peltigeralean lichens synthesise allomelanin (DHN). This research improves our understanding of the photoprotective mechanisms used by chlorophycean and cyanobacterial lichens in general. It is hoped that this work will prompt more research on the effects of climate change on the physiology of lower and higher plants.

In Chapter 3, the induction of melanin at different light regimes was investigated in the cyanobacterial lichen *Lobaria retigera*. Initially, acclimatization experiments were conducted under low ambient light conditions (50% and 70% ambient light) and thereafter an experiment carried out where lichens were placed under normal ambient light (full sunlight), beneath a -UV polycarbonate screen and an +UV acrylic screen (Figures 3.2, 3.3). Here, the acclimatization strategy was not effective, presumably due to harsh conditions compared with other studies (Matee et al., 2016); however, for some thalli melanins were slowly induced. Interestingly, NPQ in *Pseudocyphellaria aurata* increased showing a photoprotective role of dissipating excess light energy. In *L. retigera* ETR decreased after a three weeks

exposure, almost certainly because of photooxidation. Thalli of *Lobaria pulmonaria* in a transplant site synthesised melanins for four weeks (Matee et al., 2016) , while melanin in *L. retigera* was induced after three weeks.

In Chapter 4, the effect of melanisation on the photosynthetic performance of *L. pulmonaria* was studied. Here, results showed that while melanins protect the photobionts from high light, melanisation clearly reduces the photosynthetic efficiency. Melanised thalli will be significantly disadvantaged if light levels return to lower values, more typical for those habitats in which this shade adapted lichen is most abundant. A similar study in Chapter 5 was conducted in a cyanobacterial *L. retigera*, Unfortunately, as discussed above, conditions were too stressful, and conclusions could not be drawn about whether melanisation reduces the efficiency of photosynthesis in *L. retigera*. However, experiments with naturally melanised *L. retigera* showed that melanisation can protect against the photoinhibition. In Chapter 6, photoinhibition was studied in a range of lichens of pale and melanised thalli from the same population. Selectively, pale and melanised thalli were exposed to short term high light stress. In all cases, melanised thalli were significantly less sensitive to photoinhibition, sometimes very markedly so, for example *Cetraria islandica*. Overall, it was concluded that melanins play key roles in the adaptation of lichens to high light, and the results confirm that melanisation protects lichens from photoinhibition in both the wet and dry states.

Recommendations for future work

Role of melanins in cyanobacterial lichens

As discussed above, there is now excellent evidence that melanisation protects the chlorophycean lichen *L. pulmonaria* from high light stress, but can reduce photosynthesis at low light levels. However, experiments reported there with the cyanobacterial lichen *L. retigera* were unsuccessful. To study the roles of melanins in cyanobacterial species experiments will need to be carried out under less stressful conditions, e.g. in a temperate climate. Alternatively, melanised and pale thalli could be collected from the same population and compared.

Cost of melanisation

Although data presented in Chapter 4 clearly indicate that melanisation reduces photosynthetic efficiency in *L. pulmonaria* under low light levels, further work is needed to test whether this could result in a lower rate of growth should light levels fall. This would involve inducing melanisation in a lichen by exposing it to high light, and then reducing the light level e.g. to 100 µmol m^{-2} s^{-1}, and then measuring the growth rates of both pale and melanised thalli at low light levels. It could be predicted that melanised thalli may grow more slowly. The effect of melanisation on the heat balance of lichens needs much more study. It remains to be tested whether the small (3^{o}C) increase in temperature of melanised thalli reported by McEvoy et al. (2007) in *L. pulmonaria* is typical for other lichens, and what effect this will have on the energy budget of lichens e.g. photosynthesis, respiration or ultimately growth rates.

Further studies on melanisation in lichens

Further studies on the role of melanisation in lichens could include testing whether, once synthesised, melanins can be broken down. This question is particularly relevant for *Lobaria* species, which, as discussed in Chapter 4, can live for twenty years or more. This could involve inducing melanisation, and then growing thalli under low light and then measuring any changes in BRI over a time course. We still have little idea about which enzymes are responsible for melanin synthesis. Although currently extremely difficult to do, in future, it should be possible to use e.g. CRISPR technology to edit the mycobiont genome to remove certain genes e.g. tyrosinase, and then test if the modified lichens have lost the ability to melanise. Work described in this thesis has focussed on the roles of melanins in photoprotection. However, and discussed in detail in Chapter 1, melanins may play other roles in lichens e.g. protection against pathogens or grazing. Future work on melanisation in lichens needs to study other potential roles of melanins in lichen biology.

Other adaptations to high light and UV

Melanins are certainly not the only way lichens can adapt to high light. Apart from the well-known xanthophyll cycle (present in chlorophycean algae but not cyanobacteria), lichens synthesise secondary metabolites such as usnic acid (Solhaug and Gauslaa, 2012). Possibly, lichen substances may have less effect on the heat balance of lichens as they reflect rather

than absorb light. Furthermore, lichen substances can undergo seasonal variations in concentrations, implying that they can be broken down in the winter when light levels are low, and enhanced penetration of the cortex by light may increase photosynthesis. More work is needed to establish the relative advantages of lichen substances compared with melanins. Furthermore, the role of more recently discovered screening pigments such as scytonemin and mycosporines needs further study (Nguyen et al., 2013). Mechanisms to protect or repair light-induced DNA damage that have been reported to exist in other desiccation tolerant organisms (Takuma and Takekazu, 2017) should also be investigated in lichens.

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