

# Chapter 2

## Lessons from Nature: A Personal Perspective

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### Summary

This chapter highlights selected contributions to photosynthesis research made from an evolutionary and ecological perspective and, specifically, to the characterization of zeaxanthin-associated thermal energy dissipation. First, contributions of comparative ecophysiology to the discovery of different CO<sub>2</sub> fixation pathways are examined, followed by a summary of the historical developments leading to documentation of the relationship between zeaxanthin and photoprotective energy dissipation. Evergreen species exhibit exceptionally strong non-photochemical quenching of chlorophyll fluorescence (NPQ) and

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very high levels of zeaxanthin formation. This enabled an unveiling of the correlation between zeaxanthin versus NPQ and/or photosystem II quantum efficiency (as inferred from the ratio of variable to maximal fluorescence,  $F_v/F_m$ ), even prior to development of technology currently used in the assessment of these features. Results from characterization of the wide variety of different manifestations (with respect to extent and/or kinetics) of the conversion of xanthophylls, and changes in NPQ and/or  $F_v/F_m$  in different plant species and diverse environments are placed in an evolutionary and ecological context. Lastly, themes emerging from the international research community on NPQ and photoprotective thermal dissipation are summarized, and suggestions presented for how utilization of plants genetically adapted and acclimated to high levels of light stress may aid in addressing open questions.

## I Introduction

The placement of photoprotective energy dissipation into a context of the ecology and evolution of photosynthetic organisms has made contributions to the understanding and relevance of the non-photochemical quenching of chlorophyll fluorescence (NPQ), and continues to do so today as reflected in many of the chapters that follow. An ecological and evolutionary perspective examines the employment of thermal dissipation in different organisms under diverse environmental

conditions and addresses question such as: *What is the variation on the theme of photoprotective energy dissipation available to an organism and to different organisms? What parts of the system are flexible? For which of a myriad of different environmental niches is a particular variation of the theme most suited? What are the different ways of surviving in a challenging environment and how does this influence the employment of energy dissipation by different organisms? What are the costs and benefits (trade-offs) of the different variations?*

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*Abbreviations:* CAM – Crassulacean acid metabolism; Car – Carotenoid; Chl – Chlorophyll; ELIP – Early light inducible protein;  $F_m$ ,  $F_m'$  – Maximal chlorophyll fluorescence in the dark- and light-adapted state, respectively;  $F_o$ ,  $F_o'$  – Minimal chlorophyll fluorescence in the dark- and light-adapted state, respectively;  $F_v$ ,  $F_v'$  – Variable chlorophyll fluorescence in the dark- ( $F_m - F_o$ ) and light-adapted ( $F_m' - F_o'$ ) state, respectively;  $F_v/F_m$ ,  $F_v'/F_m'$  – Interpreted to be intrinsic efficiency (or quantum yield) of photosystem II in the dark and light-adapted state, respectively; hECN – 3-hydroxy-echinenone as a xanthophyll bound to the orange carotenoid protein of cyanobacteria; HLIP – High light-inducible protein; HPLC – High-pressure liquid chromatography; LHC – Light-harvesting complex; LHCI – Light-harvesting complex of photosystem II; LHCSR – Light-harvesting complex stress-related; NPQ – Non-photochemical quenching of chlorophyll fluorescence; OCP – Orange carotenoid protein of cyanobacteria; PAM – Pulse-amplitude-modulated (chlorophyll fluorometry); PS II – Photosystem II; VAZ cycle – The xanthophyll cycle involving the carotenoids violaxanthin (V) antheraxanthin (A), and zeaxanthin (Z)

## II Standing on the Shoulders of Giants

### A Comparative Ecophysiology

#### 1 Adaptation and Acclimation to the Environment

The field of comparative plant ecophysiology has provided insights into the genetic adaptations and individual flexibility (phenotypic plasticity or acclimation potential) crucial to plant survival, productivity and reproductive success. Such a comparative approach identifies features underlying the success of different species in the same habitat, multiple species across different habitats, and the same species in different habitats (Osmond et al. 1980; Lange et al. 1981a, b, c, d; Baker and Long 1986; Mooney et al. 1987; Mooney 1991; Schulze and Caldwell

1994; Larcher 2003). Some of the pioneering work in this area involved characterization of plants subjected to reciprocal transplants between contrasting habitats as well as the growth and characterization of species from different habitats under “common garden” conditions (Clausen et al. 1941, 1947; Hiesey et al. 1942; Clausen and Hiesey 1960; Mooney and Billings 1961; Mooney and Johnson 1965). Such experiments allow for identification of adaptations among plant species and varieties as well as the individual’s acclimation potential to a changing environment (for a study on the model species *Arabidopsis thaliana*, see Ågren and Schemske 2012). Comparative plant ecophysiology, furthermore, links the fields of plant molecular biology and physiology to whole-plant function and its role in communities, ecosystems, and the biosphere (Feder 2002; Beyschlag and Ryel 2007).

The evolutionary history of different plant species is reflected in their genetic *adaptations* as having provided advantages for survival and reproduction in the habitats where the respective species successfully evolved. Such traits improved plant performance with respect to the physical environment (e.g., soil conditions, climatic factors, etc.) as well as biological (e.g., herbivory, infection by viruses and fungi, etc.) challenges faced over multiple generations. In addition, the individual *acclimation* potential allows those genetic traits to be adjusted to a certain extent. For instance, as a winter annual adapted to germinate in the fall and form a rosette of overwintering leaves, followed by bolting, flowering, and seed set in the spring, *A. thaliana* grown under controlled cool versus warm temperatures develops thicker leaves with more layers of chloroplast-packed palisade mesophyll cells (Gorsuch et al. 2010; Cohu et al. 2014), higher rates of photosynthesis (Gorsuch et al. 2010; Cohu et al. 2013b, 2014), and leaf veins with more (phloem) cells for exporting sugars produced in photosynthesis (Cohu et al. 2013a, b, 2014). Such morphological and physiological plasticity within individuals represents *acclimation*.

There are, however, limits to the acclimatory range of the individual. For instance, leaf veins develop with a greater number of sugar-exporting (phloem) cells in support of higher rates of photosynthesis in *A. thaliana* plants *adapted* to the Swedish climate (Swedish ecotype) compared to plants adapted to the Italian climate (Italian ecotype) irrespective of growth temperature (Cohu et al. 2013a, b). In contrast, leaf veins develop more water-transporting (xylem) cells in response to growth under warmer versus cooler temperatures in the Italian but not the Swedish ecotype (Cohu et al. 2013b). The Italian ecotype, based on its evolutionary history in the warmer and drier climate of Italy, is thus adapted to respond to growth at warm temperature through an acclimatory response that can increase water delivery to the leaves. On the other hand, such a response of water-transporting cells was presumably not advantageous in the moister and colder climate of Sweden, while the adaptation underlying the ability to upregulate the capacity to export photosynthetically generated sugars in the face of lower temperatures (and greater phloem sap viscosity) is important for plants growing in Sweden compared to Italy.

## 2 Pioneers of Comparative Ecophysiology

The mentors of the authors (B.D.-A. and W.W.A.) were Olle Björkman and C. Barry Osmond, respectively; they both conducted pioneering comparative plant ecophysiological work on features important to the adaptation and acclimation of plants to different environmental conditions. This included leaf and photosynthetic acclimation to the light environment among plants adapted to shaded versus sun-exposed habitats (Björkman and Holmgren 1963, 1966; Björkman 1968; Björkman et al. 1973; Osmond 1983), photosynthetic adaptation and acclimation to temperature (Björkman et al. 1972; Armond et al. 1978; Mooney et al. 1978; Berry and Björkman 1980; Badger et al. 1982), characterization of plants with different photosynthetic pathways (Osmond 1967, 1970, 1971, 1974, 1978;

Björkman and Gauhl 1969; Woo et al. 1970; Kluge and Osmond 1971, 1972; Osmond and Harris 1971; Björkman et al. 1973; Osmond et al. 1973, 1980; Ehleringer and Björkman 1977), responses of plants to salinity stress (Osmond 1966; Greenway and Osmond 1972; Osmond and Greenway 1972), and responses of plants to excess light (Powles and Osmond 1978; Powles et al. 1979, 1983; Osmond 1981; Powles and Björkman 1982; Björkman and Powles 1984).

We will use the discovery and characterization of three different photosynthetic pathways, C3, C4, and crassulacean acid metabolism (CAM) to illustrate the evolution of adaptations allowing plants to diversify and colonize multiple niches in the natural world (for historical and basic aspects of C3 photosynthesis, see Benson 2005 and Bassham 2005; for C4, see Hatch 2005, and for CAM, see Black and Osmond 2005.) In a nutshell, the latter photosynthetic pathways offer distinct advantages in some environments at the cost of disadvantages in other environments, resulting in unique benefits and costs (trade-offs). Remarkably, all three pathways share the ancestral feature of fixing carbon into sugars in much the same way, while C4 and CAM plants employ an additional cycle of fixing atmospheric CO<sub>2</sub> and then concentrating it for use by the C3 pathway of photosynthesis in the chloroplast. In the case of C4, this additional cycle operates during the day in the leaf mesophyll cells and shuttles the CO<sub>2</sub> to specialized cells surrounding the veins where only the C3 pathway operates. In the case of CAM, the additional cycle operates during the night, storing the fixed CO<sub>2</sub> in the vacuoles for utilization in the C3 pathway during the subsequent day (largely behind stomata that remain closed because of the high internal levels of CO<sub>2</sub> that arise as it is released for use by the chloroplasts in the light).

The evolution of variation in the pathway for CO<sub>2</sub> acquisition presumably aided in the exploitation of many diverse habitats. Compared to C3 plants, plants utilizing the C4 and CAM pathways of photosynthesis typically require less nitrogen, exhibit little to no photorespiration, are more efficient at

capturing CO<sub>2</sub>, and consequently typically lose less water to the atmosphere for a given level of CO<sub>2</sub> fixation compared to plants utilizing the C3 pathway of photosynthesis (Osmond 1978; Winter 1985; Larcher 2003; Raghavendra and Sage 2011). On the other hand, plants using C4 and CAM have increased energetic costs associated with the use of an additional carboxylation pathway and of specialized anatomical features (modified leaf venation in C4 plants and succulent cells with large vacuoles in CAM; Björkman et al. 1973; Osmond et al. 1980; Edwards and Walker 1983; Winter 1985; Hatch 1992; Larcher 2003; Silvera et al. 2010; Raghavendra and Sage 2011; Sage and Zhu 2011). Because of their highly efficient fixation of CO<sub>2</sub>, some CAM species are able to thrive in aquatic habitats with very low CO<sub>2</sub> levels (Keeley 1998) and to fix CO<sub>2</sub> through tissues without stomatal leaf pores (Keeley et al. 1984; Cockburn et al. 1985; Winter et al. 1985). Furthermore, there are species with intermediate levels of C4 or CAM features (Holaday et al. 1981; Ku et al. 1983; Harris and Martin 1991a, b; Leegood and von Caemmerer 1994; Lüttge 2006), as well as others exploiting a greater water availability during spring as a C3 plant and transitioning to CAM developmentally and, most importantly, during the hot and dry summer (Winter et al. 1978, 2008; Winter 1985). Such variation in plant form and function among species, and even within a species, has allowed plants to thrive in an expanded range of ecological niches (specific environments) with, e.g., low levels of soil nitrogen and/or water availability. Each set of photosynthetic adaptations has its own trade-offs, or costs versus benefits, for a particular growing condition.

In addition to the three major photosynthetic pathways, many other anatomical, morphological, physiological, and developmental plant features impact plant success. For instance, in the hot deserts of North America (Mojave, Sonoran, and Chihuahuan) all three pathways (C3, C4, CAM) are found. In locations or during seasons with continuous access to water, C4 plants thrive under very hot conditions, with extremely high

rates of photosynthesis (Björkman et al. 1972; Larcher 2003; Sage and Kubien 2007). In deserts where water is periodically available, water-storing CAM plants (e.g., succulent cacti, agaves, yuccas, and euphorbs) are found (Kluge and Ting 1978; Osmond 1978; Winter 1985). C3 plants are also remarkably well represented in these deserts, as (i) extremely fast developing “ephemerals” (with high rates of growth and photosynthesis, and rapid seed set following, e.g., intense rainfall events; Patten 1978), (ii) perennials leafing out during the respective moister seasons (Mooney and Strain 1964; Nedoff et al. 1985; Yoder and Nowak 1999), (iii) as plants like palms or mesquite with deep roots tapping into the permanent water table (Nilsen et al. 1983, 1984; Sala et al. 1996; Hultine et al. 2003, 2005), and (iv) as slow-growing evergreens with low rates of photosynthetic water loss, such as the Joshua tree (*Yucca brevifolia*) and creosote bush (dominant shrub of all three North American hot deserts; Armond et al. 1978; Mooney et al. 1978; Smith et al. 1983). All species are similarly well adapted to these hot deserts, yet each by virtue of its own particular adaptation and advantage (that might be a disadvantage in other habitats, particularly in competition with other species adapted to the other habitats). Regardless of the particular photosynthetic pathway employed, all of these species share common features of photosynthetic electron transport and carbon fixation in the Calvin-Benson cycle (C3 photosynthetic pathway in the chloroplast), as well as zeaxanthin-associated photoprotective energy dissipation.

## ***B Prerequisite Contributions to Linking Thermal Dissipation and Zeaxanthin from Biochemistry and Physics***

### ***1 Pioneers in the Discovery of Rapid Xanthophyll Conversions in Plants***

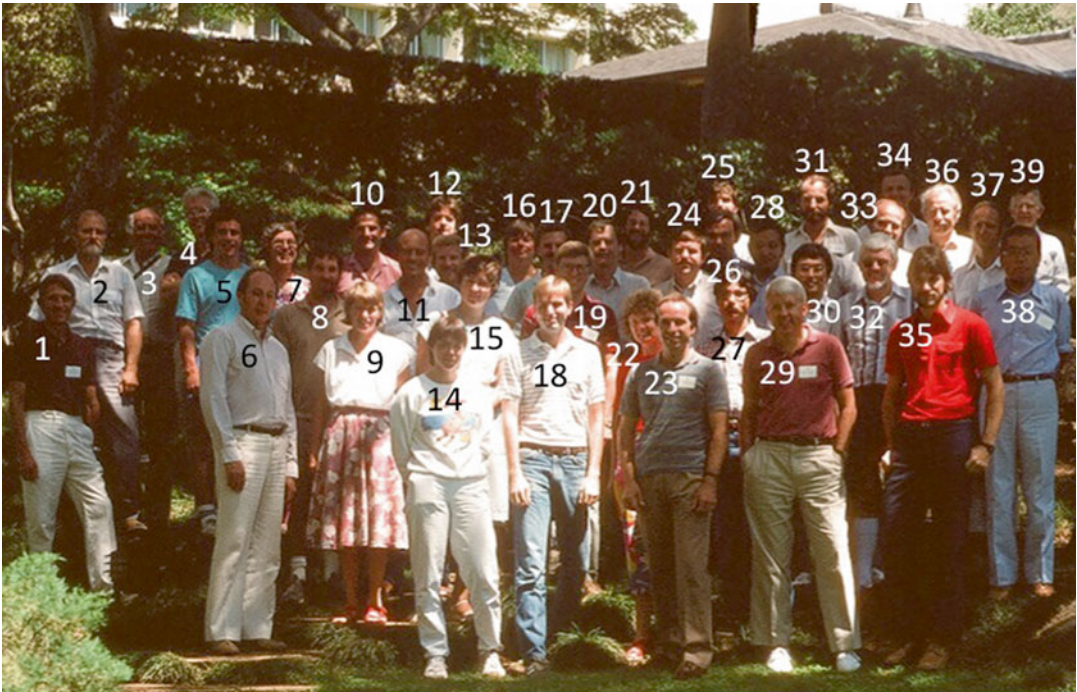
In addition to possessing chlorophyll, photosynthetically competent chloroplasts typically contain several carotenoids, including oxygen-containing xanthophylls

(neoxanthin, violaxanthin, lutein) and oxygen-free carotenes ( $\beta$ -carotene and, for some species,  $\alpha$ -carotene). The levels of most of these chloroplast pigments are relatively static over short periods of time. However, in the late 1950s it was discovered that several xanthophylls are inter-converted over short time spans (minutes) in response to changes in light level (reviewed in Sapozhnikov 1973). This observation was followed by intensive investigation of the biochemical factors controlling de-epoxidation of violaxanthin (with two epoxide groups), via the intermediate antheraxanthin (with one epoxide group) to zeaxanthin (epoxide-free), as well as factors responsible for re-epoxidation of zeaxanthin to violaxanthin (reviewed in Yamamoto 1979, 2006; Hager 1980; Yamamoto et al. 1999). This cyclical inter-conversion of the latter three xanthophylls became known as the violaxanthin cycle or xanthophyll cycle (here referred to as the VAZ cycle, where V, A and Z stand for violaxanthin, antheraxanthin and zeaxanthin, respectively, for distinction between the VAZ cycle and two other xanthophyll cycles present in some plants and algal groups; see, e.g., Esteban and García-Plazaola, Chap. 12 and Lavaud and Goss, Chap. 20). While it was known (i) that these xanthophyll conversions occurred in the chloroplast, (ii) that zeaxanthin levels increased under high light conditions, and (iii) that zeaxanthin was converted back to violaxanthin under low light, a specific function for the cycle and these carotenoids remained elusive until the late 1980s.

### ***2 History of Chlorophyll Fluorescence in Photosynthesis***

The reader is referred to other chapters (Papageorgiou and Govindjee, Chap. 1, Horton, Chap. 3, and Logan et al., Chap. 7) in this volume for detailed accounts of the historical developments in the measurement and understanding of chlorophyll fluorescence emission and factors influencing the level of fluorescence emission. For further information on the relation of chlorophyll fluorescence to photosynthesis, we refer the reader to books edited by Govindjee et al.





*Fig. 2.1* Participants in a workshop on “structure, function and photoinhibition of photosystem II in relation to plant stress” held at the East-West Center, University of Hawaii, in September of 1985. Identified individuals pictured include: (1) David Fork, (2) Robert Smillie, (3) George Cheniae, (4) Dick Dilley, (5) unidentified, (6) C. Barry Osmond, (7) Jan Anderson, (8) Neil Baker, (9) Sue Hetherington, (10) Steve Powles, (11) Jim Barber, (12) Bob Sharp, (13) Dennis Greer, (14) Robyn Cleland, (15) Barbara Demmig, (16) Kit Steinback, (17) Peter Horton, (18) William Adams, (19) Gunnar Öquist, (20) Olle Björkman, (21) unidentified, (22) Christa Chritchley, (23) David Kyle, (24) unidentified, (25) unidentified, (26) Gabriel Cornic, (27) Salil Bose, (28) Norio Murata, (29) Charlie Arntzen, (30) M.-A. Takahashi, (31) Paul Armond, (32) Merv Ludlow, (33) Don Ort, (34) unidentified, (35) Patrick Neale, (36) G. Heinrich Krause, (37) John Boyer, (38) Kozi Asada, and (39) Aubrey Naylor. Although the authors (W. W. A. and B. D.-A.) had met during the summer of 1984, this conference marked the beginning of their professional and personal relationship (see Fig. 2.4, less than 1 month later). Note: if you know the identity of #5, #21, #24, #25, or #34, please send an email to [william.adams@colorado.edu](mailto:william.adams@colorado.edu).

(1986) and Papageorgiou and Govindjee (2004). As we attempted to interpret chlorophyll fluorescence measurements in various plant species in 1984 (B.D.-A. in O. Björkman’s lab and W.W.A. in C.B. Osmond’s lab), we found the work of Kitajima and Butler (1975) particularly enlightening. Using empirical data, Butler and Kitajima had developed a model for the fates of excitation energy absorbed by photosystem II (PS II) and its antennae, including non-radiative, thermal dissipation in what he termed the chlorophyll pigment bed that offered a theoretical explanation for decreases in the level of initial or instantaneous fluorescence ( $F_0$ ) and maximal

fluorescence ( $F_m$ ). Each of us shared our findings of strong (non-photochemical) quenching of both  $F_m$  and  $F_0$  in various plant species exposed to excess light under controlled experimental conditions or in the field at a small gathering of photosynthesis investigators in 1985 (Fig. 2.1) before publishing our findings a couple of years later (Adams et al. 1987, 1988; Demmig and Björkman 1987; Adams and Osmond 1988; Demmig and Winter 1988). Upon completing a postdoctoral appointment with O. Björkman, Barbara returned to Germany, where she began exploring the relationship between chlorophyll fluorescence quenching and the VAZ cycle.

### III Contributions of Comparative Ecophysiology to the Initial Linking of Non-Photochemical Quenching of Chlorophyll Fluorescence and Zeaxanthin

#### A *The Vantage Point of Ecology and Evolution*

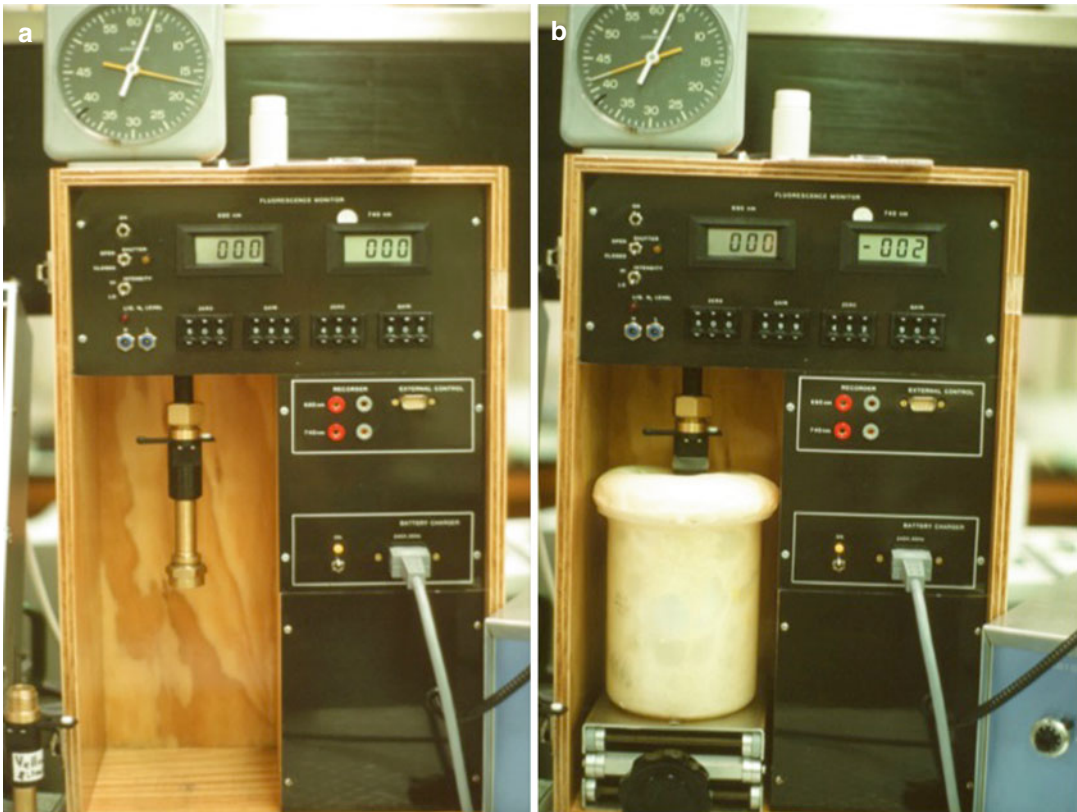
As stated in the beginning of this chapter, multiple questions lend themselves to exploration in a comparative ecophysiological framework, including: *What variation on the theme of photoprotective energy dissipation is available (i) to an organism under different environmental conditions and (ii) to different organisms? What parts of the system are flexible? For which of a myriad of different environmental niches is a particular variation of the theme most suited? What ways of surviving in a challenging environment exist and how does this influence the employment of energy dissipation by different organisms? What are the costs and benefits (trade-offs) of the different variations of thermal dissipation?*

Comparing and contrasting the features of zeaxanthin-associated energy dissipation in plants with different growth rates and life spans in response to a variety of environmental conditions unveiled a multitude of facets of the employment of photoprotective energy dissipation differing widely in, e.g., extent and kinetics. This comparative approach included different types of plants predicted to experience different demands on the extent and kinetics of their thermal dissipation, i.e., soft-leaved annuals (completing their life cycle in one growing season), soft-leaved biennials (experiencing multiple seasons over 1.5 years before reproducing and dying), deciduous species (persisting over multiple years but shedding their leaves during the harshest season every year), and evergreen species (maintaining green leaves or needles through many years). Comparing and contrasting annual and evergreen lifeforms provided examples of the predicted pronounced differences in

the magnitude of employment of zeaxanthin-associated energy dissipation as well as different modes of employment with vastly different kinetics. Many of these differences parallel, and are expected to result from, differences inherent in the annual versus evergreen lifestyle.

Annuals grow rapidly and complete their life cycle over few weeks to several months (typically before climatic conditions become unfavorable for rapid growth via, e.g., reduced water availability or growth-precluding temperatures). Since annuals are adapted for inherent high rates of growth and photosynthesis, they thrive in high-light environments under conditions favorable for rapid growth and, conversely, possess a low shade tolerance. Leaves of high light-acclimated annual species, unless subjected to other environmental stresses, utilize a relatively large fraction of the light they absorb in photosynthesis, and exhibit low maximal levels of photoprotective energy dissipation (typically ranging between 1 and 2 when quantified as  $\text{NPQ} = F_m/F_m' - 1$ ) as well as, typically, incomplete conversion of their VAZ pool to zeaxanthin (Demmig-Adams and Adams 1994; Demmig-Adams et al. 1995, 1996a, 2006; Demmig-Adams 1998; see also Demmig-Adams et al., Chap. 24).

Evergreens receive their name from the fact that they keep green leaves, with a high light-harvesting capacity, throughout both favorable and unfavorable seasons. Over their lifetime, evergreen species persist throughout multiple unfavorable seasons (e.g., summer heat in hot, dry climates or winter frost in temperate climates). Evergreens have genetically fixed low maximal rates of photosynthesis even under conditions ideal for growth. Evergreen species, therefore, successfully grow under conditions with limiting resources, but are unable to compete with rapidly growing species in resource-rich environments. Many evergreen species are highly shade tolerant, but also thrive in high-light environments, where their reproductive success is, in fact, enhanced (Adams 1988). When acclimated to high



*Fig. 2.2* Custom-built instrument for measuring chlorophyll fluorescence from photosystem II (leaf sample illuminated with blue light and fluorescence detected at 690 nm) and photosystem I (detection at 740 nm) from samples that were darkened for several minutes (**a**) then frozen to 77 K (**b**) before illumination. Tissue (leaf or cladode) samples of 1 cm diameter were appressed against one end of a quartz rod enclosed in a brass housing and allowed to equilibrate in darkness for several minutes (see lower left corner of **a**). The brass holder was screwed into the instrument and a shutter slid to the side (from right to left; compare brass holder in lower left of **a** to that in the center of **a**) to allow the other end of the quartz rod to become appressed to the fiber-optic cable that both delivered the blue light to the sample and returned the chlorophyll fluorescence to the photodiode detector (employed in this instrument) or photomultiplier (employed in other instruments). After darkening, the sample and lower portion of the brass holder and quartz rod were submerged in liquid nitrogen (**b**) for 6 min to ensure complete freezing before opening of the shutter that permitted a low level of blue excitation energy ( $1.3 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) to illuminate the sample, allowing the assessment of  $F_0$  and  $F_m$ . The signal from the photodiode or photomultiplier was captured on a strip-chart recorder (see Figs. 2.5 and 2.6). This particular instrument was built by Dr. S. Chin Wong, Win Coupland, and Peter Groeneveld at the Research School of Biological Sciences (now Research School of Biology), Australian National University. Photographs by W.W. Adams.

light, evergreen leaves are able to quickly and fully (90 % and above) convert their VAZ cycle carotenoids to antheraxanthin and zeaxanthin and reach a high maximal capacity for photoprotective energy dissipation (high NPQ levels of 4–8; Demmig-Adams and Adams 1994, 1996a; Demmig-Adams et al. 1995, 1996a, 2006; Barker and Adams 1997; Demmig-Adams 1998).

### **B Quenching of Chlorophyll Fluorescence in Evergreens**

During the mid-1980s, both authors of this chapter conducted chlorophyll fluorescence measurements on excised leaf (or cactus pad, or cladode) segments (from experimental or field-grown plants) darkened for 5 or 10 min (Fig. 2.2a) and frozen to 77 K





Fig. 2.3 (a) Barbara Demmig motoring along a waterway near the Pacific ocean on the eastern coast of Australia (just south of Brisbane) with the evergreen species *Rhizophora stylosa* (the small-stilted mangrove) growing in the background (March 1987; photograph by W.W. Adams) and (b) *Monstera deliciosa* (Swiss cheese plant), adjacent to W.W.A. and B.D.-A., growing in a glasshouse at the University of Colorado in July 2008; photograph courtesy of The Daily Camera.

(Fig. 2.2b) before determining the levels of  $F_o$  and  $F_m$  fluorescence. By virtue of the fact that evergreen species exhibit much greater decreases than annuals in the levels of both  $F_m$  and  $F_o$  fluorescence in response to exposure to excess light (e.g., much higher levels of NPQ), strongly decreased levels of  $F_m$  and  $F_o$  fluorescence were still detectable from high-light-acclimated evergreens exposed to excess light even after darkening of leaves for several minutes. Employment of evergreens was thus instrumental in the recognition of strong concomitant decreases in  $F_o$  and  $F_m$ . It is worth mentioning that the numerous data collected at 77 K invariably revealed a concomitant strong quenching of  $F_o$  and  $F_m$  (with concomitant strong decreases in  $F_v/F_m$ ) in both PS II and

photosystem I (Demmig and Björkman 1987). B.D.-A. worked predominantly with two evergreen species, Australian salt-tolerant mangroves (Fig. 2.3a) Olle Björkman had brought back to Stanford and the climbing rainforest vine *Monstera deliciosa* (Fig. 2.3b; Demmig and Björkman 1987), and W.W.A. worked with evergreen cacti (Fig. 2.4) and other CAM plants (Figs. 2.5 and 2.6; Adams et al. 1987, 1988; Adams 1988; Adams and Osmond 1988), all of which were both *adapted* and *acclimated* to highly excessive light levels and exhibited strong and sustained (non-photochemical) quenching of the  $F_m$  and  $F_o$  levels of chlorophyll fluorescence.

Shortly thereafter, the pulse-amplitude-modulated (PAM) system for room-



**Fig. 2.4** (a) View overlooking the Laboratory of Desert Biology (and luxury accommodations) in the northern reaches of Death Valley, California. The green vegetation is largely *Larrea tridentata* (creosote bush), and Stanley D. Smith is visible on the right side of the image. This trailer/laboratory was established by Frits Went (pioneer in the discovery of the growth hormone auxin; Went 1926) as part of the Desert Research Institute associated with the University of Nevada (see Went 1968); Went was still in Reno when W. W. A. began his PhD work there in 1984. (b) C. Barry Osmond, Cornelia Büchen-Osmond, Barbara Demmig, and William Adams at the Laboratory for Desert Biology in early October 1985 (prior to Barry Osmond becoming afflicted with heatstroke later that day). The laboratory was not equipped with air conditioning, and the swamp coolers were non-functional. (c) *Opuntia basilaris* (the beavertail cactus) growing nearby (see Adams et al. 1987). Photographs by W.W. Adams.

temperature fluorescence measurement from intact, attached leaves, permitting assessment of chlorophyll fluorescence quenching during illumination independently of the level of direct sunlight or artificial light, became available (Schreiber 1986; Schreiber et al. 1986). Our first use of this new system still involved darkening of leaf samples for several minutes for comparison with those fluorescence measurements obtained prior to the development of the PAM fluorometer (Demmig et al. 1987a, 1988; Adams et al. 1988, 1989,

1990a; Demmig and Winter 1988; Demmig-Adams et al. 1989d). However, it soon became clear that ascertaining the level of  $F_o$  and  $F_m$  quenching directly under (actinic) illumination or in the field (as  $F_o'$  and  $F_m'$ ) was preferable unless one was interested in characterizing only the portion of photoprotection that remained engaged for extended periods, e.g., nocturnally-sustained “photoinhibitory” depressions in PS II efficiency (Adams et al. 1995a, 2006, 2013a; see Adams et al., Chap. 23 and Demmig-Adams et al., Chap. 24).





Fig. 2.5 (a) William Adams in the midst of measuring chlorophyll fluorescence from leaf punches (darkened and frozen to 77 K) of (b) the CAM fern *Pyrrosia confluens* growing epiphytically in Dorrigo National Park (a subtropical rainforest approximately 15 km from the coast, southwest of Coffs Harbour, New South Wales, Australia). William (bundled up because the predawn temperature reached a low of  $-5^{\circ}\text{C}$  on this particular field trip [1 August 1986]) is sitting beside the strip-chart recorder and the low temperature chlorophyll fluorometer, with a dewar of liquid nitrogen on the ground and the power cords (lower right hand corner) receiving electricity from a gasoline-powered generator (off to the right, out of view). See Adams (1988). Photographs by E. Brugnoli (a) and W. W. Adams (b).

### C Zeaxanthin in Evergreens

Following the completion of her post-doctoral appointment in Olle Björkman's laboratory, B.D.-A. returned to Germany to pursue research at the Lehrstuhl für Botanik II in Würzburg. After coming across papers on VAZ cycle operation, she was intrigued by the similarity of the features of VAZ cycle conversions and her measurements of fluorescence quenching at Stanford, expressed (after Kitajima and Butler 1975) as changes in the rate constant for thermal dissipation in the pigment bed. The laboratory of professor Franz-Christian Czygan, where

separation, identification, and quantification of plant pigments was routinely carried out by experienced technician Frau Almuth Krüger, was in the neighboring Lehrstuhl für Pharmaceutical Biology. When sought out by Barbara, Franz-Christian Czygan kindly offered Frau Krüger's assistance, which led to the first concomitant analyses (in the late 1980s) of chlorophyll fluorescence quenching and leaf xanthophyll content, the latter laboriously conducted using thin layer chromatography before the development of faster and more sensitive high-pressure liquid chromatography (HPLC) methods for the separation of the close structural



Fig. 2.6 (a) Enrico Brugnoli and the 77 K chlorophyll fluorescence equipment on the beach in the shade of Middle Head (south of Scotts Head and north of Grassy Head, New South Wales, Australia) where both *P. confluens* (see Fig. 2.5b) and (b) *Hoya australis* (common waxflower) were characterized where they both grew as lithophytes on Middle Head. Photographs by W.W. Adams; see Adams 1988 and Adams et al. 1988.

isomers zeaxanthin and lutein. Once again, evergreens with their low rates of photosynthesis (and resulting high need for photoprotection) proved invaluable since thin layer chromatography requires large amounts of pigment. The evergreens conveniently generated the required large amounts of zeaxanthin and antheraxanthin, by virtue of de-epoxidizing their VAZ pool to over 90 % (versus typically only about 60 % in annuals). With the support of the German academic system that did not require young faculty members to seek their own grants for highly exploratory ideas, many hundreds of leaf carotenoid analyses (expertly performed by Frau Krüger) helped establish close relationships between VAZ pool conversion and chlorophyll fluorescence quenching indicative of photoprotective thermal energy dissipation.

Initial studies established close correlations between zeaxanthin level and sustained (for 5 or 10 min in darkness), “photoinhibitory” decreases in chlorophyll fluorescence (Demmig et al. 1987a, 1988). Subsequently, very similar correlations were established between zeaxanthin level and the rapidly reversible (high-energy state) quenching of chlorophyll fluorescence for intact evergreens and annuals under experimental treatments (Demmig-Adams et al. 1989a, b, c) or in the field (Demmig-Adams et al. 1989d) as well as for plants and green-algal lichens treated with an inhibitor of violaxanthin conversion to zeaxanthin (Demmig-Adams and Adams 1990; Bilger and Björkman 1991) and for lichens accumulating zeaxanthin either rapidly (via the VAZ cycle) or slowly (from  $\beta$ -carotene; Demmig-Adams et al. 1990b, c).



*Table 2.1* Major topics touched upon in this volume, major emerging conclusions, and the chapters in which the topics and conclusions are discussed. ELIPs and HLIPs have not been included under proteins in plants because it remains to be determined whether the correlative evidence for their involvement in sustained thermal energy dissipation in evergreen plants following sudden transfer from low to high light (Demmig-Adams et al. 2006) and during winter stress (Zarter et al. 2006a, b; Wang et al. 2009; Demmig-Adams et al., Chap. 28) can be supported by mechanistic studies

Topic	Emerging conclusions	Chapters
Identification of factors involved in NPQ	<b>LHC family proteins</b>	
	Plants	
	PsbS	3, 5, 13, 14
	Various algal groups	
	LHCSR	14, 21
	LHCSR-related proteins	11, 14, 20
	Cyanobacteria	
	OCP	14, 22
	<b>Xanthophylls</b>	
	Products of xanthophyll cycles	
	Zeaxanthin & Antheraxanthin (from Violaxanthin)	3, 4, 5, 6, 13, 14, 23, 24
	Diatoxanthin (from Diadinoxanthin)	11, 14, 20
	Lutein (from Lutein epoxide)	12
	Other xanthophylls	
	Hydroxy-echinenone	22
	Lutein (not from Lutein epoxide)	6, 15
Photophysical mechanism	Fate of excitation energy strongly affected by local environment of pigment-binding complexes; much support for Chl-Car exciton coupling	4, 6, 8, 9, 10, 16
Possible sites of quenching	Minor LHC proteins	14
	LHCII	3, 6, 10, 15
	Both minor LHCs and LHCII	5
Ecological studies	Multiple genetic adaptations to different specific environments plus strong acclimation of individuals to growth light environment	2, 12, 20, 23, 24, 25, 26, 27

## IV Additional Contributions of Ecophysiology and Evolutionary Biology to the Understanding of Photoprotection via Thermal Energy Dissipation

### A Further Contributions Since the Initial Linking

#### 1 Evolutionary Relationships: Emerging Themes and Their Variations

Table 2.1 includes a list of some of the major topics covered in this volume, some of the conclusions with support from multiple investigators, and a list of the chapters in which those topics and conclusions are discussed. In most of the large diversity of photosynthetic organisms

examined, engagement of photoprotective energy dissipation (reflected in increased NPQ and/or decreased PS II efficiency) is thought to involve both (i) a xanthophyll and (ii) a member of the light-harvesting family of proteins. Evidence for the involvement of multiple light-harvesting complex (LHC) family proteins and multiple different xanthophylls in different taxa of photosynthetic organisms is summarized throughout this book (see Table 2.1 for specific chapters). Xanthophyll-associated thermal energy dissipation is thought to be a mechanism to harmlessly dissipate excess excitation energy absorbed by chlorophyll and, thereby, prevent passing of excitation energy on to oxygen (see Demmig-Adams et al., Chap. 28). Many additional photoprotective responses of plants and algae prevent or minimize the absorption of excess light

or detoxify reactive oxygen species and other radicals that may result from exposure to excess light energy (see Logan et al., Chap. 7, for an overview of the entire suite of photoprotective mechanisms available to plants, and Havaux and García-Plazaola, Chap. 26, for differences among taxa in the employment of energy dissipation versus anti-oxidation).

## 2 Ecological Relationships

During the 1990s, we characterized (using HPLC analysis of chloroplast pigments and laboratory and portable PAM chlorophyll fluorometers) multiple plant species (including annuals, biennials, deciduous species, and evergreens) growing naturally either in open sunny locations or in the shade of tree canopies or other structures, where they typically naturally received occasional sunflecks every day. Remarkably, in all of these situations with extremely different levels of NPQ and NPQ relaxation kinetics, the same increase in NPQ and decrease in PS II light-harvesting efficiency (ascertained as the ratio of variable to maximal chlorophyll fluorescence, i.e.,  $F_v'/F_m'$  determined during illumination or  $F_v/F_m$  determined in darkness) was seen for a given VAZ cycle conversion state to zeaxanthin + antheraxanthin (Demmig-Adams et al. 1995; Demmig-Adams and Adams 1996a). We note that these inter-species comparisons apply to leaves exposed to peak light levels in clear excess of that which can be utilized in photosynthesis, thus presumably minimizing involvement of mechanisms that optimize excitation energy distribution and instead focusing on mechanisms serving to thermally dissipate excess excitation energy. From an evolutionary standpoint, the observation of uniform responses to excess light among species suggests that the underlying mechanism of quenching may be conserved, while additional, species- and growth condition-dependent factors provide diversification to meet the demands of contrasting environments. For summaries of other ecophysiological work on photoprotective

thermal dissipation and/or xanthophylls, see, e.g., Esteban and García-Plazaola (Chap. 12; see also García-Plazaola et al. 2007, 2012), Lavaud and Goss (Chap. 20), Adams et al. (Chap. 23), Demmig-Adams et al. (Chap. 24), Murchie and Harbinson (Chap. 25), Havaux and García-Plazaola (Chap. 26), and Morales et al. (Chap. 27) as well as other papers (Königer et al. 1995; Krause et al. 2004; Savage et al. 2009; Matsubara et al. 2012).

How could it be possible that the same NPQ (and  $F_v/F_m$ ) level is attained for a given VAZ cycle conversion state in a leaf performing pronounced changes in NPQ (and  $F_v/F_m$ ) over many minutes versus over only a single second – a time scale known to be too fast for VAZ cycle conversions? Leaves can contain zeaxanthin that is apparently not engaged in energy dissipation as long as leaves are not experiencing actual excessive light. This was demonstrated experimentally by inducing intact leaves to retain zeaxanthin in darkness, which was followed by rapid engagement of energy dissipation upon exposure to excess light over one to a few second(s) in zeaxanthin-preloaded leaves but not in zeaxanthin-free leaves (Demmig-Adams et al. 1989b). Likewise, leaves of two *Yucca* species experiencing the summer heat of the Mojave desert (Fig. 2.7a) were found to nocturnally retain 60 % or more of the VAZ cycle as zeaxanthin + antheraxanthin that apparently only became engaged in thermal dissipation as the sun rose (Barker et al. 2002), presumably in response to proton accumulation in the thylakoid lumen as photosynthetic electron transport was activated (see Strand and Kramer, Chap. 18). Similarly, plants under either a dense rainforest canopy (Fig. 2.7b; Logan et al. 1997) or a more open *Eucalyptus* forest (Fig. 2.7c, d; Adams et al. 1999) retain zeaxanthin + antheraxanthin overnight and throughout the day, which, during sunflecks, apparently becomes instantly engaged in thermal energy dissipation to provide photoprotection and is instantly disengaged upon return to low light limiting to photosynthesis. For plants in nature, no increases in NPQ or



Fig. 2.7 (a) *Yucca schidigera* (Mojave yucca, on the left and right side in the foreground, with CAM photosynthesis) and *Yucca brevifolia* (the Joshua tree, center foreground, with C3 photosynthesis) growing near Red Rocks Conservation Area in the Mojave Desert of Nevada (Photograph by S.D. Smith; see Barker et al. 2002), (b) *Alocasia brisbanensis* growing in the understory of a subtropical rainforest (Dorrigio National Park) during exposure to a sunfleck (Photograph by W.W. Adams; see Logan et al. 1997), and two vines from which chlorophyll fluorescence was measured and samples for pigments and antioxidants were removed during exposure to sunflecks in the understory of an open *Eucalyptus* forest south of Middle Head along the eastern coast of New South Wales, Australia, (c) *Stephanian japonica* and (d) *Smilax australis* (left side) and *Stephanian japonica* (lower right corner). Photographs in c and d by W.W. Adams; see Adams et al. 1999.

decreases in PS II efficiency in response to excess light have thus been observed in the absence of a corresponding level of zeaxanthin + antheraxanthin.

It is attractive to assume that evolution of *dual control* of thermal dissipation by *not only* (i) removal of quenching xanthophyll(s) via a xanthophyll cycle *but also* (ii) instantaneous disengagement of NPQ upon dissipation of the trans-thylakoid pH gradient allowed a swift return to a high light-harvesting capacity whenever light is limiting to photosynthesis. A central prerequisite for the evolution of land plants may thus have been the *removal* of zeaxanthin by the

VAZ cycle (with *formation* of zeaxanthin from  $\beta$ -carotene having evolved long before in the earliest oxygenic photosynthetic organisms; Hager 1980; see also Esteban et al. 2009). By this token, it would be unattractive to assume that land plants would use any xanthophyll as a quencher that is not tightly controlled by a xanthophyll cycle (see also Esteban and Garcia-Plazaola, Chap. 12; Demmig-Adams et al., Chap. 24). Similar considerations also apply to certain groups of algae employing xanthophyll cycles (see Lavaud and Goss, Chap. 20).

Walla et al. (Chap. 9) state that, “it has been suggested that even [carotenoids] other



than those involved in the VAZ cycle dissipate excess energy as a result of, for example, conformational changes within the pigment-protein complexes. However, zeaxanthin-free intact leaves never show quenching as deep as plants under excess light in nature. ... the role of [carotenoid-chlorophyll] interactions in various proposed structural rearrangements that may contribute to NPQ, such as LHCII aggregation, state transitions and other membrane restructuring, is not yet clear.” While some groups propose that zeaxanthin exerts control over putative quenching by lutein (see, e.g., Horton, Chap. 3; Krüger et al., Chap. 6), others have argued against “a direct involvement” of constitutively present lutein in thermal dissipation and instead proposed roles in optimizing antenna structure for light harvesting (Lokstein et al. 2002) and quenching of triplet chlorophyll (Dall’Osto et al. 2006; Mozzo et al. 2008). Horton (Chap. 3) concludes that, “there is little doubt about the importance of the VAZ cycle in NPQ. No theory for NPQ can be complete unless it explains the remarkable link between the extent of quenching and DES [de-epoxidation state] ... However, exactly how this link operates is still a matter of debate and disagreement.”

The results of fluorescence lifetime analyses also remain to be reconciled. Gilmore et al. (1995), in Govindjee’s laboratory, associated NPQ onset with a shift from a fluorescence component with a longer (2 ns) to one with a shorter (0.4 ns) lifetime, and concluded that this shift was responsible for a pH- and zeaxanthin-associated major component of the flexible, rapidly reversible form of NPQ. A similar conclusion was reached for the major component of flexible NPQ in diatoms: this NPQ component was proposed to be both pH- and diatoxanthin-dependent (see Holzwarth and Jahns, Chap. 5; Lavaud and Goss, Chap. 20). Gilmore and Ball (2000) extended fluorescence lifetime analysis to overwintering evergreens in the continuously dissipative state (see below) and concluded that the latter involved a zeaxanthin-dependent, but pH-independent

(and thus continuously engaged) form of NPQ. Jahns and Holzwarth (2012; see also Holzwarth and Jahns, Chap. 5), using the PsbS-deficient *npq4* mutant of *Arabidopsis*, on the other hand, proposed that zeaxanthin-dependent NPQ is not pH-dependent even in wild type exhibiting flexible NPQ. The possibility should be evaluated that zeaxanthin-dependent NPQ may revert to a pH-independent form (possibly involving a pH-independent protein factor to engage quenching) in PsbS-deficient mutants, while wild type may employ PsbS for flexible quenching involving both pH-control and zeaxanthin as a quencher as envisioned by Gilmore et al. (1995).

Although all plant species exhibit the same relationship between the level of zeaxanthin + antheraxanthin and the level of thermal energy dissipation (assessed as NPQ and decreases in PS II efficiency), different species do exhibit different absolute levels of both responses (of both of the latter parameters in concert) when growing side-by-side under the same environmental conditions. For instance, an annual species, utilizing a relatively higher proportion of absorbed light energy in photosynthesis, showed a peak level of NPQ of 2 and a decrease in PS II efficiency to 0.5 at midday, whereas an evergreen groundcover, with a lower capacity for photosynthesis, exhibited a peak NPQ of over 4 and a reduction in PS II efficiency to 0.25 at midday (Demmig-Adams et al. 1996a; see also Demmig-Adams et al. 2012 and Chap. 24).

Annuals and evergreens also differ in their response to prolonged environmental stress. While many annuals and deciduous species exhibit reduced leaf chlorophyll content, resulting in reduced levels of light absorption, in response to prolonged environmental stresses (Verhoeven et al. 1997; Logan et al. 1999; see Morales et al., Chap. 27), such a response is less common in evergreens. Instead, evergreen species arrest their VAZ cycle and maintain large amounts of zeaxanthin that either apparently become engaged in energy dissipation when light is excessive and disengaged when it is not (e.g., in



response to drought and high temperature; Barker et al. 2002) or enter into a locked-in dissipative state continuously maintained regardless of the presence of light (e.g., during winter; see Adams et al. 1995a, 2001, 2002, 2006, 2013a; Gilmore and Ball 2000; Öquist and Huner 2003; Demmig-Adams and Adams 2006; Adams et al., Chap. 23; Demmig-Adams et al., Chap. 24).

Plants respond to winter stress in different ways depending on species and environmental conditions. Soft-leaved annuals or biennials (herbaceous species) persisting through winter exhibit an upregulation of photosynthesis in response to lower versus warmer temperature (Adams et al. 1995b, 2001, 2002, 2013b; Verhoeven et al. 1999; Cohu et al. 2013b, 2014). The latter species retain zeaxanthin on colder nights in response to nocturnal maintenance of the trans-thylakoid pH gradient, exhibit an increased ATP to ADP ratio, and a continuously maintained apparent engagement of zeaxanthin in energy dissipation (reflected in continuously maintained low levels of  $F_v/F_m$ ), all of which is rapidly reversed upon warming of the leaves (Demmig-Adams et al. 1996b; Verhoeven et al. 1999). Evergreen species (conifers, bushes, and groundcovers) exhibit similar responses during the autumn-to-winter transition as temperatures drop below freezing on some days and rise above freezing on others (Adams and Demmig-Adams 1994, 1995), and throughout winters in environments with soil temperatures that intermittently reach above-freezing levels conducive to allowing plants to resume photosynthetic activity (Verhoeven et al. 1998). However, in the most extreme habitats where soil water remains frozen for long periods of time, thus precluding any intermittent photosynthetic activity, zeaxanthin (and antheraxanthin)-associated energy dissipation is not pH-dependent and is continuously maintained (does not reverse upon warming of leaves or needles; Fig. 2.8) as winter progresses, and this continuously maintained dissipative state constitutes all of the nocturnally-maintained decreases in  $F_v/F_m$  (Adams et al. 1995a, 2002, 2006; Verhoeven et al. 1996,

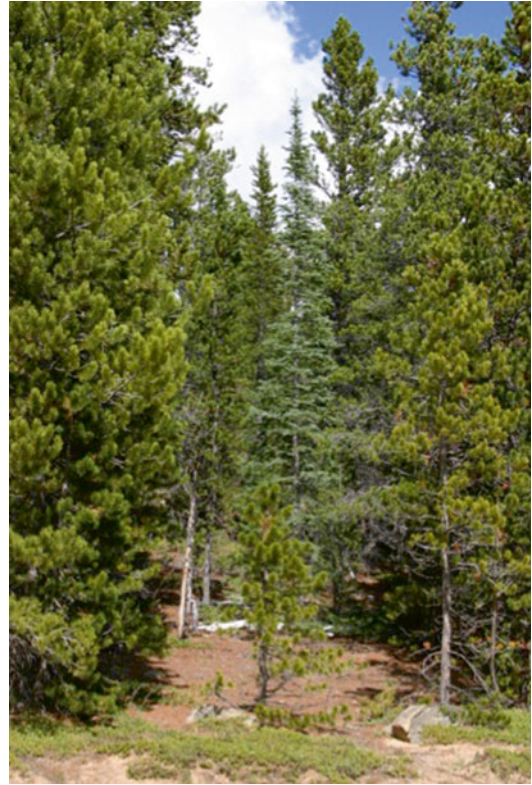


Fig. 2.8 The evergreen groundcover *Arctostaphylos uva-ursi* (bearberry, or kinnikinnick; see Zarter et al. 2006a) in the foreground with the conifers *Pinus contorta* (lodgepole pine, left and right sides) and *Picea engelmannii* (Engelmann spruce) growing in Roosevelt National Forest, Colorado (see Zarter et al. 2006b,c). Photograph by W.W. Adams.

1998; Adams and Barker 1998; Zarter et al. 2006a,b,c). Although modulation of the retention of zeaxanthin and its engagement in energy dissipation differs as dependent on species and environmental conditions, the relationship between the VAZ conversion state and PS II efficiency is the same irrespective of whether the two metrics are rapidly reversible and determined under midday exposure to sunlight in the summer or are continuously maintained in their dissipative state in winter (Adams et al. 1995a, 2006). In other words, maximal NPQ in leaves can be predicted from foliar zeaxanthin content in a range of different natural environments, while NPQ kinetics vary from seconds to minutes or days.

The latter features show that modulation of zeaxanthin-associated thermal energy dissipation is fine-tuned to closely respond to the opportunity (or lack thereof) for photosynthetic activity present in each specific environment. For instance, a continuously maintained dissipative state is seen only in environments where conditions (such as frozen soils preventing plant water uptake) continuously preclude plant carbon uptake. Algae likewise exhibit adaptation and acclimation to multiple niches with respect to the intensity and distribution of light, as well as other features of the aquatic habitats in which they are found (see Lavaud and Goss, Chap. 20). While there is no evidence that the widely variable relaxation kinetics of NPQ in nature must be due to different quenchers, there clearly are different manifestations of quenching. Evolution of multiple different manifestations of one fundamental mechanism under the selective pressures of different environments is a common theme in biology. The many different time scales of VAZ cycle operation and the several different protein and other factors correlating with these different manifestations suggest that zeaxanthin-associated NPQ may be another example for this theme.

Acclimation of plants to the degree of light stress in their growth environment involves adjustments in both xanthophylls and specific thylakoid proteins, as the two major chloroplast constituents implicated in thermal energy dissipation leading to NPQ (Table 2.1). The total VAZ pool is larger in leaves (and lichens) acclimated to higher versus lower light (Adams et al. 1992, 1993, 1996, 1999; Demmig-Adams and Adams 1992a, b, 1994, 1996b; Demmig-Adams et al. 1995; Logan et al. 1996; Demmig-Adams 1998), in winter compared to summer (Adams and Demmig-Adams 1994; Adams et al. 1995a, 2002), and in plants subjected to less than optimal soil conditions (Morales et al., Chap. 27). The level of the PS II protein PsbS (as a member of the light-stress-related subfamily of LHC proteins), involved in the flexible, pH-dependent engagement and disengagement of NPQ (Li et al. 2000,



Fig. 2.9 The evergreen groundcover *Arctostaphylos uva-ursi* (bearberry, or kinnikinnick) growing (a) in full sunlight in Gregory Canyon (City of Boulder Open Space and Mountain Parks) and (b) in a shaded site in Roosevelt National Forest, Colorado. Photographs by W.W. Adams; see Zarter et al. 2006a.

2002a, b, 2004), is likewise upregulated in leaves of evergreen species acclimated to high versus low light (Demmig-Adams and Adams 2006; Demmig-Adams et al. 2006). On the other hand, different members of the sub-family of light-stress-related LHC proteins, such as early light-inducible proteins (ELIPs) and/or high light-inducible proteins (HLIPs), were upregulated in evergreen species upon sudden experimental transfer from a low to a high light growth environment (Demmig-Adams and Adams 2006; Demmig-Adams et al. 2006) and upon the natural seasonal shift into winter (Zarter et al. 2006a, b; Wang et al. 2009), both of which transitions also caused the leaves to enter a continuously dissipative state not requiring a trans-thylakoid pH gradient (Verhoeven et al. 1998; Demmig-Adams et al. 2006). While an evergreen groundcover

overwintering in full sun exhibited no up-regulation of the PsbS protein (Fig. 2.9a), leaves of the same species overwintering in the shade did so (Fig. 2.9b; Zarter et al. 2006a; see also Ottander et al. 1995). There is also evidence for a possible involvement of thylakoid protein phosphorylation in the regulation of thermal energy dissipation (Demmig et al. 1987b; Cleland et al. 1990; Adams et al. 2001; Ebbert et al. 2001, 2005; Demmig-Adams and Adams 2006; Demmig-Adams et al. 2006), which should receive further attention (see below).

Leaves grown in the absence of excess light show very different responses than leaves grown in the presence of regular, intermittent exposure to excess light (see below). Leaves grown without excess light include leaves grown indoors under low, non-fluctuating light levels as well as plants growing outside in the extremely rare shade environment without sunflecks (e.g., leaves heavily shaded by environmental features and/or the rest of the plant). The very first exposure to excess light of such a leaf grown in the absence of excess light results in only a very low initial level of NPQ – accompanied by initial conversion of only a small fraction of the existing VAZ pool (Demmig-Adams et al. 1995, 1998; see also Demmig-Adams et al., Chap. 24). Over the course of continued exposure to excess light, NPQ levels subsequently increase very slowly and gradually – over the course of many hours – to similarly high NPQ levels as seen in plants grown under conditions including daily exposure to excess light (peak sunlight in open locations and sunflecks in otherwise shaded locations). The latter slow increase in NPQ is closely correlated with a slow increase in VAZ pool conversion to zeaxanthin and antheraxanthin, and eventually an increase in total VAZ pool size as additional zeaxanthin is synthesized from  $\beta$ -carotene (Demmig-Adams et al. 1998; see also Demmig-Adams et al. 1989e; Adams and Demmig-Adams 1992; Adams et al. 1999; Demmig-Adams et al., Chap. 24).

While the use of model species grown under controlled conditions presents

advantages with regard to understanding the underlying genetics, this approach does have its limitations. Model plant species used thus far are typically short-lived, annual mesophytes, which, among plants, are the least tolerant of environmental stress. To understand the full range of adaptation and acclimation available to plants with regard to photosynthesis and photoprotection, it is necessary to take a broader, ecophysiological approach that includes multiple species and their myriad of responses to different genetic and environmental constraints to growth (Demmig-Adams et al. 2012; García-Plazaola et al. 2012).

### *B How Ecophysiology May Aid in Addressing Open Questions*

Both adaptation and acclimation to the growth light environment determines the kinetics and capacity for NPQ (as a measure of energy dissipation). Techniques capable of exploring photo-physical mechanisms in intact leaves (Walla et al., Chap. 9) and/or the involvement of one or more quenching sites in intact leaves (via time-resolved fluorescence; Holzwarth and Jahns, Chap. 5) should be applied to leaves from plants (i) grown in environments with regular exposure to excess light (compared with plants grown in the absence of excess light) and (ii) plants genetically adapted to high versus low levels of excess light.

The tremendous utility of the many mutant studies conducted on this topic has been (i) in pinpointing factors (such as various LHC family proteins and various xanthophylls) that can be involved in NPQ and (ii) in demonstrating the astonishing flexibility of plants and other photosynthetic organisms in being able to employ apparent compensatory adjustments to tolerate full sunlight despite a missing component. Yet, the ability to compensate for missing components is, at the same time, a limitation of mutant studies that should be acknowledged. In consideration of the compensatory mechanisms employed by mutants, as evidenced by their astounding tolerance of full sunlight,



a mutant lacking, e.g., the PsbS protein may be recruiting an alternative factor to replace PsbS, albeit at some cost to the flexibility of thermal dissipation modulation (Demmig-Adams and Adams 2006; Zarter et al. 2006a, b; Wang et al. 2009).

It therefore remains to be elucidated whether or not the quenching component termed qZ, assigned to zeaxanthin and described to be pH-independent and relaxing over 20 min (rather than over a few seconds) in the PsbS-deficient *npq4* mutant (Holzwarth and Jahns, Chap. 5), is also employed by wild type. It cannot be ruled out that the wild type may still be employing PsbS to modulate zeaxanthin-dependent quenching in a pH-dependent, rapidly reversible manner, while the PsbS-deficient mutant may be forced to replace flexible modulation with a less flexible modulation of a zeaxanthin-dependent quenching, possibly via one or more other members of the stress-inducible LHC sub-family and including pH-independent manifestations. Future studies should, therefore, also address costs and benefits of the employment of different possible mechanisms and sites of thermal dissipation as well as of different extent of thermal dissipation (see Demmig-Adams et al. 2013, Chap. 28). For further insight, existing mutant studies should be complemented by studies of plant species genetically adapted to high versus low light stress (e.g., annuals versus evergreens) and leaves grown in the absence versus presence of excess light.

In the various chapters in this volume, different views are expressed as to the mechanism underlying thermal energy dissipation. Many authors argue that a xanthophyll is involved in thermal dissipation, and that this xanthophyll is formed by a xanthophyll cycle (Table 2.1). Likewise, much discussion of the photo-physical mechanisms involved in thermal dissipation centers on the ability of the local protein environment to modulate energy levels of pigments involved and on the involvement of exciton coupling between chlorophyll and carotenoids (see, e.g., Ostroumov et al., Chap. 4, Krüger et al.,

Chap. 6 and Walla et al., Chap. 9). It should prove insightful to apply these techniques, wherever possible, to plant systems both adapted and acclimated to excess light. As part of such an approach, and as proposed by Walla et al. (Chap. 9), “a quantitative study is needed that directly assesses the spectroscopic signatures of all suggested electronic interactions in a single system.”

## V Concluding Remarks

Comparative eco-physiology facilitated the uncovering of a close correlation between zeaxanthin level and non-photochemical fluorescence quenching (as an indicator for the thermal dissipation of excess excitation energy) in intact leaves. Comparison of annual and evergreen lifeforms with different inherent rates of growth and photosynthesis, furthermore, revealed stark differences in the magnitude and kinetics of zeaxanthin-associated energy dissipation. An evolutionary vantage point suggests that evolution of dual control over thermal energy dissipation, by *removal* of xanthophyll(s) via a xanthophyll cycle plus rapid *disengagement* of NPQ via dissipation of trans-thylakoid  $\Delta$ pH in environments favorable for plant growth, may have helped to maximize photosynthesis' photon yield by allowing swift return to high light-harvesting capacity during every transition from excess to limiting light over the course of a day in naturally fluctuating sunlight. Conversely, under environmental conditions less favorable for, or altogether preventing, plant growth, a cascade of different manifestations of zeaxanthin-associated thermal energy dissipation is evident especially in evergreens: each manifestation appears fine-tuned to closely match the level of opportunity for growth and photosynthesis present in each unique environment. A dissipative state continuously maintained over the entire winter season is seen only in environments where conditions (such as frozen soils) preclude plant carbon uptake for extended time periods. Likewise, both genetic adaptation and



individual acclimation to growth light environment strongly influences a plant's kinetics and maximal capacity for thermal energy dissipation (as assessed via chlorophyll fluorescence). Since leaves grown in the absence of fluctuating (intermittently limiting and excessive) light show very different responses than leaves grown in the presence of regular exposure to excess light, future research on a wide range of mechanistic questions will benefit from a comparison of plant systems adapted and acclimated to different levels of excessive light and corresponding different demands for thermal dissipation of excess excitation energy, as well as combining multiple approaches in a single system with a high capacity for NPQ.

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