Does the individual matter?

Quantifying the role of intraspecific variation and phenotypic plasticity in plant responses to climate change
Declaration

The research presented in this thesis is my own original work. All of the chapters are co-authored. The authorship indicates the intellectual input and workload. No part of this thesis has been submitted for any previous degree.

Sonya Rita Geange

February 2019
Acknowledgements

They say good things take time. Hopefully that is the case for this thesis. They also say that time is precious; make sure you spend it with the right people. In that regard, I am incredibly grateful to all those who have been involved in this amazing journey.

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Abstract

Our ability to understand the underlying morphological and physiological responses of plants to changing temperature and precipitation regimes is crucial, as we seek to construct increasingly complex models to predict how ecosystems may respond to climate change. Here, I investigate trait variation and phenotypic plasticity at the species and population level using glasshouse experiments, field transplant manipulations, and large scale, multi-habitat, multi-species observational work.

Climate change models predict warming temperatures and increasingly variable precipitation and snow cover across the Australian Alps. Plasticity in water use traits and responsiveness to extremes in temperature may become important for the establishment and persistence of Australian alpine plants. Plants from relatively lower elevations inhabit a more heterogeneous environment with more frequent frosts, greater temperature extremes, and higher evapotranspiration. To test whether adaptive plasticity may be more common at lower elevations, I investigated the extent of plasticity and its adaptive value using a glasshouse and field experiment.

To test the responses of reduced water availability and determine if plasticity varies across elevation, seeds of three alpine species from low and high elevation sites were grown under ample and water-limited conditions in a glasshouse. Patterns of plasticity were highly variable among species and among traits within species, however, responses were independent of elevation. Furthermore, there were few instances of adaptive plastic responses. Given the lack elevational variation in plant responses, there is need to understand the extent to which microhabitat variation within a given elevation may be important in shaping the persistence of these alpine species.

Climatic changes leading to decreasing snowfall and earlier snowmelt in alpine areas may expose the underlying plants to frosts and wide range of thermal extremes. To test how populations from different elevations vary in their capacity to respond to such thermal extremes, I conducted a field manipulation using alpine seedlings in open-top chambers (OTCs). I proposed that seedlings from environments with greater thermal ranges would have a greater capacity to acclimate to warming temperatures and tolerate freezing events. The warmer conditions provided by the OTCs significantly increased seedling
mortality, but seedlings that survived grew slightly taller. Warming did not affect freezing resistance, leaf production or photosynthetic efficiency. There was little evidence of intraspecific variation. A warming climate exposing plants to extreme events may lead to a reduction in seedling establishment and survival, although survivors may not exhibit any ongoing detrimental effects.

The links between ecology and evolution are driven by the variation in species traits, and these inform our capacity to predict species and community responses to changing conditions. In this final section, I sought to determine: 1) the extent of variation in trait values and plasticity at habitat to site scales, and among species and individuals; 2) whether patterns of variation were consistent across plant functional traits, and; 3) whether trait variation was associated with increased fitness consistent with adaptive plastic responses. I used field data from three habitats, six sites, 36 species, and repeated sampling of 30 individuals per species, resulting in over 200,000 leaf samples. Differences between species explained the largest component of variation in trait values and trait plasticity. A large proportion of variance in plasticity was explained by among individual variation, which, as the level at which selection acts, is important. That said, there were very few instances where indices of plasticity correlated with measures of fitness, providing little evidence of adaptive plasticity across the study.

I discuss the logistical and biological difficulties in defining relevant plasticity indices and fitness proxies, which are further explored in the synthesis chapter. Here, I take a broader look at the different research approaches and recent data capture and analytical advances that are improving our ability to predict species persistence in a changing world.
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Introduction

Phenotypic plasticity and its role in a changing climate
Climate change is resulting in substantial shifts in our natural ecosystems (IPCC 2014), influencing biodiversity through changing species’ capacities to persist and thrive. Effects upon organisms range from shifts in phenology, growth, and physiology with flow on effects to competitive ability, distribution, community structure, and ultimately ecosystem function (Travis et al. 2013, Alexander et al. 2016, Gray and Brady 2016, Tansey et al. 2017, Alexander et al. 2018, Harris et al. 2018). Thus, one of the major ecological challenges of our day is the need to enhance our capacity to predict which species are likely to be influenced by climate change, and to what extent. Consequently, new and improved species distribution models, and species or ecosystem risk assessment techniques are rapidly developing (Willis et al. 2015, Foden et al. 2018). However, within these approaches, intraspecific variation, acclimation and phenotypic plasticity remain difficult to accurately account for (Benito Garzon et al. 2019). This is despite our increased awareness of how these factors can shape ecosystem responses to climate change (Chevin and Hoffmann 2017, Fox et al. 2019).

**Phenotypic Plasticity**

Phenotypic plasticity, the capacity for a given genotype to express different phenotypes under varying environmental conditions, is an important mechanism for adapting to spatial and temporal heterogeneity (Schlichting 1986, Schlichting and Levin 1986, Bradshaw and Holzapfel 2006). Traditionally, phenotypic plasticity was viewed as background noise, or a mechanism which ‘piggybacked’ upon other processes (Bradshaw 1965, Schlichting 1986). Confusion around the exact nature of phenotypic plasticity persists for a number of reasons, including the challenges of: quantifying its existence in the field; isolating active responses (which require a signal transduction system) from passive responses; distinguishing plasticity from the effects of ontogeny; and also of linking observed phenotypic changes to underlying genetic processes (van Kleunen and Fischer 2005, Valladares et al. 2006, Valladares et al. 2007, Auld et al. 2010, Murren et al. 2015). Recent technological developments in genetic methodology, in conjunction with increased research in restoration ecology, invasion processes and the potential impacts of climate change upon species distributions and survival, have led to an increase in the number of studies specifically aimed at understanding plasticity and its significance to

1. **Adaptive Phenotypic Plasticity**

Not all phenotypic plasticity is adaptive. In order for it to be considered adaptive, trait plasticity must improve lifetime fitness when taking into consideration environmental heterogeneity, or maintain fitness under changing environments such as changes in temperature or water availability (Schlichting 1986, Matesanz et al. 2010, Nicotra and Davidson 2010, Davidson et al. 2011, Nicotra et al. 2015, Hendry 2016). Thus, despite often held assumptions within the literature, not all observable changes in traits correspond to adaptive plasticity, some may be maladaptive or neutral (van Kleunen and Fischer 2005, Ghalambor et al. 2007, Valladares et al. 2007, Arnold et al. 2019). In other words, homeostasis or canalization may be an adaptive response to environmental variation in some scenarios (Ghalambor et al. 2007).

The expression of adaptive plasticity is constrained both by the strength and reliability of environmental cues (Schlichting 1986, Dewitt et al. 1998, Auld et al. 2010). Consequently, the ability of an organism to produce a plastic response depends upon its ability to detect environmental changes and the cost-benefit of responding. A poor ability to sense change, or if information is untimely, unreliable, or incurs a long lag time before a plastic change occurs, may result in poor phenotypic-environment matching; or mal-adaptive phenotypes (Dewitt et al. 1998, van Kleunen and Fischer 2005, Alexander et al. 2018). However, the maintenance of sensory and regulatory mechanisms associated with plasticity may impose a cost or limitation upon the organism. Therefore a reduction in fitness may arise as result of plastic rather than fixed development (van Kleunen and Fischer 2005). Because of these costs and limitations, plasticity is predicted to evolve
under spatially and temporarily heterogeneous environments (Bradshaw 1965, Baythavong and Stanton 2010). In contrast, coarse scale spatial heterogeneity, in which offspring are likely to experience similar conditions and selective pressures as parental plants, should favour genetic differentiation. Accordingly, our knowledge of species’ responses to environmental change is contingent upon knowing what environmental drivers are important, how costly it is to respond to changes in these drivers, and to what extent phenotypic plasticity as opposed to local adaptation is likely to play a role in driving patterns of persistence in the short term, and therefore drive evolutionary processes.

2. Between and Within Species Variation in plasticity

Phenotypic plasticity may vary both between and within species. Among species variation is often examined in the context of comparisons between communities, generalists and specialists, pioneers or old growth, or invasive vs natives (Davidson et al. 2011, Griffith and Sultan 2012). There has been much effort to establish trait databases that capture among species variation (e.g. TRY plant database, Kattge et al. 2019; and the more recent sPlot, Bruelheide et al. 2019), which have been used to create more mechanistic understandings of how ecological communities and global biodiversity patterns are shaped by mechanistic processes (Kattge et al. 2011, Bruelheide et al. 2019). In these applications, species responses are often generalized and do not consider within-species variation in phenotypic plasticity (Schlichting and Levin 1986, Thomas et al. 2004, Davidson et al. 2011, Reed et al. 2011, Catullo et al. 2015, Cochrane et al. 2015); though greater awareness of the limitations of current approaches are being documented (Beaumont et al. 2016, Peterson et al. 2019) and more sophisticated models developed (Benito Garzon et al. 2019). Conversely, investigations into within species variation often focus upon distribution characteristics such as latitude (Molina-Montenegro and Naya 2012, Li et al. 2016), elevation (Briceño et al. 2014, Lajoie and Vellend 2015, Halbritter et al. 2018), and range or resource gradients (Davidson et al. 2011, Briceño et al. 2014, Valladares et al. 2014, Geange et al. 2017). Of the few studies that have attempted to incorporate within-species detail, they have indicated that finer scale understanding is important for obtaining a more holistic and realistic prediction of species distribution and survival patterns (Chevin et al. 2010, Valladares et al. 2014). Within-individual investigations typically span elements of localized spatial variation, i.e. leaves in the sun vs shade leaves, or the canopy vs. understory (Martin et al. 2017, Bloomfield et al. 2018).
Few studies explicitly investigate how within-individual variation may occur across longer timescales, and this has important implications when considering how we design our experiments (Araya-Ajoy et al. 2015, Cleasby et al. 2015, Westneat et al. 2015).

3. **Plant Functional Traits**

Plant functional traits are quantitative traits used to correlate plants responses to environmental conditions, and as such are good indicators of a species’ ecological strategy including its dispersal, establishment, size or persistence (Westoby 1998, Westoby et al. 2002, Reich et al. 2003, Wright et al. 2004, Wright et al. 2005, Grime 2006a, Nicotra et al. 2010, Díaz et al. 2015, Garnier et al. 2017). Functional traits are defined by morphological, physiological and phenological differentiation, provide useful insight into the dispersal, establishment and persistence of plants, and thus may provide insight into interactions at community and ecosystem levels (Grime 2006b, Albert et al. 2011). Whilst a wide variety of traits could be considered to describe ecological functioning e.g. Grime (2006a), not all are useful. Some may have tight correlations with particular functions e.g. Farquhar et al. (1980), whilst others only service as indirect indicators. Furthermore, the selection of traits may be constrained by the amount of information produced, as well as the costs associated with acquiring that information for each trait. For example, plant physiological traits are highly dynamic, and thus likely plastic, however, this dynamic nature may also make them prohibitive to study for any length of time. In contrast, morphological traits, such as specific leaf area, are relatively easy to measure, but are less plastic (Martin et al. 2017, Bloomfield et al. 2018). These limitations are pertinent when considering the range of plant forms, lifespans and environmental conditions that exist (Lavorel and Garnier 2002), and which must be considered to identify plant functional traits that display adaptive plasticity. Thus while we understand the ecological significance of plant functional traits across species, we know less about how those traits vary within species or individuals or which traits are likely to show adaptive plasticity responses (Nicotra et al. 2010).

4. **Bridging the Gap between Lab and Field**

Given the complex nature of phenotypic plasticity, it is perhaps unsurprising that the majority of research to date focuses upon examining plant responses under controlled situations. Studies in growth cabinets and glasshouses allow for direct manipulation of
key environmental variables, selection of important genotypic lines, and investigations into underlying mechanisms. However, the disadvantage of such approaches is often at the sacrifice of biological realism. Plant performance in the field is rarely driven by a single factor. Variation, be it in environmental conditions over time or space, or amongst genotypes or with ontogeny and phenology, is what allows for natural selection. The costs and trade-offs balanced by organisms within natural systems is likely to mean estimates of plasticity, or their adaptive value, under controlled conditions are over- or underestimated. Conversely, working under field conditions creates challenges as well. Disentangling the influence of varying abiotic and biotic factors is difficult, as is establishing large enough sample sizes, due to limited genotype replication. The contribution of ontogenetic change further limits the choice of individuals within field populations. In spite of these challenges, from the perspective of predicting the impact of climate change, and understanding the importance of plasticity in determining these, we require methodologies that allow us to assess phenotypic plasticity in the field and across multiple species with multiple life forms and histories.

Aims and Objectives of the Thesis

Given the rapid rate of environmental change, identifying which species, or ecotypes are ill-adapted for future climate conditions is critical. For many organisms, and in particular plants, persistence is likely to primarily be determined by an in-situ capacity to withstand or cope with change (Gienapp et al. 2008). Thus, the role of phenotypic plasticity as a mediating mechanism of environmental change, is not only of interest from a pure ecology or evolutionary biology perspective, but is also incredibly relevant from an applied conservation management position as well (Foden et al. 2018). The research within this thesis endeavors to make a significant contribution to our understanding of the knowledge gaps currently found n intraspecific and phenotypic plasticity literature.

This thesis comprises three projects that together address the overarching questions: 1. Does the individual matter? And 2. Can we quantify the role of intraspecific variation and phenotypic plasticity to altered plant growing conditions? In this thesis I investigate these concepts using glasshouse experiments, field transplants and manipulations, and large scale, multi-habit and multi-species observational work.
Overall, the main research elements within the thesis were:

1. To identify if there are predictable patterns plastic responses between or within species;

2. To assess if functional traits differ in expression and adaptive value of plasticity; and

3. To ascertain whether one can extrapolate from controlled environmental measures of plasticity to predictions about real-world field responses?

Thesis Structure

This thesis contributes a novel multi-faceted approach to extending our understanding of how phenotypic plasticity, and intra-specific variation in plasticity, might contribute to species’ capacities to persist under rapidly changing environmental conditions. This thesis contains published work and work prepared for publication that has been co-authored with collaborating researchers. As such, these chapters can be read independently, and some duplication of concepts or ideas may arise. All the data presented within this work is original research and the contribution of each co-author is stated below. No part of this thesis has been submitted for any previous degree.

Chapter 1: Thesis Introduction

Presents the background introduction to material covered within the thesis. I am the sole author of this chapter with editorial assistance from my PhD supervisors.

Chapter 2: Phenotypic plasticity and water availability: responses of alpine herb species along an elevation gradient

In Chapter Two I use a glasshouse trial to establish what influence reduced water availability is likely to have upon three Australian alpine herb species. Specifically, I
investigate if populations of species differ in their responses as a result of elevation; an oft-used space-for-time substitute. Within this chapter, I not only document morphological, physiological and biochemical trait responses, but also conduct a selection gradient analysis to assess whether or not plastic response are actually correlated with fitness outcomes, i.e. adaptive plasticity.


J.A.R-V and A.B.N formulated the ideas for the study, established the design and developed the hypothesis. S.R.G, V.F.B.N., N.C.A., and A.B.N collected the data. S.R.G conducted all the statistical analysis and drafted the manuscript. A.B.N and M-M.H-P contributed substantial conceptual and editorial advice. All authors contributed to writing and approved the final manuscript.

**Chapter 3: *Aciphylla glacialis* mortality, growth and frost resistance: a field warming experiment**

In Chapter Three, I employed a field transplant approach in conjunction with simulated climate warming (open top chambers). This research assessed how an Australian alpine plant species may respond to early snow melt and increased exposure to extreme thermal events such as frosts, on balance with higher maximum temperatures. Building upon Chapter Two, I also examined how intra-specific variation in plasticity might play a role in determining a species’ capacity to persist in a rapidly changing environment. In this instance, I contrasted mortality, growth, photosynthetic efficiency and freezing tolerance responses for populations from four thermally different sites.

S.R.G, V.F.B, M-M.H-P and A.B.N formulated the ideas for the study, established the design and developed the hypothesis. S.R.G collected the data and conducted all the statistical analysis and drafted the manuscript. M-M.H-P, V.F.B and A.B.N contributed conceptual and editorial advice. All authors contributed to writing and approved the final manuscript.

Chapter 4: Does the individual matter? Quantifying intraspecific variation and phenotypic plasticity across alpine, semi-arid and coastal plant communities

Within Chapter Four, my aims were to: 1) determine the extent of variation in trait values and trait plasticity at ecological scales ranging from habitat to site, species and individual; 2) assess whether patterns of variation were consistent across plant functional traits; and 3) investigate whether trait variation was associated with increased fitness consistent with adaptive plastic responses. With three habitats, six sites, 36 species, and multiple visitations to 30 individuals per species, resulting in over 200,000+ leaf samples, this project represents the largest body of work to date examining the extent to which intraspecific variation, and specifically within-individual variation, contributes to our understanding of plant responses over and above that of species level variation.

This chapter is formatted for submission to *New Phytologist*. The authorship will be: Geange, S.R., Aitken, N.C., Holloway-Phillips, M-M., Matesanz, S., Robertson, M., Richards, C.L., Valladares, F., van Kleunen, M. and Nicotra, A.B.

A.B.N, F.V., M.vK. and C.R formulated the ideas for the study, established the design and developed the initial hypotheses. S.R.G, N.C.A, and A.B.N collected data for Australian field sites. S.R.G modified hypotheses, conducted the analyses, and drafted the initial manuscript. M-M.H-P, C.R. and A.B.N contributed conceptual and editorial advice.
Chapter 5: Thesis synthesis

Finally, I summarize the key points of each of my thesis chapters, drawing together key themes and elements. Given that the preceding chapters are formatted as stand-alone publications, with complete discussions and references to the literature, I do not present another synthesis of that work. In this chapter I will mostly focus upon drawing attention to how I see the fields of intraspecific variation and phenotypic plasticity progressing into the future.

I intend for elements of this chapter to become a published commentary or opinion piece. I am the sole author of this chapter with editorial assistance from my PhD supervisors.


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Chapter Two

Phenotypic plasticity and water availability: responses of alpine herb species along an elevation gradient

Authors: Sonya R. Geange¹, Veronica. R. Briceno², Nicola. C. Aitken¹, Jose A. Ramirez-Valiente³, Meisha-Marika Holloway-Phillips¹ and Adrienne B. Nicotra¹

1. Research School of Biology, The Australian National University, Canberra, ACT 2601, Australia
2. Instituto de Ciencias Ambientales y Evolutivas, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile.
3. Department of Integrative Ecology, Donana Biological Station (EBD-CSIC), Avda, Americo Vespucio s/n, 41092, Seville, Spain

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Abstract

Alpine regions are particularly vulnerable to the effects of climate change. The Australian Alps are potentially more so than other mountain regions, as they cover a very small geographic area (<0.05% of mainland Australia), with a low maximum elevation (2228m). Therefore, response to climate change will be primarily determined by the ability of species to survive in-situ through local adaptation or phenotypic plasticity. Existing climate change models project not only warming but increasingly variable precipitation and snow cover across the Australian Alps. Thus, plasticity in water use traits may become increasingly important for the establishment and persistence of Australian alpine plants. Given that plants from lower elevations inhabit a more heterogeneous environment with more frequent frosts, greater temperature extremes, and higher evapotranspiration, we predict plasticity – and particularly adaptive plasticity – may be more common at low relative to high elevation. To test these predictions we investigated the extent of plasticity and the adaptive value thereof in water use traits in three herbaceous Australian alpine plant species. Seeds were collected from low and high elevation alpine sites and grown at ample and limiting water availability under common-garden conditions. For morphological and physiological traits, we compared both their means and phenotypic plasticity across treatments and elevations.

Responses of morphological and physiological traits to water availability were in accord with many previous studies of water response. Although previous work in the same environment demonstrated greater plasticity in response to temperature for low elevation populations, plasticity in response to water availability in our study showed markedly little variation as a function of elevation. Rather, patterns of plasticity were highly variable among species and among traits within species, with few instances of adaptive plastic responses.

We discuss the difficulties in observing adaptive plasticity and the importance of microhabitat variation in shaping the persistence of these Australian alpine species.
Introduction

Global climate change predictions include increasing temperatures, changing precipitation patterns and an increase in the frequency of extreme events (IPCC 2014). Given the limited spatial and elevation scale of alpine landscapes, they are likely to be vulnerable to climate change (Theurillat and Guisan 2001, Hughes 2003). Indeed, particularly rapid rates of environmental change within alpine areas have been observed (Bergert et al. 2005). The Australian alpine region is likely to be particularly impacted because it has a small geographic area (<0.05% of the Australian continent), a limited elevation gradient of approximately 400m above tree-line, and low summits (maximum of 2228m.a.s.l) (Nicholls 2005, Hennessy et al. 2008, Theobald et al. 2016).

Alpine plants therefore have limited options for range shift, and in addition, some are characterized by poor dispersal capacity (Gottfried et al. 2012, Pauli et al. 2012). In conjunction with slow growth rates and often fragmented habitats responses to climate change are therefore expected to be primarily determined by the ability to survive *in-situ* through local adaptation or phenotypic plasticity (Nicotra et al. 2010, Hoffmann and Sgro 2011, Gottfried et al. 2012, Pauli et al. 2012). Phenotypic plasticity is the capacity for a given genotype to express different phenotypes under heterogeneous environments (Schlichting 1986, Bradshaw and Holzapfel 2006). Plasticity may be an important adaptation to spatial and temporal heterogeneity and may allow species to better cope with climate change (Schlichting 1986, Bradshaw and Holzapfel 2006, Ghalambor et al. 2007). The extent to which plasticity is adaptive, whereby a fitness benefit is conferred, is expected to vary among and within species as a function of both environmental conditions and evolutionary history (van Kleunen and Fischer 2005, Ghalambor et al. 2007). Whether plasticity can facilitate adaptation under changing conditions, through allowing for short-term adjustments to novel conditions, is still debated (Hoffmann and Sgro 2011). As such, more information is required to understand the capacity for, and variability in, adaptive phenotypic plasticity across species within vulnerable habitats, and how this may impact on future distributions and persistence (Nicotra et al. 2010, Nicotra and Davidson 2010).
Intra-specific variation in traits and tolerances is increasingly seen as a potentially fundamental factor in understanding community responses to climate change (Valladares et al. 2014). Relative performance along environmental gradients, often using elevation as a proxy, is often used as an indicator of potential responses to climate change. At lower elevations, for instance, organisms are exposed to higher mean temperatures, greater temperature fluctuations, longer growing seasons and increasingly variable water availability (Körner 2003, Sierra-Almeida et al. 2009). For that reason, lower elevations are often seen as indicative of the changes that will potentially occur at higher elevations (Byars et al. 2007). Thus, one may ask whether low elevation populations, which are already exposed to high levels of environmental heterogeneity, are more likely to display greater levels of adaptive phenotypic plasticity? The answer to this question may provide an indication of whether such plasticity will be favoured under climate change.

In contrast to temperature, water availability in alpine regions often varies in a mosaic-like pattern, as a function of many factors, including climate and topography, soil composition, snow layer presence and aspect (Körner 2003, Venn and Morgan 2009). Water availability has not generally been considered a driving factor in the evolution of alpine plants (Körner 2003). However, climate change scenarios predict not only higher mean temperatures and a greater variability in temperatures, but also changing spatial and temporal patterns of precipitation and snowfall, thus the present mosaic-like variation in water availability may further intensify (Hughes 2003, IPCC 2014). As such, understanding the underlying morphological and physiological responses of plants to varying water availability, not only on a species by species but also at a within-species level, may help inform predictions on distributions and persistence into the future for this vulnerable plant community (Parmesan 2006, Carlson et al. 2013).

Among Australian alpine herbs, lower elevation populations of *Wahlenbergia ceracea* and *Aciphylla glacialis* have been shown to have a greater plasticity in a range of traits in response to warming temperatures (Nicotra et al. 2015) and a greater ability to acclimate to cold temperatures than their higher elevation counterparts (Briceño et al. 2014). In both cases, greater plasticity resulted in a fitness advantage (Briceño et al. 2014, Nicotra et al. 2015). However, it is possible the evolution of adaptive responses for one abiotic variable may be maladaptive or neutral for another (Auld et al. 2010, Baythavong and
Stanton 2010). Consequently, it is important to assess the responses of alpine plants to variable water availability, in order to see whether they display similar patterns of plasticity in response to water availability along elevation gradients as were seen for temperature. Alternatively, given that water availability may vary more as a mosaic within the alpine landscape than as a continuous gradient, there may be less pressure for the evolution of plasticity in response to water availability along elevation gradients.

Here we examine whether three Australian alpine herb species show within-species variation in plastic response to water availability. For two of these species prior studies have shown such variation in plastic response to temperature (Briceño et al. 2014, Nicotra et al. 2015). We grew plants under common garden conditions exposed to either well-watered or water-limited conditions and examined plant functional traits that are known to vary as a function of water availability, including morphological and physiological traits (Reich et al. 2003). Specifically, we looked to answer the following questions: 1) How are plant traits and fitness influenced by reduced water availability, and how does this vary depending upon source elevation? 2) Is there any evidence that the water responses represent adaptive plasticity? Or 3) Are the traits themselves under directional selection?

We predicted that plant biomass, leaf count, leaf size and specific leaf area (SLA) would be lower under limited water availability. Also, we expected plants grown under low water availability to have more conservative water use strategies, and thus to exhibit reduced stomatal conductance and consequently reduced photosynthetic rates, and potentially greater instantaneous water use in comparison to plants grown under high water availability (Körner et al. 1986, Wright et al. 2004). Furthermore, as plants decrease photosynthesis in response to reduced water availability, less discrimination of $^{13}$CO$_2$ in favor of $^{12}$CO$_2$ occurs due to the reduced CO$_2$ concentration within the leaf. This results in a less negative $\delta^{13}$C (an integrated measure of water use efficiency) compared to a plant with stomata more open (Farquhar et al. 1982). Finally, we expected leaf nitrogen content (N) to decrease under water limitation (Chapin et al. 1987).

With regards to intra-specific variation attributable to elevation, we considered two alternative hypotheses; a) that we would observe similar patterns of plasticity for water
availability as previously observed for temperature along the elevation gradient (Briceño et al. 2014, Nicotra et al. 2015). As such, plants sourced from environmentally more heterogeneous lower elevations are predicted to display greater plasticity and more evidence of adaptive plastic responses. Alternatively, that b) as a result of the inherently more mosaic-like distribution of water availability within the Australian alpine landscape, responses to water availability would be measurable but few elevation-based differentiation in traits responses would be observed.

Methods and Materials

Seed Collection and Germination

Seeds from three perennial alpine herbs; *Aciphylla glacialis* (F. Muell.) Benth. (Apiaceae), *Oreomyrrhis eriopoda* (DC.) Hood.f. (Apiaceae), and *Wahlenbergia ceracea* Lothian (Campanulaceae), were collected from Kosciuszko National Park, New South Wales, Australia between December 2009 and April 2010 (Appendix Figure 1). For each species, seeds were collected from 30 individual plants within their natural distribution (ranging from 1600-2200 m a.s.l.). Seeds were germinated over winter between May and August 2010 under controlled conditions at the Australian National Botanic Gardens, Canberra. From these seedlings, replicates of four to six maternal lines from the upper and lower 25% of the elevation distribution (henceforth referred to as high and low elevation) of each species were selected for the experiment (Table 1). Seedlings were grown under common conditions in 50 mm/0.2 l pots containing commercial seed raising mix with micronutrients (Debco Pty Ltd, Victoria, Australia) in the nursery for approximately six months with some variation among species depending upon germination timing.
Table 1 Study species and their elevation-based sampling distributions within Kosciuszko National Park, NSW, Australia. Numbers within brackets indicate sample sizes for various trait analyses, in order: individuals used for tissue chemistry analysis, individuals used for specific leaf area, individual used for physiological trait analysis.

<table>
<thead>
<tr>
<th>Species</th>
<th>Source Elevation</th>
<th># of Maternal Lines</th>
<th>High Water Individuals</th>
<th>Low Water Individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aciphylla glacialis</strong></td>
<td>Low (1906-1937m)</td>
<td>4</td>
<td>27 (11/15/22)</td>
<td>28 (11/17/22)</td>
</tr>
<tr>
<td></td>
<td>High (2162-2225m)</td>
<td>5</td>
<td>11 (10/10/10)</td>
<td>10 (8/8/10)</td>
</tr>
<tr>
<td><strong>Oreomyrrhis eriopoda</strong></td>
<td>Low (1733-1746m)</td>
<td>6</td>
<td>36 (9/33/17)</td>
<td>39 (9/28/18)</td>
</tr>
<tr>
<td></td>
<td>High (1974-2225m)</td>
<td>6</td>
<td>36 (9/29/18)</td>
<td>38 (9/31/18)</td>
</tr>
<tr>
<td><strong>Wahlenbergia ceracea</strong></td>
<td>Low (1610-1720m)</td>
<td>6</td>
<td>35 (9/31/17)</td>
<td>31 (9/27/16)</td>
</tr>
<tr>
<td></td>
<td>High (1875-2128m)</td>
<td>5</td>
<td>34 (9/28/17)</td>
<td>35 (9/31/18)</td>
</tr>
</tbody>
</table>

**Experimental Design: Glasshouse Conditions**

In March 2011 (autumn), 370 seedlings were transplanted from the nursery into glasshouse conditions with ambient light conditions and a temperature set to cool alpine conditions of 20/10 °C day/night with a natural light cycle. Seedlings were potted into 175mm diameter/2.8 l pots containing a mix of 80:10:10; commercial potting mix: field soil: and washed river sand with added slow release osmocote for natives (Debco Pty Ltd, Victoria, Australia). Field soil was used to provide the seedlings with a natural inoculum, and was collected from a site within the Kosciuszko National Park where all three species co-occur. Initial leaf number was counted and seedlings of uniform size were selected from each maternal line to minimize variance within and between lines for each species. Following time to adjust to transplanting, pairs of seedlings were allocated to three blocks in a stratified design (May 2011); one member of each pair was randomly assigned to a well-watered treatment, and the other to a dry treatment. Seedlings were placed on a bench in a randomized design (minimum n=2 per block), however, due to variation in germination and establishment success the design was not perfectly balanced (Table 1).

High and low water treatments were applied using a Water-Pro vapour pressure deficit (VPD) controlled system (MicroGrow GreenHouse Systems, Temecula, California), with
each plant watered individually by a dripper at soil level (Nicotra et al. 2008). Dripppers were calibrated to a standard flow rate that was checked at the beginning and end of the experiment. Watering events were triggered when pre-set VPD targets were reached. High water plants received ~160 ml per watering (enough to saturate the soil); low water plants received ~80 ml per watering. Watering occurred roughly once per week for low water plants and every 2–3 days for high water plants, depending on VPD in the glasshouse. Under this regime, low water plants received ~25% of that which the high-water plants received.

**Physiological and Leaf Chemistry Measurements**

In order to understand how the soil moisture treatments affected plant physiology, we measured changes in gas exchange rates, leaf morphology, and tissue chemistry on a representative subset of plants, the number of which varied among species (Table 1). Gas exchange was measured using a LiCor 6400 portable photosynthesis system (LiCor, Lincoln, Neb) after 6 months of growth (26/11/2011 to 16/12/2011). On each plant, a young fully expanded leaf was selected for measurement. Measurements were taken at two time points; on the morning following a watering event (wet cycle) when the plants were at their least water-limited, and on the final day of a watering cycle (dry cycle) when the low water plants were at the driest point in the cycle. Block temperature was set at ~20°C, CO₂ was 400ppm, relative humidity was maintained around 60% and light levels were 500 µmol m² s. Preliminary assessment indicated that this light level was saturating. Photosynthetic measurements were done between 8:00 till 13:00 hrs, before stomatal closure.

Leaf samples were taken to measure leaf size and specific leaf area (SLA, cm² g⁻¹) at the time of gas exchange measurements. Collected leaves were placed with petioles positioned in saturated florist foam and kept at 4°C in the dark overnight before being weighed to determine saturated weight, scanned on a flatbed scanner, and dried at 60°C for 72 hours before being weighed again to determine dry weight (Perez-Harguindeguy et al. 2013). Leaf size was calculated as an area (cm²/ per leaf), and specific leaf area was calculated as area/dry mass (cm² g⁻¹).
For each species by elevation combination that had been measured for photosynthesis, replicates from three to four maternal lines were selected for isotope analysis. From these lines, three replicates from each of the high and low water treatments were chosen to be measured from each block (n = 112). Carbon isotopic ($\delta^{13}$C) and elemental analysis (%N) were performed on an isochrome continuous flow stable isotope ratio mass spectrometer (Micromass, Middlewich, England).

**Harvest Measurements**

In November 2011 (late-spring) all plants were harvested. The total number of leaves on each plant was counted. Plant leaves, stems and roots were separated and dried in an oven at 60°C for 72 hours before weighing using a Mettler-Toledo AB304-S Analytical Balance (Mettler-Toledo, Switzerland). For the few plants that flowered, total flower number was counted and reproductive biomass weighed. Above-ground biomass was calculated as the sum of leaves and stem dry weight (g), with reproductive biomass included for those that flowered.

**Statistical Analysis**

Trait means were compared using a restricted maximum likelihood (REML) model that included elevation (categorical), treatment, and their interaction as fixed factors. Random factors were block and maternal line. Individual replicates were nested within maternal line, which itself was nested within elevation. For traits where there was not sufficient replication at a given hierarchical level (e.g. block or maternal line) to fit the model, the term was excluded. Each analysis was conducted independently for each of the three species. Comparisons between wet and dry cycle traits revealed no significant difference, so only dry cycle results are presented henceforth.

Traits were examined for conformity to the assumptions of normality, and transformed as required. Data outliers were excluded when associated with data entry, or where for example drippers had failed to deliver the allocated water dose for the treatments (Cochrane et al. 2014).

To calculate plasticity across watering treatments, plants were paired within maternal line x treatment combinations within each block. This was performed per each species.
Then, we calculated the plasticity index (PI) as: (largest trait value – smallest trait value)/largest trait value. Not all plants could be paired, e.g. if the individual in the other treatment had died. Where more than one pair was possible within a maternal line*block, plants were paired by proximity on the glasshouse bench to minimize differences in conditions. As a result of the study design the number of pairs varied across the three species (A. glacialis = 25, O. eriopoda = 62 and W. ceracea = 58).

The potential for adaptive, maladaptive, or neutral plasticity was analyzed using selection gradient analysis, where the average fitness of plants across environments (or a proxy thereof) is regressed against the index of trait plasticity (van Kleunen and Fischer 2005). We standardized plasticity indices and trait values both to a mean of zero. We used total biomass as a fitness proxy and calculated relative fitness as the mean total biomass for a pair of plants divided by the mean of all pairs, and then subsequently log (e) transformed (Van Kleunen and Fischer 2001, Nicotra et al. 2015). Selection differentials were assessed separately for each species. Using a REML model (lmer, package lme4, R) (Bates et al. 2015), the selection gradient model assesses relative fitness regressed against trait plasticity as a fixed factor, with maternal line nested within elevation as a random factor. We also assessed plasticity against relative fitness for each water treatment individually to investigate which treatment, if any, may be driving adaptive plasticity. Here, relative fitness was again regressed against plasticity, using the maternal line as a random factor. Using the same models as above, we also investigated how trait values regressed against fitness to see if there was selection upon the trait mean itself.

Results

Responses to Water Availability

Reducing water availability to 25% of saturated conditions affected many morphological and physiological traits across the three species. As predicted, total biomass was significantly lower under water limitation for O. eriopoda and W. ceracea whilst in A. glacialis this was found only to be true for the high elevation plants(Figure 1, a-c). In conjunction with reduced growth, a greater investment into water-sourcing root relative
to shoot biomass was observed in all species under water limitation and was significant for both *A. glacialis* and *W. ceracea* (Figure 1, d, f). Total leaf number was lower under water limitation in all but the *A. glacialis* low elevation plants (Figure 1, g-i). Reductions in leaf size were also observed under water limitation and were significant for *A. glacialis* (Figure 1, j). We predicted that SLA would also be lower under water limitation, but changes in SLA were varied across the three species, with no clear main treatment response observed (Figure 1, m-o; Table 2).

Physiological and tissue chemistry responses to water limitation were more varied. As predicted, all three species had significantly lower stomatal conductance (*gs*) under water-limiting conditions (Figure 2, a-c). Rates of photosynthesis were also lower under water-limiting conditions, though these differences were not statistically significant (Figure 2, d-f). Consequently, instantaneous water use efficiency (*WUEi*), being the ratio of photosynthesis to stomatal conductance, while slightly greater under water limitation was not significantly so (Figure 2, g-i). Similarly, this pattern was also observed across species using the longer term indicator of water use efficiency, δ13C, although less negative values, indicating increased water use efficiency, were observed for water-limited plants within *W. ceracea* (Figure 2, l). Leaf nitrogen content was lower under water limitation for *A. glacialis* (Figure 2, m).

**Elevation Effects and Interactions**

We found little evidence of intra-specific variation in traits or response to water limitation along the elevation gradient (Table 2), in keeping with our alternative hypothesis. *Wahlenbergia ceracea* plants sourced from low elevation produced fewer leaves throughout the experiment (Figure 1, i), and in general, these leaves had higher levels of stomatal conductance (Figure 2, c), and correspondingly more negative δ13C (higher water use efficiency) (Figure 2, l). Low elevation sourced *O. eriopoda* plants also displayed more negative δ13C values (Figure 2, k).

We predicted that low elevation sourced plants would show greater plasticity than high elevation plants but found few significant interaction terms (Table 2). For *A. glacialis*, low elevation sourced plants consistently maintained homeostasis in total biomass across treatments, whereas high elevation plants showed a decline in biomass under water-
limiting conditions, our proxy for fitness (see below) (Figure 1, a, g). For SLA, high elevation sourced plants maintained homeostasis between treatments (Figure 1, m). The same pattern was also observed for δ13C, whereby low elevation sourced plants displayed a more negative δ13C and a lower water use efficiency across treatments in comparison to high elevation plants (Figure 2, j). Finally, for *O. eriopoda* we found a significant treatment by elevation (TxE) interaction for SLA, whereby high elevation sourced plants increased SLA under low water conditions, while low elevation sourced plants decreased SLA under low water conditions (Figure 1, n).

**Selection Gradient Analyses**

For traits that showed significant plasticity in response to water treatment and/or elevation effects, we examined whether the plastic response was associated with increased fitness. However, despite widespread treatment effects few of these plastic shifts were shown to have adaptive value. The single instance of selection for adaptive plasticity observed was total leaf count in *O. eriopoda* (Table 3). In contrast, selection towards homeostasis was observed for more traits: stomatal conductance in *A. glacialis*, percentage nitrogen in *O. eriopoda*, and total leaf count in *W. ceracea*, the latter for high elevation sourced plants only (Table 3). Instances whereby plasticity significantly influenced fitness were primarily observed where selection differentials were in opposing directions within high and low water treatments (Appendix Table 1).

Significant directional selection gradients on trait means were also observed in some cases, but again varied among species, treatment and elevation (Table 4). For *A. glacialis* larger leaf size was significantly correlated with increased fitness (Table 4), and this was primarily driven by variation among high water treatment plants (Appendix 1). For *O. eriopoda*, increased percentage nitrogen was negatively correlated with fitness, and also primarily driven by variation among plants in the high water, rather than low water treatment (Appendix 1). For *W. ceracea*, where significant elevation effects on trait means were more common, increased total leaf count was positively correlated with fitness, and this was driven by variation among the high elevation sourced plant responses (Table 4). Finally, less negative δ13C (higher water use efficiency) was associated with increased fitness (Table 4) and was driven by variation in the response of
high elevation sourced plant grown under high water treatment in *W. ceracea* (Appendix Table 1).

While slightly more cases of selection upon plasticity than would be expected by chance alone, likely arising from false significant effects due to multiple testing, the 5/22 traits displaying significant results provide only very weak evidence that water response traits and plasticity therein are under selection in these species.
Figure 1: Comparison of morphological traits comparing responses of low (dashed) and high elevation (solid lines) across high and low water treatments for each species. Species and trait responses by column are: a) *Aciphylla glacialis*, b) *Oreomyrrhis eriopoda* and c) *Wahlenbergia ceracea*. Significance bars are mean differences. Significant results are represented for treatment (T), elevation (E) and treatment by elevation (T x E) by bolded and italic font.
Figure 2: Comparison of physiological traits comparing responses of low (dashed) and high elevation (solid lines) across high and low water treatments for each species. Species and trait responses by column are: a) *Aciphylla glacialis*, b) *Oreomrynchis eriopoda* and c) *Wahlenbergia ceracea*. Significance bars are mean differences. Significant results are represented for treatment (T), elevation (E) and treatment by elevation (T x E) by bolded and italic font.
Table 2: Summary of significant trait responses to treatment, elevation or treatment by elevation interaction for three species Aciphylla glacialis (Ag), Oreomyrrhis eriopoda (Oe) and Wahlenbergia ceracea (Wc). Significant responses are shown by black dots (<0.05).

Table 3: Selection gradient analysis on the paired dataset of trait plasticity and relative fitness on traits which displayed significant treatment responses for A) Aciphylla glacialis, B) Oreomyrrhis eriopoda and C) Wahlenbergia ceracea. Selection differentials were calculated combining data from plants of different elevations. In cases where elevation effects were significant (Figure 2 and 3), additional analysis were performed for data from plants of the different elevations separately. Positive selection differentials indicate adaptive responses, while negative values indicate maladaptive responses. Significant responses are indicated by bolded p-values, n is the number of pairs within each analysis.

<table>
<thead>
<tr>
<th>Species</th>
<th>Trait</th>
<th>Plasticity Differentials</th>
<th>P value</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) A. glacialis</td>
<td>Root Mass Ratio</td>
<td>0.01</td>
<td>0.940</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Leaf Size</td>
<td>-0.08</td>
<td>0.532</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Stomatal Conductance (Dry)</td>
<td>-0.25</td>
<td>0.031</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>%N</td>
<td>0.92</td>
<td>0.477</td>
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</tr>
<tr>
<td>B) O. eriopoda</td>
<td>Leaf Mass Ratio</td>
<td>-0.05</td>
<td>0.420</td>
<td>53</td>
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<td>Leaf Size</td>
<td>0.18</td>
<td>0.157</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>%N</td>
<td>-0.18</td>
<td>0.016</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Total Leaf Count</td>
<td>0.12</td>
<td>0.036</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>Water Use Efficiency</td>
<td>0.11</td>
<td>0.247</td>
<td>30</td>
</tr>
<tr>
<td>C) W. ceracea</td>
<td>Photosynthesis (Dry)</td>
<td>0.11</td>
<td>0.063</td>
<td>34</td>
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<tr>
<td></td>
<td>Root Mass Ratio</td>
<td>0.00</td>
<td>0.996</td>
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<td>High Elev</td>
<td>0.12</td>
<td>0.234</td>
<td>29</td>
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<tr>
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<td>Low Elev</td>
<td>-0.06</td>
<td>0.592</td>
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<tr>
<td></td>
<td>Stomatal Conductance (Dry)</td>
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<tr>
<td></td>
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<td>Low Elev</td>
<td>0.03</td>
<td>0.838</td>
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<td>High Elev</td>
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<td>0.023</td>
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<td>Low Elev</td>
<td>-0.03</td>
<td>0.639</td>
<td>29</td>
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<tr>
<td></td>
<td>$\delta$ 13C</td>
<td>-0.13</td>
<td>0.654</td>
<td>16</td>
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<td>High Elev</td>
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<td>Low Elev</td>
<td>-0.20</td>
<td>0.443</td>
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Table 4: Selection gradient analysis on the paired dataset of trait value and relative fitness of functional traits which displayed significant treatment responses for A) *Aciphylla glacialis*, B) *Oreomurus eriopoda* and C) *Wahlenbergia ceracea*. Selection differentials were calculated combining data from plants of different elevations. In cases where elevation effects were significant (Figure 2 and 3), additional analysis were performed for data from plants of the different elevations separately. Positive selection differentials indicate adaptive responses, while negative values indicate maladaptive responses. Significant responses are indicated by bolded p-values, n is the number of pairs within each analysis.

<table>
<thead>
<tr>
<th>Species</th>
<th>Trait</th>
<th>Plasticity Differentials</th>
<th>P value</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) <em>A. glacialis</em></strong></td>
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<td>-0.20</td>
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<td>23</td>
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<td></td>
<td>Leaf Size</td>
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<td><strong>&lt;0.001</strong></td>
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<tr>
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<td>Stomatal Conductance (Dry)</td>
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<td>0.090</td>
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<tr>
<td></td>
<td>%N</td>
<td>0.01</td>
<td>0.940</td>
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<tr>
<td><strong>B) <em>O. eriopoda</em></strong></td>
<td>Leaf Mass Ratio</td>
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<td>0.707</td>
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<td>-0.25</td>
<td><strong>0.025</strong></td>
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<td>Total Leaf Count</td>
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<td>Water Use Efficiency</td>
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<td>0.245</td>
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<td><strong>C) <em>W. ceracea</em></strong></td>
<td>Photosynthesis (Dry)</td>
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<td>0.000</td>
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<td></td>
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<td>Low Elev</td>
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<td></td>
<td>Total Leaf Count</td>
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<td>Low Elev</td>
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<td>δ 13C</td>
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<td><strong>0.007</strong></td>
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<td>Low Elev</td>
<td>0.21</td>
<td>0.067</td>
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Discussion

In this study we examined the effects of reduced water availability on morphological and physiological trait means and plasticity in three Australian alpine herb species; *Aciphylla glacialis*, *Oreomyyrrhis eriopoda*, and *Wahlenbergia ceracea*. Specifically, we asked whether there was evidence of adaptive plasticity in water response and/or intra-specific variation between low and high elevation populations in trait plasticity to water availability. We found widespread evidence of plasticity in morphological and physiological traits in response to water limitation in all three species. As expected, under water limitation, plants accumulated less biomass, had an increased investment into root structures, and consequently a reduction in both the number and size of leaves (Figure 1). In addition, reduced stomatal conductance, and in some instances higher integrated water use efficiency, measured according to δ13C, were observed under low water availability (Figure 2). Despite this, we observed little evidence that the phenotypic plasticity in response to water availability was adaptive, nor did we detect much variation in either trait means or trait plasticity as a function of elevation (Tables 3 and 4). Here we discuss why, despite eliciting a significant water treatment, little evidence of adaptive plasticity or elevation-based differentiation in trait values or plasticity was observed within our study.

*Why doesn’t elevation affect water response patterns?*

The use of elevation gradients within ecological studies is increasingly common, particularly as a method of assessing responses to future climate change conditions. Our first elevation-based hypothesis predicted that low elevation sourced plants would display greater variation in trait mean, and therefore increased plasticity as a result of occupying a more heterogeneous environment (Sultan 2001, Bradshaw and Holzapfel 2006). For the high elevation populations it was predicted that little evidence of plasticity would be observed (Baythavong and Stanton 2010, Gugger et al. 2015), though see Frei et al. (2014) and Pohlman et al. (2005) for opposing perspectives. We found relatively few significant elevation responses, and, in many instances within our study low elevation sourced plants displayed a greater degree of trait homeostasis across our water
treatment (Figures 3 and 4). In contrast, previous studies have demonstrated differences in plastic responses along elevation gradients and associated these with differing selection pressures at each elevation, such as increased temperature (Vitasse et al. 2013, Briceño et al. 2014, Nicotra et al. 2015), increased frost risk due to earlier melting snow (Goldstein et al. 1985, Bannister et al. 2005, Bannister 2007), and competition from upward shift in low elevation alpine plants (Walther et al. 2005). Likewise, past research has assessed elevation-based changes in traits such as; growth rate, biomass (Wright et al. 2004), leaf size (Byars et al. 2007), stomatal conductance (Körner et al. 1986) and carbon isotope discrimination (Xu et al. 2015).

Generally within alpine regions, decreasing soil depth and vegetation cover with increasing elevation may mean that soil moisture deficits, particularly during summer, may be higher at high elevations (Korner and Cochrane 1985, Körner 2003). However, others suggest that because water availability within an alpine landscape may be more likely to vary over micro-scales than across an elevation gradient per se we considered an alternative hypothesis: that patterns of plasticity in response to water availability might be consistent across elevations, and in this differ from plasticity patterns in response to temperature. Thus, we would predict plenty of plasticity in response to water availability, but not differentiation in the pattern of that plasticity across elevations. Water availability within alpine ecosystems is not only a function of precipitation, or evapotranspiration, but may be determined by prevailing weather conditions, snow melt, localised topography, aspect, soil structure, and/or vegetation cover (Körner 2003, Körner 2007). In our tall alpine herb field, heavy vegetation cover may act to minimize evaporative moisture loss during the summer period such that variation with elevation is moderated (Körner 2003). Thus, it appears that these alpine plants have plasticity in response to water in expected directions, but that there has been no selective pressure for differentiation in that plasticity across elevation. (Körner 2007, Carlson et al. 2013).

Water availability is just one component of microhabitat heterogeneity that may influence the evolution of adaptive plasticity in physiological and morphological traits, consequently conflict in selective pressures are likely to arise. Shifts in abiotic factors may co-occur and act synergistically, or alternatively antagonistically, such that the fitness landscape may be highly complex (Baythavong and Stanton 2010). Given the significant
differentiation in distribution of adaptive plasticity in response to water between populations of different elevation found by Nicotra et al. (2015) and Briceño et al. (2014), we expected to detect adaptive plasticity in water response, and potentially elevation differences as well. The paucity of such differentiated responses and limited evidence of adaptive plasticity in response to water thus could reflect such conflicts (Valladares et al. 2007, Auld et al. 2010). This finding highlights the fact that adaptive plasticity in response to a given trait may reflect a neutral or even maladaptive response for another, thereby limiting the development of a phenotypic optimum in response to any one environmental trait (Auld et al. 2010, Davidson 2014, Murren et al. 2015, Münzbergová et al. 2017).

Furthermore, given that phenotypic plasticity is predicted to evolve when environmental cues are reliable (Scheiner and Holt 2012), it is important to consider both spatial and temporal scales of different environmental parameters (Murren et al. 2015). Follow-up experiments could therefore investigate the simultaneous effects of water and temperature, perhaps incorporating elements of frequency and duration of deficit events to fully understand whether and how selective conflicts and trade-offs exist within this system.

We found little evidence that the observed plasticity in water-use traits was adaptive (Tables 3 and 4), and this may reflect both evolutionary history and the different ways phenotypic plasticity may influence fitness. If selection for plasticity in water response has been strong and consistent across the landscape, inability to detect an adaptive signal may simple reflect loss of variation in that plasticity, not that the plastic response is not adaptive per se. Observations of positive correlations between fitness and plasticity may indicate an ability to capitalize upon favourable conditions, or alternatively, observations of high plasticity under unfavourable, or stressful conditions may result in the maintenance of fitness homeostasis (Sultan 2001, Molina-Montenegro et al. 2016). Furthermore, such responses are likely to be trait and species specific. It is also important to note that plasticity may not always be advantageous (Schlichting 1986, Auld et al. 2010). There are costs and limits associated with plasticity that may limit not only its development, but also its extent and effectiveness. It is also the case that costs and adaptive value of plasticity are not always easy to assess under experimental settings, or on naturally occurring genotypes (Dewitt et al. 1998, DeWitt and Schiener 2004, van...
Kleunen and Fischer 2005, Nicotra et al. 2007, Auld et al. 2010, Murren et al. 2015). Finally, plasticity may operate in a non-linear manner and it is possible that our study represents a smaller portion of a much larger and complex reaction norm (Murren et al. 2014). Thus, further research to identify the genetic architecture of the observed plastic response to water availability might improve our understanding of its history and adaptive role.

Conclusions

As we increasingly strive to predict species’ responses to changing climatic conditions, the use of space-for-time substitution, including the use of elevation gradients, is becoming more common. While these methods may in certain cases inform us about the influence of particular abiotic factors such as temperature (Briceño et al. 2014, Nicotra et al. 2015), we caution against broad generalizations as responses to differing abiotic factors vary. Future investigation into climate change responses within alpine ecosystems should seek to understand how shifts in co-occurring abiotic factors may act synergistically or antagonistically upon fitness, but may also vary as a function of time and space. Moreover, investigations into not only the mean changes in abiotic variables, but also the periodicity and frequency of extreme events, will be of increasing importance (Schwinning and Ehleringer 2001, Murren et al. 2014). Finally, when considering the capacity for adaptive phenotypic plasticity to buffer climate change, it is also important to assess how observed trait changes may translate into population-level responses (McLean et al. 2016). An increased understanding of variability in phenotypic plasticity, in particular, adaptive plasticity, may allow us to better assess the evolutionary potential of species. Knowledge of how plasticity varies as a function of differing abiotic pressures will aid in developing informative models of more complex, realistic climate change scenarios.
Acknowledgements

We would like to especially thank Deb Segal, Tobias Hayashi and Nicholas Wilson for assistance with the field and lab work, and also the members of the Nicotra Lab for their feedback on draft manuscripts. The authors would also like to acknowledge New South Wales Parks and Wildlife for providing a Scientific License for plant collection (License Number: S10873). Finally, we would like to thank two reviewers for their thoughtful insights into the manuscript.


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Supplementary material

**Figure A1:** Study species *Aciphylla glacialis* (top left), *Wahlenbergia ceracea* (top right), and *Oreomyrrhis eriopoda* (bottom). Photo credit: Sonya Geange and Veronica Briceno.
Table A1: Within treatment selection gradient analysis on trait value and relative fitness upon functional traits which displayed significant treatment responses for; A) *Aciphylla glacialis*, B) *Oreomyrrhis eriopoda* and C) *Wahlenbergia ceracea*. Selection differentials were calculated within each treatment, *High Water* and *Low Water*, combining elevations unless elevation was also significantly different, in which case elevation subsets are presented below the combined values. Positive selection differentials indicate adaptive responses, while negative values indicate maladaptive responses. Significant responses are indicated by bolded p-value. n = number of individuals within each analysis.

<table>
<thead>
<tr>
<th>Species</th>
<th>Trait</th>
<th>Trait Selection: Treatment</th>
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<tr>
<td></td>
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<td><strong>High Water</strong></td>
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<td><strong>C) W. ceracea</strong></td>
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<td>Low Elev</td>
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Chapter Three

Aciphylla glacialis mortality, growth and frost resistance: a field warming experiment

Authors: Sonya R. Geange, Meisha-Marika Holloway-Phillips, Veronica. R. Briceno, and Adrienne B. Nicotra

1. Research School of Biology, The Australian National University, Canberra, ACT 2601, Australia
2. Instituto de Ciencias Ambientales y Evolutivas, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile.

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Abstract

Decreasing snow depth and earlier snowmelt in alpine regions are expected to expose plants to a greater range of thermal extremes. Thus, paradoxically, in addition to increasing mean temperatures, aseasonal frost is likely to emerge as a major determinant of plant survival and development under a warming climate.

Here, through the use of open top chambers, we assessed the effects of simulated warming on seedlings of the alpine herb Aciphylla glacialis (F. Muell.) Benth sourced from provenances that vary in thermal characteristics. We hypothesized that warm-grown seedlings would have reduced survival and lower freezing resistance, but increased growth. Further, seedlings sourced from the lower elevation sites, where temperatures varied more, would exhibit fewer negative impacts of warming conditions than those from sites with narrower thermal ranges.

Warmed conditions significantly increased seedling mortality but seedlings that survived warming grew slightly taller. Warming did not affect freezing resistance, leaf production, or photosynthetic efficiency. Contrary to our expectations, there was little evidence of intraspecific variation. Our results indicate that a warmer climate with more extreme events may lead to a reduction in seedling establishment and survival. However, seedlings that survive and establish exhibit no lingering detrimental effects.
Introduction

When asked to picture climate change, often one envisages the future as a warmer environment (IPCC 2014). For most ecosystems, this is true; for others, such as alpine ecosystems, increasing temperatures may paradoxically increase the risk of frost (Inouye 2000, Ball et al. 2012). This is because snow acts as an insulating blanket protecting vegetation from freezing winter temperatures, and providing a source of water during spring and early summer (Billings and Bliss 1959, Körner 2003). Predictions of increased temperatures and variable patterns of precipitation may substantially reduce snow pack development and persistence, resulting in earlier snow melt (Inouye 2008, Rixen et al. 2012, Luca et al. 2017). In an ecosystem where plant growth is constrained by low temperatures and a truncated growing season, increased mean temperatures and earlier snow melt may present an opportunity for increased plant growth (Galen and Stanton 1993, 1995). However, models of projected climate change also predict increased risks of temperature extremes (IPCC 2014, Harris et al. 2018), which may instead stunt or reduce growth through damage to the photosynthetic apparatus, or in extreme cases, result in the loss of leaves, or plant death. This, along with the increased risk of otherwise aseasonal freezing events suggests a need to understand how resilient alpine plants are to both warming and frost (Inouye 2000, Beniston 2003, Wipf et al. 2009, Larcher et al. 2010, Sierra-Almeida and Cavieres 2012, Gerdol et al. 2013). Given the often limited capacity for plant migration at higher elevations, due to restricted land availability, and dispersal syndromes often resulting in poor dispersal capacities (Gottfried et al. 2012, Pauli et al. 2012), understanding plasticity in plant responses may be key to understanding the in-situ capacity of alpine species to survive (Nicotra et al. 2010).

The ability of a plant to persist across variable spatial or temporal landscapes may arise through phenotypic plasticity, which is the capacity for a given genotype to express different phenotypes across heterogeneous conditions (Schlichting 1986, Bradshaw and Hardwick 1989, Bradshaw 2006, Bradshaw and Holzapfel 2006). Within sub-alpine, alpine and arctic landscapes, plasticity in phenological traits has been widely documented (Anderson et al. 2012, Vitasse et al. 2013, Prevey et al. 2017, Richardson et al. 2017). However, we know markedly less about how underlying traits of growth and freezing
resistance vary across the alpine environment or influence plant survival. Furthermore, the extent of plasticity may vary not only among traits but also between populations of a given species, and within populations of a species. These changes occur as a function of both environmental conditions and evolutionary history making it difficult to generalise about the capacity of a species to respond to environmental changes (van Kleunen and Fischer 2005, Ghalambor et al. 2007).

In the highly heterogeneous alpine environment, locations in which snow melts earlier may be characterised by greater exposure to spring freezing events, summer heat events, longer growing seasons, and more growing degree days than sites where snow typically melts later (Briceño et al. 2014). Although depending upon site specific factors, seasonal mean temperatures may in fact be cooler, and overall less variable if late snowmelt sites release plants directly into the hottest summer temperatures, whilst truncating spring and autumn freezing points (Körner 2003). One could hypothesize that populations from locations with different thermal ranges may have a different capacity to express plastic responses appropriate to future climatic conditions. Indeed, within the Australian Alps, seedlings from more source locations with greater thermal ranges, be they low elevation or early snow melt sites, have been shown to exhibit greater plasticity in functional traits in response to warming temperatures (Nicotra et al. 2015). Furthermore, Briceno et al. (2014) showed that for the native herb Aciphylla glacialis, both adults and their glasshouse grown progeny sourced from thermally variable provenances had a greater capacity to acclimate to cold conditions (Briceno 2014). Importantly, variation in trait expression among individuals may reflect current growing conditions, genetic differentiation, and also parental environmental condition during seed development (Auge et al. 2017).

Temperatures within the Australian Alps have increased by 0.8 °C since the 1950’s (Hennessy et al. 2008), and precipitation, snowfall, and snow cover are becoming increasingly variable (Osborne et al. 1998, Hughes 2003, Nicholls 2005, Hennessy et al. 2008, Green and Pickering 2009, Davis 2013, Harris et al. 2016, Theobald et al. 2016). In addition, there has been a decrease in the duration of snow cover by 8.5 days during this period (Sánchez-Bayo and Green 2013). We seek to understand how simulated warming affects survival, growth, photosynthetic efficiency and freezing resistance in Aciphylla
glacialis, through the use of open top chambers (OTCs). Furthermore, we explore intra-specific variation, asking whether more low elevation sites with larger thermal ranges, as characterised on a local climatic scale by Briceno (2014), produce seedlings with a greater capacity to acclimate to warming temperatures and tolerate freezing events, such as would be indicative of locally adapted ecotypes. Specifically, we hypothesize that seedlings of Aciphylla glacialis grown under simulated warming would display higher rates of mortality, but that the warmer conditions under OTCs would also promote growth and plant health in the survival seedlings, as indicated by photosynthetic efficiency (Fv/Fm). With regard to intra-specific variation, we hypothesized that seedlings sourced from the lower elevation sites where thermal variation over days and seasons was greater would display greater trait plasticity when exposed to warmer than ambient temperatures in the open top chambers. This would result in higher freezing resistance and heat tolerance, and thus have higher overall survival, growth and photosynthetic performance in the field.

Methods and Materials

Study species and study sites

Aciphylla glacialis (F. Muell.) Benth (Apiaceae) is a perennial, dioecious, Australian alpine herb. Individuals often form clumps in the field, with individuals around 30–70cm in height when flowering. Within Kosciuszko National Park, NSW, Australia, the natural distribution of A. glacialis is within the functionally alpine region ranges from 1800 to 2228m.a.s.l (Costin et al. 2000).

Aciphylla glacialis seeds were collected in Autumn 2012 from Kosciuszko National Park from four populations that were chosen to reflect differences in thermal regimes, arising due to differences in elevation and snow duration patterns (Briceno 2014). These collections are hereafter referred to as provenances and called: Charlotte Pass (1842m), Snowy River (1972m), Seamans Hut (2030m) and Kosciuszko Summit (2225m). The summer-time conditions of the two lower elevation sites, Charlotte Pass and Snowy River, have greater temperature ranges and longer growing seasons than the two higher
elevation sites. Specifically, Charlotte Pass is characterised by higher mean and maximum temperatures, frequent high temperature events, and a relatively longer growing season (based on growing degree days), and Snowy River has the greatest frequency of frosts, and also more severe frost events, as well as lower minimum temperatures. The higher elevation sites, Seamans Hut and Kosciuszko Summit, were exposed to a narrower thermal range, both within days and across seasons and generally shorter growing seasons. A prolonged snow pack presence at Seamans Hut results in fewer growing degree-days than at Kosciuszko Summit (Briceno 2014).

Seed collection and germination

Seed at each provenance was collected from 10 mature plants of a similar size (maternal lines) that were at least 3 m apart. Collected seeds from each maternal line were kept separate, cleaned and stored in a seed-drying room at ~15 % relative humidity prior to cold stratification. Seeds were sown into Petri dishes 9 cm in diameter containing 1 % plain water agar. To alleviate morphological and physiological dormancy, seeds were germinated by exposing them to conditions that mimicked the natural progression of post-dispersal temperatures through summer, early autumn, late autumn and winter. Germination in *Aciphylla glacialis* occurs after exposure to winter conditions (Briceno 2014, Hoyle et al. 2014). Following germination, seeds were transferred to 68 mm square pots with Native Planting Mix (Martins Fertilizer Australia) and Yates Nutricote Grey Pellets (16N:4.4P:8.3K) fertilizer (Briceno 2014). Seedlings were then left to grow in a glasshouse under warm summer alpine conditions 25/15 °C day/night temperature regime with natural daylength conditions until they had 4 true leaves and were approximately 25 mm in height and so large enough to establish and survive transplant into the field (approx. two years). Further, by allowing the seedlings to establish under controlled conditions we sought to minimise the potential for maternal provisioning effects to be expressed as differences among provenances. At the commencement of the field trial, the plants were two years old and generally at the four-leaf stage. As these small plants were pre-reproductive and still would not flower for several years, we refer to them as seedlings throughout.
Open top chamber field trial

In March 2014, seedlings were transplanted into the field at Kosciuszko National Park, near Merritt’s Creek (1950m a.s.l, -36.462431, 148.305765). Small polycarbonate open top chambers (OTC) were used to simulate climate warming (Cochrane et al. 2015). Chambers were circular with a 45cm basal diameter, 32cm opening diameter, and 15cm central height. The experimental design consisted of five blocks, where a block is composed of 10 plots (Supplementary Figure 1). A plot included one open top chamber and one equivalently sized ambient control subplot (5 blocks by 10 plots by 2 subplots (one per treatment) = 100 subplots; 50 OTC and 50 ambient). Within each OTC or ambient subplot, four individual *A. glacialis* seedlings were planted, one per provenance. Maternal lines were randomized within each provenance using a stratified design across blocks, plots and subplots. Within each OTC or ambient subplot, seedlings were randomly assigned to the cardinal directions. Seedlings were located approximately 15cm away from one another, and encroaching vegetation was removed at each visit. Paired subplots were orientated in east/west pairings and separated from adjacent subplots by at least one meter. Plots as a whole were arranged in a generally north/south orientation, though positions and orientations were modified as dictated by the topography (e.g. large rocks or dense existing vegetation). We controlled as best as possible for constant slope and aspect across blocks and plots.

Field measurements

Seedling survival, growth and photosynthetic efficiency were assessed up to 11 times during the growing seasons between March 2014 and April 2016. Growth was measured as the height of the plant with the longest leaf extended (mm). We also counted the number of fully expanded leaves. The maximum quantum efficiency of Photosystem II (Fv/Fm) was used as an indicator of photosynthetic health of the seedlings throughout the experiment (Murchie and Lawson 2013). Fv/Fm was measured using a Photosynthetic Efficiency Analyser (PEA Meter, Hansatech Instruments, UK). Measurements were taken after 20 minutes of dark adaptation on a single leaf per seedling, using a leaf clip (Hansatech Instruments, UK).
Environmental characteristics

We monitored soil moisture and air temperature throughout the experiment. Soil relative water content was measured to 10cm depth in each of the 100 subplots using a Delta-T Thetaprobe (Delta-T Devices) at each site visit (n = 11, subject to variable weather). Temperature was measured for each subplot at three hourly intervals from March 2014 – April 2016 using iButtons (Maxim Integrated). Buttons were hung from stakes within each subplot 5cm above the soil. Freeze-thaw activity caused some of these to sink further into the soil so that buttons were 1-5cm above the soil, at roughly same height range as the seedlings. The buttons were held on plastic clips that partially shaded them while hanging, but the possibility remains that some were exposed to direct sun for portions of the experiment. At each return visit, we carefully checked and re-orientated/positioned each ibutton. As a result, we screened the ibutton data and excluded values greater than 50°C in both treatments which indicated periods of direct radiation on the buttons not air temperature. Unfortunately, it is not possible to separate the relative effects of soil temperature and air temperature from our iButton data, but soil temperature effects are likely to be major determinants of the thermal microclimate of these small plants given their short stature.

To assess differences between our treatments, we divided the ibutton temperature data into day (9am, 12pm, 3pm and 6pm readings) and night (9pm, 12am, 3am and 6am readings) during the growing season. The growing season was defined as any period of time without snowpack formation, where the date of snow pack formation was defined as four consecutive days of iButton data with a mean day-time temperature of 0°C, and date of snow melt was the date when mean temperature exceeded 0°C. We focus on the growing season because when the plots were under snow there were no differences in the temperatures between treatments.

Ice Nucleation

Freezing resistance was assessed as the temperature at which ice formation occurs in leaves (Taschler and Neuner 2004). These measurements were conducted in late summer/early autumn just over one year after planting (14 – 15th of April 2015) and followed the protocol of Briceño et al. (2014). Seedling were considered too small for
measurements in the first spring (2014) and few samples remained in the spring of 2015. For each treatment, leaf tissue was sampled from 5 plants per treatment per provenance, giving a total of 40 samples (2 treatments by 4 provenances by 5 replicates = 40). Due to high mortality the previous summer, there was insufficient replication to completely balance blocks or maternal lines within sites. The largest healthy leaf from each plant was collected in the field and kept damp at a temperature of 2 - 4 °C in the dark during transportation back to the laboratory in Canberra for use within the next 10-48 hours, depending upon the run.

Leaves were trimmed to a standard size of 2cm length before each sample was placed into an individual chamber in an insulated polystyrene box connected to a water bath (Julabo labortechnik, Seelbach, Germany). Temperature within the insulated box was reduced from 20 °C to -25 °C at a rate of 2 °C per 20 minutes following Briceno et al. (2014). Temperatures for each leaf sample were recorded at 1 second intervals using copper-constant thermocouples and a Datataker DT500 (Biolab Aust Pty Ltd., trading as Datataker Scoresby, Australia). The point of freezing was indicated by detecting an exotherm, or sudden rise in leaf temperature, which is a result of the heat produced through the freezing process. Nucleation temperature was defined as the lowest temperature reached before the occurrence of the exothermic reaction. The insulated box held 20 samples so samples were randomly allocated to each of two runs, with each run treatments and sites were equally represented.

**Harvest measurements**

In late April 2016 all surviving seedlings were harvested to assess biomass allocation and specific leaf area (SLA). Seedlings were carefully excavated and all loose dirt shaken from root systems. Plants were stored in ziplock bags with damp paper towel and transported back to the laboratory in Canberra. Seedlings were separated into above and below ground components. Roots were washed to remove any fine soil particles. Leaf size (cm²) of the three largest leaves for each individual was determined using a flatbed scanner and analysed using ImageJ (Schneider, 2012). Above and below ground biomass and leaf area samples were then oven dried for 72 hours at 60 °C and then weighed using an
analytical balance (Mettler-Toledo, Switzerland). SLA was calculated as the total leaf area (cm²) / mass (g) for the three scanned leaves (Perez-Harguindeguy et al. 2013).

**Statistical analysis**

Comparisons of iButton air temperature between treatments were conducted using an ANOVA, with fixed terms treatment, season (spring, summer, autumn, winter), year and their interactions. Terms for block and plot were not included due to iButton loss throughout the experiment. Tukey’s post-hoc testing was used to compare levels. Only those subplots for which continuous data were available were analysed e.g. excluding plots in which iButtons failed. Day and night-time temperatures were assessed independently.

Analysis of mortality over the experimental duration was conducted using the R package “survival” (Therneau 2015). We used a binomial restricted maximum likelihood model to test for differences between treatments and provenances using the function ‘glmer’ within the R package “lme4” (Bates et al. 2015). To explore what factors might contribute to mortality we similarly applied a binomial restricted maximum likelihood model with treatment and its interaction with soil moisture, as well as plant height, leaf number, and Fv/Fm from the preceding visit as fixed factors to predict mortality at the following trip. Block and individual plant ID nested within provenance were included in the model to account for individual variation through time.

Differences in ice nucleation temperatures were examined using a restricted maximum likelihood model (REML) and the function ‘lmer’ within the R package “lme4” (Bates et al. 2015). Treatment, provenance and their interaction were considered as fixed factors, while block and maternal line (nested within provenance), were treated as random factors.

To assess variation in plant height, leaf number, and Fv/Fm throughout the experiment, a number of restricted maximum likelihood models (REML) were developed. Comparisons were constructed using the fixed factors treatment, provenance, sample date, and soil moisture, along with possibly interactions. Random factors were block and individual plant ID, which was nested within provenance. Plant height and Fv/Fm were
assessed using ‘lmer’, whilst leaf number was assessed using ‘glmer’ and a poisson distribution. Model comparisons were then conducted and the best model was identified based on AICc scores using the package “AICcmodavg” (Mazerolle 2016). In cases where multiple models fell within four AICc values, model averaging was conducted. Note, this analysis was conducted only upon individuals that survived the experimental duration.

Analysis of final trait measurements for treatment and provenance effects was conducted using a restricted maximum likelihood model (REML), for leaf number a ‘glmer’ model with a poisson distribution was used, for all other plant traits a ‘lmer’ approach was taken. Treatment and provenance were fixed factors. We included block and maternal line as random effects. Data were natural log transformed when they did not meet the assumptions of normality.

Results

Open top chambers increased the average day-time temperature at plant height during the growing season by 2.5 K (CI: 1.6 – 3.4) (Figure 1, Table 1a). Night-time temperatures were also warmed on average by 1.1 K (CI: 0.58 – 1.57), though this was not a statistically significant difference (Figure 1, Table 1b). The onset of snowpack development was not influenced by the presence of OTCs. However, the occurrence of mid-season melts, and earlier melt out in spring did occur (Table 2). Snowpack duration under OTCs was reduced by 11 days during the first year and 44 in the second, which although consistent in direction, with n of 2 years not something we can test statistically. Daytime temperatures in excess of 30 °C occurred on 20-30 more days under OTC than ambient conditions. Less influence of the OTCs was noted for minimum temperatures (below -10 °C) with infrequent occurrences observed and no significant difference between treatments, noting that these extreme cold events occur only prior to formation and after melt of the snowpack. There was no significant difference in soil moisture between treatments.

Within our study, high levels of seedling mortality were observed during the first summer period (Dec 2014 – Feb 2015). Mortality was higher under open top chambers at 76% (alive n =44), compared with 56% (alive n =79) under ambient conditions (p = 0.012,
Comparing environmental conditions and plant traits from the measurement period preceding mass mortality, we found that mortality was high where soil moisture was comparatively low (Table 3). Likewise, seedlings that were either small, had few leaves, or had low Fv/Fm were more likely to have died (Table 3). These trends were consistent irrespective of treatment or provenance. Following this mortality event, instances of mortality stabilized as did Fv/Fm (Figure 5c, Table 5), and in general plants that survived this initial event persisted until the end of the experiment (Figure 2).

We assessed cold resistance in the autumn following the high mortality event (April 2015), and found that *A. glacialis* seedlings were highly freezing resistant. On average, ice formation was initiated at -9.4 °C (Figure 3); there were no significant differences in freezing resistance between treatments or provenances (Table 4).

There were no significant effects of treatment or provenance on SLA, leaf number, total biomass, or above ground biomass assessed at the end of the experiment (Table 5). However, surviving seedlings grown under the OTCs were significantly taller at harvest time irrespective of source provenance (Figure 4, Table 5).
Figure 1: a) Illustration of the mean temperature difference between ambient and warmed (OTC) treatments across the course of the transplant experiment from April 2014 to April 2016 at Merritt’s Creek, Kosciuszko National Park, NSW, Australia. Day-time temperature differences shown as an orange line, while night-time temperature differences are shown as a black line. Temperature readings taken at 3-5cm above ground level. Grey bars represent periods under snow cover. B) Average temperature difference between ambient and warmed (OTC) plots during the growing seasons for day and night-time temperatures.

Figure 2: Kaplan-Meier mortality curve for *Aciphylla glacialis* seedlings grown under ambient (A) and warmed OTC conditions (W). Solid line represents means for each treatment averaged across the four provenances, whilst coloured bands are the 95% confidence intervals.
Figure 3: The temperature of ice nucleation (the formation of internal ice) for *Aciphylla glacialis* seedlings grown under ambient (blue) and warmed OTC conditions (orange). For completeness, each provenance is displayed as a panel, and ordered from left to right in ascending elevation for reference. Error bars represent 95% confidence intervals.
Figure 4: *Aciphylla glacialis* plant traits observed during the growing season on 11 visits between March 2014 and April 2014 for all surviving seedlings, grown under either ambient (blue) conditions or warmed OTC conditions (orange). Means and error bars (CI) represent modelled values representing for each of a) plant height (mm), b) the number of expanded leaves and c) photosynthetic efficiency (Fv/Fm). Points are slightly offset horizontally for clarity. Lines between means are to help visualise trends over time. Dark and light shading in the background represent the beginning of winter and summer seasons, respectively, for each year of the study.
Table 1: ANOVA comparison of means for a) day-time and b) night-time temperature differences between ambient and warmed (OTC) plots during the snow free growing seasons between April 2014 and April 2016.

A) Day-time Temperature Model
Model: Mean day-time Temperature ~ Treatment * Field Year * Season

<table>
<thead>
<tr>
<th></th>
<th>Df</th>
<th>Sum of Sq</th>
<th>RSS</th>
<th>AIC</th>
<th>F value</th>
<th>Pr (&gt;F)</th>
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<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>405</td>
<td>63862</td>
<td>4526</td>
<td>6.96</td>
<td>0.008</td>
</tr>
<tr>
<td>Field Year</td>
<td>2</td>
<td>1319.1</td>
<td>64776</td>
<td>4539.8</td>
<td>11.33</td>
<td>&gt; 0.000</td>
</tr>
<tr>
<td>Season</td>
<td>3</td>
<td>9135.5</td>
<td>72593</td>
<td>4664</td>
<td>52.33</td>
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<tr>
<td>Treatment: Field Year</td>
<td>2</td>
<td>112.1</td>
<td>63569</td>
<td>4518.9</td>
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<td>0.382</td>
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<tr>
<td>Treatment: Season</td>
<td>3</td>
<td>205.3</td>
<td>63663</td>
<td>4518.6</td>
<td>1.18</td>
<td>0.318</td>
</tr>
<tr>
<td>Field Year: Season</td>
<td>3</td>
<td>61.2</td>
<td>63518</td>
<td>4516</td>
<td>0.35</td>
<td>0.789</td>
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<td>Treatment: Field Year: Season</td>
<td>3</td>
<td>203.3</td>
<td>63660</td>
<td>4518.5</td>
<td>1.16</td>
<td>0.322</td>
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</table>

B) Night-time Temperature Model
Model: Mean night-time temperature ~ Treatment * Field Year * Season

<table>
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<th>Sum of Sq</th>
<th>RSS</th>
<th>AIC</th>
<th>F value</th>
<th>Pr (&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>22.5</td>
<td>19420</td>
<td>3210.8</td>
<td>1.26</td>
<td>0.261</td>
</tr>
<tr>
<td>Field Year</td>
<td>2</td>
<td>56.6</td>
<td>19904</td>
<td>3236.1</td>
<td>14.30</td>
<td>&gt;0.000</td>
</tr>
<tr>
<td>Season</td>
<td>3</td>
<td>1931.3</td>
<td>21329</td>
<td>3310.8</td>
<td>36.24</td>
<td>&gt;0.000</td>
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<td>Treatment: Field Year</td>
<td>2</td>
<td>0.5</td>
<td>19398</td>
<td>3207.5</td>
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<td>0.985</td>
</tr>
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<td>Treatment: Season</td>
<td>3</td>
<td>15.4</td>
<td>19413</td>
<td>3206.4</td>
<td>0.29</td>
<td>0.833</td>
</tr>
<tr>
<td>Field Year: Season</td>
<td>3</td>
<td>239.2</td>
<td>19637</td>
<td>3219.1</td>
<td>4.49</td>
<td>0.004</td>
</tr>
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<td>Treatment: Field Year: Season</td>
<td>3</td>
<td>5.3</td>
<td>19403</td>
<td>3205.8</td>
<td>0.09</td>
<td>0.961</td>
</tr>
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</table>

Table 2: Comparing days of snow duration, maximum temperatures exceeding 30 °C and minimum temperatures below -10 °C for each year of the field experiment for either ambient or warmed (OTC) conditions.

<table>
<thead>
<tr>
<th></th>
<th>Year 1 Ambient</th>
<th>Year 1 Warm</th>
<th>Year 2 Ambient</th>
<th>Year 2 Warm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snow Duration (days)</td>
<td>114</td>
<td>104</td>
<td>158</td>
<td>115</td>
</tr>
<tr>
<td>Days with max. exceeding 30 °C</td>
<td>130</td>
<td>169</td>
<td>155</td>
<td>172</td>
</tr>
<tr>
<td>Days with min. below -10 °C</td>
<td>2</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 3: Analysis of Deviance table (Type II Wald Chi-square tests) for survival of *Aciphylla glacialis* seedlings at the point of mass mortality (Nov – Jan 2015) compared to plant traits measured at the preceding time point (October 2015): plant height (mm), leaf number, Fv/Fm, soil moisture and the treatment, either warmed (OTC) or ambient. Significant values are indicated by bolded p-values.

<table>
<thead>
<tr>
<th>Factors</th>
<th>ChiSq</th>
<th>Df</th>
<th>Pr (&gt; Chisq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height</td>
<td>0.034</td>
<td>1</td>
<td>0.854</td>
</tr>
<tr>
<td>Leaf Number</td>
<td>13.410</td>
<td>1</td>
<td>0.000</td>
</tr>
<tr>
<td>Fv/Fm</td>
<td>23.540</td>
<td>1</td>
<td>0.000</td>
</tr>
<tr>
<td>Treatment</td>
<td>6.569</td>
<td>1</td>
<td>0.010</td>
</tr>
<tr>
<td>Provenance</td>
<td>4.630</td>
<td>3</td>
<td>0.201</td>
</tr>
<tr>
<td>Soil Moisture</td>
<td>6.659</td>
<td>1</td>
<td>0.009</td>
</tr>
<tr>
<td>Treatment: Provenance</td>
<td>0.924</td>
<td>3</td>
<td>0.819</td>
</tr>
<tr>
<td>Treatment: Soil Moisture</td>
<td>0.480</td>
<td>1</td>
<td>0.488</td>
</tr>
</tbody>
</table>

Table 4: Restricted Likelihood Estimate (REML) comparing the temperature at which ice nucleation occurred (intracellular freezing) for *Aciphylla glacialis* leaves harvested in April 2015 from warmed (OTC) or ambient conditions across the four provenances.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Sum Sq</th>
<th>Mean Sq</th>
<th>NumDF</th>
<th>DenDF</th>
<th>F. value</th>
<th>Pr (&gt; F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Provenance</td>
<td>0.615</td>
<td>0.205</td>
<td>3</td>
<td>26</td>
<td>0.204</td>
<td>0.893</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.232</td>
<td>0.232</td>
<td>1</td>
<td>26</td>
<td>0.231</td>
<td>0.635</td>
</tr>
<tr>
<td>Trt:Provenance</td>
<td>2.701</td>
<td>0.910</td>
<td>3</td>
<td>26</td>
<td>0.906</td>
<td>0.452</td>
</tr>
</tbody>
</table>

Table 5: Comparison of final harvest traits (plant height, leaf number, Fv/Fm, total biomass, total above ground biomass (ABG, log transformed), and total below ground biomass (BG, log transformed), for *Aciphylla glacialis*. Comparisons include treatment, provenance and their interaction.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Factors</th>
<th>Sum Sq</th>
<th>Mean Sq</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F. value</th>
<th>Pr (&gt; F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height</td>
<td>Treatment</td>
<td>2711.04</td>
<td>2711.04</td>
<td>1</td>
<td>73.22</td>
<td>13.34</td>
<td>&lt;0.000</td>
</tr>
<tr>
<td></td>
<td>Provenance</td>
<td>1344.30</td>
<td>448.10</td>
<td>3</td>
<td>30.99</td>
<td>2.21</td>
<td>0.107</td>
</tr>
<tr>
<td></td>
<td>Trt:Provenance</td>
<td>602.73</td>
<td>167.58</td>
<td>3</td>
<td>71.35</td>
<td>0.82</td>
<td>0.484</td>
</tr>
<tr>
<td>Leaf Number</td>
<td>Treatment</td>
<td>1.02</td>
<td>1.02</td>
<td>1</td>
<td>67.96</td>
<td>1.01</td>
<td>0.152</td>
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<tr>
<td></td>
<td>Provenance</td>
<td>0.73</td>
<td>0.24</td>
<td>3</td>
<td>23.02</td>
<td>0.24</td>
<td>0.266</td>
</tr>
<tr>
<td></td>
<td>Trt:Provenance</td>
<td>2.67</td>
<td>0.89</td>
<td>3</td>
<td>66.03</td>
<td>0.89</td>
<td>0.085</td>
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<tr>
<td>Fv/Fm</td>
<td>Treatment</td>
<td>0.0001</td>
<td>0.0001</td>
<td>1</td>
<td>75.46</td>
<td>0.06</td>
<td>0.799</td>
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<tr>
<td></td>
<td>Provenance</td>
<td>0.0047</td>
<td>0.0016</td>
<td>3</td>
<td>33.40</td>
<td>0.94</td>
<td>0.430</td>
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<tr>
<td></td>
<td>Trt:Provenance</td>
<td>0.0051</td>
<td>0.0017</td>
<td>3</td>
<td>73.61</td>
<td>1.03</td>
<td>0.385</td>
</tr>
<tr>
<td>Total Biomass</td>
<td>Treatment</td>
<td>0.71</td>
<td>0.71</td>
<td>1</td>
<td>70.13</td>
<td>1.25</td>
<td>0.267</td>
</tr>
<tr>
<td></td>
<td>Provenance</td>
<td>0.61</td>
<td>0.20</td>
<td>3</td>
<td>32.12</td>
<td>0.36</td>
<td>0.873</td>
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<tr>
<td></td>
<td>Trt:Provenance</td>
<td>1.48</td>
<td>0.47</td>
<td>3</td>
<td>69.64</td>
<td>0.84</td>
<td>0.479</td>
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<tr>
<td>ABG (log)</td>
<td>Treatment</td>
<td>1.79</td>
<td>1.80</td>
<td>1</td>
<td>70.59</td>
<td>2.64</td>
<td>0.109</td>
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<tr>
<td></td>
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<td>0.59</td>
<td>0.20</td>
<td>3</td>
<td>32.12</td>
<td>0.29</td>
<td>0.833</td>
</tr>
<tr>
<td></td>
<td>Trt:Provenance</td>
<td>1.99</td>
<td>0.66</td>
<td>3</td>
<td>70.09</td>
<td>0.97</td>
<td>0.410</td>
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<tr>
<td>BG (log)</td>
<td>Treatment</td>
<td>0.05</td>
<td>0.05</td>
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<td>69.35</td>
<td>0.09</td>
<td>0.769</td>
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<tr>
<td></td>
<td>Provenance</td>
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<td>0.28</td>
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<tr>
<td></td>
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<td>1.40</td>
<td>0.46</td>
<td>3</td>
<td>68.84</td>
<td>0.88</td>
<td>0.455</td>
</tr>
</tbody>
</table>
Discussion

The Australian Alps are predicted to warm by 0.4 – 2.0 °C by 2030 and 1 – 6 °C by 2070 (Hennessy et al. 2008). To simulate a warmer future, we used open top chambers and successfully increased mean daytime temperature by 2.5 K. In doing so, we observed increased mortality, but no detrimental effects on growth or physiological freezing tolerance for surviving seedlings. Below we discuss potential mechanisms that could explain differences and similarities in key traits, and what this may mean for how alpine plant species respond in the future.

Mortality rates during seedling establishment are generally high, and the degree of mortality may vary substantially depending on factors such as the frequency and magnitude of extreme events, along with species-specific characteristics (Walck et al. 2011, Williams et al. 2015). In our experiment, seeding mortality was high with the highest mortality observed under warmed conditions, which is consistent with prior research exploring artificial warming (Cochrane et al. 2015); though forested habitats often contrast this, see Piper et al. (2013). In conjunction with warmer temperatures, low soil moisture has previously been shown to increase the incidence of mortality (Aragon-Gastelum et al. 2017). In our case, we found that both warming and low soil moisture were correlated with increased risk of mortality, but no interaction between these factors was detected. That said, as our soil moisture data were collected every three months, we were not able to test for an interactive effect during the intervening period between measurements when the mortality actually occurred. However, in support of this idea, nearby weather station data for the relevant period indicates minimum temperatures were warmer than usual and rainfall levels were substantially lower (Australian Bureau of Meteorology). Seedlings were also susceptible if they were short, had few leaves or had low Fv/Fm in the period before the mortality sampling point. Taken together, this suggests that smaller seedlings may not have established large enough root systems to enable them to persist under drier and warmer conditions (Aragon-Gastelum et al. 2017). When placed in the context of other Australian alpine plants, particularly shrubs, the higher mortality of Aciphylla glacialis under warmed conditions could reflect one driver of the predicted shift from tall-alpine herb communities to shrub-land, with Camac et al.
highlighting shrubs suffered little mortality in general, and had doubled growth rates under OTC conditions.

A critical component of alpine plant establishment and persistence is freezing resistance. Frosts may occur within the Australian alpine area throughout the year, though not necessarily at lethal levels, and not surprisingly many Australian alpine plants exhibit relatively high levels of freezing resistance year-round (Bannister 2007, Venn et al. 2013). Low profile plants, such as *A. glacialis*, are not only exposed to high daytime leaf temperatures, due to the decoupling from ambient air temperature (Körner 2003), but also possibly cooler than ambient night-time temperatures as, particularly on calm days, long wave energy is dissipated (Jordan and Smith 1994). We found that *A. glacialis* exhibited high levels of freezing resistance, with ice nucleation occurring at approximately -9 °C, which is comparable to both *A. glacialis* adults in the field (Briceno 2014) and glasshouse grown seedlings (Briceño et al. 2014). We note our conservative approach to evening the playing field, through growing our seedlings in the glasshouse for two years may question these comparisons. However, seedlings were in the field for nearly as long again, thus providing ample time to adjust to local conditions. In addition, the upright leaf angle (Körner and Hiltbrunner 2018), waxy leaves (Körner 2003, Wisniewski et al. 2014), and divaricating leaf structure of *A. glacialis* may contribute further to resistance to freezing under natural conditions (Hacker and Neuner 2008).

We allowed a full year for the plants to recover from transplant shock and measured freezing tolerance in late summer/early autumn before plants had a chance to acclimate to severe frosts. Thus, our measurements explore differences in freezing resistance between treatments in plants prior to cold hardening when treatment effects might be most pronounced. Our treatments resulted in significantly warmer days, and warmer nights but not significantly so. Perhaps the lack of treatment effects reflects the similarity of night-time low temperatures. Regardless, given the relative paucity of freezing events below -10 °C within the Australian Alps, it seems unlikely that frost damage is a major risk for this species, particularly when the plants are cold-hardened. Still, it is notable that no evidence of plasticity for freezing resistance in response to treatment conditions or differentiation among provenances was detected.
In terms of the potential benefits of warmer temperatures, we predicted that seedlings that established would be larger and more vigorous as a result of either the direct impact of increased mean temperatures under the OTCs, or indirectly through the extension of the growing season (Galen and Stanton 1993, 1995). Growth promotion has been found in other studies utilising OTCs (Kudo and Suzuki 2003, Kudernatsch et al. 2008, Camac et al. 2017), and similarly with the use of infrared lamps (Rustad et al. 2001) and underground heating, but see Wheeler et al. (2014). We observed modest increases in height, but not in other traits, though we certainly did not find any evidence of detrimental effects of warming, such as might be expected given the higher mortality.

Our study suggests that survival, of both leaves and individuals, may not necessarily rely on an intrinsic capacity to withstand thermal events outright, but rather an ability to sacrifice, regenerate or repair (Körner 2003, Taschler and Neuner 2004). This is because our estimation of plant growth in *A. glacialis* may be slightly under-estimated as we did not document leaf turnover and we observed that it did occur over the course of sampling. Assessing leaf longevity and turnover may have provided a more accurate measure of biomass investment (Venn et al. 2018), particularly given our increase in plant height was not correlated with an increase in seedling biomass. In addition, it’s possible that our removal of a leaf for ice nucleation may have differentially influenced the final growth biomass for the 40 individuals sampled. However, we note that sampling occurred after the mass mortality event, and so would not have affected survival, and given it was applied across treatments and provenances, we believe the underlying patterns remain consistent. Finally, herbaceous species, such as *A. glacialis*, are known to display a greater lag time in response to simulated warming than graminoids or grasses (Kudernatsch et al. 2008). We therefore conducted this study over two years, and none-the-less suggest, as many others have (Lindenmayer et al. 2012), that the establishment of even longer-term monitoring of field based climate manipulation studies is required.

At the outset, we hypothesized that provenances from sites exposed to more frequent high and low temperature events (our lower elevation sites) would be more plastic in their trait responses, in line with previous studies on this species (Briceno 2014, Briceño et al. 2014), and others in the region (Bannister et al. 2005, Nicotra et al. 2015). Strikingly, we found no evidence of such intra-specific variation. Whilst this contrasts with recent
literature for this species, our result is in line with results of several other studies conducted on alpine plants, which have shown similar degrees of freezing tolerance in individuals growing along elevation, or snowmelt gradients (Bannister et al. 2005, Marquez et al. 2006, Sierra-Almeida et al. 2009, Wheeler et al. 2014). It is possible our power to detect these results may have been reduced by high mortality, and so reflects just one window in the plants’ development, but the number of surviving seedlings was similar to those in other experiments showing significant within species differentiation (Venn and Morgan 2009). Thus, we consider this null result is most likely biologically genuine, and therefore interesting for several reasons. There exists the potential for a ‘developmental hangover’ effect to be present, whereby during the seed and early seedling developmental stages maternal provisioning may contribute to provenance differentiation (Bischoff and Müller-Schärer 2010), which we aimed to remove by growing under controlled conditions in early life. Therefore, the work on seedlings by Briceno (2014) may reflect parental imprint in early life stages, and at the adult stage perhaps reflects acclimation to local conditions Briceño et al. (2014). Our study may reflect a mid-way point between these processes. Our glasshouse growth period may have mitigated the provisioning influence as we intended, but acclimation to the variable field conditions may have overwhelmed any lingering small differences that would indicate ecotypic differentiation among provenances. Such differences might be apparent under controlled conditions, but harder to detect against the noise of real field circumstances.

Understanding if, and when, intraspecific variation may play an important role in species establishment, growth, and survival is increasingly relevant to conservation in view of climate change (Parmesan 2006). Modelling efforts not incorporating either intra-specific variation, or a relevant scale of micro-habitat variability may over estimate species loss into the future (New et al. 2002, Nogués-Bravo et al. 2007, Randin et al. 2009, Moran et al. 2016) or underestimate it (Valladares et al. 2014). It is possible micro-habitats of suitable scale and stability may act as refugia, promoting in-situ persistence and possibly the evolution of locally adapted ecotypes, particularly within topographically complex alpine regions, or where species have restricted dispersal, as is the case for many Australian alpine species (Morgan and Venn 2017), (Scott Armbruster et al. 2007,
Scherrer and Koerner 2010, Scherrer and Korner 2011, Opedal et al. 2015, Lenoir et al. 2017). Defining microhabitat variability may however be contentious. Not only is an understanding of the interplay between abiotic and biotic drivers over spatial and temporal scales required, but also knowledge of their relative contribution towards the development of the phenotype, which we know is rarely in response to any one given driver. For instance, in our system we know *A. glacialis* populations along an elevation gradient varied little in their response to experimentally reduced water availability (Geange et al. 2017). Taken together these studies demonstrate that one should neither assume nor ignore the potential for within species variation in establishment and growth traits, but also that assessing these under biologically realistic conditions can be extremely challenging.

Seedling establishment and growth is a crucial, but often understudied aspect of alpine plant ecology. Within our study, initially high levels of mortality indicated *A. glacialis* seedlings might be susceptible to the negative impacts of climate warming. However, continued monitoring revealed that despite the mortality, surviving seedlings showed high levels of resilience to the altered growing conditions, regardless of provenance. Our seedlings were sourced along the entire distribution of the species within this area: from treeline to summit and all provenances showed equivalent capacity to tolerate warming and associated frost events. In this regard, the effects of simulated climate change upon *A. glacialis* can be summarized as ‘what doesn’t kill you makes you stronger’. Understanding how in-situ persistence will influence alpine plant distributions into the future will require a greater acknowledgement of not only the role of micro-habitats, but also the rate at which acclimation occurs.
Acknowledgements

We would especially like to thank Tobias Hayashi, Nicholas Wilson, Anissa Satyanti, Pia Cunningham, Cassia Cunningham and Abby Widdup for assistance with the field and lab work; the NSW National Parks Service for allowing us access to the field site; and, Marilyn Ball and Jack Egerton for assisting in the establishment and running of the ice nucleation experiment. We acknowledge members of the Nicotra Lab for their feedback on draft manuscripts.
References


Supplementary Figure 1. 1) Study species *Aciphylla glacialis*. 2) Schematic roughly indicating location of; a) the source populations, which were in ascending elevation order, Charlotte Pass and Snowy River (the more climatically variable sites), Seamans Hut and Kosciuszko Summit (the less variable sites), b) the block layout at the transplant site near Merritt’s Creek, and c) a representative illustration of the layout of 10 plots, consisting of an ambient control plot and an OTC.
Chapter Four

Does the individual matter?
Quantifying intraspecific variation and phenotypic plasticity across alpine, semi-arid, and coastal plant communities

Authors: Sonya R. Geange¹, Nicola C. Aitken², Meisha-Marika Holloway-Phillips¹, Silvia Matesanz³, Marta H. Robertson³, Christina L. Richards³, Fernando Valladares²,⁴, Mark van Kleunen⁵, and Adrienne B. Nicotra¹

1. Australian National University, Research School of Biology, Canberra, Australia
2. Museo Nacional de Ciencias Naturales, Madrid, Spain
3. University of South Florida, Department of Integrative Biology, Tampa, FL, USA
4. Spanish National Research Council (CSIC), Madrid, Spain
5. University of Konstanz, Department of Biology, Konstanz, Germany

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Abstract

Phenotypic plasticity is touted as a buffering mechanism, enabling continued persistence of organisms that would otherwise be at risk in the face of climate change. But little is known about how within-individual plastic variation compares to the variance at other scales (within and among individuals, across species, and communities) or its evolutionary role. We conducted a field study assessing variation in leaf and biomass allocation traits across three habitats: alpine, coastal and semi-arid to: 1) determine the extent of variation in trait values and trait plasticity at ecological scales ranging from habitat to site, species and individual, 2) assess whether patterns of variation were consistent across plant functional traits, and 3) investigate whether trait variation was associated with increased fitness consistent with adaptive plastic responses.

Traits were measured on six different species per site (two sites per habitat) seasonally for two years on 30 individuals per species. Variance partitioning of trait values and measures of trait plasticity (CV, coefficients of variation), were assessed using a hierarchical mixed-model approach, across habitat, sites, species and individuals. The relationship between the CV and fitness was examined to assess evidence of adaptive plasticity.

Differences amongst species explained the largest component of variation in trait values. For some traits differences among habitat comprised a large component of trait variation. Relatively less variation was explained at the among- or within-individual level, though this varied among traits. However, when partitioning of variation in plasticity of those trait values was specifically examined, most of the variance lay at the species level, very little was explained by habitat, and a surprisingly large proportion was explained by among individual variation. Thus, the results indicate that there is substantial variation in plasticity between species and that individuals vary in the extent of plasticity they display. Nonetheless, there were very few instances where indices of plasticity correlated with our proxies of fitness, providing little evidence for adaptive plasticity across the study.

Models of plant persistence under changing climates need to consider how and when plastic responses occur and whether ultimately, they translate to fitness outcomes.
However, in order to achieve this, advances in developing fitness metrics and plasticity indices for comparative studies are needed.
Introduction

Climate change is occurring at a rapid rate, necessitating unprecedented responses from organisms (IPCC 2014). A potential mechanism for organisms to mediate or buffer the effects of rapid environmental change is phenotypic plasticity - the capacity for a given genotype to express multiple phenotypes under varying environmental conditions (Bradshaw 1965, Nicotra et al. 2010a). Phenotypic plasticity operates over shorter timescales than genetic differentiation, potentially enabling individuals, or populations, to rapidly adjust to novel conditions. These adjustments may allow the short term persistence of the organism, potentially enabling evolution in the longer term as organisms with advantageous adjustments are selected for (Scheiner 1993). For many organisms, slow rates of dispersal prevent effective tracking of suitable climate niches and may result in phenotype-climate mismatches (Jump and Penuelas 2005). In these cases of mismatch, the persistence of an organism is highly likely to be determined by its in-situ responses, which are mediated by genetic variation and phenotypic plasticity in key functional traits (Bradshaw 1965, Nicotra et al. 2010b, Munzbergova et al. 2017).

Functional traits are defined as the readily measurable morphological, physiological and chemical attributes of plants that reflect interactions with abiotic and biotic drivers (Grime 2006b, Albert et al. 2011, Drenovsky et al. 2012). Plant functional traits are quantitative traits used to correlate plants’ responses to environmental conditions with the fitness and success of individuals, and as such, are good indicators of a species’ ecological strategy (Westoby 1998, Westoby et al. 2002, Reich et al. 2003, Wright et al. 2004, Wright et al. 2005, Grime 2006a, Nicotra et al. 2010b, Diaz et al. 2015, Garnier et al. 2017). The ‘leaf economics spectrum’ is a group of plant functional traits that is used to describe a gradient of trade-offs associated with resource acquisition; the gradient runs from species with more rapid resource acquisition to those that are more conservative (Wright et al. 2004, Read et al. 2014). Species with high rates of resource acquisition are typically characterized by high specific leaf area (SLA) and high rates of leaf turnover (Shipley 2004). Within and among species, the leaf economics spectrum may also be reflected across ‘stress gradients’, whereby individuals or species within
more stressful environments, such as extreme low or high temperatures, or reduced rainfall, display low SLA, thicker leaves, and increased leaf longevity (Wright et al. 2004).

The most widely measured plant functional traits gained prominence, in part, due to variation at the individual level being considered small relative to that expressed at the species level (Albert et al. 2011). These traits therefore have the potential to reveal broader ecological processes occurring at a community or landscape scale. However, recent work has drawn attention to the oft overlooked potential contribution of within-individual and within-species variation to model predictions of species persistence and distribution (Violle et al. 2012, Niinemets et al. 2015, Siefert et al. 2015, Martin et al. 2017). Models are likely to be under- or overestimating species’ capacities to respond to environmental challenges when within-individual or within-species variation is ignored (Albert et al. 2011, Valladares et al. 2014, Forsman 2015, Keenan and Niinemets 2016). Overall, differences among species will be greater than those within species as a general rule: otherwise species would not be distinguishable. However, substantive within-species, i.e. population and even individual level, trait variation may occur at a range of spatial (microhabitats such as light, soil moisture, temperature), or temporal (seasonality, ontogeny), scales (Reich et al. 1999, Garnier et al. 2001, Albert et al. 2011). Recently, fine-scale spatial investigations into trait variation are beginning to be conducted (Molina-Montenegro et al. 2009, Suggitt et al. 2011), however, less research has been conducted into how these traits may change over the course of time.

Studies examining the role of plasticity in ecological and evolutionary processes occurring in the complex and variable conditions found within natural landscapes are challenging and thus less common than experiments in highly controlled conditions (Nussey et al. 2007, Valladares et al. 2007, Poorter et al. 2016). The mechanistic underpinnings of plastic responses are generally explored using experimental assessments in highly controlled conditions. Even though extrapolating from controlled conditions to species responses in the field is hindered by the lack of realism, direct field validation of these concepts is rare (Poorter et al. 2016).
Decisions regarding which traits to investigate, the extent of the environmental gradient to examine, study length, or what constitutes a fitness proxy, may all influence the conclusions drawn regarding the consequences of plastic responses (Valladares et al. 2007, Brommer 2013). Thus, to understand the relevance of plasticity under natural, non-controlled, conditions requires the development of approaches that can be applied with relative ease across different systems and species in a natural environment.

Simply quantifying plasticity is not sufficient to determine its role in the capacity of a population or species to persist. To achieve this, investigations need to assess the adaptive nature of plasticity (Chevin et al. 2010, Nicotra et al. 2010b, Nicotra et al. 2015). For plasticity to be adaptive individuals or genotypes with high plasticity must incur a fitness benefit. Fitness can be measured as lifetime reproductive success, or more-often-than-not, for (long-lived) plants growth is used as a proxy (Younginger et al. 2017). Not all plastic responses are adaptive, some may be neutral, others may even be maladaptive (van Kleunen and Fischer 2005). Whilst trait variation has been relatively well documented among species, and assessed against many abiotic drivers, less evidence for within-species trait variation exists, and fewer studies again have attempted to link functional trait variation within-individual to fitness.

Phenotypic variation can be conceptualised hierarchically, especially in particularly labile traits. For example, variation can be partitioned into various explanatory strata, such as within individual, between individuals within populations, between populations within species, and between species within habitats (Westneat et al. 2015). As such, a variance partitioning approach is ideal for assessing the relative extent of plasticity at each of these aforementioned levels, where the fraction of total variance explained is obtained for each level (Ackerly and Cornell 2007, Lepš et al. 2011, Violle et al. 2012, Messier et al. 2017). Variance partitioning provides a tractable approach to measuring drivers of phenotypic plasticity, and is quickly gaining prominence in ecology research (Messier et al. 2017). However, progress has been hindered by the logistical constraints of working across different scales, e.g. within and among individuals and species (Poorter et al. 2009).

The objectives of this study were 1) to quantify how trait variation in plants is partitioned across scales i.e. at the habitat, site, species, or individual level, 2) to assess whether the
observed patterns of variation are consistent across plant functional traits, and 3) to determine whether variation in trait plasticity itself follows similar patterns. Lastly, we assessed whether 4) plasticity in plant functional traits is adaptive, that is to say associated with fitness benefits.

Materials and Methods

Field Sampling and Lab Processing

We established a multi-habitat (3), multi-site (2 for each habitat type = 6 sites), multi-species (6 per site = 36 species) comparative study. Our sites spanned alpine, coastal and semi-arid habitats. Each habitat was represented once within Australia, and again overseas, in order to provide a global perspective (Figure 1; Table 1). We selected these three habitat types because although they are very different, each is exposed to water limitation of some form. Semi-arid sites are of course prone to frequent high temperature events, high evapotranspiration and restricted water availability. Alpine environments in contrast are characterised by long periods of snow cover, a truncated growing season, and frequent exposure to cold temperatures and high winds where water limitation comes primarily in the form of drought-induced conditions during freezing events. Finally, in coastal sites, high levels of salinity within salt marsh and mangrove systems, or low water content in coastal sand dune soils create water limitation despite relatively moderate temperatures and year-round precipitation. Thus, we considered these three habitats would all be exposed to water limitation and thus expected all to show plasticity in leaf traits and allocation patterns (Nicotra and Davidson 2010).
Figure 1: Hierarchical experimental design incorporating a nested structure of habitats, sites within habitats, species within sites, individuals within-species, and individuals across time. Individuals were tracked across the course of the two year experiment for leaf traits and biomass allocation patterns.

The six perennial species selected for monitoring in each site were chosen to be representative of community dominant species and life forms. Within each site one Poaceae and one Asteraceae species was included as these two families were well represented in all sites. We conducted repeated measures of key plant functional traits on 30 individuals per species over two years at times of peak flowering (spring) and fruiting (late summer) at each location.

At each field sample time point we collected leaves for assessment of specific leaf area (SLA) and leaf size. Leaves were chosen from the most recently expanded shoots in each case, though seasonality of growth meant that leaf age varied. Leaves were stored in a ziplock bag with damp paper towel under cool, dark conditions (~5°C) until returning to the lab. In the lab 5-30 fully expanded, undamaged leaves were selected (depending on leaf size) and scanned on a flatbed scanner, and then dried at 60°C to constant weight (minimum 72 hours). Images were analysed in ImageJ (Abràmoff et al. 2004). Bulked leaf area and dry leaf weights were used to calculate an average SLA (cm²/g⁻¹). Leaf number along with SLA was used to calculate average leaf size.
Table 1: The habitats, sites and species used within the study. For each species family and growth form are listed along with an abbreviation used within figures and table outputs. The 18 Australian species were used for all aspects of the analyses. The species from the northern hemisphere (b), were used in trait means and plasticity partitioning for SLA and leaf size, but not for leaf to stem mass ratio as biomass metrics or the adaptive plasticity assessments as the overseas dataset did not provide branch samples or fitness indicators needed for these analyses.

<table>
<thead>
<tr>
<th>Region</th>
<th>Habitat</th>
<th>Site</th>
<th>Species name</th>
<th>Family</th>
<th>Growth form</th>
<th>Abrv.</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Australia</td>
<td>Semi arid</td>
<td>Loughnan, NSW</td>
<td>Hakea leucoptera</td>
<td>Proteaceae</td>
<td>Shrub to small tree</td>
<td>HL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Melaleuca uncinata</td>
<td>Myrtaceae</td>
<td>Shrub to small tree</td>
<td>MeUn</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Oleaeria pimeleoides</td>
<td>Asteraceae</td>
<td>Small shrub</td>
<td>OP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Prostanthera aspalathoides</td>
<td>Lamiaceae</td>
<td>Small shrub</td>
<td>PA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Senna artemisioides</td>
<td>Fabaceae</td>
<td>Medium-large shrub</td>
<td>AR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Triodia scariosa</td>
<td>Poaceae</td>
<td>Grass</td>
<td>TS</td>
</tr>
<tr>
<td>Coastal</td>
<td>Jervis Bay, ACT</td>
<td></td>
<td>Acacia sophorae</td>
<td>Fabaceae</td>
<td>Shrub to small tree</td>
<td>AcSo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Aegiceras corniculatum</td>
<td>Myrsinaceae</td>
<td>Shrub to small tree</td>
<td>AM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Austrosteca littoralis</td>
<td>Poaceae</td>
<td>Grass</td>
<td>AuLi</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Leucopogon parviflorus</td>
<td>Ericaceae</td>
<td>Shrub</td>
<td>LePa</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Olearia axillaris</td>
<td>Asteraceae</td>
<td>Shrub</td>
<td>OlAx</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Westringia fruticosa</td>
<td>Lamiaceae</td>
<td>Shrub</td>
<td>We</td>
</tr>
<tr>
<td>Alpine</td>
<td>Kosciuszko, NSW</td>
<td></td>
<td>Aciphylla glacialis</td>
<td>Apiaceae</td>
<td>Herb</td>
<td>AcGl</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chionochloa frigida</td>
<td>Poaceae</td>
<td>Grass</td>
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</tr>
<tr>
<td></td>
<td></td>
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<td>Epacris paludos</td>
<td>Ericaceae</td>
<td>Shrub</td>
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</tr>
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<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Oxylabium ellipticum</td>
<td>Fabaceae</td>
<td>Shrub</td>
<td>OxEl</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Prostanthera cuneata</td>
<td>Lamiaceae</td>
<td>Shrub</td>
<td>PrCu</td>
</tr>
<tr>
<td>b) Northern</td>
<td>Semi arid</td>
<td>Madrid, Spain</td>
<td>Astragalus alopecuroides</td>
<td>Fabaceae</td>
<td>Herb</td>
<td>AA</td>
</tr>
<tr>
<td>Hemisphere</td>
<td></td>
<td></td>
<td>Centaurea hissopifolia</td>
<td>Astereaceae</td>
<td>Herb</td>
<td>CH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Helianthemum squamatum</td>
<td>Cistaceae</td>
<td>Small shrub</td>
<td>HS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lepidium subulatum</td>
<td>Brassicaceae</td>
<td>Shrub</td>
<td>LS</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Stipa tenacissima</td>
<td>Poaceae</td>
<td>Grass</td>
<td>ST</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Thymus zygis</td>
<td>Lamiaceae</td>
<td>Small shrub</td>
<td>TZ</td>
</tr>
<tr>
<td>Coastal</td>
<td>Florida, USA</td>
<td></td>
<td>Avicennia germinans</td>
<td>Acanthaceae</td>
<td>Small tree</td>
<td>Avge</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Iva imbricata</td>
<td>Asteraceae</td>
<td>Shrub</td>
<td>IvIm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Juncus roemerianus</td>
<td>Juncaeeae</td>
<td>Rush</td>
<td>JuRo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rhizophora mangle</td>
<td>Rhizophoraceae</td>
<td>Small tree</td>
<td>Rhma</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spartina alterniflora</td>
<td>Poaceae</td>
<td>Grass</td>
<td>Spal</td>
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<td></td>
<td></td>
<td></td>
<td>Uniola paniculata</td>
<td>Poaceae</td>
<td>Grass</td>
<td>UnPa</td>
</tr>
<tr>
<td>Alpine</td>
<td>Davos, Switzerland</td>
<td></td>
<td>Anthoxantum alpinum</td>
<td>Poaceae</td>
<td>Grass</td>
<td>Aa</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Epilobium angustifolium</td>
<td>Onagraceae</td>
<td>Herb</td>
<td>Ea</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gentiana punctata</td>
<td>Gentianaceae</td>
<td>Herb</td>
<td>Gp</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Potentilla rupestris</td>
<td>Rosaceae</td>
<td>Herb</td>
<td>Pr</td>
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<tr>
<td></td>
<td></td>
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<td>Rhododendron ferrugineum</td>
<td>Ericaceae</td>
<td>Shrub</td>
<td>Rf</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Senecio incanus</td>
<td>Asteraceae</td>
<td>Herb</td>
<td>Si</td>
</tr>
</tbody>
</table>
For the Australian species we also marked two stems from each plant and collected these 6 months later to determine leaf to stem mass ratio of new growth and to calculate growth increment (g/day) as a proxy for fitness. Leaf to stem mass ratio reflects relative investment into photosynthetic material as opposed to supporting structures. Branches were taken from the northern facing side of the plant for southern hemisphere sites. The base and tip of each stem was marked with paint and a wire tag was attached for identification. The initial stem length, and number of leaves, buds, flowers and/or fruits were recorded. We aimed to harvest the preceding season’s branches and mark the next season’s branches at the point of peak flowering and then when the fruits were generally mature at each site, depending on local seasonality (e.g. flowering was much earlier in the arid sites than the alpine ones). At each sampling time point the marked branches were harvested and a new pair were marked. This was done four times, i.e. flowering and fruiting season in 2013 and 2014.

Harvested branches were stored in ziplock bags under cool, dark conditions until returning to the lab. Branches were then divided into ‘old’ and ‘new’ growth, where ‘old’ growth was determined by the previous tagging measurements along with the presence of marking paint, with all additional material designated as ‘new’ growth. In each instance, stem length (including branches) was measured, and the number of fully expanded leaves and reproductive structures counted. Old and new material was then dried separately at 60°C for a minimum of 72 hours before being weighed.

Due to the range of life forms and species represented (herbs, graminoids, shrubs and trees) some modifications to the sampling regime were required. For graminoids plant dimensions i.e. width and length of the whole plant were recorded instead of marking branches for measures of fitness. Some shrubs had densely packed leaves at the nodes such that it was difficult to distinguish individual leaves in the field; thus, nodes were counted instead of leaves. Some species had either very few or very large leaves and for these a representative portion of the leaf was used as a sample for SLA. In such a large field-based experiment it was inevitable that some plants died or were lost. Thus, only those plants for which we were able to collect data for at least 3 of the 4 key time points were included in the analyses.
Environmental Monitoring

In the Australian field sites, soil moisture measures were taken for each individual using a Delta ML3 ThetaProbe meter (Delta-T Devices Ltd, England) in order to characterize individual microhabitats at each sampling date. Individual plants were ranked based on relative water status relative to: a) sample time points, b) other individuals within-species, and c) all individuals from the same site (across species) to correlate plastic trait responses to underlying changes in water availability. Unfortunately, because the measures didn’t integrate across time, which would have allowed us to more accurately reflect the environmental conditions during branch growth, or leaf development, we found they presented too narrow a snapshot to be useful. We also explored the use of climate layers at a habitat scale (i.e. site-based air temperatures obtained via the Australian Bureau of Meteorology data), but these were not a fine enough scale to explain variation within an individual over time or among individuals in a small geographic area. Thus, we present our results in the context of variation across time, without implying specific causation from an underlying environmental variable.

Statistical Analyses

Variance Components Analyses

We used a linear mixed effects modelling approach to partition the variance in trait values among the different hierarchical strata in our design: habitats, sites, species and among individuals. Each of the strata were included as a separate random effect (but are recognised as nested), with no fixed effects specified. Residual variance includes and is interpreted as within-individual variation (e.g. plasticity) though of course it also includes other sources of unexplained variance. To partition variance with respect to plasticity in trait values, plasticity was calculated as the coefficient of variation (CV, standard deviation/ mean) and the models included terms for habitat, site, and species; here residual variance includes among-individual variation in plasticity as well as unexplained variance. Note that when modelling CV, higher values within the variance partitioning equate to greater variation in plasticity, not higher values of plasticity per se.
Fixed Effects Models of Trait Means and Plasticity

To explicitly test whether there were significant differences in trait values or trait plasticity among our different strata, we conducted a fixed-effects analysis. Linear mixed effects models included habitat, site, and species as fixed effects. Individual plant ID was included as a random effect for the trait value model. Data for trait means were transformed as needed to meet assumptions of normality. Where transformed variables are referred to as such. In order to assess whether trait values themselves influenced plasticity, i.e. whether species with larger trait means displayed greater plasticity, we also regressed log transformed trait values against trait plasticity.

Regression of Plasticity against Fitness

For plasticity to be adaptive, it must confer a fitness advantage. For each species, we used all available individuals to assess the relationship between our average measure of plasticity and fitness using regression analysis. The dataset included 13 of the 18 Australian species as these were the ones for which we could calculate a fitness proxy. The graminoids and seasonal herbs could not be included because we could not collect a comparable fitness measure on a given individual. Fitness was calculated as a relative biomass growth increment as follows:

\[
\frac{\log(\text{total biomass at harvest}) - \log(\text{biomass of initially tagged ‘old’ material})}{\text{days of growth for each harvest period}}
\]

Biomass was the sum of leaf, stem and reproductive material (where present). The coefficient of variation (CV) was used as the measure of trait plasticity. Trait plasticity and fitness were scaled for each species to a mean of 0, and standard deviation of 1. Cases of adaptive plasticity were indicated if regression slopes were positive and differed from 0, maladaptive plasticity was indicated when slopes were negative and differed from 0, and neutral plasticity was inferred when slopes did not significantly differ from 0. To examine the relationship, R-squared values were used.

All analysis was completed within R Studio. Variance partitioning was calculated with an initial fixed effects model using ‘lmer’ in “lme4” (Bates et al. 2015) and then extracted via the ‘icc’ function in “mertools” package. All other mixed effects analysis were assessed
using ‘lmer’ function. Linear models were conducted with base ‘lm’ function. R version 3.5.1 (R Core Team Developers, 2018).

Results

Variance partitioning

Partitioning of variance in trait values revealed that most of the variation in SLA and leaf size was at the species level: explaining 54% and 80% of the total variation respectively, whilst the species term explained a lesser proportion for the leaf to stem mass ratio (just 9%, Figure 2a). Habitat and site within habitat explained 21 and 3% of the variation in SLA and 17 and 0% of the variation in leaf size; however, neither habitat nor site explained detectable components of variation for leaf to stem mass ratio. Among-individual variation (Plant ID) did not explain the variation in SLA or leaf size and explained just 7% of the variation in leaf to stem mass ratio. Lastly, the residual variation, which represents both within-individual plastic variation and other sources of variance accounted for 23 and 3% of the variance in trait values for SLA and leaf size but comprised a surprisingly large component of the variance for leaf to stem mass ratio (84%).

When considering trait plasticity (CV) the term for individual plant ID drops out of the model leaving habitat, species, and residual terms. Here, variance partitioning indicated that differences in plasticity values amongst species explained the largest component of variation in SLA and leaf size (77% and 68%, respectively, Figure 2b). Little evidence of habitat level variation in plasticity was observed for SLA or leaf size and habitat comprised only 2% of variation for leaf to stem mass ratio. Residual variation, which here represents variation in plasticity among individuals of a given species (plus unaccounted sources of variance), was high across all trait: 23% in SLA, 32% in leaf size and 90% in the leaf to stem mass ratio.
Figure 2: Stacked bar chart showing variance partitioning outcomes in a) trait values and b) plasticity therein (CV) for SLA, leaf size and leaf to stem mass ratio. On the left, two hypothetical partitioning outputs are presented for reference: an equal variance model, assuming equal partitioning across levels, and a species-dominated model which proposes that the greatest variation is among species, and less variation is due to habitat and among/within-individual levels. Variance partitioning values (%) are based on a hierarchical mixed-modelling approach, where separate random effects were specified for habitat, sites within habitat, species within sites within habitats, and individuals within species within sites within habitats. Residual variation was attributed to variation within individual, i.e. sampling variation across time, in addition to any measurement errors. Leaf to stem mass data were available only for Australian sites (i.e. one site per habitat type, where growth was measured.)
Table 2: Model outputs for a) trait values and b) trait plasticity (CV) for SLA, leaf size and leaf mass to stem mass ratio. Linear mixed effects models included Habitat and Species nested within Habitat as fixed factors, with individual plant ID considered as a random effect for the trait value analysis.

<table>
<thead>
<tr>
<th>Trait Value</th>
<th>Level</th>
<th>Df</th>
<th>Sum Sq</th>
<th>Mean Sq</th>
<th>F value</th>
<th>Pr (&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLA</td>
<td>Habitat</td>
<td>2</td>
<td>916814</td>
<td>458407</td>
<td>497.14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Habitat:Species</td>
<td>28</td>
<td>3220610</td>
<td>115022</td>
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</tr>
<tr>
<td>Leaf size</td>
<td>Habitat</td>
<td>2</td>
<td>91</td>
<td>45.4</td>
<td>4.96</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>Habitat:Species</td>
<td>25</td>
<td>114032</td>
<td>4561.3</td>
<td>499.82</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Leaf to stem mass ratio</td>
<td>Habitat</td>
<td>2</td>
<td>829.9</td>
<td>414.9</td>
<td>5.32</td>
<td>0.005</td>
</tr>
<tr>
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<td>11</td>
<td>4215.6</td>
<td>383.2</td>
<td>4.92</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Are differences in trait values and plasticity among species statistically significant?

The fixed effects models demonstrated that trait means varied significantly among species and habitat (Figure 3, Table 2). There were significant habitat effects for both SLA and leaf size, with the alpine habitat displaying both a higher average SLA and leaf size compared with the other habitats, but there was considerable spread among species in each habitat (Table 3). Variance in trait values between species was higher within the alpine region for SLA and leaf size (Figure 3), with the coastal region displaying less variation between species with respect to SLA, and the arid region displaying less variation with respect to leaf size. In contrast, leaf to stem mass ratio varied little across habitats, or between species within habitats (Figure 3c; Table 2,3).
Figure 3: Estimates of trait values for a) SLA, b) leaf size and c) leaf to stem mass ratio. Estimates are organized from left to right in order of mean plasticity for a given species nested within its habitat. Species are only presented if trait values were able to be captured across at least three of four key time points.
Table 3: Fixed effects model outcomes comparing trait values in SLA, leaf size and leaf to stem mass ratio across habitats (alpine, coastal and semi arid) along with the species therein. For SLA and leaf size this analysis involved all species, whilst for leaf to stem mass stem ratio it was Australian species only.

<table>
<thead>
<tr>
<th>Habitat and Species</th>
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<th>Leaf Size</th>
<th>Leaf to stem mass ratio</th>
</tr>
</thead>
<tbody>
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Figure 4: Estimates of trait plasticity for a) SLA, b) leaf size and c) leaf to stem mass ratio. Estimates are organized from left to right in order of mean plasticity for a given species nested within its habitat. Species are only presented if trait values were able to be captured across at least three of four key time points.
Table 4: Fixed effects model outcomes comparing trait plasticity (CV) in SLA, leaf size and leaf to stem mass ratio across habitats (alpine, coastal and semi arid) along with the species therein. For SLA and leaf size this analysis involved all species, whilst for leaf to stem mass ratio it was Australian species only.

<table>
<thead>
<tr>
<th>Habitat and Species</th>
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<th>Leaf Size</th>
<th>Leaf to stem mass ratio</th>
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<td>Est.</td>
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<td>0.21</td>
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<td>Centaurea hyssopifolia</td>
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<td>0.24</td>
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<td>Hakea leucoptera</td>
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<td>Helianthemum squamatum</td>
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<td>Thymus zygis</td>
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<td>Triodia scariosa</td>
<td>0.17</td>
<td>0.13</td>
<td>0.20</td>
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</tbody>
</table>
As suggested by the variance partitioning exercise, the fixed effects comparisons indicated significant differences in plasticity among species, particularly among the species from alpine and coastal habitats (Figure 4). Far less variation in SLA and leaf size plasticity was observed for the semi-arid species (Figure 4; Table 4). Plasticity in leaf to stem mass ratio did not significantly differ either as a result of habitat, or species within habitats.

Do trait means correlate with plasticity across species?

To determine whether differences in plasticity might simply reflect differences in mean trait values we examined the correlation between these. For SLA and leaf to stem mass ratio trait there was no significant relationship SLA: \( R = 0.044, p = 0.8 \) and leaf mass ratio: \( R = -0.063, p = 0.83 \) respectively. For leaf size, species with increasing trait mean on average displayed higher rates of plasticity (\( R = 0.49, p = 0.0046 \)) (Figure 5).

Figure 5: Comparisons of trait value means (logged) against trait plasticity for a) SLA, b) leaf area and c) leaf to stem mass ratio. Correlations were conducted using all species with available data for a given trait across a minimum of three out of the four census points.
Does greater plasticity confer greater growth potential?

For the Australian species, when plasticity (CV) was regressed against fitness (biomass increment) at the species level within habitats, we found just 2 out of 32 cases displayed significant slopes (for SLA and leaf size, but in different species). One of these was positive, indicative of a potentially adaptive response, and one was negative, or potentially maladaptive. However, given the multiple comparisons we caution against over-interpreting in these two marginally significant relationships. We do note that overall there were more negative slopes (15/32) than positive, perhaps even indicating selection for homeostasis (Figure 6; Table 5).
Figure 6: Fitness, measured as biomass growth increment, versus plasticity (CV) in a) SLA, b) leaf size (LA), and c) leaf to stem ratio (mass based) across three habitats; alpine, coastal and semi-arid. Both plasticity value and fitness values were scaled to a mean of 0 and standard deviation of 1. Relationships across habitats are displayed for mean fitness per branch harvest in dark grey, with species specific relationships displayed in underlying coloured lines. Adaptive plasticity is indicated by positive relationships between plasticity and fitness, mal-adaptive plasticity as a negative relationship, and neutral relationships exist when slopes are no different from 0. Lines do not indicate significance, but are included to infer direction.
Table 5: Linear regression comparing plasticity in a) SLA, b) leaf size and c) leaf to stem mass ratio with fitness, measured as relative change in total biomass produced (leaf, stem and reproductive material) over a relative growing period. Regressions were conducted for each species separately with slope coefficient (estimate and standard error), significance (p-value) and fit (R²) presented.

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<th>Species</th>
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Discussion

Our study was an ambitious endeavour to assess variation in trait means and plasticity across habitats and species to examine how trait variation is partitioned and whether that variation could be associated with adaptive benefit. Habitat, site, species, and individual explained plant functional trait variation to differing degrees. As expected, species level variation was prominent for mean trait values for SLA and leaf size, but the results for leaf to stem ratio did not follow expectation. The strongest predictor of variation in trait plasticity was also at the species-level. Thus, in addition to varying in trait values, species differ in trait plasticity. We also showed that a large proportion of plasticity was explained by among individual variation, which is critical if natural selection is to act upon plasticity. Our data provide minimal indication that trait plasticity was associated with adaptive outcomes. The discussion that follows draws attention to the challenges of testing ecological and evolutionary theory in natural, realistic field conditions: specifically, how developments in microhabitat measurements, fitness proxies and measures of plasticity could further advance the field.

Variance partitioning of trait means and plasticity

Variance partitioning of trait means revealed trait-specific patterns, a trend consistent with prior work (Albert et al. 2010, Siefert et al. 2015, Ander egg et al. 2018, Bloomfield et al. 2018). For SLA we found 50% of trait variation lay at the among-species level. This result aligns with findings in leaf mass area (LMA, the inverse of SLA) reported by Bloomfield et al. (2018) and to a lesser extent with Fajardo and Siefert (2018); though it is double that in Messier et al. (2010), who found relatively equivalent variance across their six ecological scales. Leaf size had minimal intraspecific variation within our study, with similar findings reported in a meta-analysis by Siefert et al. (2015) examining plant communities; though it is lower than the contribution reported by Fajardo and Siefert (2018).

Few studies have examined variance partitioning in leaf to stem ratios, which had the highest levels of within-individual variation in our study (as illustrated by the residual in Figure 2a and b). This variability is possibly a statistical artefact arising because the leaf
to stem mass ratio incorporates many factors: i.e. the number of leaves, size of the leaves and the relative contribution of supporting stem structures. Alternatively, the variability reflects a biologically interesting point: the pattern of allocation of resources to photosynthetic area versus support structures is highly flexible and poses an opportunity for the expression of complex plastic resource economies (Westoby et al. 2002). Thus, plasticity in leaf to stem mass ratios provides an enticing area for further investigation.

Our work focused upon morphological traits, which putatively demonstrate reduced variation compared to physiological traits (Martin et al. 2017). Morphological traits are constrained by environmental conditions during early development, in combination with underlying genetic variability. In contrast, physiological traits, such as gas exchange, often exhibit higher rates of intraspecific variation as a result of substantial temporal stochasticity in microhabitat conditions, i.e. temperature, light availability etc. and a capacity to adjust over relatively short time scales (Martin et al. 2017, Bloomfield et al. 2018, Fajardo and Siefert 2018). Thus, potentially if we had included in our study physiological measures then the variation apportioned to among and within-individual levels would have been even higher.

Previous studies comparing patterns of variance partitioning across morphological, physiological and chemical traits indicate that growth form may also explain a substantial component of trait variation and that woody species, which dominate our study, are more likely to vary in the extent of within-species variation (Siefert et al. 2015). Although when examined within the context of a broader hierarchy, Anderegg et al. (2018) highlight that for woody species, a large proportion of trait variation is explained at the within and between family level. Even with 36 species, our power to detect differences among growth forms, families, types of traits is necessarily limited.

Nonetheless, our work does present one of the broadest studies to date to take into account plasticity in key functional traits. Furthermore, to our knowledge, this is the first study to explicitly apply variance partitioning approaches to analysing trait plasticity. Our results demonstrate that there is substantial variation in plasticity among species, both within and between sites. Habitat, in contrast, explains little of the observed variation in
trait plasticity. Our results also demonstrate that the scale of variation both within and among individuals is substantial, which has two implications.

Firstly, it reinforces the conventional wisdom that some of the wide spread variation in functional trait values among species within a given community reflects plasticity and will vary across season. Secondly, it demonstrates that there is significant plasticity in core functional traits and that this plasticity may therefore have potential to respond to selection and to have an adaptive component independent of selection on the mean. Of course, to realise that potential the observed differences must be heritable, and associated with a fitness benefit. That we found little explanatory power at the habitat level implies there is no ‘broad brush’ approach to predicting which communities, or species therein, are likely to display plasticity. Therefore, perhaps future efforts could investigate patterns at the species level, i.e., perhaps elements such as plant height, size, or growth form, may be better predictors of plasticity.

**Adaptive plasticity**

We found only the most minimal evidence that plastic responses in SLA, leaf size or leaf to stem mass ratio resulted in increased fitness; in the few cases where this relationship was observed, it was weak. However, given that the overall trend was negative, this may indicate that homeostasis, rather than plasticity, could be beneficial for certain traits, as found for SLA responses to shading by Liu et al. (2016). Other studies also highlight that increased plasticity does not always translate to fitness outcomes (Matesanz et al. 2017). In fact, several meta-analyses found that adaptive responses may occur, or be detectable, in only around 30-50% of cases (Van Buskirk and Steiner 2009, Davidson et al. 2011, Palacio-Lopez and Gianoli 2011, Arnold et al. 2019b). At this stage, it remains unclear whether our data indeed suggest a lack of evidence for adaptive plasticity for the traits assessed in this study, whether our fitness proxy was insufficient to test for such associations, or whether our samples did not explore a sufficient breadth of the actual reaction norm. Considering the challenges of field assessments of plasticity, below we discuss three areas that need to be further developed if we are to obtain good measures of plasticity and have the power to explore its relevance to past and contemporary
evolution: the challenges of microhabitat measurement, measures of plasticity, and the importance of fitness proxies in establishing adaptive responses.

**Real world vs controlled environment settings**

To better elucidate drivers of plasticity and ensure that we have elicited a similar portion of the potential reaction norms of these traits for these species we ideally would have precise documentation of microhabitat variation. Given the global context of our study, spanning three continents, six sites, 36 species, 1080 individuals and two years of data surveys, it was, unfortunately, logistically impossible to record microhabitat data for this large dataset, despite our best efforts. However, we note that recent developments in microhabitat loggers (Wild et al. 2019) and fine scale modelling approaches (Kearney and Maino 2018), make it increasingly possible to map microhabitat variation at the individual scale across multiple sites, species and years; these methods could be useful for future studies.

Quantifying plasticity is critical if we’re to accurately assess species’ persistence capacities. In spite of rapid developments within the field, there is little consensus on the ‘best’ approach or metric for plasticity, especially in complex multi-site, or multi-species investigations, or where repeated trait measures exist. As highlighted within Valladares et al. (2006), a plethora of indices exist. However, most of these indices share common limitations: comparisons between species are often statistically limited, and many of these indices are calculated across two controlled but often arbitrary environments, i.e. high or low water, or ambient and warmed temperatures. Currently, the phenotypic plasticity index (maximum mean – minimum mean / maximum mean) (Valladares et al. 2006), is perhaps the most commonly applied. This metric is useful when comparing two level factorial designs, with high/low contrasts for instance, however, with a study such as ours, it overlooks much of the available data collected by only singling out just the largest and smallest values (Murren et al. 2014, Murren et al. 2015). In a world where non-linear reaction norms, complex factorial designs, and repeated surveys are becoming increasingly utilized, more sophisticated metrics for plasticity will need to be developed, and tested (Arnold et al. 2019a).
For plasticity to be adaptive plasticity it must be associated with increased fitness; however, fitness remains an elusive metric, particularly in long lived species (Younginger et al. 2017). We aimed to capture variation in fitness by using a growth increment at the branch level. With 36 species, six sites, and a global distribution, capturing standard proxies, such as seed set, flowering, fruiting, and even biomass outputs presented a logistical challenge. But perhaps in long-lived species like ours this measure simply didn’t capture the longer-term context. Something closer to a lifetime perspective might be captured with longer term fitness outputs, i.e. fecundity in a single or multiple seasons.

Our challenge is not unique; a recent review by Younginger et al. (2017) found that for 150 plant species examined, most papers focused upon fitness estimates for herbs or annuals, with only 7% of studies examining trees, and 6% shrubs, - both growth forms that dominated our study habitats, and, by in large, represent the greatest biomass, and carbon storage in terrestrial plants. Further, of studies comparing biomass estimates of fitness to actual fecundity measures, 30% found a positive correlation, whilst 24% found insignificant or neutral relationships. Thus, the utility of measures such as biomass or size as universal fitness proxies is ambiguous. Given additional complexity may be introduced by factors such as plant life span, asexual and sexual reproductive strategies, large pollen and seed dispersal distances, and the often hermaphroditic breeding systems of plants (Younginger et al. 2017), we suggest that a crucial next step in functional or trait-based ecology, is to establish a set of standardized protocols for establishing if, and when, fitness proxies actually correlate to fecundity, and the success of the next generation.

**Conclusion**

Our study is among the first to quantify the relative importance of within species and within individual trait variation and phenotypic plasticity using a hierarchical approach, on a global scale spanning multiple habitats, sites, species and individuals through time. Furthermore, we not only investigated trait variation, but also explored whether this plasticity correlated with fitness outcomes; an essential step if we’re to begin incorporating intraspecific variation or plasticity in dynamic vegetation models. We established that within-species variation for plasticity in key plant functional traits, can be substantial. While our study indicates the potential for selection on plasticity is weak
within these species, it also highlights the challenge of assessing both fitness and plasticity. To further this work, and determine what the evolutionary significance of this substantial variation in plasticity within and among species is, requires big advances in our capacity to quantify plasticity, characterize the environment under realistic conditions, and to assess fitness proxies in a reliable way. Do our results indicate that plasticity is not adaptive? Possibly, but having substantiated our claim that both among and within species variation in plasticity is measurable, we now must turn to the question of why such variation is present, and whether we have the power to detect its potential adaptive value.
Acknowledgements

The authors would like to acknowledge the immense work of a large group of researchers, in particular; Abigail Widdup, Tobias Hayashi, Nicholas Wilson, Timo Scheu, Marta Rodríguez Pérez, along with the many volunteers for assistance in the field and lab. Thanks also to Alyssa Weinstein for helpful comments on the manuscript. This work would not be possible without the assistance of Australian Research Council Discovery Grant (DP120100945) to Adrienne Nicotra, Fernando Valladares, Mark van Kleunen and Christina Richards, and an Australian Government Research Training Scheme Scholarship to Sonya Geange.
References


Synthesis

The relevance of microhabitat, fitness proxies and adaptive plasticity in enhancing our understanding of plant responses to climate change
Rapid rates of environmental change are necessitating the evaluation of species’ capacities to persist. Phenotypic plasticity provides a potential buffer to climate change impact and also may affect evolutionary response, but plasticity is difficult to quantify and responses may vary across drivers, habitats, species and even populations within species. Since capturing individual-level measures of plasticity across such scales is unrealistic, it is critical to understand the levels at which phenotypic plasticity varies, and the significance of this plasticity in terms of plant performance under new conditions. Further, careful consideration is needed about how best to capture this information to improve the predictive potential of vegetative models.

Within this thesis I examined two main research themes: within-species variation and phenotypic plasticity (within-individual variation). Specifically, my research aimed to: 1. identify whether there are predictable patterns of plastic responses between or within species; 2. assess whether functional traits differ in plasticity and adaptive value thereof; and 3. determine whether one can assess plastic responses and their adaptive nature in the field under natural, uncontrolled conditions. These concepts were investigated using glasshouse experiments, field transplants and manipulations, and large scale, multi-habitat and multi-species field work.

I show that within the Australian alpine plant community, species’ responses to reduced water availability (Chapter Two, hereafter Geange et al. 2017) and altered temperature regimes (Chapter Three, hereafter Geange et al. in review), are likely to substantially impact upon species’ persistence, in what is an already vulnerable ecosystem. Furthermore, in both studies, little evidence of within-species variation was found, indicating resilience to changing climatic conditions is unlikely to vary throughout the study species’ range.

Looking more broadly, I conducted a comparative ecological study on phenotypic plasticity using a multi-site, multi-species design spanning both Australian and overseas
sites (Chapter 4, hereafter Geange et al in prep). I found between species level variation explained the majority of trait value differences, and surprisingly also trait plasticity. Also noteworthy was the relatively high degree to which plasticity varied among individuals, especially as within-individual variation is less often explicitly examined as a factor of interest, despite being the level at which selection acts. Despite the high level of within-individual variation, the data provide little, if any, evidence for adaptive plasticity within these species.

In considering the results of the experiments in my thesis together, four themes emerged. First, that the relative merits of lab and field work still deserve careful consideration. Second, that there is a need to better address how time and space – the duration and scale of environmental characterization – may influence interpretations of plasticity. Third, these experiments also demonstrated the immensely challenging nature of quantifying plasticity and fitness, and finally, in the process of considering the above, they led me to think deeply about the new tools and approaches available that will allow us to better tackle these problems. I expand on these below.

1. From the lab to the field and beyond

From the perspective of predicting the impact of climate change and understanding the role of plasticity therein, we require methods that allow us to assess phenotypic plasticity across multiple species with varying life histories – in the field (Merila and Hendry 2014, Parmesan and Hanley 2015). Glasshouse studies and field manipulations have played an important role in improving our understanding of the complex mechanisms underlying plasticity (Bell and Lechowicz 1991, Sultan and Bazzaz 1993). However, there are complex trade-offs in trait responses that result from the plethora of interactions between abiotic and biotic factors in the real-world (Tilman 1989, Sultan 2000). These complexities mean it is difficult to estimate the importance of plastic responses measured in controlled environments on ecological and evolutionary processes in the field (Poorter et al. 2016, Xu et al. 2019).

While field assessments provide the most realistic measures of plasticity, they present multiple logistical complexities (Korner 2018). It is interesting to note that there is an apparent decline in fieldwork based science, relative to uptake of modelling and data
synthesis approaches (Ríos-Saldaña et al. 2018). Arguably, any effort in data modelling or synthesis is reliant upon ground-truthed data, and theories developed from work in the field. Similarly, model predictions need to be validated in the field (Augusiak et al. 2014). The disparity between the goals of academia and conservation may be the cause of the decline in field-based projects (Knight et al. 2008, Ríos-Saldaña et al. 2018). Irrespective of the underlying cause, the ecological and evolutionary insights that can be generated through field approaches are critical to assessing plastic responses of species to natural conditions (Anderson 2017). We risk creating assessments that don’t fully capture the complexity of real-world conditions in predicting plant responses to climate change, and this was one of the major motivations for my work.

2. The concepts of time and space within plasticity research

Time frames of studies

Regardless of whether lab, field manipulation, or observational approaches are taken, experimental designs for plasticity studies need to consider the timescales over which underlying ecological processes occur. Short-term responses (e.g. of one life stage) are not always reliable predictors of long-term responses, and such discrepancies hamper our capacity to accurately predict species’ responses over generations (Hollister et al. 2005, Saccone and Virtanen 2016). For example, in Geange et al. (in review, Chapter Three), we show that despite high initial mortality rates in seedlings under open top chambers, surviving seedlings actually performed well in the long term. Had the experiment been truncated earlier on, the positive impacts of open top chambers upon growth would have been missed, and the study conclusions very different.

Another key consideration, particularly for field manipulations and observational work, is that time-frames that extend over more than one season or encompass the lifetime of the organism allow for the possibility of detecting asynchrony in species’ responses to climate drivers (Parmesan and Hanley 2015, Alexander et al. 2016). Variation in a species capacity to respond, or to display plastic responses, may result in asynchronous rates of migration, phenology and developmental, and thus influence community composition (Alexander et al. 2015, Alexander et al. 2016, Alexander et al. 2018). By encapsulating all-of-life timeframes, plasticity studies can also better assess how the impact of climatic
variability varies across developmental stages. This approach also allows for plasticity to be considered in the context of tissue or organ-level duration, i.e. an economic return on investment (Korner 2018). Furthermore, well-structured studies of this nature would allow for the integration of phenotypic plasticity of demographic parameters to be included in demographic models. This is a critical element when considering trees and other woody perennials, which could ultimately function as phytometers (measures of ecological change) through the use of dendrochronology (Dietrich et al. 2013)

Environmental Characterization

The ability to interpret trait responses is often dependent upon a capacity to identify or manipulate several key components of an organism’s environment. This is relatively straightforward in a controlled environment, tractable but not simple in a field manipulation, and extremely challenging under natural field conditions. To quantify response relative to environment a researcher must: 1) correctly identify the major environmental or biotic drivers within the system and then measure or manipulate these, 2) measure or manipulate these drivers in such a manner that they reflect natural variations, and ideally threshold points at which the environment elicits a response. Or, 3) in the case of an uncontrolled field studies if it is not possible to document environmental variation directly at an appropriate scale then high-resolution modelling approaches may provide the best opportunity to represent variation in environmental conditions.

Characterizing environmental conditions in the field in order to provide insight into specific plant responses has long been a recognized challenge. The need for multiple sensors at high density leads to logistic constraints, thus there is need for an approach that makes nuanced measures attainable. Issues common to field studies include: a need for more accurate and localized recording of environmental data and also sampling at high spatial and temporal resolution. Better indices of localized temperature and soil moisture regimes, for example, would allow for more direct assessments of how environmental variables may drive systems, and allow for the identification of critical environmental thresholds. In Geange et al (in review), leaf temperature in addition to air temperatures could have been measured to provide a more direct indicator of the
thermal stresses to which seedlings were exposed (Leigh et al. 2012, Körner and Hiltbrunner 2018), and an increased sampling rate would allow identification of how the number of hot or cold events may have acted as critical thermal thresholds for mortality (IPCC 2014, Ummenhofer and Meehl 2017). Finally, reporting absolute timings and frequencies of events may over- or underestimate the relative importance of environmental drivers. A shift towards reporting relative frequencies and durations, i.e. against an organisms lifespan, may address these concerns (Korner 2018).

Often, correlations between trait values and environmental conditions are examined in the context of historical and annual means. Such coarse-scale measures derived from weather station data can be used to differentiate habitat level variation, but they are unlikely to provide sufficient understanding of localized microhabitats (Scherrer et al. 2011, Graae et al. 2012, Körner and Hiltbrunner 2018). Microhabitat variation is likely to be incredibly important in the context of climate change responses, as it elicits plasticity or local adaptation processes, or both (Opedal et al. 2015, Graae et al. 2018). Microhabitat variation can also provide micro-refugia (Suggitt et al. 2011, De Frenne et al. 2013, Lenoir et al. 2017) in which individuals are not pushed beyond tipping points. Characterizing individual microhabitat variation, through ranking individuals relative to conspecifics both at a given time point, along with at species, or site levels, allows for a more thorough means of evaluating plastic responses, and linking them to underlying drivers. In Geange et al. (in prep, Chapter Four), we sampled soil moisture conditions 3-4 times per year but these finite snapshots of environmental variability were ineffectual in reflecting conditions underlying plant development or even characterizing the relative availability of soil moisture at a given site over the course of the year. But to deploy permanent loggers at a sufficient spatial scale to accurately characterize the environment is prohibitively expensive. Advances in technology and lowered costs of high resolution microclimate loggers may provide a solution, allowing for increased temporal and spatial resolution of environmental variation to be captured (Wild et al. 2019). To supplement direct environmental monitoring, particularly in cases where data loggers may still be impractical, i.e. tough terrain, extensive areas for deployment and/or prohibitive costs relative to scale, rapidly developing modelling approaches may provide essential insights.
High resolution spatial models are becoming increasingly available. Resources may range from global in scale e.g. WorldClim2 (Fick and Hijmans 2017), to continental based, e.g. Australian fine scale moisture models by Kearney and Maino (2018), or localized to a specific ecosystem, such as the Australian alps (Harris et al. 2016). Localized resources are particularly important within topographically complex habitats, where traditional weather station data poorly reflects climate conditions even short distances away (Vitasse et al. 2017). As technology advances, tools such as satellite photos (Moore et al. 2016), deployable phenocams (Brown et al. 2016) and gigapixel cameras (Flynn et al. 2016), will make high resolution data acquisition and modelling of plastic responses to changes in underlying environmental conditions much easier (Boyle et al. 2014, Pettorelli et al. 2014, Pasquarella et al. 2016).

3. The challenges of quantifying measures of plasticity and fitness

Phenotypic Plasticity Indices

There is little consensus on the ‘best’ approach or metric for assessing plasticity. Indeed, since the review of Valladares et al. (2006), in which a plethora of indices were highlighted, core issues still remain unsolved. In particular, comparisons between species are often limited, and additionally that many indices are constrained to two environmental conditions. For example, metrics such as the phenotypic plasticity index (maximum mean – minimum mean/ maximum mean) (Valladares et al. 2006) dominate the literature, especially in cases where two-level contrasts are employed, i.e. high and low water availability, or ambient and warmed temperatures (Gugger et al. 2015, Nicotra et al. 2015b, Hamann et al. 2016, Li et al. 2016, Chmura et al. 2017, Geange et al. 2017, Matesanz et al. 2017, Munzbergova et al. 2017). However, when considering complex study designs such as presented within Geange et al. (in prep, Chapter Four) where repeated measures are taken, metrics such as this waste much of the available data because they only incorporate two values. In contrast, the coefficient of variation, as a unit-less measure, not only provides a platform with which different traits and species can be compared (Ruprecht et al. 2013, Malyshev et al. 2016, Christiane et al. 2018), but also enables all available data points to be utilized (Valladares et al. 2006).
With an increased awareness of how microhabitat variation may influence phenotypic responses, it is clear we need to move away from traditional factorial designs with high/low environmental contrasts (Via et al. 1995). Given that researchers rarely justify their choice of contrasts (e.g. the specific water or light levels imposed), employing approaches that span broader environmental conditions – current and likely future – has merit. The development and use of non-linear analyses and random-regression mixed-effects models are also useful for experimental designs where plasticity is examined along environmental gradients and is potentially non-linear (Murren et al. 2014, Arnold et al. 2019a). These approaches are discussed further below.

*Adaptive Plasticity & Fitness*

To answer fundamental questions in climate change ecology, we need to not only understand how key traits respond to environmental change, but also to understand whether trait changes are correlated with fitness outcomes (Merila and Hendry 2014). Specifically, fitness outcomes not only at the individual level, but through to success at population, species and communities levels as well, i.e. continued persistence (McLean et al. 2016). Despite the often-held assumption that plastic responses result in increased adaptive outcomes, few studies actually take the additional step of evaluating fitness, and those that do find mixed support (Geange et al. 2017, Chapter Two, Matesanz et al. 2017) or indicate that whilst they are adaptive, the rate of change may be too slow to keep up with climate change (Franks et al. 2014). To date, several meta-analyses indicate that adaptive responses are detectable in 20-50% of cases (Van Buskirk and Steiner 2009, Davidson et al. 2011, Palacio-Lopez and Gianoli 2011, Arnold et al. 2019b). We do not yet understand whether the relative paucity of evidence for adaptive plasticity reflects that plasticity in certain plant functional traits is not generally adaptive, or whether it reflects that we haven’t quite developed the right method to assess it. In addition to the questions of how to best estimate plasticity, it is critical that researchers quantify fitness effectively.

It is not until plastic responses are examined in the light of fitness outcomes that management and conservation actions can be employed. Defining a fitness outcome however is not without challenges. As highlighted by Hendry et al. (2018), fitness is a
rather vaguely defined construct, most simply construed as the ‘success’ of a biological unit. Thus far, a metric that allows for equivalency in comparison across diverse life forms, especially in the context of time, is elusive. Lifetime reproductive output and survival are common fitness proxies, (see references in Anderson (2016)); however, these responses may only be suitable for species with short life spans and/or rapid reproductive rates. Survival has the additional complexity that differences in rates of survival at early vs. late life stages may be substantial, which may confound conclusions if treated equivalently (Hendry et al. 2018). Furthermore, as highlighted earlier, the duration of many ecological studies is short in comparison to the focal organisms’ lifespan. As such, other oft-used proxies, such as biomass or growth estimates, may be inadequate to represent lifetime fitness (Younginger et al. 2017). As highlighted in Geange et al. (in prep, Chapter Four), comparisons using biomass growth estimates are difficult as investments into photosynthetic tissue, supporting structure or reproductive material are subject to ontogeny and resource availability, vary across an individual plant, and differences in patterns of growth among different growth forms make it difficult to design a widely applicable metric of growth (Westoby et al. 2002). Prior meta-analysis and systematic reviews have considered short term fitness consequences in the field (Anderson 2016), or focussed exclusively on biomass as a proxy (Younginger et al. 2017), but a comparison of fitness metrics across glasshouse, field manipulation and observation studies may be useful.

One last element critical to assessing adaptive plasticity, is moving from a modular, or organism level assessment, to predictions of population and species success, especially if outcomes are to be used in conservation practice (McLean et al. 2016, Shipley et al. 2016). This element is intriguing, as much like the assumptions around plasticity being adaptive, it cannot be assumed that population- or species-level success can be predicted by lower order data such as demographic rates or phenotypic trait expression. The relative strength of relationships between individual traits and fitness is not well documented, particularly under complex, real world conditions (Shipley et al. 2016). Empirical support for these relationships is required as there is an inherent assumption that chosen study attributes, such as specific plant functional traits or environmental gradients, are reflective of key drivers of fitness outcomes, and thus in the longer term,
community assembly (Read et al. 2017, Korner 2018). In reality, the choice of trait or driver may in fact reflect relative ease of measurement, and a given correlation may not be reflective of a single trait response, but rather a trait complex (Korner 2018). To this end, structural equation models are a tool that allow for the influence of indirect and direct processes to be compared against fitness outcomes (Grace et al. 2010, Lefcheck 2016, McLean et al. 2016). To date, such approaches tend to be found within animal literature, where a greater emphasis upon collecting demographic data, and long term data-sets is found (Scheiner and Callahan 1999). However, increasing capacity for large scale data manipulations and analyses, along with the development of global databases (Kattge et al. 2011a, Bruelheide et al. 2019), provides the potential for multi-faceted data analysis such as structural equation models to become more commonplace.

4. New approaches for assessing and applying plasticity in a climate change context

Gaining critical insights into the ecological and evolutionary role of intraspecific variation and phenotypic plasticity in climate change responses requires new analytical approaches and an element of generalization. This acknowledgement that generalisations must be safely made, along with an increased emphasis on evidence-based policy and management practices, has resulted in a rapid development of open-source code for statistical analyses and uptake of large-scale data-synthesis tools, such as systematic reviews and meta-analyses (Stewart 2010, Lortie 2014, Nakagawa et al. 2019). In addition, a variety of approaches to assessing the adaptive capacity of species are being explored, including the application of Expert Knowledge elicitation exercises to parameterise models and direct research priorities.

New statistics for plasticity research

Above I highlighted the importance of incorporating broader assessments of the reaction norms of trait responses and non-linear responses in particular. The uptake of these approaches will be enhanced by the rapid development of open access statistical packages, and well-illustrated tutorials. For example, given the complexity in experimental design required to capture phenotypic plasticity, especially across hierarchical levels, automated tools such as the R package for power analysis “SIMR” by Green and MacLeod (2016), or the R Shiny App “SQuID” (Statistical Quantification of
Individual Differences) by Allegue et al. (2017) may ensure sampling is sufficiently rigorous to enable quantification or plasticity, without incurring resource limitations. As gradients of exposure are being examined rather than discrete treatments, non-linear assessments of plasticity will become more common (Via et al. 1995, Murren et al. 2014). In such instances, random regression models are touted as the way forward, as highlighted by Araya-Ajoy et al. (2015) and their R package “MultiRR”, and the recent guide by Arnold et al. (2019a).

Data Synthesis Approaches

If intraspecific variation and/or phenotypic plasticity are to be more readily incorporated in species distribution models or Dynamic Global Vegetation Models, there needs to be a concerted effort into developing data repositories sufficiently capable of storing variable data types (Albert et al. 2011, Moran et al. 2016). For example, trait value data that varies among populations or even across seasons for individuals of a given population. We know trait data is predominantly collected at the individual level, yet most repositories still maintain amalgamated averages for species of functional types (Kattge et al. 2011a, Kattge et al. 2011b). It is therefore encouraging to see new approaches to data storage being developed, for examples see Kotta et al. (2019) and Benito Garzon et al. (2019) which attempts to explain species distribution ranges based on phenotypic plasticity and local adaptation of fitness related traits. Once such resources are established, opportunities are opened for data-synthesis techniques such as systematic reviews and meta-analyses to be used, to highlight knowledge gaps and seek generalisations and theoretical frameworks (Nakagawa et al. 2019).

Systematic reviews may be used to surmise the current research landscape, highlight knowledge gaps within the current literature, and identify the extent to which certain hypotheses, methodological approaches or study systems have been explored (Lortie et al. 2004, Lortie 2014). For example, I am currently conducting a systematic review (Geange et al. (in prep), see appendix) to assesses whether techniques for assessing how thermal tolerance of land plants (and the plasticity therein) varies across ecological and agricultural systems, biomes, and life forms. The review demonstrates that research approaches within the field are highly siloed, and that it is unclear whether or how
generalities could be achieved. It also highlights how important it is to establish cross-disciplinary bridges to assess thermal tolerance and plasticity therein with maximal efficiency and effect in the face of rapid climate change. Secondly, the review emphasizes that standardized assessments of thermal tolerance and the effect of growth, treatment and measurement conditions are critically needed, for data to be useful in comparative evaluations of the plasticity of thermal tolerance (e.g. such as meta-analyses).

Meta-analyses allow researchers to statistically explore how the size of difference, or strength of a relationship, relative to its variability, compares amongst traits, species, or biomes (Stewart 2010, Gurevitch and Nakagawa 2015, Nakagawa et al. 2015). Over the last few years a plethora of new meta-analysis toolboxes have appeared, including open source R packages such as ‘Metagear’ (Lajeunesse 2016) and ‘OpenMEE’ (Wallace et al. 2017). Of particular note, these tools are increasingly recognising that data synthesis will be more-often-than-not a collaborative effort as expert knowledge is drawn in from a diverse range of fields and backgrounds. For instance, in Geange et al. (in prep, appendix), collaborator knowledge of thermal plasticity required incorporating perspectives from ecology and agriculture disciplines, and experience from alpine to arid biomes.

An important caveat across each of these approaches, is that all data syntheses are only as informative and reliable as the information contained within the literature and databases that support them (Parker et al. 2016). Meta-analyses of plasticity research are additionally challenging because the lack of standardised assays (as highlighted above for thermal tolerance), and fitness metrics, make comparisons across studies difficult. Progress in this area can be accomplished through establishing global research networks with clearly outlined methodological practices and reporting structures. Many such networks already exist with e.g. International Tundra Experiment (ITEX) (Henry and Molau 1997), rainfall exclusion experiments (droughtNet) (Smith et al. 2016), the Global Observation Research Initiative in Alpine Environments (GLORIA) (Pauli et al. 2005), and the Tundra Trait Team (TTT) (Bjorkman et al. 2018). Increasing the focus on intraspecific trait variation within these existing project, or establishing new ones will be immensely useful going forward. Finally, increased requirements around data transparency in extracting values for comparison (e.g. across environmental treatments), and article-level
metadata, is helping mitigate concerns around data comparisons (Parker et al. 2016, Westgate et al. 2018).

**Adaptive Capacity Frameworks**

Our ability to address knowledge gaps in climate change responses is time critical. Traditional experimental approaches to investigating climate change responses, including assessments of phenotypic plasticity, may be prohibitively slow and costly especially when applied at community scales. Thus, we need to supplement these classical research approaches with alternative tools such as species vulnerability assessments or frameworks (Foden et al. 2018). Frameworks, such as employed by NatureServ’s Climate Change Vulnerability Assessments (CCVAs) (Young et al. 2012), the IUCN red list for species (IUCN 2012) and similarly ecosystems (Bland et al. 2017), evaluate species susceptibility to climate change drivers. In many of these assessments, species’ capacities to persist are evaluated across three factors: exposure, vulnerability and adaptive capacity. In particular, the element of adaptive capacity, which reflects the intrinsic capacity of a species to cope or adjust to climate change, remains elusive, possibly due to confusion around what exactly it is, and how to assess it (Beever et al. 2015, Nicotra et al. 2015a). Adaptive capacity is generally accepted to be a function of life-history traits, genetic diversity and phenotypic plasticity (Nicotra et al. 2015a).

To address the confusion surrounding assessing adaptive capacity, in Thurman et al. (in prep, Appendix), colleagues and I have developed an attribute based framework to provide guidance on how to evaluate a species’ adaptive capacity across a suite of species ranging in life histories. The creation of this tool should improve critical evaluations of species’ responses to climate change, through providing a clarified pathway for incorporating adaptive capacity into existing management practises. This is especially pertinent, because adaptive capacity is currently alluded to in principal in many vulnerability assessments, but seldom actually incorporated in any meaningful way (Thompson et al. 2015). Briefly, in Thurman et al. (in prep, Appendix) we argue that the capacity of an organism to move through a landscape (referred to as Shift in Space (SiS)), is characterized by ‘distribution’ and ‘movement’ complexes. The ability of an organism to withstand changing climates *in situ* (referred to as Persist in Place – PiP), is
characterized by ‘evolutionary potential’ and ‘ecological role’ of organisms. These attributes, along with their ‘abiotic limits’, can be used to inform both ends of the PiP/SiS spectrum. Within many of these attributes, understanding the scope for plastic response is clearly highly important. For each attribute, species can be evaluated on a simple “low-moderate-high” scale. Scales are either based on well-established climate change vulnerability assessments (e.g., NatureServe’s CCVI; Young et al. 2012) and IUCN Red List criteria (IUCN 2012), or are derived from previous findings about characteristic relationships to adaptive capacity. To reflect the role that within-individual and within-species variation may play, our framework also allows for comparison of populations within a species. Thus, the framework is applicable for a range of conservation practices, from continental scale threat assessments to local park management.

**Expert Elicitation Pathways**

It is becoming increasingly apparent that evaluating and incorporating adaptive capacity into management plans and actions can’t always wait for the relevant data to be published in the literature. For threatened species or communities, the rapid rate of environmental change necessitates alternative approaches, such as expert elicitation. Expert opinion is often sought in scenarios where risks are imminent, data is deficient, and resources are scarce. In spite of this, the process by which expert estimates and uncertainties are quantified is rarely subjected to testing or verification, and so the uptake of expert elicitation within ecological fields has been relatively slow to date. The development of structured expert elicitation processes is helping to change this by: asking questions with clear operational meanings; establishing transparent methodological rules; anticipating psychological and motivational biases and mitigating these where possible; and most importantly, enabling the potential for empirical evaluation and validation (Hanea et al. 2016, Hemming et al. 2018). To this end, in Nicotra et al. (in prep), we address fundamental questions about the quantification of adaptive capacity (including potential for phenotypic plasticity) of an organism and its functional importance (the relative contribution of an organism within a system), with direct practical application to climate-ready management of the vegetation communities within Australia’s iconic high mountain regions. This is further supplemented by a parallel project by Umbers et al. (in prep), where we examined adaptive capacity and functional
importance within Australia’s alpine animals and lead naturally to empirical tests, for example of whether estimates of adaptive capacity correlate with breadth of thermal tolerance and extent of adaptive plasticity.

Concluding Remarks

In summary, future assessments of phenotypic plasticity should consider the relative merits of lab and field work. Is the goal a greater understanding of the mechanistic underpinnings, or is it to quantify how prevalent adaptive plastic responses are under complex, real-world conditions, and will be under novel climate regimes? Irrespective of the approach taken, there is much potential to address how time and space – the duration and scale of environmental characterization – may influence our interpretations of plasticity. Rapid developments in low-cost, reliable microclimate loggers, and high-resolution modelling tools will greatly enhance our capacity to directly correlate environmental drivers with phenotypic plasticity. In particular, these developments will allow assessments of phenotypic plasticity to be extended to better reflect our knowledge of micro-refugia and allow for incorporation of non-linear reaction norms. However, an understanding of the true role played by phenotypic plasticity in enabling species’ persistence in a changing climate will only come about if we make a concerted effort to determine whether or when plastic responses are indeed adaptive, and this requires critiquing our current estimates of fitness. Finally, whilst our theoretical understanding of intraspecific variation and phenotypic plasticity is increasing rapidly, our capacity to translate this knowledge to management and conservation actions is lagging behind. Future research endeavours should work towards breaching this divide, and given the often rapid rates of change, should incorporate synthesis and other emerging approaches as the opportunity arises.
References


Persist in place or shift in space: evaluating the adaptive capacity of species to a changing climate.

Authors: Lindsey Thurman\textsuperscript{1,2}, Bruce Stein\textsuperscript{2,3}, Erik Beever\textsuperscript{1,2,4}, Wendy Foden \textsuperscript{2,5}, Sonya Geange\textsuperscript{6}, Nancy Green\textsuperscript{7}, John Gross\textsuperscript{8}, David Lawrence\textsuperscript{8}, Olivia LeDee\textsuperscript{9}, Laura Thompson\textsuperscript{11}, and Bruce Young\textsuperscript{2,12}.

\textsuperscript{1} U.S. Geological Survey, Northern Rocky Mountain Science Center, Bozeman, MT, USA;
\textsuperscript{2} International Union for Conservation of Nature, Species Survival Commission, Climate Change Specialist Group, Gland, Switzerland;
\textsuperscript{3} National Wildlife Federation, Washington, DC, USA;
\textsuperscript{4} Montana State University, Department of Ecology, Bozeman, MT, USA;
\textsuperscript{5} University of Stellenbosch, Department of Botany & Zoology, Matieland, South Africa;
\textsuperscript{6} Australian National University, Research School of Biology, Canberra, Australia;
\textsuperscript{7} U.S. Fish and Wildlife Service, Ecological Services Program, Washington, D.C., USA
\textsuperscript{8} National Park Service, Climate Change Response Program, Fort Collins, CO, USA;
\textsuperscript{9} U.S. Geological Survey, Northeast Climate Adaptation Science Center, St. Paul, MN, USA;
\textsuperscript{10} University of Washington, School of Aquatic & Fishery Sciences, Seattle, WA, USA;
\textsuperscript{11} U.S. Geological Survey, National Climate Adaptation Science Center, Reston, VA, USA;
\textsuperscript{12} NatureServe, Arlington, VA, USA
Abstract

Species vulnerability assessments – typically evaluating the factors of exposure, sensitivity, and adaptive capacity – can inform both adaptation planning and climate-smart conservation. Adaptive capacity (AC) is a species’ ability to cope with or adjust to changing climatic conditions and is the least understood and implemented of these components. Evaluations of AC have been inconsistent and underutilized. We propose an attribute-based framework and guidance for evaluating species’ AC, and in doing so identify two general classes of AC responses: “persist in place” and “shift in space”. “Persist in place” attributes enable species to survive and reproduce in situ. “Shift in space” emphasizes attributes that allow tracking of suitable bioclimatic conditions. We provide guidance for assessing AC attributes and demonstrate applications for suites of species with similar life histories. Results illustrate the broad applicability of this first generalized framework for assessing AC and reveal opportunities and challenges to conserving species amidst a changing climate.
Plant thermal tolerance:

A global synthesis for future research

Authors: Sonya Geange\textsuperscript{1}, Pieter Arnold\textsuperscript{1}, Alexandra Catling\textsuperscript{1}, Onoroide Coast\textsuperscript{1}, Alicia Cook\textsuperscript{2}, Kelli Gowland\textsuperscript{1}, Andrea Leigh\textsuperscript{2}, Rocco Notarnicola\textsuperscript{1}, Brad Posch\textsuperscript{1}, Susanna Venn\textsuperscript{3}, Lingling Zhu\textsuperscript{1}, and Adrienne Nicotra\textsuperscript{1}.

\textsuperscript{1}Australian National University, Research School of Biology, Canberra, Australia
\textsuperscript{2}University of Technology Sydney, School of Life Sciences, Sydney, Australia
\textsuperscript{3}Deakin University, School of Life and Environmental Sciences, Melbourne, Australia
Abstract

Researchers seek to understand patterns of thermal tolerance of plant species in order to breed crops for a growing population, to predict responses of native species to a changing climate, and to gain fundamental insight into evolutionary and ecological processes. Heat and cold tolerance are of interest, particularly in those areas where plants may experience both these extremes. But after over 100 years of plant thermal tolerance research there are remarkable gaps in our knowledge. We conducted a systematic review to describe the range of research techniques used and their distribution across systems, biomes and life forms. The vast majority of thermal tolerance studies focus on frost tolerance of agricultural species and only a trivial percentage considered both heat and cold tolerance. Our understanding of heat tolerance is rudimentary; worrying in a warmer world with increasingly frequent heat waves. In terms of experimental approach we show that many studies take advantage of chance events and post hoc interpretation, rather than testing the impacts of exposure to defined temperature events a priori. While valuable, these opportunistic studies do not lend themselves to determination of critical temperatures or to comparison across studies because the methods are not standardised. Indeed, only a relatively small portion of studies report a thermal metric that can be compared across work. We advocate for increased research attention on heat tolerance, adoption of standardised methods, and further consideration of the underlying mechanisms of thermal response.
Appendix 3
Assessing adaptive capacity in the Australian Alps:
An expert elicitation process

Authors: Adrienne Nicotra¹, Sonya Geange¹, Anca Hanea², Keith McDougall³, Ary Hoffmann², Susanna Venn⁴, James Camac², Peter Vesk², Emma Burns¹, Michael Doherty¹, Louise Gilfedder⁵, Lydia Guja⁶, Geoffrey Hope¹, David Keith³, Sandra Lavorel⁸, Joe McAulliffe⁶, Scott Mooney³, James Shannon⁹, Mel Shroder³, Rachel Slatyer¹, Elaine Thomas¹⁰, Kate Umbers¹¹, Neville Walsh¹², Jennie Whinham¹³, Genevieve Wright³, and John Morgan⁹.

1. Australian National University, Canberra, Australia
2. University of Melbourne, Melbourne, Australia
3. NSW Office of Environment and Heritage, Sydney, Australia
4. Deakin University, Melbourne, Australia
5. Tasmania Parks, Tasmania, Australia
6. Australian National Botanic Gardens, Canberra, Australia
7. University of Western Sydney, Sydney, Australia
8. Laboratoire d’Ecologie Alpine, France
9. LaTrobe University, Melbourne, Australia
10. Victoria Parks, Melbourne, Australia
11. Western Sydney – Hawksbury Institute for the Environment, Sydney, Australia
12. Melbourne Botanic Garden, Melbourne, Australia
13. University of Tasmania, Hobart, Tasmania
Abstract

Increasingly conservation and management practitioners are required to make decisions about allocation of resources based on vulnerability assessments that incorporate exposure risk and adaptive capacity of species. But there is little agreement on how to quantify that capacity efficiently or rigorously. Further, resource allocation decisions cannot be based on adaptive capacity alone; the relative importance of the organism to ecosystem function must also be considered for conservation resources to be effectively allocated. Species with high functional importance and low adaptive capacity require urgent management attention, whereas those with both low adaptive capacity and functional importance most likely not. But where is the science to enable such decisions to be made objectively? Expert opinion is emerging as a way to augment empirical resources in a time of rapid change. We applied the IDEA framework which allows for rigorous, structured assessment of expert reliability, and imposes clear protocols for elicitation of information. We will report on the outcomes of a working group involving expert botanists, ecologists and land managers, where we assessed the application for expert elicitation to predict the adaptive capacity of plant species within Australia’s critically threatened alpine and mountain biomes over the next 25 - 50 years.

Preliminary analysis suggests experts have a high level of certainty around predicting community level shifts, and some species level shifts. For example, species within snow-patch communities were predicted to decline in cover, and woodlands to increase. However, predictions for species from other biomes were more variable. With respect to functional importance, there were some species of considerable functional importance for which experts predicted little adaptive capacity, and some of low functional importance for which high adaptive capacity was noted. Interestingly, there are some species of significant functional importance about which experts agreed the trajectory was highly uncertain. Continued investigations will focus upon how predictions of adaptive capacity reflect hypothesis around plant traits, population dynamics and environmental change. The use of robust expert elicitation processes not only provide insights into where experts agree on likely impacts of climate change upon species performance, but also provide direction for where research priorities should be focused.
This may be of particular importance within biomes and communities where trait collection is both difficult and time consuming, and the relative risk of inaction is high.