Assessing Water Use Variation Among the Cytotypes of the Autopolyploid Southwestern Desert Creosotebush (*Larrea tridentata* [DC.] COVILLE: ZYGOPHYLLACEAE)

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ASSESSING WATER USE VARIATION AMONG THE CYTOTYPES OF THE AUTOPOLYPLOID SOUTHWESTERN DESERT CREOSOTEBUSH (*LARREA TRIDENTATA* [DC.] COVILLE: ZYGOPHYLLACEAE)

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ABSTRACT

Genome duplication, or polyploidy, has played an important role in the diversification of flowering plants, but the ecological and evolutionary consequences of polyploidy still remain unclear. Polyploidy is known to either cause or facilitate phenotypic changes, and ploidy-specific phenotypic differences may lead to the exploitation of novel niche space. Many studies reporting phenotypic variation between cytotypes have been observational, and may not reflect ecological adaptation by diploids and polyploids to different habitat. One such trait, water use, may play an outsized role in survival and population expansion into novel habitat. To test whether water use differs among ploidy levels, we grew field-collected diploid, autotetraploid, and autohexaploid cytotypes of the characteristic North American desert plant, *Larrea tridentata* (DC.) Coville, under greenhouse conditions. We measured whole plant water use gravimetrically over six wk, standardizing water use with measures of total stomatal area. We found that water use was positively correlated with stomatal area, but the cytotypes had similar total stomatal areas and did not differ significantly in mean water use/total stomatal area. Cytotype-specific water use responses through time were also not significantly different. Taken together, these results suggest that the cytotypes have similar water relationships, and possibly fitness outcomes, with respect to water use in common environments.

Key Words: desert plant, leaf area, polyploidy, stomatal area, stomatal density, water use.

Paleontological and genomic studies indicate the evolutionary history of angiosperms is punctuated by repeated polyploidization, or whole genome duplication events (Masterson 1994; Ramsey and Schemske 1998; Soltis et al. 2009). Contemporary opinion implicates genome duplication as having played an important role in the ancient diversification of flowering plants (*Amborella* Genome Project 2013). As an evolutionary force, polyploidy continues to play a role in plant diversification with approximately 15% of speciation events involving genome duplication (Wood et al. 2009). Genome duplication may also cause phenotypic alterations between polyploids and their diploid progenitors. Differences in herbivore resistance, phenology, reproductive system, cell size, and drought tolerance have been documented between diploids and polyploids (Bretagnolle and Lumaret 1995; Segraves and Thompson 1999; Maherali et al. 2009; Ramsey 2011). Arising either as a direct effect of increased chromosomal complement (Levin 1983, 2004), or from adaptative responses to extrinsic factors (Maherali et al. 2009; Ramsey 2011; Madlung 2013), such differences have been argued to facilitate polyploid expansion into novel habitat by broadening ecological amplitude (Brochmann et al. 2004; Martin and Husband 2009; McIntyre 2012; but see Buggs and Pannel 2007) and may lead to ecological speciation (Levin 2004; Ramsey 2011; Martin and Husband 2013).

Though polyploidy may recurrently introduce genetic and phenotypic variation into populations, the ecological consequences of genome duplication for adaptation remain unclear (Ramsey and Ramsey 2014; Soltis et al. 2014). Many studies reporting phenotypic variation between cytotypes have been observational, focused on climatic correlates with diploid and polyploid distributions, for example, or on simple trait differences between cytotypes (Ramsey and Ramsey 2014; Soltis et al. 2016). Ecological niche modeling studies of diploid and autotetraploid *Chamerion angustifolium* (L.) Holub indicate tetraploids are found in warmer and drier habitats than diploids, consistent with ecological adaptation associated with polyploidization (Thompson et al. 2014). Recent experimental efforts building upon observational studies have made great strides in characterizing the fitness consequences of apparent ecological adaptation by polyploids. For example, water relations, particularly important for plant adaptation and ecological niche differentiation (Heschel et al. 2002; McKay et al. 2003; McKay et al. 2008; Engelbrecht et al. 2007), have been investigated in a number of systems comprising multiple cytotypes. Allotetraploid *Brachypodium hybridum* Catalán, Joch. Müll., Hasterok & G. Jenkins is more often found in arid habitats than diploid *Brachypodium distachyon* (L.) P. Beauv., and greenhouse studies show that the tetraploids have greater water use efficiency than diploids (Manzaneda et al. 2012;

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Manzaneda et al. 2015). Similarly, reciprocal transplant experiments with tetraploid and hexaploid *Achillea millefolium* L. demonstrate ecological adaptation by the two cytotypes (Ramsey 2011), and common garden experiments document differences in altitudinal adaptation (Martin and Husband 2013) and water relations (Maherali et al. 2009) between diploid and tetraploid *C. angustifolium*. However, such relationships are not universal. Reciprocal transplant studies of *Allium oleraceum* L. indicate less clear ecological differentiation among cytotypes (Duchoslav et al. 2016). Rather, individuals tended to show some evidence of local adaptation, regardless of ploidy, and that tetraploids, pentaploids, and hexaploids may exhibit enough phenotypic plasticity to cope with the ecological variation present throughout the entire range of the species. Thus, what seems clear from the emerging literature exploring diploid and polyploid ecological differences is the need for additional experimental evaluations of ecologically relevant phenotypic differences.

Phenotypic alterations associated with genome duplication that alter water relations may play an outsized role in facilitating range expansion, ecological adaptation, and community assembly (Cavender-Bares et al. 2004; Levin 2004; Ramsey 2011; McIntyre 2012). Numerous taxa comprising intraspecific ploidy levels are known to have distributions correlated with climatic or other ecological gradients (e.g., *Ambrosia* L., Raven et al. 1968; *Larrea* Cav., Yang and Lowe 1968; *Melampodium* L., Stuessy 1971; *Tolmiea* Torr. & A. Gray, Soltis 1984; *Chamerion* [Raf.] Raf. ex Holub, Husband & Schemske 1998; *Galax* Sims, Burton and Husband 1999; *Achillea* L., Ramsey et al. 2008; *Campanula* L., Sutherland and Galloway 2016). However, in most cases inter-cytotype phenotypic differences that could facilitate niche partitioning along ecological gradients, including water use, remain poorly known and often have not been examined in common environments. Traits such as water use efficiency (water use/carbon gain) can play an important role in determining species distributions by influencing carbon assimilation and fitness (Hetherington and Woodward 2003; McKay et al. 2008; Hodgson et al. 2010; Kropp and Ogle 2015). However, measurements of water use efficiency typically involve sensitive equipment, may be influenced by slight methodological variations, and are often difficult to extrapolate to whole-plant measures of water use efficiency under natural conditions for large perennial plants that may exhibit interleaf variability in transpiration and photosynthetic rates, or when age and ecological history is unknown (Pearcy et al. 1989; Cirelli et al. 2012). In contrast, estimates of whole plant water use using gravimetric methods can provide a snapshot of plant water relations under a range of conditions despite lacking an estimate of carbon assimilation (Cirelli et al. 2012; Medrano et al. 2015). Here, we investigated water use differences between the cytotypes of one archetypal autopolyploid desert plant in an effort to more clearly understand the role genome duplication has played in ecological differentiation.

Larrea tridentata (DC.) Coville (Zygophyllaceae) is a widespread and ecologically dominant species of the North American warm deserts (Hunziker et al. 1977; Lewis 1980). The ecological success of *L. tridentata* in arid environments has been attributed to its ability to maintain photosynthesis during extreme heat and drought (Ogle and Reynolds 2002). For example, studies in the Chihuahuan Desert indicate *L. tridentata* can sustain pre-dawn water potentials (Ψ_{pd}) in excess of −10 Mpa (Cunningham and Burk 1973), while maintaining positive net photosynthesis (Odening et al. 1974). Similarly, plants in the Mojave Desert have been recorded maintaining positive net photosynthesis while experiencing water potentials as low as −60 atm (Bamberg et al. 1973). Not considered in these studies is that *L. tridentata* comprises three autopolyploid chromosome races distributed throughout the Chihuahuan (diploids; $2n = 2x = 26$), Sonoran (predominantly tetraploids $2n = 4x = 52$), and Mojave (predominantly hexaploids; $2n = 6x = 78$) Deserts of the southwestern U.S. and northern Mexico (Yang 1967, 1970; Yang and Lowe 1968; Barbour 1969; Hunter et al. 2001; Laport et al. 2012; Laport et al. 2013; Laport and Ramsey 2015). Though hypothesized to have facilitated adaptation to increasingly arid environments (Barbour 1969; Hunziker et al. 1977), the influence of genome duplication on the ecological success of *L. tridentata* remains essentially untested for many ecologically relevant traits (Laport et al. 2016).

Prior studies of *L. tridentata* indicate the cytotypes evolved relatively recently (ca. $\leq$1 mya; Laport et al. 2012), but occupy climatically distinct habitats even in areas where they come into contact (Barbour 1969; Yang 1970; Laport and Ramsey 2015). Differences in leaf, flower, and whole-plant traits among the cytotypes (Laport and Ramsey 2015) support hypotheses that repeated genome duplications have contributed to phenotypic differentiation (Barbour 1969), and facilitated rapid ecological adaptation (Yang 1970; Hunziker et al. 1977). However, these morphological differences do not always vary linearly with increasing ploidy, and may be uncoupled from physiological traits related to water use. For example, Barbour (1969) observed that diploid seedlings were more drought tolerant than tetraploid and hexaploid seedlings. Laport et al. (2013) found that tetraploids and hexaploids occupy climatic niches that are significantly different from their diploid and tetraploid progenitors, respectively, but also that the cytotypes tend to occupy climatically similar areas within their respective deserts. Combined, these prior observations leave unclear the degree to which genome duplication has altered cytotype-specific water use and facilitated adaptation to the unique ecological conditions of the Chihuahuan, Sonoran, and Mojave Deserts. To investigate whether water use differs among cytotypes of *L.*

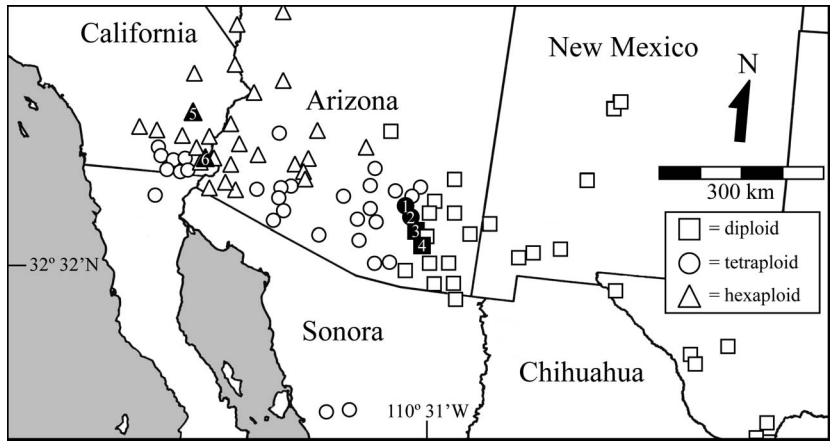

FIG. 1. Map of live *L. tridentata* collection localities. The distributions of diploid (open square), tetraploid (open circle), and hexaploid (open triangle) *L. tridentata* throughout the southwestern US and northern Mexico was previously determined by Laport et al. (2012) and Laport and Ramsey (2015) using flow cytometry to infer the ploidy of >20 adult plants at permanent study sites. Filled site markers (numbered 1–6) indicate where seedlings were collected for water use analyses. Numbering of sites corresponds to Table 1.

tridentata, we grew field-collected plants of known ploidy under greenhouse conditions and measured whole plant water use gravimetrically. Making these investigations in *L. tridentata*, an ecologically dominant member of the North American desert flora, will help shed light on the evolutionary significance of polyploidy to ecological adaptation, incipient speciation, and patterns of biodiversity, as well as elucidate the structuring of desert plant communities.

MATERIALS AND METHODS

Live Plant Collections

In the spring of 2014, we collected small, naturally occurring diploid, tetraploid, and hexaploid plants (ca. 5–15 cm tall) from each of two previously identified sites of known ploidy (six sites total, Fig. 1, Table 1; Laport et al. 2012; Laport and Ramsey 2015). We were unable to make collections at additional sites because seedling recruitment and survival is episodic for *L. tridentata* (Chew and Chew 1965). Seedlings were transported to the University of Nebraska-Lincoln greenhouses in 7.5 cm peat pots containing native soils. Of the plants that survived being transported we re-potted (along with some of the native soil) 15 diploids, 15 tetraploids, and 15 hexaploids into 13 cm plastic pots with a growing media consisting of five parts Sunshine MVP (formerly Metro-Mix 200, www.hummert.com), three parts washed horticultural river sand, and three parts calcined clay (Turface MVP, www.hummert.com). Plants were irrigated once a week and fertilized once per month for nine months under common greenhouse conditions prior to water use assessments.

Water Use and Transpiration Determination

To determine if water use differed among the three cytotypes, we measured whole plant water loss gravimetrically every week for six weeks. We assigned potted plants of each ploidy to shuttle trays (Fig. 2A). Each week, the potted plants were given 200 ml of water, ensuring no water leaked from the bottom of the pots. After watering, we determined the mass of each pot ("initial mass"), and then allowed the plants to transpire for 72 hr. We then

TABLE 1. Environmental conditions at collection localities for live *L. tridentata* plants. All environmental data from WorldClim (www.wordclim.org; Hijmans et al. 2005). Site names and cytotypes (in parentheses) correspond to permanent collection sites established by Laport et al. (2012) and Laport and Ramsey (2015).

Sites	Latitude (°N)	Longitude (°W)	Elevation (m)	Mean annual temperature (°C)	Mean annual precipitation (mm)
1. San Pedro 1 (4*x*)	32.633983	-110.55865	809	19.5	351
2. San Pedro 2 (4*x*)	32.616083	-110.5386	780	19.3	347
3. San Pedro 4 (2*x*)	32.538133	-110.50795	815	19.1	356
4. San Pedro 5 (2*x*)	32.555917	-110.516267	810	19.3	348
5. Joshua Tree (6*x*)	33.67395	-115.800983	481	20.4	122
6. Algodones S4 (6*x*)	32.8886	-114.842067	166	22.2	74

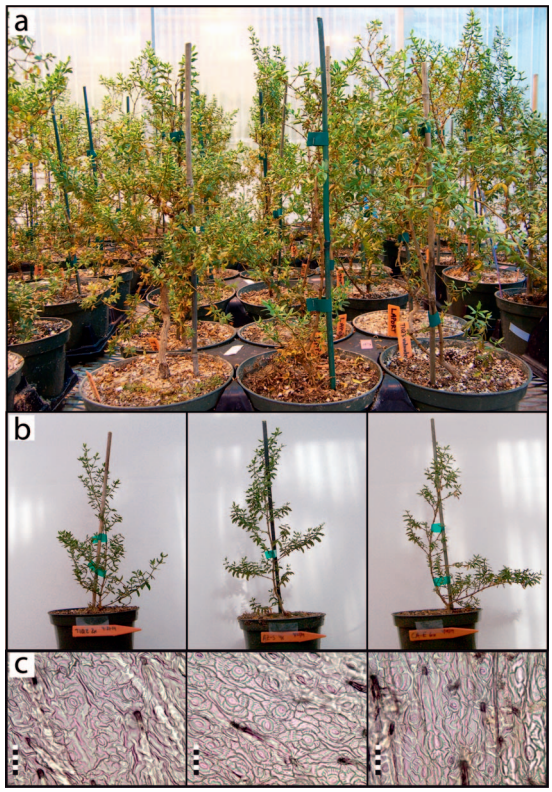

FIG. 2. Wild-collected *L. tridentata* in the experimental water use setup. A) Diploids, tetraploids, and hexaploids randomly arrayed in shuttle trays for assessment of whole plant water use. B) From left to right, diploid (2×), tetraploid (4×), and hexaploid (6×) plants, against a white background for leaf area assessments. Total leaf areas were approximately equal among cytotypes. C) From left to right, images of diploid, tetraploid, and hexaploid epidermal impressions used for stomatal area and density measures (400× total magnification). Stomatal size increased with ploidy, but stomatal density decreased with increased ploidy. Scale bars in each epidermal impression image measure ~40 μm.

determined the mass of each of the potted plants again ("final mass"). After determining the final mass, the shuttle trays were shifted to reduce positional bias in lighting and airflow within the greenhouse between trials, as some trays did not contain all of the cytotypes. The plants were then watered lightly to provide sufficient water until the beginning of the next trial. We determined total whole plant water loss as the difference between the initial and final masses.

To estimate the whole plant water use, we corrected for water lost to evaporation using four control pots containing only the growing media. The control pots were distributed among the shuttle trays in a representative way, and were watered and massed in the same manner as the potted plants. The change in mass among the control pots was averaged each week and subtracted from the weekly total water loss by each of the potted plants as evaporated water. We considered the remaining value as total water use by the potted plants.

Total Leaf Area Determination

Transpiration in *L. tridentata* is sensitive to leaf area (Ritchie 1972; Bird 2010). To reduce the influence of leaf area on water use across individuals, we lightly pruned the plants periodically to attain approximately similar overall sizes prior to water use assessments. We estimated the total leaf area for each of the potted plants during the third week of the experiment to capture the leaf area at the midpoint of any growth that may have occurred over the six-week experiment. To estimate the total leaf area, we photographed each plant against a white background along with a scale of known length ensuring visible leaf area appeared representative for a two dimensional image (Fig. 2B). Each image was then imported into ImageJ (Schneider 2012) and transformed into a binary image. We set the scale for each image using the length of the known scale in each image, and then determined the total leaf area of the plant image in mm^2.

Stomatal Size and Density

Stomatal size and density are correlated with ploidy (Masterson 1994; Hunter et al. 2001; Beaulieu et al. 2008), and play an important role in water use efficiency (Hetherington and Woodward 2003). We made adaxial epidermal leaf impressions at the conclusion of the study (week six) by coating the surface of three haphazardly chosen pressed and dried leaves with a thin layer of clear nail polish. The nail polish was allowed to dry prior to being peeled off the leaf with clear plastic tape and affixed to a glass microscope slide for determination of stomatal area and density with a compound light stereomicroscope (Carl Zeiss Axiostar Plus) under 400× total magnification (Barbour et al. 1974; Hunter et al. 2001; Fig. 2C). Digital photographs for a representative field of view for each leaf were made using a digital camera affixed to the microscope (Leica EC3 paired with Leica Acquire software v3.2). We counted stomatal density and measured the area of three sets of stomatal guard cells for three leaf impressions for each of the 45 greenhouse-grown plants (405 total stomatal guard cell areas) using ImageJ (Schneider 2012). Area measures were converted to μm^2 using a calibrated stage micrometer viewed at the same magnification. All measurements were averaged for each plant and used to estimate total stomatal area for each plant by multiplying by the leaf area estimates. This allowed us to standardize measurements of water use across plants by determining the mean water use/total stomatal area (grams/hr/μm^2) for each plant.

Data Analysis

We evaluated the relationship between water use and total stomatal area, with a linear regression. We tested for cytotype differences in leaf area, stomatal area, and stomatal density with a MANOVA model that included ploidy as a categorical effect. Individual differences were evaluated with ANOVA and a Tukey HSD test for post hoc comparisons between cytotypes. To test whether the cytotypes differed in mean water use/total stomatal area (averaged across the six wk of the study) we used an ANOVA model that included ploidy as a categorical effect and a Tukey HSD test for post hoc comparisons between cytotypes. To examine changes in water use over time we implemented a repeated measures ANOVA model that included ploidy, date, and ploidy × date as effects. Stomatal areas and densities, and mean water use/total stomatal area were natural log transformed prior to statistical analyses to improve the distribution of residuals. All statistical analyses were performed in JMP Pro (v.12; SAS Institute Inc., Cary, NC, USA).

Results

The small plants in this experiment were collected near the distributional contacts between the three cytotypes (Fig. 1). Though generally abiotically similar, the diploid collection sites tended to be at higher elevations with slightly cooler mean annual temperatures and slightly higher mean annual precipitation than the tetraploid and hexaploid collection sites. The hexaploid collection sites occurred at the lowest elevations and had the highest mean annual temperatures and lowest mean annual precipitation (Table 1). The cytotypes had significantly different leaf and stomatal features (Wilks' $\lambda = 0.225$, $F_{6,80} = 14.772$, $P < 0.0001$). Although the cytotypes did not differ in total leaf area ($F_2 = 0.186$, $P = 0.831$), they were significantly differentiated by individual stomatal area ($F_2 = 65.156$, $P < 0.0001$) and density ($F_2 = 25.131$, $P < 0.0001$). Tetraploids had the greatest mean leaf area (20,873.79 mm^2, SE = 2320.57 mm^2), followed by diploids (19,836.12 mm^2, SE = 1356.22 mm^2) and hexaploids (19,451.33 mm^2, SE = 1237.14 mm^2). Diploids had the lowest individual stomatal area (167.006 μm^2/stomata, SE = 3.647) and the highest stomatal density ($4.203\times10^{-4}/\mu m^2$, SE = 1.347×10^{-5}), followed by tetraploids (199.279 μm^2/stomata, SE = 3.472; $3.800\times10^{-4}/\mu m^2$, SE = 1.271×10^{-5}), and hexaploids (275.257 μm^2/stomata, SE = 7.348; $2.803\times10^{-4}/\mu m^2$, SE = 1.406×10^{-5}). These measures differ from those reported previously by Barbour et al. (1974) and Hunter et al. (2001), which may be related to the genetic variability expressed at the specific localities we sampled, or to having grown the plants for an extended period in the greenhouse. Nevertheless, we observed the same relative size and density relationships among cytotypes. Mean water use was positively correlated with total stomatal area ($R^2 = 0.125$, $P = 0.017$), despite a large spread in water use values (Fig. 3A). However, while diploids had slightly higher mean water use/total stomatal area than tetraploids or hexaploids, the cytotypes did not differ significantly in mean water use/total stomatal area in our ANOVA model ($F_2 = 0.3975$, $P = 0.675$; Fig. 3B).

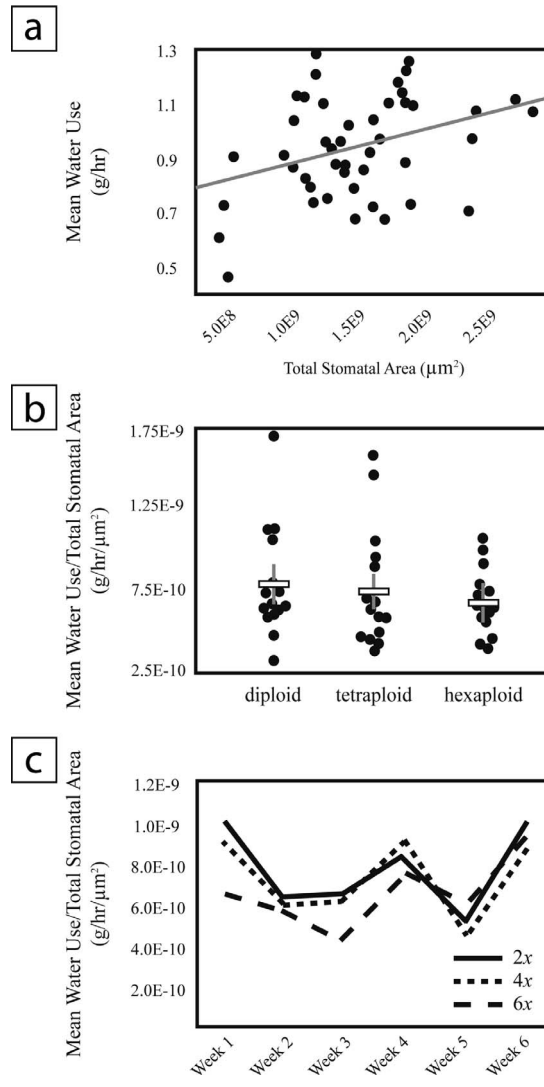

FIG. 3. Water use relationships for the three cytotypes of *L. tridentata*. A) Mean water use was positively correlated with total stomatal area. B) Diploids, tetraploids, and hexaploids did not differ in mean water use/total stomatal area. Each point indicates an individual plant mean over the six weeks of the experiment. Mean values are indicated with a rectangle, and error bars indicate ± 1 SE. C) Diploids, tetraploids, and hexaploids varied in mean water use/total stomatal area over the course of the experiment. However, cytotype-specific responses over time were not significantly different in a repeated measures analysis.

Over the six weeks of the experiment, the mean water use/total stomatal area varied differentially among the three cytotypes (Fig. 3C). While water use fluctuated approximately equally up and down from week one to six, diploids and tetraploids initially had slightly higher mean water use/total stomatal area than hexaploids, but by the end of the experiment this relationship disappeared. Despite these fluctuations, the repeated measures model indicated that none of the cytotypes significantly differed in mean water use/total stomatal area × date interactions (Wilks' $\Lambda = 0.700$, $F_{10,\ 76} = 1.485$, $P = 0.161$). Thus, the cytotypes had similar water use responses over time (Fig. 3C).

Discussion

Studies over the last few decades make clear that polyploidy is an important evolutionary mechanism in angiosperms (Soltis et al. 2009; Madlung 2013; Ramsey and Ramsey 2014; Husband et al. 2016). Since the discovery of polyploidy, evolutionary biologists have sought to determine whether observed trait differences between diploids and polyploids are an intrinsic consequence of genome duplication or result from ecological adaptation (Levin 1983; Ramsey and Ramsey 2014; Soltis et al. 2014). Though important for understanding the evolution of polyploid species, this question has proven difficult to parse. Extant polyploid species often comprise cytotypes that have already diverged over many generations, and the artificial creation of neopolyploids may not adequately represent the combination of alleles present at the formation of natural polyploids (Husband et al. 2016). Ecological differences may also exist among allopolyploid cytotypes that are not observed immediately upon formation among autopolyploid cytotypes since allopolyploids combine two diverged genomes (Ramsey and Schemske 1998). For example, allopolyploids may more often exhibit patterns of ecological niche differentiation at broad scales than autopolyploids (Glennon et al. 2014; Marchant et al. 2016). Nevertheless, comparisons of relatively recently diverged cytotypes in common garden experiments and reciprocal transplants have been instrumental in better understanding the role genome duplication plays in ecological divergence (Ramsey 2011; Martin and Husband 2013; Duchoslav et al. 2016; Segraves and Anneberg 2016).

The renewed focus on studying polyploid evolution has resulted in the recognition that intraspecific ploidy levels often represent novel genetic and phenotypic entities (Ramsey and Ramsey 2014). Recent studies have characterized cytotype-specific genetic (Vallejo-Marin and Lye 2013; Fehlberg and Ferguson 2012; Glennon and Church 2015; Laport et al. 2016), climatic (Glennon et al. 2014; Thompson et al. 2014), ecological (Ramsey 2011; Martin and Husband 2013), pollinator (Husband and Schemske 2000; Nghiem et al. 2011; Borges et al. 2012; Roccaforte et al. 2015), and herbivore (Thompson and Merg 2008) differences, but such differences have been investigated for relatively few species in common environments, and the underlying mechanisms for observed differences remain understudied. As a measure of physiological performance, water use relations have proven useful for understanding cytotype divergence. In allopolyploid species, such as *Brachypodium* Brid., pronounced cytotype-specific differences in water use may have facilitated niche partitioning by diploids and tetraploids in the Mediterranean basin (Manzaneda et al. 2012; Manzaneda et al. 2015). However, differences in water use between diploid and autotetraploid *C. angustifolium* are less evident (Maherali et al. 2009), and may only have a limited effect on intercytotype competition even when water is limited (Thompson et al. 2015). Such observations suggest polyploid physiological adaptation may be complex, and that other ecological interactions may play a significant role in cytotype divergence resulting in idiosyncratic outcomes with respect to the ecological setting or whether auto- or allopolyploidy was involved.

Water use has been assessed previously in *L. tridentata* to test hypotheses related to ecological adaptation for arid environments (Barbour 1969; Barbour et al. 1974; Meinzer et al. 1990; Ogle and Reynolds 2002; Kropp and Ogle 2015). These studies have investigated the influence of diurnality, seasonal shifts, soil type, and neighboring plants on water use in *L. tridentata*, as well as differences in water use between *L. tridentata* and its South American relatives. For example, tetraploid *L. tridentata* had a much lower transpiration rate than the diploid South American *L. divaricata* Cav. (Barbour et al. 1974) under greenhouse conditions, and soil texture and composition played an important role in determining transpiration across seasons for tetraploid *L. tridentata* near Tucson, AZ (Ignace and Huxman 2009). Moreover, daily and seasonal fluctuations in temperature and moisture (Barker et al. 2006; Ogle et al. 2012), and the composition of neighboring plant communities (Kropp and Ogle 2015), strongly influenced transpiration for *L. tridentata* growing in the Mojave and Sonoran Deserts, while supplemental water and fertilization influenced water use for plants in the Mojave and Chihuahuan Deserts (Meinzer et al. 1988; Lajtha and Whitford 1989; Franco et al. 1994). However, these studies have typically been restricted to only diploids, or tetraploids, or hexaploids, leaving the relationship between water use and ploidy unclear despite the potential for relative water use to be a major contributor to fitness differences between cytotypes and the evolution of this important desert plant.

One exception to this single cytotype focus is an investigation by Barbour (1969) into the leaf wilting points for lab-grown seedlings of the three cytotypes collected from across the range. Barbour found that diploid seedlings wilted at a lower (more negative) water potential than the tetraploids or hexaploids,

counter to expectations of higher ploidies being better adapted for the more arid environments of the Sonoran and Mojave Deserts. In contrast, Ogle et al. (2012) conducted a study of stomatal conductance and transpiration for multiple common desert species across the Great Basin, Mojave, Sonoran, and Chihuahuan Deserts. *Larrea tridentata* in the Mojave Desert (probable hexaploids) was found to have lower transpiration than plants in the Chihuahuan Desert (probable diploids). Though these studies provide some evidence that ploidy influences water use in *L. tridentata*, the counterintuitive results and comparisons made between plants experiencing unique ecological conditions in disjunct ecoregions makes it difficult to interpret the role of genome duplication in facilitating ecological adaptation and the geographic expansion of *L. tridentata* into the many distinct habitats in which it is found.

In our controlled greenhouse experiment, we found that mean water use/total stomatal area did not differ significantly among diploids, tetraploids, and hexaploids (Fig. 3B). Despite the positive relationship between water use and stomatal area (Fig. 3A), the lack of a significant difference in water use/stomatal area may be due to a compensatory relationship between lower stomatal density with increased stomatal area. By pruning the plants to have approximately equal canopy sizes, we controlled for total leaf area among diploids, tetraploids, and hexaploids, resulting in similar total stomatal areas. Moreover, despite fluctuations in water use over the six weeks of the experiment, none of the cytotypes exhibited significantly different water use over time (Fig. 3C). Taken together, these results suggest that in areas of cytotype contact, diploid, tetraploid, and hexaploid *L. tridentata* may have similar water use, and would be expected to have similar survival and fitness outcomes in the absence of other ecological interactions affecting water relations.

While these findings bring into question assertions that the cytotypes are differentially ecologically adapted (Yang 1967, 1970; Barbour 1969; Hunziker et al. 1977; Lewis 1980), we did not quantify water use efficiency, and it is unknown if the three cytotypes differ in biomass accumulation despite similar water use. There are also many other factors that can influence water use in natural settings that we did not examine here. In our study, we controlled for canopy size and stomatal density of the three cytotypes. However, naturally occurring cytotype-specific differences in leaf and canopy architecture may influence relative stomatal densities and water use dynamics, altering fitness outcomes for competing naturally sympatric cytotypes (Laport and Ramsey 2015). For example, as shown here and by others (Barbour 1969; Barbour et al. 1977; Hunter et al. 2001), stomatal size and density vary with genome size in *L. tridentata*, and leaf and canopy size differs significantly between naturally sympatric cytotypes (Laport and Ramsey 2015). Thus, even in areas of cytotype contact subtle cytotype-specific differences in stomatal size, density, and leaf area, have the potential to be major determinants of water use (Barbour et al. 1977; Kropp and Ogle 2015).

Diploids, tetraploids, and hexaploids also inhabit unique abiotic and biotic niche space that could influence water relations. Prior soil analyses and ecological niche models indicate the cytotypes occur in edaphically and climatically differentiated habitat (Laport and Minckley 2013; Laport et al. 2013), and community analyses indicate the cytotypes occur with distinct species associations in areas of contact (Laport et al. 2016). Differences in soil texture, nutrients, or co-occurring species could influence water relations over even relatively short distances. Such edaphic and climatic niche differences may also help structure the unique plant communities in which closely parapatric cytotypes occur (Laport et al. 2016). While the effect of differing inter-specific interactions on the three cytotypes in natural settings remains unknown, community structure has previously been shown to influence the water relations of *L. tridentata* (Kropp and Ogle 2015), and unique inter-specific interactions could influence the ability of the three cytotypes to accumulate water.

Complex cytotype-specific interactions with the ecological setting are likely common. For example, Maherali et al. (2009) found that while diploid and tetraploid *C. angustifolium* had similar photosynthetic rates, indicating similar water use, tetraploids were able to maintain photosynthesis longer than diploids due to better soil moisture depletion. Moreover, this difference appears to only partially derive intrinsically from genome duplication, as artificially generated neotetraploids included in the study only partially recapitulated the physiological differences observed for natural tetraploids, consistent with postpolyploidization adaptation playing an important role in driving evolution in polyploid species (Husband et al. 2016). In *L. tridentata*, similar complex trait interactions may affect water relations. For example, soil nutrient or microbiota differences in the native soil attached to the roots of our wild-collected plants could have altered water use of the three cytotypes in our greenhouse study and should be investigated more generally for polyploid species (Powell and Doyle 2016; Segraves and Anneberg 2016). Moreover, Meinzer et al. (1990) found that epicuticular leaf resin was an important antitranspirant in *L. tridentata* and greater quantities of leaf resin among greenhouse-reared plants collected from a wild population in Nevada (probable hexaploids) were correlated with reduced stomatal conductance. Field and greenhouse observations suggest that tetraploids and hexaploids tend to more often have obviously resinous or hairy leaves (Barbour et al. 1977; Laport personal observation), and diploid plants have more often been observed succumbing to extreme drought conditions than polyploids when regular watering was inadvertently neglected (Laport personal observation). It remains unclear whether the

three cytotypes produce different amounts of epicuticular leaf resin or leaf hairs and whether this may have affected our results, but such observations suggest other traits, such as drought tolerance, may be more important for determining fitness or survival outcomes than water use as evaluated here (Yang 1967; Barbour 1969; Maherali et al. 2009).

Although our results do not support major differences in water use among diploid, tetraploid, and hexaploid *L. tridentata* when grown in a common environment under relatively mesic conditions, our observations do not preclude the possibility that complex abiotic, biotic, and genetic interactions play a significant role in determining cytotype occurrence and community structure in natural settings. We did not measure traits such as epicuticular resin or soil moisture depletion and drought tolerance, which may modulate water relations and survival independent of leaf area or stomatal attributes, and the traits we did measure were widely variable among cytotypes. This variability potentially reflects genetic variation harbored among our wild-collected experimental plants and suggests the sample sizes we employed in our greenhouse study may have been too small to detect subtle cytotypic differences in water use, especially if water use is a phenotypically plastic trait that is able to vary over a wide range of conditions. While differences in water use and other traits may exist between cytotypes, as suggested by prior field studies, additional investigations are required in a common environment to eliminate environmental and genotype- or cytotype-by-environment effects. Such investigations of inter-cytotype differences may reveal multi-character phenotypic differentiation among ploidy levels in *L. tridentata* informing our understanding of polyploid adaptation and evolution.

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