# Understanding the roles of polyploidy and the environment on nordihydroguaiaretic acid variation in *Larrea tridentata*

by

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**Abstract**

Nordihydroguaiaretic acid (NDGA) is the principal compound in the resinous leaf coating of *Larrea tridentata* (creosote bush), the dominant shrub of North American deserts. *L. tridentata* exists as three polyploid races: diploid (2X = 26), tetraploid (4X = 52), and hexaploid (6X = 78). The distributions of these ploidy levels are strongly associated with the three major deserts of the region where diploids primarily reside in the cooler, wetter Chihuahuan desert, tetraploids in the Sonoran desert, and hexaploids in the hot, dry Mojave desert. NDGA is a secondary metabolite of creosote bush that functions to protect plants from biotic and abiotic stressors such as extreme drought, harmful UV radiation, and herbivory. Here, I investigated the role of polyploidy and environmental variables on the production of NDGA by quantifying concentrations from field and greenhouse-grown polyploids. Citizen scientists were utilized to facilitate simultaneous sampling across the entire distributional range of this species, for one full year. Under natural conditions, shrubs produced significantly higher NDGA concentrations than when removed from the harsh desert environment. In field and greenhouse treatments, hexaploids exhibited higher NDGA concentrations than diploids or tetraploids. Within the diploid cytotype, I documented environmental influences on NDGA concentration based on comparisons between a field site experiencing severe drought, a watered field site, and greenhouse-grown diploids. Principal components analysis revealed that NDGA response to environmental variables successfully predicts the current ploidy

distribution of this species. These observations highlight the complexity of plant-environment-genotype interactions and suggest that evolution in production of secondary metabolites may be driven by long-term changes in environmental conditions, and potentially influence species distribution regimes.

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## Introduction

Vegetation is important for ecosystem functions including energy input for food webs, carbon cycling, nutrient cycling through the soil, and water availability (Power 1992, Wolkovich et al. 2012). As environmental conditions are never static, plants must have the capacity to exhibit an immediate response to combinations of variation in resource availability or exposure to environmental extremes, suggesting that a certain degree of phenotypic/genotypic plasticity must exist (Walbot 1996, Lande 2009, Agarwal et al. 2011). These responses can range from short-term physiological changes or relatively fixed longer-term adaptations, which can be important indicators of how abiotic factors influence the traits of individual species, populations, or even whole communities (Chapin III 1987, Diaz and Cabido 1997, Suding et al. 2008). For example, prolonged exposure to cold can delay flowering via down-regulation of transcription factor genes, an effect that is reversible in the next generation (Wang et al. 2003, Reeves et al. 2007, Franks and Weis 2008). Seed traits such as decreased dormancy, slower growth rates, and shorter longevity can arise through high temperature, maternal stress, elevated $CO_2$, and/or rainfall events during development (Nicotra et al. 2010). These effects can either increase or decrease the likelihood of germination depending on the circumstances (Huxman et al. 1998, Meyer and Allen 1999, Steadman et al. 2004, Kochanek et al. 2010). Whether or not these seed traits are adaptive has yet to be determined (Nicotra et al. 2010). Variance in soil nitrogen content can directly influence plant productivity factors such as

number of flowers, foliar density, foliar nitrogen content, total foliar resin concentration, etc. that affect interactions with other plant species (Tilman 1987, Huenneke et al. 1990, Brooks 2003) and higher trophic level organisms (Lightfoot and Whitford 1989, Tripler et al. 2002, Tomas et al. 2011). Over time, selection can prompt the formation of adaptive traits across landscapes, leading to entire suites of traits being exhibited by groups of species occupying similar niches, such as high temperature/water limited ecosystems (Chapin III et al. 1993). For example, smaller leaves are a common feature exhibited by plants in drier environments that must balance carbon intake with water loss (Givnish 1979, Dudley 1996). This phenomenon has recently been documented in bluebunch wheat grass, *Pseudoroegneria spicata*, where populations in warm, arid environments produce narrower leaves compared with conspecifics in milder climate zones (Bradley St. Clair et al. 2013). Root to shoot ratios are generally larger for plants in dry environments as water and nutrient uptake are constant limiting resources for plant growth (Pallardy 1981, Chapin III et al. 1993). Thus, examining how extreme environmental conditions have shaped overall physiological responses serves as a strong indicator of how xerophytic plants respond to abiotic stimuli over evolutionary time.

Polyploidization (whole genome duplication) has the potential to increase heterozygosity and phenotypic plasticity, which may lead to increased fitness and ecological tolerances that afford competitive advantages over their diploid

progenitors (Hijmans et al. 2007, Prentis et al. 2008, Maherali et al. 2009, Chen 2010, Jackson and Chen 2010). This may increase survival and successful propagation in harsher environments (Hancock and Bringhurst 1981, Masterson 1994, Otto and Whitton 2000, Fawcett et al. 2009, Parisod et al. 2010). It is estimated that 30% - 80% of all angiosperms have polyploidy in their history, rendering it a rather common phenomenon (Grant 1981, Otto and Whitton 2000, Soltis et al. 2003, Blanc and Wolfe 2004, Soltis et al. 2009). The consequences of polyploidization on gene expression have been examined in natural and artificial polyploids to better understand what triggers whole genome duplication in natural populations, as well as how increased genetic information influences gene expression. In *Rosa* sp., high frequency of diploid gamete production has been induced by high temperatures, suggesting that adverse environmental conditions could facilitate polyploidization events (Pecrix et al. 2011). It has also been shown that colchicine-induced polyploids of the plant *Petunia* 'Mitchell' exhibited not only increased leaf and cell size, but also increased concentrations of specific secondary metabolites in the flavonol profiles of the polyploid species (Griesbach and Kamo 1996). Recently, differential abundances of secondary metabolites in allotetraploid *Arabidopsis* sp. were associated with targeted microRNA regulation of the gene for small molecule methyltransferase (a key enzyme in metabolite synthesis) (Ng et al. 2011). Differences in ploidy-induced genetic variability often contribute to habitat differentiation between polyploid cytotypes and their progenitors (Soltis et al. 2003). High degrees of habitat

differentiation may result in cytotypes that occupy different geographic ranges with very little overlap (Soltis 1984, Soltis and Soltis 1989). Such a polyploidization/differentiation event has occurred in the deserts of southwest North America where the xerophytic shrub, *Larrea tridentata* (creosote bush), dominates the landscape. This is the sole member of the genus existing outside of South America, where there are four sister species *L. divaricata, L. cuneifolia, L. nitida,* and *L. ameghinoi* (Hunziker et al. 1978, Lia et al. 2001, Laport et al. 2012). *L. tridentata* exists in three ploidy levels: diploid (2X = 26), tetraploid (4X = 52), and hexaploid (6X = 78) and the distribution of these three cytotypes is strongly associated with the ranges of the three major deserts of the region. Diploids are associated with the Chihuahuan Desert, tetraploids with the Sonoran Desert, and hexaploids with the Mojave Desert (Barbour 1969, Yang 1970, Hunziker et al. 1972, Hunter et al. 2001). During the time of the last major glacial maximum in the Pleistocene epoch, from >40,000 ybp to about 11,000 ybp, the majority of the current *L. tridentata* high elevation distribution range was occupied by evergreens (juniper and pine). The immense climactic shift that occurred into the Holocene, following the retreat of the Wisconsin Glaciation, caused the region to become drastically hotter and drier. This allowed the rapid expansion of *L. tridentata* from southern glacial refugia throughout the newly formed desert, as the evergreen woodlands retreated (Wells and Hunziker 1976). The mode of ploidy separation across the North American deserts is poorly understood, but one hypothesis is that differentiation of the cytotypes evolved as a

result of exposure to increasingly higher temperatures and aridity during the early-Holocene north-westward range expansion (Hunter et al. 2001).

*L. tridentata* produces the phenolic compound, nordihydroguaiaretic acid, hereafter, NDGA (Gisvold 1948, Duisberg 1952). This compound is the most prevalent component of its waxy resinous leaf coating (Mabry 1977). The resin functions to reduce evapotranspiration, protect from damaging solar wavelengths, and deter herbivores (Rhoades 1977), and comprises 10-20% of the dry leaf mass (Seigler et al. 1974, Mabry 1977). Phenolic compounds compose the majority (80%) of total leaf resin, of which NDGA is the major component. Overall, this compound alone accounts for approximately 5-10% of dry leaf mass (Mabry 1977). NDGA also has relevance outside of plant biology as its derivatives exhibit a variety of medicinal functions as powerful antioxidants, as well as in the treatment of cancer, diabetes, viral infections, etc. (Lambert et al. 2004, Lambert et al. 2005, Meyers et al. 2009, Lu et al. 2010). Previous studies have quantified NDGA content in natural populations of creosote bush to document its occurrence, abundance, and where it resides within the shrub (Gisvold 1948, Duisberg 1952, Hyder et al. 2002). NDGA be found throughout the plant in flowers, leaves, green stems, and woody stems of the shrub, with the highest concentrations in leaves and green stems (Hyder et al. 2002). One study analyzed the effects of experimental water and nutrient supplementation on seasonal NDGA production for period of 21 months, at one site near Palm Desert, California and reported addition of water to shrubs significantly reduced foliar NDGA

concentrations (Gonzalez-Coloma et al. 1994). Downum et al. (1988) monitored how NDGA concentration varied within and between populations throughout the growing season in the Sonoran Desert. These authors reported both geographical and seasonal variation in NDGA with decline in concentration from northern to southern latitudes, and significant decrease between the months of April and July. These studies have provided insight on how shrubs growing under natural conditions may respond to changes in their environment in terms of secondary metabolite (NDGA) production, however, these studies are lacking in the breadth of their sampling. I investigated this same fundamental concept by employing a more informative means to understand how the species is responding to its environment through simultaneous sampling from all cytotypes, throughout its entire distributional range, for an entire year. Then I will compare the responses of shrubs in natural conditions to their ploidy equivalents growing in a common garden environment. To date, no studies have analyzed NDGA variation in *L. tridentata* in this way.

The aim of my study is to quantify NDGA concentrations across the polyploid races of this extreme drought-tolerant desert shrub. Specifically, I am interested in understanding: (i) if ploidy level is directly correlated to NDGA concentration, (ii) how environmental variables (temperature, precipitation, solar irradiation) influence NDGA concentration, and (iii) if any interactions between these factors may be an influence on current ploidy distribution.

## Materials and Methods

*Sample collection*

I selected *L. tridentata* shrubs for field sampling based on central locations within their polyploid distribution ranges. Two plants were selected at each of 20 sites in Arizona, California, Nevada, New Mexico, and Texas. At each site, plants were tagged with a unique sample ID to designate plant number (1 or 2). Each of the three polyploid races were represented; diploid $n = 4$, tetraploid $n = 4$, hexaploid $n = 12$ (Table 1). Representative plants from each region were tested to confirm ploidy level following guard cell measurement protocol (Hunter et al. 2001). I employed a total of 22 Citizen Scientists to facilitate simultaneous collection of leaves from each plant at monthly intervals from October 2010 through September 2011. These individuals were selected through various methods including previous collaboration with academic advisors, petitions to botanical organizations, and/or outside associations with other participating collegaues. This sampling method was ideal as Citizen Scientists were able to collect leaves efficiently, allowing the compilation of a sample set that encompassed the entire range of the species for a full calendar year. Each month, collectors pulled leaves from the two assigned plants at each site. The collectors placed the fresh leaves in a small manila envelope labeled "A" (plant 1) or "B" (plant 2), which was then mailed in a standard letter envelope directly to our laboratory in the Department of Biological Sciences at Salisbury University. Collectors often sent variable numbers of leaves from each plant. I weighed leaves

from each of the two plants (A and B) and sorted them to generate three replicates per plant (one leaf = one replicate), labeled (A/B 1, 2, and 3).

I selected individuals for greenhouse analysis from a previously established group of plants. I only selected shrubs that were grown from seeds collected at centrally located sites within the polyploid distribution ranges for this study. Each of the three polyploid races were represented equally: diploid $n = 10$, tetraploid $n = 10$, hexaploid $n = 10$ (Table 1). These shrubs were grown from *L. tridentata* seeds collected from field sites in Arizona, California, Nevada, New Mexico, and Texas from August 1992 – October 2005. Dr. Richard Hunter and a group of undergraduate researchers facilitated germination, planting, and watering of seeds. The seeds were germinated on agar plates made from 1X Hoagland's Solution, 6g Difco Purified Agar, and 1.0mL Plant Preservative Mixture (PPM). Once germinated, seedlings were planted in 9.0 ounce plastic containers from July 2004 – June 2006. Containers were filled with a soil preparation consisting of 1.89 L mixed sand, 0.05 L lime, and 0.41L garden soil. Containers were filled with soil up to 0.50 inches from the top brim, and then soil was wetted with 50mL Hoagland's solution before germinated seedlings were inserted. All plants were watered every 2-4 days with tap water and kept in a common area of the greenhouse at Salisbury University. As shrubs grew larger, they were transplanted to larger containers (18 ounce plastic containers, 24 ounce plastic soda bottles, or 2L plastic soda bottles) as needed. The watering regimen varied based on the size of each independent shrub at each growth stage, and the varying ambient

temperature and hours of sunlight in the greenhouse throughout the year. This process was designed to eliminate water stress for each individual by adjusting the administered volume at each watering based on the moisture level of the soil. Shrubs in 9 ounce containers received 10-20mL when soil was damp and 30- 50mL when dry, Shrubs in 18 ounce containers/24 ounce bottles received 10-30mL when soil was damp and 40-60mL when dry. Shrubs in 2L bottles received 60-65mL when soil was damp and 175-200mL when soil was dry. I continued this watering regime throughout the period of the study. At the onset of sampling, I chose individuals based on current health condition in that there were a sufficient number of open, green leaves. Health of shrubs was monitored throughout the study to ensure that the stress of removing leaves was not so great as to kill the plant. I collected two leaves from each plant, monthly, from October 2009 through August 2010.

*NDGA extraction and HPLC analysis*

I selected entire leaves (both leaflets) for processing from both greenhouse and field collections. In cases where leaves were broken, I assembled fragments to approximate the size of an intact leaf. I weighed each leaf, placed it in a 1.5 mL microcentrifuge tube, and then stored it in a dark drawer. NDGA was extracted from leaves by solubilization in 100% HPLC-grade methanol. First, I ground leaves with $SiO_2$ sea washed sand (Fisher Scientific, CAS 14808-60-7) using a 2mL microcentrifuge tube and a Kontes pestle. Once homogenized, I added 1 mL of 100%

MeOH to the mixture and ground again. This mixture was centrifuged at 13,000 rpm for 5 minutes. I transferred the resulting supernatant to a new 1.5 mL microcentrifuge tube and stored at -20°C until further analysis. Prior to HPLC, I filtered samples using Discovery® DSC-C8 1mL (100mg) solid phase extraction filters (Supelco Analytical Cat. No. 52707-U) purchased from Sigma-Aldrich. Samples were allowed to reach room temperature before filtering. I used HPLC-grade methanol for all preparations. For each sample, I wetted a filter with 80% MeOH, pushed the sample through into a clear, 1mL Fisherbrand screw thread vial (12 x 32mm), and sealed with a Fisherbrand blue 9mm PTFE/Rubber closure. All dilutions were made in 80% MeOH.

I measured nordihydroguaiaretic acid concentrations on a Perkin-Elmer Series 200 HPLC system with a Spheri-5 RP-8 5 μm (30 x 4.6 mm) column at 40.0 °C and UV detection at 218 nm. TotalChrom Chromatography Data System (CDS) software was used to record the data. E-pure water was used as solvent A, and HPLC-grade methanol (100%) for solvent B. Sample injection volume was 20.0 μL. The pump equilibrated at 8.0 minutes with 40%A/60%B at a flow rate of 1.2 mL/min. At 10.0 minutes, solvent concentration was increased on a linear gradient to 100.0%B and maintained for the duration of the mobile phase (end time 15.0 minutes)

I determined NDGA concentrations in field ($n$= 1391) and greenhouse ($n$= 620) sample solutions using a curve generated from standard solutions at the following concentrations from a 1.0 mg/mL stock solution: 0.01, 0.02, 0.05, 0.075, 0.10, 0.15, 0.20, and 0.30 mg/mL. NDGA generally eluted between 1.0 and 2.0

minutes in one and/or two peaks (occasionally peak splitting occurred and a third peak was measured). I included an internal 0.075 mg/mL standard with all sample runs for comparison to experimental samples to ensure consistent identification of NDGA peak position. I report final concentration as μg NDGA/mg dry leaf weight.

*Climatological data*

I obtained climatological information for the focal year (October 2010-September 2011) of the field study using the Climatology Resource for Agroclimatology Daily Averaged Data site (NASA/POWER). Records for precipitation (mm/day), average air temperature (degrees C), and solar irradiation (measured as insolation incident on a horizontal surface in $kWh/m^2/day$) were collected for this duration. I entered GPS coordinates into the database in order to obtain information from an area geographically closest to each of the sampling sites.

I compiled climatological information for temperature and precipitation for long-term weather analysis from the Western Regional Climate Center database. I chose six weather stations from central locations within each desert range: Chihuahan desert (diploid): five stations in New Mexico and one station in Texas; Sonoran desert (tetraploid): six stations in Arizona; Mojave Desert (hexaploid): four stations in California and two stations in Nevada. The periods of records range from 1892 – 2012 (Table 2).

*Statistical analysis*

All analyses were conducted using STATISTICA Version 10 (Stat Soft, Inc., 2011). Unforeseen environmental parameters experienced by shrubs within the diploid distribution range at the Big Bend National Park and El Paso sites prompted separate analyses for these two sites. The Big Bend site experienced severe drought conditions, while the El Paso site was located on a university campus and was regularly watered by the irrigation system. Based on these conditions, I devised a diploid comparison analysis to compare mean monthly and annual NDGA concentrations of shrubs from Big Bend and El Paso to that of greenhouse-grown diploids. NDGA concentrations, as well as mean annual precipitation and temperature for the field sites, were analyzed by one-way analysis of variance (ANOVA) and Tukey's HSD test for multiple comparisons. Data were examined for deviations from normality and homogeneity of variance using Box-Cox tests. To avoid skewing data for the other diploid collection sites, the Big Bend and El Paso sites were removed from further analyses as it was known that these shrubs were exposed to inconsistencies not present throughout the remainder of the region.

I averaged the monthly mean NDGA concentrations between select field sites based on relative geographic proximity to one another (site grouping). Two sample sites in the 4X distribution range (Tucson), and nine sample sites in the 6X distribution range (Inyokern (2)/Ridgecrest (2) and Las Vegas (3)/Boulder City (2)) were grouped. No sites from the 2X distribution range were grouped. Thus, from the

20 original field sites, site grouping allowed analysis of a total of two sites in the Chihuahuan desert (2X), three in the Sonoran desert (4X), and five in the Mojave Desert (6X).

I calculated monthly mean NDGA concentration for each greenhouse plant by taking the mean concentration of the two leaves sampled each month. I calculated monthly mean concentration for each field site by taking the mean of the six measurements at each site (three replicates per plant, two plants per site). NDGA concentration is reported as μg/mg leaf weight ± SEM. I calculated mean annual concentration for each ploidy race by taking the mean of all monthly measurements for each plant within that ploidy. Data were examined for deviations from normality and homogeneity of variance using Box-Cox tests. For analysis within treatments, I transformed greenhouse shrub NDGA concentration values using Box-Cox transformation ($\lambda = 0.489$), and I transformed field NDGA concentration values using the Box-Cox ($\lambda = -0.283$). For analysis between treatments, I transformed all NDGA concentration values using the Box-Cox transformation: ($\lambda = -0.191$). I analyzed the results from each ploidy level (2X, 4X, 6X), within and between each treatment (greenhouse or field), by one-way (ANOVA) and Tukey's HSD test for multiple comparisons.

For field samples, I conducted simple linear regression separately for each environmental variable (temperature, precipitation, and solar radiation) against NDGA concentration. Environmental variable values were transformed using Box-

Cox transformations for temperature ($\lambda = 0.046$) and solar radiation ($\lambda = 0.855$). Precipitation values were transformed using square root transformation. I conducted comparisons using one-way ANOVA with univariate tests for significance. Effects were considered significant at $\alpha = 0.05$. To determine the influence of environmental variables on mean annual NDGA concentrations, I conducted a principal components analysis (PCA), grouping variables by ploidy.

I used climatological information for the focal year of the field study (temperature, precipitation, and solar radiation) to compare the mean annual temperatures (°C) ± SEM, mean annual precipitation (mm/day) ± SEM, and mean annual solar irradiation ($kWh/m^2/day$) for each desert/ploidy distribution range using one way ANOVA and Tukey's HSD test for multiple comparisons. I also compared climatological information for each desert/ploidy range using data for the focal year and long-term years for the following: mean annual precipitation, mean July temperature, mean January temperature, mean summer temperature (June-August), and mean winter temperature (December-February). Analysis was conducted using one-way ANOVA and Tukey's HSD test for multiple comparisons. I made these comparisons to document any differences between the focal year and the long-term weather patterns of the region.

**Results**

For the field samples, NDGA concentrations were 55.026 ± 4.313 SEM μg/mg leaf weight (2X), 52.875 ± 2.383 SEM μg/mg leaf weight (4X), and 65.977 ± 3.155 SEM μg/mg leaf weight (6X). The three ploidy levels exhibited significantly different mean annual NDGA concentrations ($F_{2, 114} = 7.884$; $p < 0.001$). The mean annual concentrations for 2X and 4X plants were not significantly different ($p = 0.711$), while the mean annual concentration for 6X plants was significantly higher than both 2X plants and 4X plants ($p < 0.05$ and $p < 0.001$, respectively). For the greenhouse samples, NDGA concentrations were 20.315 ± 1.169 μg/mg leaf weight (2X), 17.660 ± 0.914 μg/mg leaf weight (4X), and 25.543 ± 0.981 μg/mg leaf weight (6X). There were significant differences in the mean annual concentrations between the ploidy levels ($F_{2, 316} = 17.896$; $p < 0.0001$). The differences between mean annual concentrations followed the same pattern as observed in field samples: 2X and 4X plants were not significantly different ($p = 0.213$), 6X plants exhibited significantly higher concentration than 2X and 4X ($p < 0.001$ and $p < 0.0001$, respectively). Overall, mean annual field NDGA concentrations were significantly different from greenhouse concentrations ($F_{1, 434} = 436.09$; $p < 0.0001$). NDGA concentrations observed in field samples were higher than greenhouse samples ($p < 0.0001$) (Figure 1).

Mean annual temperature and precipitation at the Big Bend and El Paso sites were not significantly different from each other, or from the other samples sites

within the Chihuahuan desert ($p > 0.05$). There were significant differences between mean annual NDGA concentrations ($F_{2, 124} = 38.954, p < 0.0001$). Greenhouse-grown 2X plants and El Paso site plants were not significantly different ($p = 0.095$), while both exhibited significantly lower annual NDGA concentrations than the field plants at Big Bend ($p < 0.0001$). Mean NDGA concentration of plants at Big Bend were consistently higher than El Paso or greenhouse-grown 2X across all months of the year, with the exception of April (Figure 3).

Simple linear regression revealed that precipitation alone was not a significant predictor of NDGA concentration, while temperature and solar irradiation were significant predictors (Table 3). Principal components analysis revealed that field sample sites group together by ploidy when NDGA is analyzed as a response to interactions of environmental variables. The shrubs at the El Paso site grouped closely with other field diploids, while shrubs at the Big Bend site fell between diploid and tetraploid groupings (Figure 4 and Table 4).

Temperature and precipitation data for the focal year of the field study and for the long-term period were analyzed. Long-term climate data reveal that the three desert ranges exhibited significantly different mean annual temperatures ($F_{2, 15} = 12.426, p = 0.00066$). Mean annual temperatures in the Sonoran (4X) and Mojave (6X) deserts were not statistically different ($p > 0.05$), while the mean annual temperature in the Chihuahuan (2X) desert was significantly lower than the Sonoran and Mojave deserts ($p < 0.001$ and $p < 0.05$, respectively). Temperature differences

during the focal year of the field study did not exhibit the same patterns as long term climate records ($F_{2,7} = 15.786$, $p = 0.00255$). Mean annual temperatures in the Chihuahuan (2X) and Mojave (6X) deserts were not statistically distinct ($p > 0.05$). Mean annual temperature in the Sonoran (4X) desert was statistically higher than in both the Chihuahuan and Mojave deserts ($p < 0.05$ and $p < 0.005$, respectively). Long-term records show that the Chihuahuan and Sonoran deserts did not exhibit statistically different mean annual precipitation values ($p > 0.05$); however, both of these deserts do have higher mean annual precipitation values than the Mojave desert ($p < 0.0005$ and $p < 0.005$, respectively). For the focal year, the Chihuahuan desert experienced significantly lower mean annual precipitation values than both the Sonoran and Mojave deserts ($p < 0.05$ and $p < 0.005$, respectively). The Sonoran and Mojave deserts did experience significantly different mean annual precipitation ($p < 0.05$).

## Discussion

The data I present here are the result of a novel and integrative sampling method, carried out through the development of a unique experimental design which no previous studies have done. I employed a dedicated group of Citizen Scientists who facilitated simultaneous sample collection over a vast geographic span for a substantial time period. Additionally, comparison to a common garden environment was made possible through sampling of a previously established and accessible group of healthy *Larrea tridentata* polyploids. My successful incorporation of these factors allowed me to analyze this system in a way that was never before possible. Here, I highlight the four key results from my study: First, I have documented variation in NDGA concentration in naturally growing individuals that successfully predicts the current ploidy distribution of *Larrea tridentata*; an effect that is explained by interactions between the environmental variables of temperature, precipitation, and solar radiation (Figure 4). Second, by comparing variation in NDGA production within select shrubs of the same ploidy level (Big Bend and El Paso) to their greenhouse equivalents, I have also demonstrated that the environment can play a significant role in NDGA production independent of ploidy level, where consistent watering at the El Paso site influenced the production of NDGA levels similar to greenhouse-grown shrubs (Figures 2 and 3). Third, I have documented that when grown in a common garden environment, shrubs produce significantly lower NDGA concentrations than when grown under natural, field conditions. Fourth, while there

are differences in NDGA concentrations between ploidy levels, they do not occur in a step-wise fashion with increase in ploidy; however, in both the field and the greenhouse, hexaploid shrubs exhibited significantly higher NDGA concentrations than diploids or tetraploids (Figure 1). Overall, these results reveal that there is great deal of complexity involved in how this species responds to environmental cues, and how this correlates to increases in genetic information. It is important to note that while climatological data show the focal year of the study did not exhibit the same patterns as long-term records, PCA still separated the ploidy levels based on NDGA production and the interactions of environmental variables. This suggests that while deviations from the average within a one year period may contribute to variation in NDGA concentration, they are not likely to be strong enough to alter distributional patterns within this system. This lends support to the idea that sustained climatic shifts may be driving ploidy differentiation and subsequent changes in phenotypic expression in *L. tridentata* that are directly related to NDGA production.

Understanding how *Larrea tridentata* has evolved to deal with extreme environmental conditions through the production of NDGA is a complex problem that may be dependent upon gene expression. We first must consider that plants are sessile in nature and therefore cannot avoid constant and simultaneous exposure to various biotic and abiotic stresses. Instead, individuals must respond to adverse conditions within their tolerance ranges by coordinating changes in physiological and biochemical mechanisms (Chapin III 1987, Agarwal et al. 2011, Osakabe et al. 2012).

Such responses are regulated by changes in gene expression; a process that is mainly controlled by transcription factors (TFs) (Singh et al. 2002, Agarwal et al. 2011). One of the dominant TF families found in higher plants is the WRKY family which has been documented to play key roles in plant responses to stress (Eulgem et al. 2000, Rushton et al. 2010, Agarwal et al. 2011) that induce WRKY mRNA, WRKY proteins, and DNA-binding activity (Wang et al. 1998, Hara et al. 2000). Interest in the extreme drought tolerance of *L. tridentata* prompted researchers to search for genes encoding drought stress-induced TFs in this species. Ten such genes were identified, one of which is *LtWRKY21*, a gene localized to the nucleus and highly expressed in seeds. LtWRKY21 was found to be an activator of abscisic acid (ABA) signaling (Zou et al. 2004), a hormone pathway that plays key roles in seed development, dormancy, germination, and cell division, as well as to environmental stresses such as drought, UV radiation, and elevated $CO_2$ (Zeevaart and Creelman 1988, Rock 2000). Zou et al (2007) documented that *LtWRKY21* expression is induced by elevated $CO_2$, water stress, and ABA in a way that activates the ABA (stress) signaling pathway. Also, LtWRKY21 enhanced expression of *HVA1*, a stress-inducible gene, and suppressed genes required for seed germination. This suggests that *LtWRKY21* is functioning to induce stress tolerance under extreme environmental conditions in *L. tridentata*. Given the results of my study which document increased NDGA concentration in field shrubs and hexaploids, future studies may investigate a potential connection between this and the increased expression of *LtWRKY21* in

shrubs under adverse environmental conditions. As polyploidization has the potential to cause additive or non-additive expression of genes in a dosage dependent manner (Jackson and Chen 2010), an increase in ploidy level has the potential to alter *LtWRKY21* expression across the cytotypes of *L. tridentata*, increasing tolerance to more extreme conditions. Additionally, WRKY proteins have also been implicated in the biosynthesis of secondary metabolites (Eulgem et al. 2000), which may provide a mechanistic link between polyploidization, plant stress response, and NDGA production. The synthesis of secondary metabolites is associated with an array of enzymes to facilitate chemical modifications in the biosynthesis pathway. In *L. tridentata,* (+)-larreatricin hydroxylase is a known polyphenol oxidase (PPO) that participates in the biosynthetic pathway of NDGA. (+)-larreatricin hydroxylase facilitates conversion of the precursor molecule larreatricin to hydroxylarreatricin, *in vivo*. Researchers were able to clone the encoding gene for this PPO (Cho et al. 2003). As this study did not specify the polyploid level of the plants used for analysis, future studies of gene expression could be conducted across cytotypes using this model of NDGA biosynthesis to determine the role of ploidy in expression of key secondary metabolite synthesis genes, and then draw parallels to expression of key stress response genes such as *LtWRKY21*. Such studies may provide a genetic explanation for the success of increased ploidy cytotypes in deserts with higher temperatures and aridity.

As a whole, plant responses are the result of genotypic/phenotypic plasticity, often evolved from long-term adaptations to changes in local or global climactic conditions (Dynesius 2000, Prentice 2000). Laport et al (2013) used ecological niche modeling to determine that cytotype distributions in *Larrea tridentata* were structured via multiple factors including ecological adaptation, where mean annual temperatures and the timing of precipitation regimes across the three deserts made the largest contributions to the individual cytotype models. Thus, understanding how *L. tridentata* has adjusted to these long-term environmental differences, is important for predicting how entire biomes will adapt to global warming and the subsequent shifts in regional weather patterns in the future. Many studies have focused on examining the ways plant distributions have changed in response to changing regional/global climactic conditions (Davis 1981, Dynesius 2000, Prentice 2000). For example, range expansion of the Dutch John Mountain population of pinyon pine, *Pinus edulis*, was documented to occur over a multidecadal time scale due to alternating rain-drought events (Gray et al. 2006). At the onset of the Holocene, tree species across the globe began to expand their ranges northward and into higher altitudes in concert with glacier retraction (Davis 1981, Bennett et al. 1991, Montoya et al. 2007). During this period, mean global temperature increased, leading to large increases in atmospheric $CO_2$, opening novel niches and allowing invasions of new habitats and ranges (Adams et al 1990, Clark et al. 2009). In North America, the onset of deglaciation triggered the northward range expansion of *Larrea tridentata* throughout the southwest region

of the continent. Over time, increased temperatures, high aridity, overgrazing by livestock, and overall decrease in land quality has allowed this extremely tolerant shrub to dominate the landscape (Wells and Hunziker 1976, Hunter et al. 2001, Lia et al. 2001, Duran et al. 2005, Laport et al. 2012). As global air temperature has increased by 0.74 ± 0.18 °C over the past century, and is predicted to increase by another 1.8°C to 4°C by the end of the next century (Pecrix et al. 2011), predicting future range expansion in this species is of particular interest due to its potential widespread dominance under future adverse conditions. Shafer et al (2001) investigated the potential shifts in North American tree and shrub taxa distributions in the coming century using several predictive climate models. According to this study, *L. tridentata* distribution is simulated to extend its range far past its current northern limits in southern Nevada and Utah, and potentially exist as far north as areas of southern Washington. As *L. tridentata* exhibits allelopathic properties (Elakovich and Stevens 1985), expansion of its range to novel areas may exert negative effects on natural flora of northern latitudes. These models also predicted areas where *L. tridentata* may not exist within the next century, including parts of southern California, Arizona, Texas, and the Baja peninsula. Extinction of *L. tridentata* from current distributional ranges may exert negative effects on natural fauna of the region as creosote bush dominance and evergreen foliage make the species central to mammalian and insect life in providing food, water, and shelter (Meyer and Karasov 1989, Cane et al. 2006). It is interesting to note that both *Neotoma lepida* (desert

woodrat) and *Liguirotettix coquilletti* (desert grasshopper) preferentially forage on and colonize creosote shrubs that exhibit lower NDGA concentrations than neighboring shrubs (Greenfield et al. 1989, Sorensen et al. 2005). Based on my results, shrubs occupying the harshest climate zones produce overall greater amounts of NDGA. Thus, increasing global temperature and decrease in frequency of precipitation regimes may result in increased NDGA production, directly affecting plant-plant and higher trophic level interactions. Additionally, polyploid distribution may no longer be associated with the three dominant deserts of southwest North America. The implications of creosote expansion to new and extinction from current ranges may have profound impacts on entire ecosystems in future scenarios.

Overall, this study has revealed novel insight into the roles of secondary metabolites in plant distribution regimes and highlights the complexity of plant-environment-genotype interactions. By quantifying NDGA production in naturally growing *Larrea tridentata* shrubs I have documented that current ploidy distribution appears to be associated with interactions of environmental variables and response via secondary metabolite production. It is also suggested that polyploidy plays a determining factor in NDGA production, as the higher ploidy levels produced significantly higher NDGA concentrations. Due to the unique distribution regime of this species, differential NDGA production may be exerting unique landscape-level effects across the dominant southwest deserts. Observations that greenhouse-grown polyploids produce lower total NDGA concentrations suggest that extreme

environmental conditions experienced by individuals in the field induce increased secondary metabolite production. Thus, these landscape-level effects may be altered in future climate change scenarios as increased temperatures and aridity may heighten such stress-responses in this dominant species. The methods of this study highlight the importance of integrative research, such as utilizing unique chemical procedures to address complex ecological questions, as well as incorporating a sampling regime in Citizen Science to efficiently sample across large distances, simultaneously. Moving forward, we must learn to be multi-disciplinary in our research and to be open to new techniques in order to expand our abilities to not only ask big questions, but also have the means to address them. Specifically, observing that hexaploid shrubs in both the field and greenhouse produced the highest amounts of NDGA, in concert with the successful prediction of the current ploidy distribution regime using PCA suggest that there may be underlying genetic mechanisms and/or long-term environmental adaptation involved in this particular stress response. Future studies on the effects of polyploidy will enhance our understanding of these interactions as we focus on stress-induced/stress-response genes, as well as genes involved in NDGA biosynthesis.

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**Table 1. Sample Collection Information**

| Ploidy | | Sample Name | Latitude (N) | Longitude (W) | Elevation (m) |
|---|---|---|---|---|---|
| **Diploid** | **Greenhouse** | Rincon, NM 3 | 32°41'43" | 106°59'57" | 1233 |
| | | Paguate, NM 5 | 35°05'36" | 107°20'35" | 1347 |
| | | Belen, NM 6 | 34°15'01" | 107°11'50" | 1704 |
| | | Carrizozo, NM 10 | 33°43'58" | 105°51'42" | 1655 |
| | | Deming, NM 13 | 32°17'33" | 107°42'34" | 1500 |
| | | Socorro, NM 61 | 34°02'36" | 106°36'48" | 1707 |
| | | Las Cruces, NM 72 | 32°17'33" | 106°45'45" | 1185 |
| | | Las Cruces, NM 80 | 32°17'33" | 106°45'44" | 1185 |
| | | McNary, TX 4 | 31°14'05" | 105°45'59" | 1088 |
| | | Presido, TX 32 | 29°34'21" | 104°23'13" | 850 |
| **Tetraploid** | **Greenhouse** | Why, AZ 9 | 32°15'47" | 112°44'12" | 552 |
| | | Picture Rocks, AZ 11 | 32°20'47" | 111°27'33" | 784 |
| | | Picture Rocks, AZ 13 | 32°20'23" | 111°27'54" | 768 |
| | | Picture Rocks, AZ 16 | 32°22'16" | 111°24'23" | 674 |
| | | Picture Rocks, AZ 18 | 32°20'46" | 111°27'10" | 768 |
| | | Marana, AZ 19 | 32°23'36" | 111°17'35" | 619 |
| | | Avra Valley, AZ 20 | 32°22'16" | 111°22'17" | 643 |
| | | Pictrure Rocks, AZ 21 | 32°21'15" | 111°12'59" | 664 |
| | | Lake Havasu City, AZ 36 | 34°28'30" | 114°21'01" | 153 |
| | | Wikieup, AZ 74 | 34°43'53" | 113°41'27" | 722 |
| **Hexaploid** | **Greenhouse** | Inyokern, CA 8 | 35°41'07" | 117°55'04" | 914 |
| | | Ridgecrest, CA 41 | 36°16'52" | 116°25'35" | 695 |
| | | San Bernardino, CA 4 | 34°13'52" | 117°29'34" | 900 |
| | | Yermo, CA 10 | 34°54'20" | 116°49'16" | 762 |
| | | North Edwards, CA 44 | 35°00'54" | 117°57'08" | 755 |
| | | Lucerne Valley, CA 713 | 34°27'35" | 116°54'06' | 886 |
| | | Beatty, NV 2 | 36°54'30" | 116°45'33" | 1007 |
| | | Las Vegas, NV 24 | 36°09'28" | 115°08'16" | 606 |
| | | Las Vegas, NV 39 | 35°58'92" | 114°51'37" | 823 |
| | | Las Vegas, NV 41 | 35°58'92" | 114°51'37" | 823 |

| Ploidy | | Sample Name | Latitude (N) | Longitude (W) | Elevation (m) |
|---|---|---|---|---|---|
| **Diploid** | **Field** | Las Cruces, NM 18 A | 32° 16'11" | 106° 43'02" | 1244 |
| | | Las Cruces, NM 18 B | 32° 16'12" | 106° 42'60" | 1244 |
| | | Alamogordo, NM 19 A | 32° 44'53" | 105° 54'50" | 1261 |
| | | Alamogordo, NM 19 B | 32° 44'01" | 105° 55'31" | 1261 |
| | | Big Bend N. Park, TX 20 A | 29° 19'16" | 103° 12'31" | 1175 |
| | | Big Bend N. Park, TX 20 B | 29° 19.16' | 103° 12'31" | 1174 |
| | | El Paso, TX 23 A | 31° 46'11" | 106° 30'17" | 1179 |
| | | El Paso, TX 23 B | 31° 46'09" | 106° 30'22" | 1180 |
| **Tetraploid** | **Field** | Tucson, AZ 1 A | 32° 16'34" | 110° 56'22" | 719 |
| | | Tucson, AZ 1 B | 32° 16'39" | 110° 56'22" | 722 |
| | | Scottsdale, AZ 2 A | 33° 35'04" | 111° 47'48" | 491 |
| | | Scottsdale, AZ 2 B | 33° 35'04" | 111° 47'47" | 494 |
| | | Phoenix, AZ 3 A | 33° 27'43" | 111° 56'30" | 383 |
| | | Phoenix, AZ 3 B | 33° 27'43" | 111° 56'31" | 387 |
| | | Tucson, AZ 4 A | 32° 14'39" | 111° 10'04" | 865 |
| | | Tucson, AZ 4 B | 32° 14'32" | 111° 10'04" | 853 |
| **Hexaploid** | **Field** | Inyokern, CA 6 A | 35° 37'24" | 117° 48'50" | 766 |
| | | Inyokern, CA 6 B | 35° 37'23" | 117° 48'50" | 762 |
| | | Ridgecrest, CA 7 A | 35° 37'04" | 117° 38'50" | 702 |
| | | Ridgecrest, CA 7 B | 35° 37'04" | 117° 38'50" | 702 |
| | | Inyokern, CA 8 A | 35° 37'35" | 117° 48'57" | 759 |
| | | Inyokern, CA 8 B | 35° 37'35" | 117° 48'57" | 764 |
| | | Yermo, CA 9 A | 34° 54'26" | 116° 49'28" | 605 |
| | | Yermo, CA 9 B | 34° 54'24" | 116° 49'30 | 605 |
| | | Apple Valley, CA 10 A | 34° 33'38" | 117° 09'43" | 930 |
| | | Apple Valley, CA 10 B | 34° 33'39" | 117° 09'46" | 924 |
| | | Desert Hot Springs, CA 11 A | 33° 56'01" | 116° 29'55" | 262 |
| | | Desert Hot Springs, CA 11 B | 33° 56'01" | 116° 29'55" | 268 |
| | | Ridgecrest, CA 12 A | 35° 35'17" | 117° 39'04" | 745 |
| | | Ridgecrest, CA 12 B | 35° 35'17" | 117° 39'04" | 743 |
| | | Boulder City, NV 13 A | 35° 58'38" | 114° 48'56" | 759 |
| | | Boulder City, NV 13 B | 35° 58'38" | 114° 48'96 | 761 |
| | | Boulder City, NV 14 A | 36° 03'04" | 114° 49'25" | 408 |
| | | Boulder City, NV 14 B | 36° 03'04" | 114° 49'26" | 409 |
| | | Las Vegas, NV 15 A | 36° 12'25" | 115° 03'49" | 552 |
| | | Las Vegas, NV 15 B | 36° 12'25" | 115° 03'50" | 557 |
| | | Las Vegas, NV 16 A | 36° 01'46" | 115° 16'15" | 819 |
| | | Las Vegas, NV 16 B | 36° 01'44" | 115° 16'12" | 819 |
| | | Las Vegas, NV 17 A | 35° 58'25" | 115° 15'16" | 830 |
| | | Las Vegas, NV 17 B | 35° 58'25" | 115° 15'15" | 830 |

**Table 1. Sample Collection Information.** Record of name, ploidy, latitude, longitude, and elevation for all samples. Greenhouse: latitude, longitude, and elevation correspond to location where seed was collected. Field: latitude, longitude, and elevation correspond to location of sampled shrub.

**Table 2. Weather Stations: Long-Term Climate Data**

| Desert Region | City, State | Station | Period of Record |
|---|---|---|---|
| Chihuahuan | Alamogordo, NM | 290199 | 1909 - 2009 |
| | Las Cruces, NM | 294799 | 1897 - 2012 |
| | El Paso, TX (WSO AP) | 412797 | 1947 - 2012 |
| | Socorro, NM | 298387 | 1893 - 2011 |
| | Sierra, NM (Caballo Dam) | 291286 | 1936 - 2012 |
| | Carlsbad, NM | 291469 | 1900 - 2012 |
| Sonoran | Phoenix, AZ (WSFO AP) | 026481 | 1933 - 2012 |
| | Florence, AZ | 023027 | 1982 - 2009 |
| | Tucson, AZ (Univ. of AZ) | 028815 | 1894 - 2008 |
| | Mesa, AZ | 025467 | 1896 - 2012 |
| | Chandler, AZ | 021511 | 1912 - 1980 |
| | Laveen, AZ (3 SSE) | 024829 | 1948 - 2012 |
| Mojave | Palm Springs, CA | 046635 | 1903 - 2012 |
| | Inyokern, CA | 044279 | 1940 - 2010 |
| | Lucerne Valley, CA (1 WSW) | 045182 | 1919 - 1973 |
| | Las Vegas, NV (WSO AP) | 264436 | 1937 - 2012 |
| | Boulder City, NV | 261071 | 1931 - 2004 |
| | Daggett, CA (FAA AP) | 042257 | 1948 - 2012 |

**Table 2. Weather Stations: Long-term Climate Data.** List of location, station number, and period of record for each weather station used for long-term temperature and precipitation records.

**Table 3: Simple Linear Regression Significance Results**

| Effect | *SS* | *df* | *MS* | *F* | *p* |
|---|---|---|---|---|---|
| **Temperature** | 0.050 | 1 | 0.050 | 4.079 | 0.046 |
| **Solar Radiation** | 0.144 | 1 | 0.144 | 12.435 | 0.001 |
| **Precipitation** | 0.014 | 1 | 0.014 | 1.120 | 0.292 |

**Table 3. Simple Linear Regression Significance Results** Simple linear regression of all monthly mean NDGA concentration values (Box-Cox transformed) for the field study. Analysis conducted against each environmental variable independently. Monthly mean temperature and solar radiation values Box-Cox transformed, and precipitation values square root transformed.

**Table 4. Principal Components Analysis**

| | Eigenvalue | % Total Variance | Cumulative Eigenvalue | Cumulative % |
|---|---|---|---|---|
| **Factor 1** | 2.289 | 76.284 | 2.288 | 76.284 |
| **Factor 2** | 0.531 | 17.707 | 2.820 | 93.992 |
| **Factor 3** | 0.180 | 6.008 | 3.000 | 100 |

**Table 4. Principal components analysis**. Breakdown of variances and eigenvalues. Variables analyzed: mean annual temperature, precipitation, and solar radiation. Response variable: mean annual NDGA concentration. Grouping factor: polyploid level.

**Figure 1.**

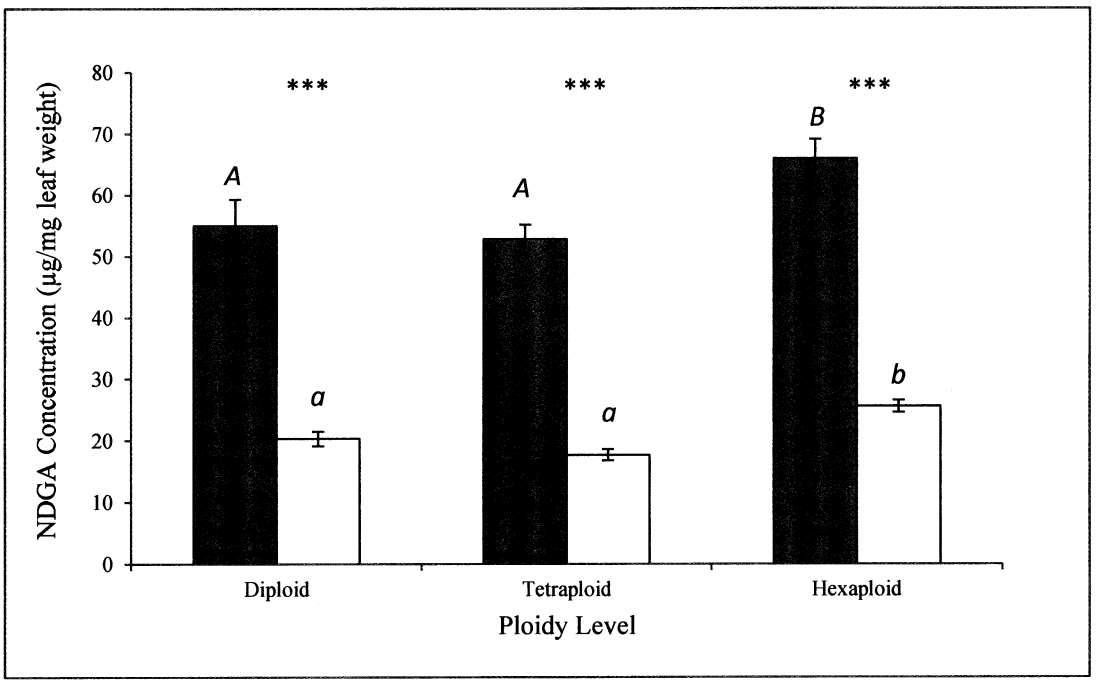
NDGA Concentration (µg/mg leaf weight)
80
70
60
50
40
30
20
10
0
***
***
***
A
A
B
a
a
b
Diploid
Tetraploid
Hexaploid
Ploidy Level

**Figure 1. Mean annual NDGA Comparisons.** Comparison between the mean annual NDGA concentration values of greenhouse v. field samples across the ploidy levels. Shaded bars represent field sample values; open (un-shaded) bars represent greenhouse sample values. Significant differences between treatments within the same ploidy level are indicated by asterisks (*** = $p < 0.0001$). Significant differences between ploidy levels within the same treatment are indicated by a different letter. Field: 2X - 4X, $p = 0.711$; 6X - 2X, $p < 0.05$; 6X - 4X, $p < 0.001$. Greenhouse: 2X - 4X, $p = 0.213$; 4X - 6X, $p < 0.0001$; 2X - 6X, plants $p < 0.0001$. Error bars represent ± SEM.

**Figure 2.**

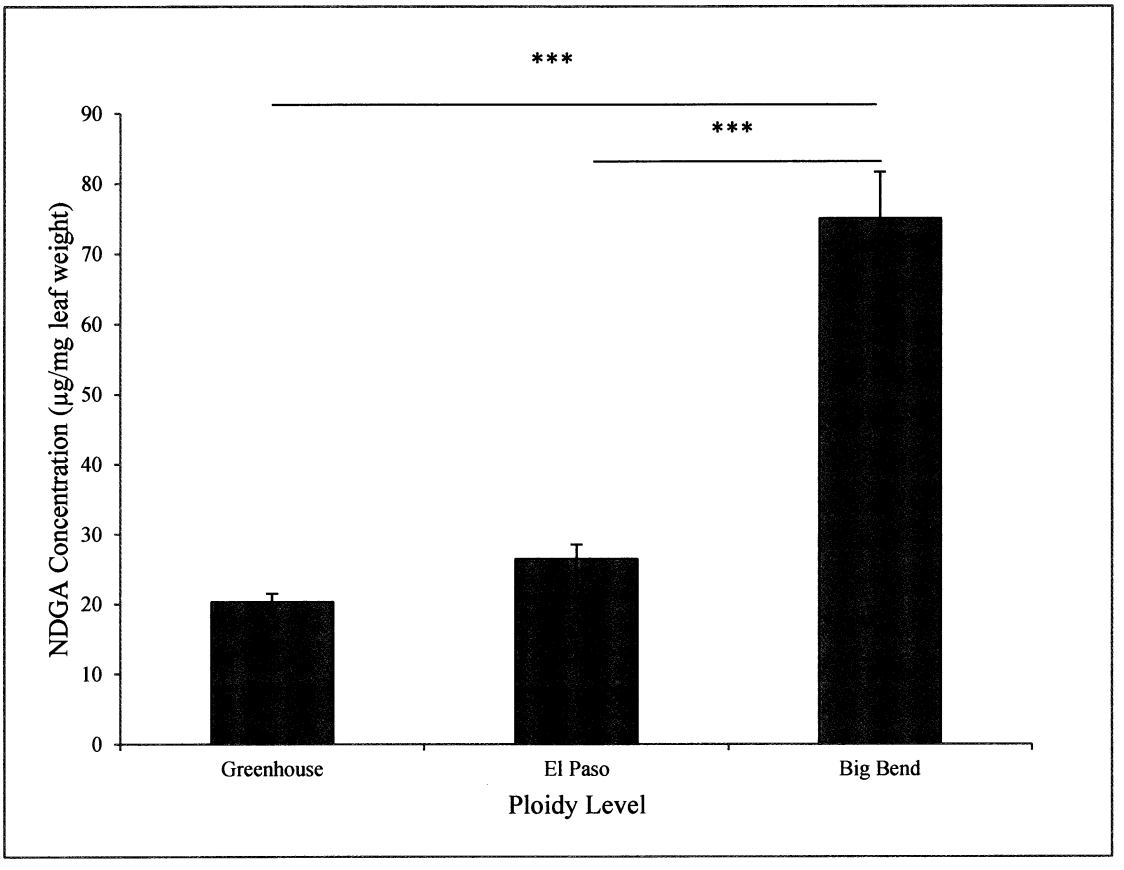
***
***
90
80
70
60
50
40
30
20
10
0
NDGA Concentration (µg/mg leaf weight)
Greenhouse
El Paso
Big Bend
Ploidy Level

**Figure 2. Diploid Comparison Analysis – Mean Annual.** Comparison of mean annual NDGA concentration values between greenhouse diploids, El Paso samples, and Big Bend samples. Error bars represent ± SEM. Significant differences are indicated by asterisks (*** = $p < 0.0001$).

**Figure 3**

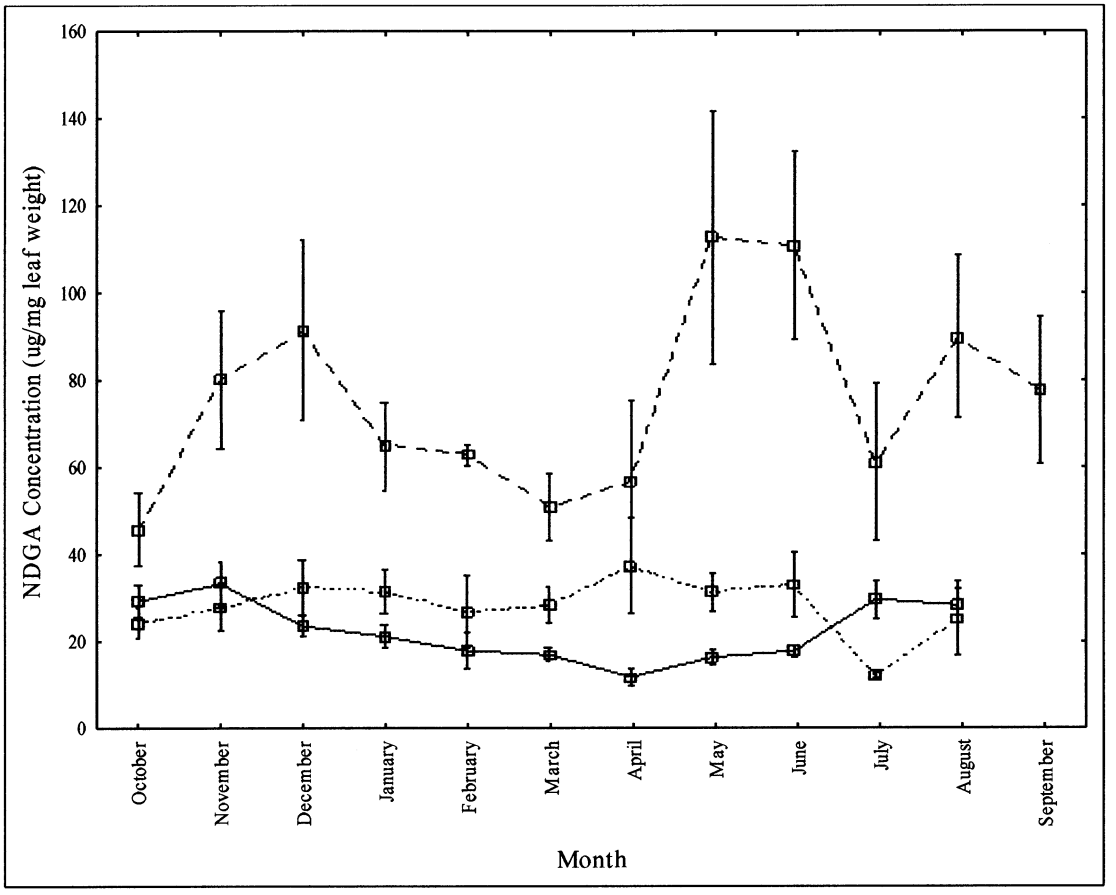
NDGA Concentration (ug/mg leaf weight)
160
140
120
100
80
60
40
20
0
October
November
December
January
February
March
April
May
June
July
August
September
Month

**Figure 3. Diploid Comparison Analysis – Mean Monthly.** Comparison of monthly mean NDGA concentration values of greenhouse-grown diploids, field samples from Big Bend Nat'l Park, and field samples from El Paso. Greenhouse sample year: October2009 – August 2010. Field sample year: October 2010 – September 2011. Error bars represent ± SEM. Green dashed line: Big Bend; Blue dotted line: El Paso; Solid red line: Greenhouse diploids.

**Figure 4**

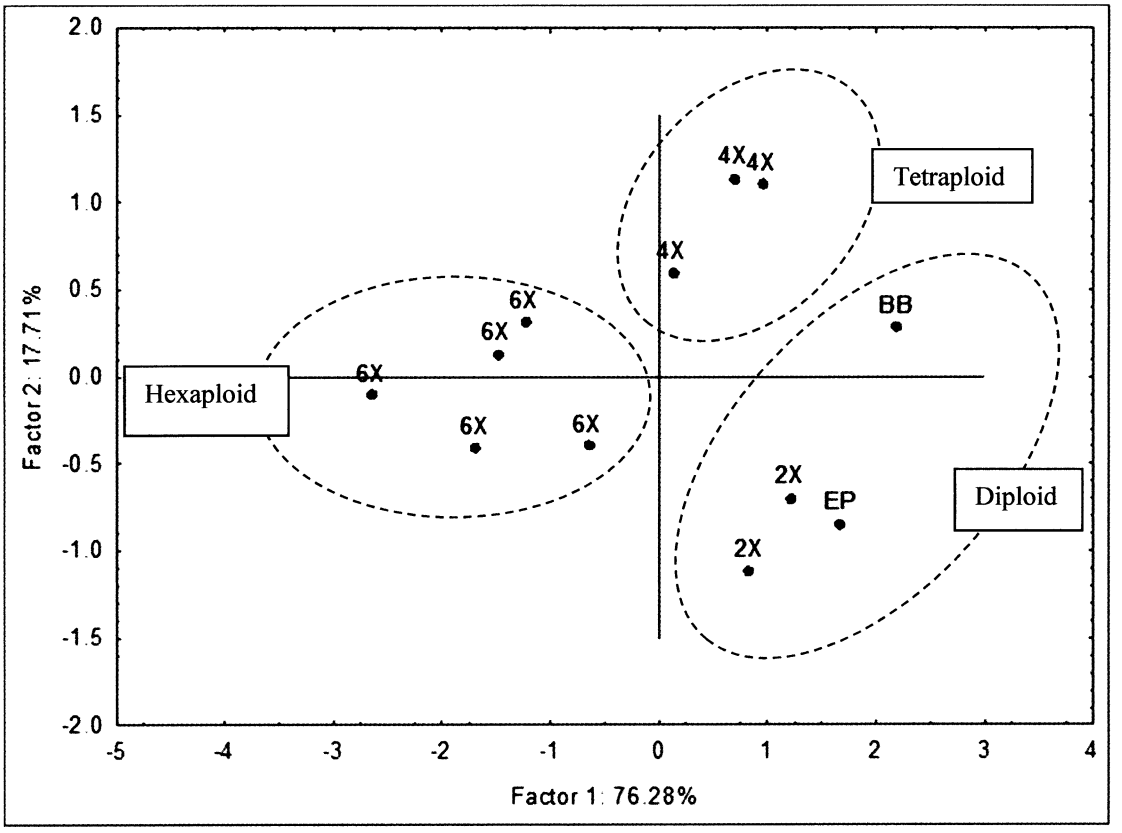
Factor 2: 17.71%
Factor 1: 76.28%
Tetraploid
Hexaploid
Diploid
4X
4X
4X
6X
6X
6X
6X
6X
BB
2X
EP
2X

**Figure 4. Principal Components Analysis**. Mean annual NDGA concentration plotted as a response to the annual means of the environmental variables at each study site. Diploid, tetraploid, and hexaploid plants group together in space relative to the environmental variables, temperature, precipitation, and solar radiation. Outlier sites EP (El Paso) and BB (Big Bend) are included to highlight the environmental influence on responses of shrubs of a particular ploidy level.