Gas exchange in *Ranunculus* species and the evolution of alpine herbaceous plant physiology

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Abstract

Buttercup species (*Ranunculus*) in the Australian Alps vary in their leaf morphology and soil moisture preference, but little is known about their rates of gas exchange. Gas exchange rates of three buttercup species, *R. graniticola, R. muelleri* and *R. dissectifolius*, were measured under three conditions (ambient CO_2, unlimited CO_2 (to test maximum photosynthetic rate) and decreased water vapour (to test rates under water stress)) using an infrared gas analyser. There was no statistically significant variation in gas exchange rates between any of the species for any of the conditions. This then begs the question of why morphology has undergone such clear divergent evolution while physiology has not. This paper argues that every morphological 'decision' a plant makes has trade-offs and the result of this is the existence of many optimal morphologies that are advantageous in different ways. However, because physiology can change instantaneously to optimise plant growth in different environmental conditions, it does not face the same sort of trade-offs and thereby, plants have fewer options for diverse advantageous physiologies.

Key Words

Gas exchange, leaf morphology, *Ranunculus*

Introduction

Ranunculus, a genus of herbaceous plants commonly known as buttercups, occupies a diverse range of habitats across the globe. Three species, *R. graniticola, R. muelleri and R. dissectifolius*, exist across a range of environmental conditions in Kosciuszko National Park in the Australian Alps. These species vary in their distribution, with *R. graniticola* occurring in grassland with well-drained to damp soils, *R. muelleri* occurring in grassland and feldmark and *R. dissectifolius* occurring in wet grasslands and *Sphagnum* bogs (Briggs 1962).

The three species can be distinguished on the basis of their leaf morphology. Species vary in their leaf size, shape and hairiness, all of which are potentially structural strategies for overcoming the different environmental pressures faced by each species. Leaves of *R. graniticola* are oval shaped, 1–4 cm long with three lobes and are covered in long, fine, soft hairs (Briggs 1962). The habitat of *R. graniticola* is often sloping hillsides with soil moisture ranging from moderate to low. These areas have relatively high levels of soil nutrients and protection in comparison to other alpine environments (Costin *et al.* 1979). Of the three species tested, *R. graniticola* has the largest leaf area and this could be evidence of an ability to allocate resources to maximising photosynthesis rather than building protective structures due to the relatively sheltered nature of its environment in comparison to other alpine environments. Leaf lobes might assist in temperature control during hot weather as the lobes thin the leaf boundary layer, allowing faster movement of heat away from the leaves (Nicotra *et al.* 2011).

Leaves of *R. muelleri* are oval shaped, 0.8–3 cm in length and relatively narrow with long, coarse hairs covering the upper surface of leaf. *Ranunculus muelleri* is found in similar locations to *R. graniticola*, but a variety of this species also occurs on the feldmark which is characterised by very harsh conditions: exposure to extreme temperatures, very strong winds, intense solar radiation, low nutrient soils and limited water availability (Costin *et al.* 1979). Leaf hair is an important protective structure of *R. muelleri* leaves and is a common structural feature of plants in low water environments (Ehleringer 1982). A high density of leaf hair drastically decreases leaf absorbance and this results in a decrease of leaf temperature which in turn lowers water loss. Thus, leaf hair assists in survival of high temperature extremes in low water environments (Ehleringer 1982). Leaf hair is also known to act as a protective

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agent against UV damage and herbivory as well as increasing heat and drought tolerance (Roy *et al.* 1999). *Ranunculus muelleri* also have the smallest leaf area in comparison to the other two species. Small leaf area may contribute to heat loss from leaves in high temperature extremes as small leaves have a thinner boundary layer and thus heat can move away from the leaf faster, however, this also means that smaller leaves could be exposed to more frequent low temperature excursions (Nicotra *et al.* 2011). Small leaf size may therefore be advantageous in the low resource, high stress conditions of the Alps in summer, but not necessarily winter. *Ranunculus muelleri* can also have one tooth on either side of the leaf tip. There is a link between leaf growth in cold environments and being toothed (Dkhar and Pareek 2014). It is thought that toothing may assist in fast leaf growth in the early stages of leaf development (Nicotra *et al.* 2011) thus decreasing time the delicate early stage is exposed to extremes, but the extent of toothing in *R. muelleri* is minimal (Roy *et al.* 1999; Dkhar and Pareek 2014).

Leaves of *R. dissectifolius* are 1–4.5 cm long with highly divided structure and a fine hair covering on the underside and no hair on the upper side (Briggs 1962). This lack of hair on the upper surface of the leaf suggests that transpiration, rather than reflectance, is the major mechanism for temperature control in this species (Ehleringer 1982). This is possible as *R. dissectifolius* grows in the most waterlogged environment of the three species (Briggs 1962).

Gas exchange rates in plants are influenced in many ways by environmental factors. Availability of photosynthetic substrates is often a limiting factor for rate of gas exchange, rather than speed of leaf photosynthetic machinery. This allows plants to increase rates of gas exchange instantaneously when there are environmental changes conducive to greater levels of gas exchange, namely when there is an increase of the substrates light, water and CO_2 in the environment (Amthor 1994). Environmental stresses also cause changes in gas exchange rates (Amthor 1994). There is little written in the literature about the physiology, and in particular gas exchange, of Australian alpine buttercups. As *R. dissectifolius* does not experience water as a limiting factor for gas exchange rate while *R. muelleri* and *R. graniticola* do, I predicted that *R. dissectifolius* would have the highest rate of gas exchange and the stress-adapted *R. muelleri* the lowest. Production of excess photosynthetic machinery comes at a high energy cost and nutrient input, particularly nitrogen. Thus, I predicted that the amount of nitrogen in the plants would be proportional to the average gas exchange rates so as to allow the plant to work optimally when the environment allows it but limit wastage from producing far too much machinery. Therefore, I predict the maximum rate of photosynthesis (A_{MAX}) to be highest in *R. dissectifolius* and lowest in *R. muelleri*. The gas exchange rates at ambient conditions (A_{SAT}) were expected to be highest in *R. dissectifolius* and lowest in *R. muelleri* for the reasons described above.

During photosynthesis, plants face a trade-off in the degree to which the stomata are open. Open stomata allow diffusion of CO_2 into the leaf while allowing high levels of transpiration. Closed stomata prevent water loss through transpiration while also preventing CO_2 entering the leaf, thereby stopping photosynthesis. Plants must control stomatal conductance to maximise photosynthesis while limiting water loss (Beer *et al.* 2009). This can be measured as the intrinsic water use efficiency (IWUE), which is the amount of carbon assimilation per unit stomatal conductance (Beer *et al.* 2009). In this experiment IWUE is measured as intercellular CO_2/ambient CO_2 (C_i/C_a).

Increasing stomatal conductance increases rate of water loss through transpiration to a greater extent than it increases carbon assimilation (Geber and Dawson 1990). This means that plants with lower stomatal conductance per unit carbon assimilated have a higher intrinsic water use efficiency. Based on the assumption that *R. dissectifolius* has the greatest stomatal conductance (this assumption is made on the fact that this species has the greatest water availability and can therefore afford to transpire at a high rate), it is therefore hypothesised that it will have the lowest IWUE. *Ranunculus muelleri* and *R. graniticola* both occur in low to moderate soil moistures and thus it is hypothesised they will have similar, high, IWUEs.

This experiment tested the gas exchange rates of the three species of *Ranunculus* under varying conditions to identify the presence of physiological differences between the species that may exist as a result of the different environmental pressures faced by each species. I hypothesised that:

1. Photosynthetic rate measured at roughly ambient conditions (A_{SAT}, CO_2 of 400 ppm; flow of 250 µmol s^{-1}), unlimited CO_2 conditions (A_{MAX}, CO_2 of 1,200 ppm; flow of 250 µmol s^{-1}), and

arid conditions (CO_2 of 400 ppm; VPD of 2) will be highest in *R. dissectifolius* and lowest in *R. graniticola*.

2. Rate of photosynthesis at unlimited CO_2 (A_{MAX}) will be higher than rate of photosynthesis at A_{SAT} for all *Ranunculus* species.
3. As photosynthetic rate often scales with stomatal conductance, stomatal conductance will be highest in *R. dissectifolius* but similar between *R. muelleri* and *R. graniticola*.
4. Intrinsic water use efficiency (C_i/C_a) will be highest in R. *muelleri* and *R. graniticola*, and lowest in *R. dissectifolius*.

Methods

Sample collection

Twelve individual plant samples were collected in total (Table 1). Plants were dug out of the ground with a spade, ensuring the root system was still intact, and placed in a zip-lock bag. Plants were brought back to the lab and placed in the fridge (temperature between 1°C and 4°C with no light) until ready to be used. Only samples that could be identified as one particular species with a high level of certainty (i.e. not hybrids) were used in this experiment.

Table 1: Collection details of *Ranunculus* plant samples.

Species	Collection location description	Collection location GPS coordinates	Date of collection	No. samples collected
R. dissectifolius	Base Pulpit T-bar in Charlotte Pass Village	36.43331°S, 148.33208°E	10/12/16	1
	Upper Spencers Creek	36.42849°S, 148.34940°E	6/12/16	1
	Lower Spencers Creek	36.42958°S, 148.36021°E	7/12/16	1
	Betts Creek - Kosciuszko Rd intersect	36.42509°S, 148.37694°E	12/12/16	1
R. graniticola	Base Pulpit T-bar in Charlotte Pass Village	36.43373°S, 148.33229°E	10/12/16	1
	Upper Spencers Creek	36.42849°S, 148.34915°E	6/12/16	1
	Lower Spencers Creek	36.42958°S, 148.36021°E; 36.42958°S, 148.36049°E	7/12/16	2
R. muelleri	Upper Spencers Creek	36.42849°S, 148.34919°E	6/12/16	3
	Lower Spencers Creek	36.42958°S, 148.36025°E	7/12/16	1

Gas exchange

Plants were placed in a tray of water or repotted and placed in the sun for at least 30 minutes prior to measurements being taken. The largest leaf from each sample plant was used for gas exchange measurements. A portable Licor LI-6400 (Li-Cor Inc, USA) infrared gas analyser system was used to make gas exchange measurements. The block temperature was maintained at 20°C and 1,200 µmol photon $m^{-2}s^{-1}$ was used during photosynthetic measurements. Leaf area was estimated through visual observation and placed into the gas analyser chamber.

Samples were run under three conditions:

1. CO_2 of 400 ppm; flow of 250 µmol s^{-1} – this is a representation of ambient conditions used to determine the basal rate of molecular processes measured, A_{SAT}.
2. CO_2 of 1,200 ppm; flow of 250 µmol s^{-1} – a measurement of the maximum photosynthetic rate achievable by the leaf over a short time period (about 10 ± 5 minutes), known as the sprint test, A_{MAX}.
3. CO_2 of 400 ppm; VPD (vapour pressure deficit) of 2 – a measurement of rate of molecular processes under arid conditions (there is unlimited water available to the roots however).

Data was logged for each leaf under each condition once rate of photosynthesis had stabilised. This usually took approximately 10 minutes. Differences among species and treatments were assessed with *t*-tests.

Results

Part 1: Photosynthesis

Contrary to expectation, no significant difference was found between the species in the rates of photosynthesis under any of the three conditions (Figure 1). Under ambient conditions (CO_2 at 400 ppm, flow at 250 µmol s^{-1}) rate of photosynthesis in µmol CO_2 m^{-1} s^{-1} was 11.0 ± 2.9 (average ± standard deviation) for *R. dissectifolius*, 13.3 ± 3.6 for *R. graniticola* and 9.7 ± 3.1 for *R. muelleri* (Figure 1A). Under the unlimited CO_2 condition (CO_2 at 1,200 ppm, flow at 250 µmol s^{-1}) rate of photosynthesis in µmol CO_2 m^{-1} s^{-1} was 23.6 ± 3.0 for *R. dissectifolius*, 25.4 ± 13.2 for *R. graniticola* and 23.1 ± 11.6 for *R. muelleri* (Figure 1B). Under arid conditions (CO_2 at 400 ppm, VPD of 2) rate of photosynthesis in µmol CO_2 m^{-1} s^{-1} was 9.5 ± 3.6 for *R. dissectifolius*, 12.5 ± 3.9 for *R. graniticola* and 9.1 ± 3.4 for *R. muelleri* (Figure 1C).

However, the measurement conditions did result in large differences in photosynthetic rates. The average rate of photosynthesis across all species under ambient conditions was 11.3 ±3.2 µmol CO_2 m^{-1} s^{-1} while the average rate of photosynthesis across all species under unlimited CO_2 conditions was 24.0 ± 9.4 µmol CO_2 m^{-1} s^{-1} (Figure 2, $p = 0.002$).

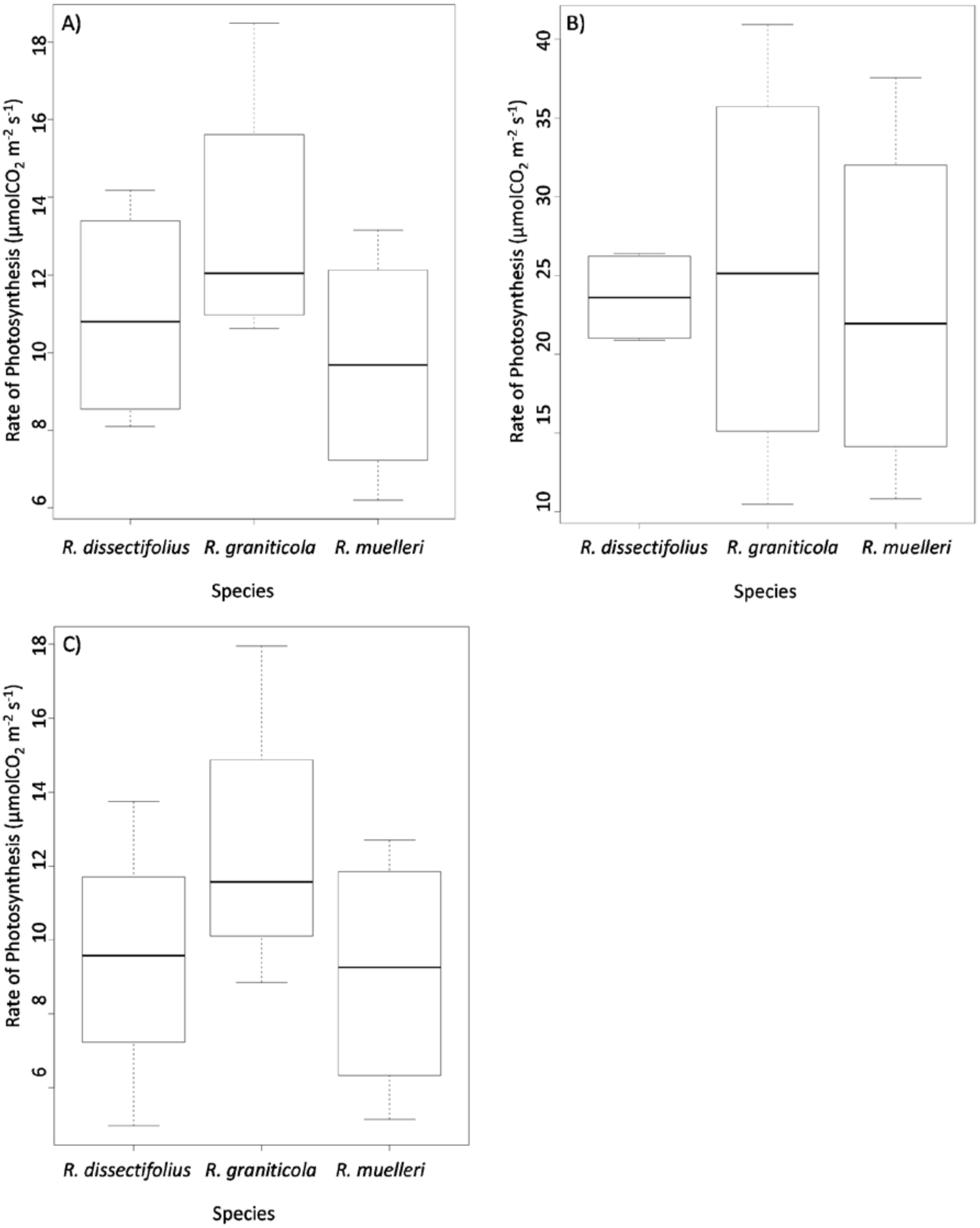

Figure 1: Comparison of rates of photsynthesis between the three species under A) ambient conditions (CO_2 at 400 ppm, flow at 250 µmol s^{-1}) (net photosynthetic capacity), $p = 0.314$; B) unlimited CO_2 (CO_2 at 1,200 ppm, flow at 250 µmol s^{-1}), $p = 0.945$; and C) arid conditions (CO_2 at 400 ppm, VPD of 2), $p = 0.387$.

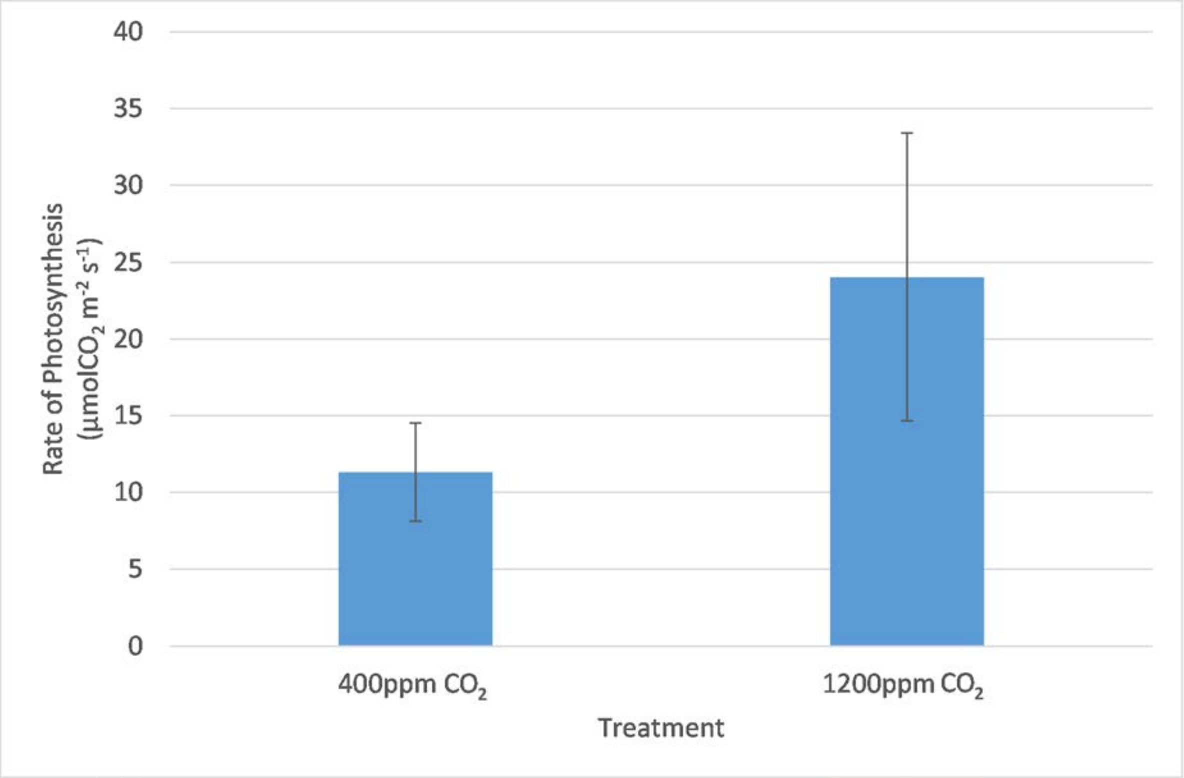

Figure 2: Average rate of photosynthesis irrespective of species for ambient CO_2 (400 ppm) and unlimited CO_2 (1,200 ppm). Difference in rate of photosynthesis between the two treatments is very significant ($p = 0.0002$). Error bars show standard deviation.

Part 2: Intrinsic water use efficiency and stomatal conductance

Stomatal conductance was highest in *R. dissectifolius* and lowest in *R. muelleri* under all three conditions, though the difference between species was not statistically significant (*p*-value = 0.2, Figure 3). Likewise, no significant difference was found between the species in their intrinsic water use efficiency (IWUE) under any of the three conditions. Under ambient conditions (CO_2 at 400 ppm, flow at 250 µmol s^{-1}), average IWUE was 0.756 ± 0.045 (average ± standard deviation) for *R. graniticola*, 0.768 ±0.033 for *R. muelleri* and 0.762 ± 0.079 for *R. dissectifolius*. Under the unlimited CO_2 condition (CO_2 at 1,200 ppm, flow at 250 µmol s^{-1}) average IWUE was slightly higher at 0.832 ± 0.046 for *R. graniticola*, 0.816 ± 0.062 for *R. muelleri* and 0.809 ± 0.070 for *R. dissectifolius*. Under arid conditions (CO_2 at 400 ppm, VPD of 2) average IWUE was 0.735 ±0.048 for *R. graniticola*, 0.769 ± 0.047 for *R. muelleri* and 0.709 ± 0.104 for *R. dissectifolius* (Figure 4).

The IWUEs were not statistically different between the species under ambient conditions, unlimited CO_2 conditions or arid conditions, nor were there significant differences between measurement conditions.

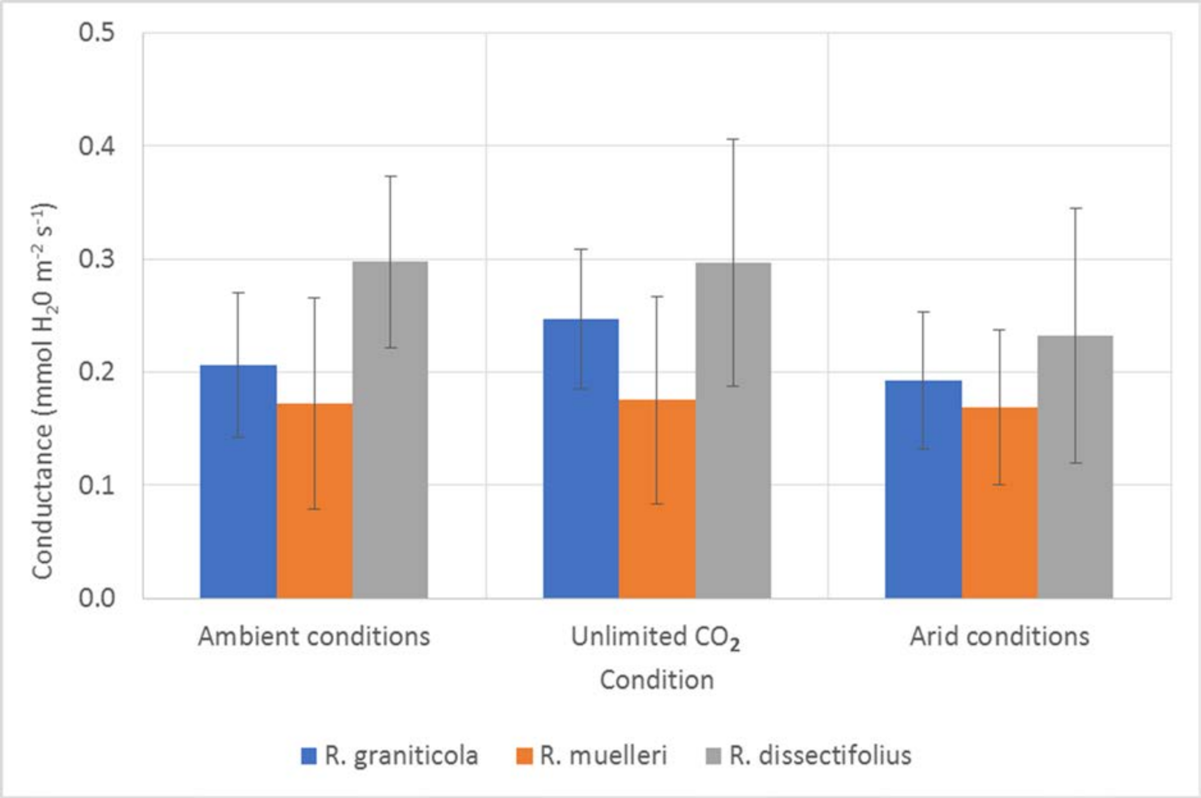

Figure 3: Average conductance of each species at the three testing conditions. R. *dissectifolius* had the highest conductance in every instance and *R. muelleri* had the lowest conductance in every instance but species differences were not statistically significant (*p*-value = 0.2). Error bars show standard deviation.

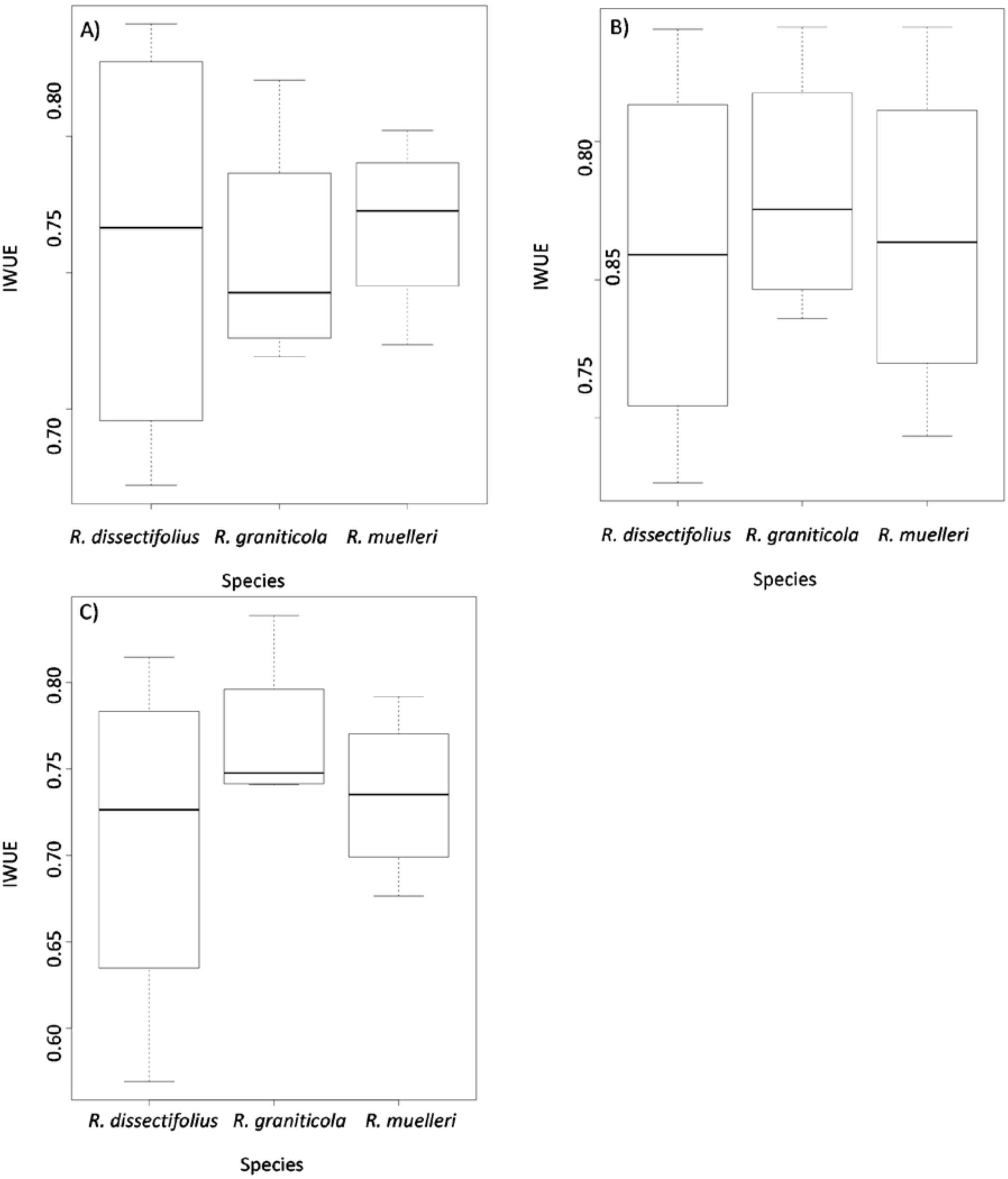

Figure 4: Comparison of the intrinsic water use efficiency (IWUE) of the three species. A) IWUE between species under ambient conditions, $p = 0.959$; B) IWUE between species at 1,200 ppm CO_2, $p = 0.858$; and C) IWUE between species with VPD at 2, $p = 0.524$.

Discussion

The three species of *Ranunculus* face different environmental pressures due to variation in growth location in the Australian Alps. The leaf morphological differences between different species may have arisen to help the plants survive in their respective environments. We hypothesised that the three species of *Ranunculus* occurring in different environmental conditions would also exhibit physiological differences which optimise their function in their respective environments. However, under all testing conditions, there was no significant difference in photosynthetic rate between the species. Further, despite average conductance being highest in *R. dissectifolius* across treatments (as predicted) and lowest in *R. muelleri* across treatments, the variation was not statistically different. Finally, there was no significant difference between species across treatments for IWUE and thus our final hypothesis was not upheld. Despite divergence in the leaf morphology and soil moisture preference in the three *Ranunculus* species, this experiment suggests there has been no substantive divergence of gas exchange rates. This may be an unsurprising result as plants of the same functional type often share similar physiological characteristics (Reich *et al.* 1999). However, this then begs the questions of why it is that there is so much more variety in morphology compared to physiology and why the evolution of morphology appears to occur much more rapidly than the evolution of physiology?

The answer may lie in trade-offs. Because plant morphology cannot change in response to every change in the ambient environment, it cannot be perfectly adapted to every environmental pressure it faces and trade-offs must be made. For example, a morphological characteristic that assists in heat retention may be helpful on a cold day in winter, but detrimental on a hot day in summer, and vice versa for a morphological trait that cools the plant down (Nicotra *et al.* 2011). Because of this, a species may evolve a morphological characteristic that assists in optimal function in, for example, a cold environment while another plant may evolve a morphological characteristic that assists in optimal function in a warm environment. The two species have very different morphologies and different times when they function optimally, but over their entire lifetime, could have equal levels of reproductive success and so both morphologies are equally advantageous and persist in their respective microenvironments. This is seen less so in physiology as physiology can change almost instantaneously in response to a change in the ambient environment (as shown in leaf response to unlimited CO_2 compared to ambient CO_2, Figure 2). In an environment like the Australian Alps where there are immense fluctuations in environmental conditions, it may be possible for many morphologies to coexist and be equally successful overall in particular microclimates. Trade-offs may not be as apparent in physiology, however, because photosynthetic machinery has evolved so that when environmental conditions change, so does the physiology. This would result in less diversifying selection on plant physiology which may be why physiology appears to be identical across these *Ranunculus* species.

There were several logistical constraints in this experiment that bear consideration. Leaf morphology in *Ranunculus* exists on a continuum as most of the described species have been shown to interbreed to form hybrids with intermediate leaf morphology (Briggs 1962). As samples in this experiment were selected based purely on observation of the leaf morphology, there may have been mistakes in species identification, with hybrids being incorrectly identified as pure species, and this was a weakness of this experiment. When DNA sequencing become cheaper and more ubiquitous in the future, it may be used as a method for more accurate species identification. Further, investigation of subtle differences between the hybrids could be of interest in future. Also, some samples were tested days after being dug up while other were tested the same day. A more consistent method in this regard would also improve the experiment. Finally, sample sizes were quite small and thus our estimates of rates were quite variable. It is possible that higher replication would have revealed more differentiation.

We suggest that future research could be directed at root physiology, including respiration, as that might better establish correlation between *Ranunculus* physiology, evolution and adaptation to different soil moisture conditions. Root respiration is an extremely important part of plant physiology and survival, especially in extreme environments. A recommended future experiment would involve measuring the effects of environmental manipulations on root respiration (Aenone III and Körner 1997). In addition, research on evolution of morphology is much more in-depth than research on evolution of physiology because it is possible to study the morphology of plants that existed long ago but not study their physiology. Furthermore, accurate measurements of physiology have only been possible with the development of modern technologies. Long-term studies on the effect of environmental manipulations over many generations may give insight into evolutionary mechanisms acting on plant physiology.

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