1. Introducing ecological proteomics

The decade has seen a step change in our ability to investigate the molecular bases of biological adaptation and evolution. Rapidly progressing ‘omics’ technologies now allow researchers to characterise complete sets of biological molecules in non-model organisms (Wang, Gerstein & Snyder 2009; Ekblom & Galindo 2010; Armengaud *et al.* 2014). The field of transcriptomics in particular has made substantial progress in describing responses to environmental stimuli at the molecular level, and comparative analysis of mRNA abundances has become a standard method in molecular ecology (Alvarez, Schrey & Richards 2015; Diz & Calvete 2016). Extent of protein expression determines an organism’s capacity to perform biochemical functions where the rate at which work is done is a function of the amount of protein doing the work (ref – Michaelis & Menten?). In photosynthesis, for example, light energy captured by light harvesting proteins is used to catalyse carbon uptake from the atmosphere by enzymatic carboxylation. The rate at which plants are able to perform this process depends directly on the abundance of photosynthetic proteins in their leaves (Evans 1989?). Transcript data is a relatively weak proxy for protein abundance, however, as protein abundance is primarily controlled at the level of translation (Gygi *et al.* 1999; Schwanhausser 2011). As such, direct quantification of protein amounts using proteomics methods can provide better information about functional responses of organisms to their environment (Diz & Calvete 2016).

Proteomic applications in ecological and evolutionary research are increasingly being facilitated by the availability of genomic resources for non-model organisms (Armengaud *et al.* 2014; Baer & Millar 2016; Diz & Calvete 2016). Comparative data on the abundances of proteins or groups of proteins which perform key biochemical functions measured under different environmental conditions could provide mechanistically explicit insight into organism-environmental relationships. Comparative ecological proteomics would require rapid, scalable methods for absolute quantification of protein abundances in wild organisms. To date however, absolute quantification of plant proteins has proved to be challenging: proteins are difficult to extract from plant tissues and the required data-dependent mass spectrometric methods remain novel (refs from Steve).

1. A continental-scale field study of the molecular composition of leaves

We have developed proteomics methods which allow comprehensive extraction of leaf proteins and absolute quantification of the top 2000-3000 most abundant proteins. This allows us to compare protein abundances between samples, which has been demonstrated in model organisms under controlled environments (need refs from Steve) but not in a large-scale, fully replicated ecological study in wild plants.

Using this new technology, we have conducted (a/the first) continental-scale ecological proteomics experiment to characterise the influence of biogeographic and environmental controls on leaf protein expression. We analysed 320 eucalypt leaves across 32 species sampled from Tasmania, New South Wales, and Queensland, spanning large gradients of mean annual precipitation and temperature (200-3200 mm and 5-27 °C) (Fig. 1).

1. How do leaves construct their photosynthetic apparatus in different environments?

Given the factorial possible relationships between abundances of different protein functional categories and environmental variables, we have concentrated our initial analysis of the resulting dataset on photosynthesis, as it represents one of the most important and abundant set of biochemical reactions in the biosphere (Blankenship & Hartman 1998; Raven 2013). The 500 most abundant proteins account for >90% total leaf protein by weight and the majority of these are involved in photosynthesis and photorespiration (see Fig. X in *Methods*).

Much of what is known about variation in photosynthetic capacity in wild plants is derived from measurements of leaf nitrogen content, on the basis that photosynthetic proteins comprise the largest pool of nitrogen in leaves (which ref?). A more nuanced understanding of how the photosynthetic apparatus is optimised under varying environmental conditions requires quantifying differential investment in its subcomponents (Niinemets 2007).

Mathematical models of photosynthesis describe two important processes in photosynthetic carbon assimilation: carboxylation of ribulose-1,6-bisphosphate (RuBP) by the enzyme Rubisco, and regeneration of RuBP using energetic products derived from the light reactions of photosynthesis (Farquhar, von Caemmerer & Berry 1980; Farquhar, von Caemmerer S & Berry 2001). Theoretically, either of these processes can limit the rate of photosynthesis, depending on whether leaves are light or CO2 limited. According to the co-ordination hypothesis of C3 photosynthetic acclimation, proportional allocation of protein resources to the light capturing photosystem complexes and carbon fixing Calvin cycle enzymes should be optimised such that carboxylation and regeneration of RuBP are co-limiting in leaves under average daytime conditions (Haxeltine & Prentice 1996; Chen *et al.* 2009; Maire *et al.* 2012). Previously, leaf chlorophyll content expressed in nitrogen equivalents has been used as a proxy for investment in light capturing machinery (Niinemets & Tenhunen 1997), while Rubisco abundance has typically been estimated using gas exchange methods to estimate rates of carboxylation (ref). Our leaf protein abundance dataset provides the opportunity to directly test hypotheses about molecular adaptation of the photosynthetic apparatus to environmental conditions.

Following co-ordination theory, we derived a set of predictions about differential investment in light capture and carbon assimilation along gradients of temperature, precipitation and light availability (see Fig. 1b):

1. Investment in both Calvin cycle enzymes and photosystems should increase towards colder environments, to make up for the associated thermodynamic reduction of biochemical reaction rates (Hikosaka *et al.* 2006).
2. Investment in Calvin cycle enzymes should be greater at drier sites, to effect greater internal CO2 drawdown at low stomatal conductance; i.e. to reduce the water cost of photosynthesis for dryland plants (Wright *et al.* 2005) (other more ecophys oriented refs?). No direct effect of precipitation on investment in photosystem proteins is expected, although cross-correlation between precipitation and vegetation canopy density could influence this relationship.
3. Investment in photosystem complex proteins should be greatest where photosynthesis is light-limited (Niinemets 2007), and investment in Calvin cycle enzymes should increase with light availability, since capacity for carboxylation of RuBP determines the rate of light-saturated photosynthesis (Farquhar *et al.* 1980).

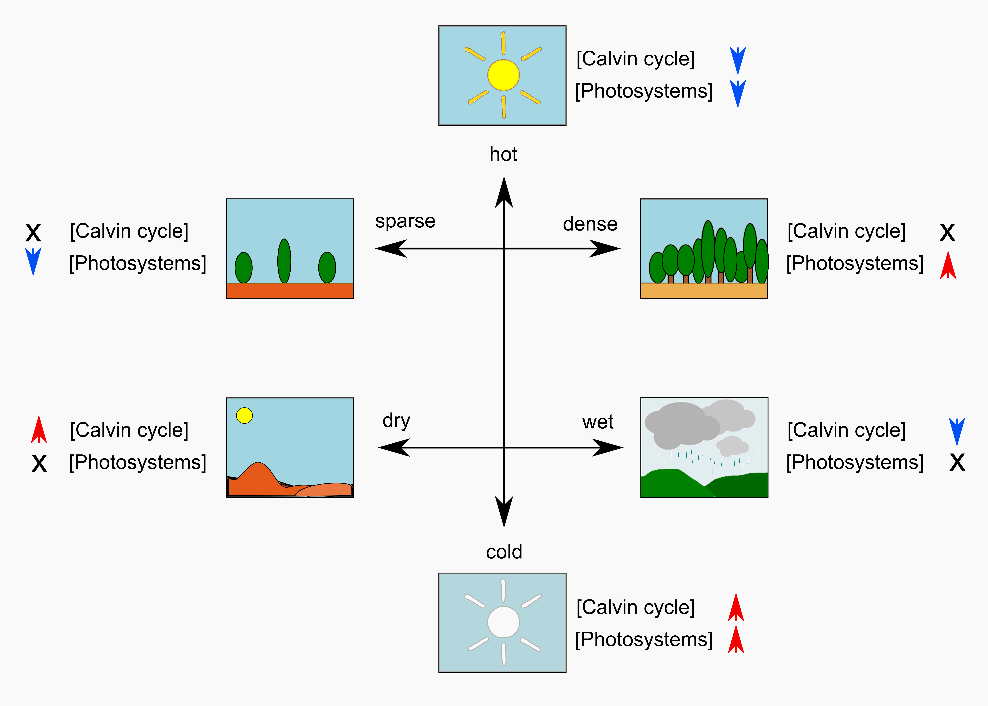
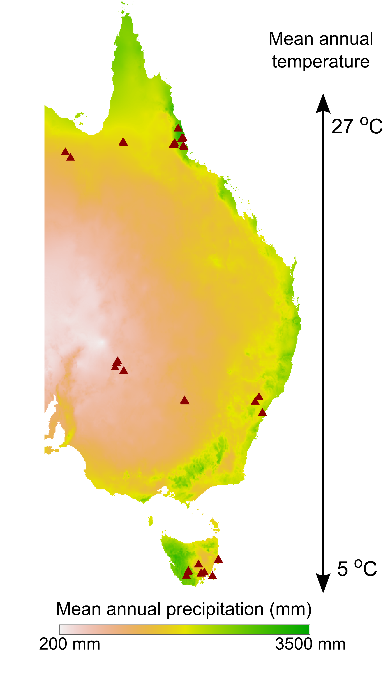


Figure 1.) (left) Location of sampling sites across eastern Australia. Sites are marked by red triangles; 2.) (right) Hypotheses about differential investment in light capture and carbon assimilation proteins along gradients of temperature, precipitation and light availability (represented here as canopy density). Red up arrows indicate a predicted increase, blue down arrows indicate a predicted decrease, black ‘X’ indicates no predicted trend. The environmental gradients described here can be more or less overlaid across the map in Fig. 1. It is worth noting that although distinct mechanisms underlie hypotheses regarding canopy density and precipitation, the two variables are strongly related. [this caption needs refining, also need to standardise display of units – ‘mean annual precip (mm)’ vs ‘mean annual temperature’]

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