6-30-2019

Polyploidy and Water: Relations Traits in Rubus

Tori Crumrine
Western Oregon University

Polyploidy and Water Relations Traits in *Rubus*

By Tori Crumrine

An Honors Thesis Submitted in Partial Fulfillment of the
Requirements for Graduation from the
Western Oregon University Honors Program

Dr. Ava Howard and Dr. Kristin Latham-Scott
Thesis Advisors

Dr. Gavin Keulks
Honors Program Director

June 2019

Acknowledgements

Thanks to Western Oregon University's Walker Award for funding this research, Dr. Jill Bushakra and USDA's NCGR in Corvallis OR for all of the subjects of the study, Drs. Ryan Contreras (OSU), David Woodruff (USDA-FS) and Frederick Meinzer (USDA-FS) for access to labs and equipment, and finally, my advisors Drs. Gavin Keulks, Ava Howard and Kristin Latham-Scott for guidance and support.

Table of Contents

Abstract

Unlike many other organisms, plants have the ability to survive and even thrive with more than two sets of chromosomes, known as a condition called polyploidy. How this impacts the physiology of those plants is convoluted and needs further study. In my attempt to relate polyploidy to various water relations traits, I completed pressure-volume curves on plants within the blackberry family, *Rubus* Subgenus *Rubus* with ploidies ranging from 2-10. As supported by many previous studies, stomatal anatomy characteristics did vary significantly with ploidy. I also found that nighttime, but not daytime, water potential showed significant variation due to ploidy. There was not significant variation due to ploidy in any of the pressure-volume measurements studied. Additional collection of gas exchange data would provide more insight into these results. These studies will begin to fill a knowledge gap that currently exists in plant physiology. Additionally, results from this research could aid current agricultural dilemmas, such as feeding the growing human population in a changing climate.

Introduction

The effects of polyploidism, or additional complete sets of chromosomes, on the physiology of plants is not yet fully understood and is a topic of current interest. These effects include impacts on plant morphology and physiology. Previous studies find larger anatomical traits to be common in many polyploids (Buggs et al. 2007; Maherali et al. 2009; Liu et al. 2010; Van Laere et al. 2010; Manzaneda et al. 2015). Additionally, some studies suggest that polyploid plants may have increased drought tolerance (Maherali et al. 2009; Liu et al. 2010; Van Laere et al. 2010; Manzaeda et al. 2015). With confirmation from more extensive and complete studies, we may be able to induce polyploidism in our crops to allow them to grow in our increasingly warm and dry climate. Furthermore, the larger anatomical features characteristic of polyploids could be utilized to increase crop yield. This area of study has many important implications to society and is in need of more research.

Plants are able to survive and thrive with polyploidism much easier than animals. In fact, very few animals are able to function with any other ploidy than the most common state, diploid, in which there are two sets of chromosomes; therefore, the wide majority of polyploid studies, including this one, use plants as subjects. Any plant with additional sets of chromosomes is considered a polyploid. There can be as few as one additional set, or over ten additional sets. In order to be polyploid, the sets must be complete. Extra or missing individual chromosomes, not sets of chromosomes, is called aneuploidy. This is an important distinction, because the total amount of genetic material will be doubled for instances of the polyploid condition of tetraploid, whereas

the genetic material may be increased by less than 10% under aneuploid conditions. At the cellular level, this makes a large difference in cell size and function.

Furthermore, polyploidy can be separated into autopolyploidy and allopolyploidy. In autopolyploidy all of the genetic material originates from one individual. This can occur when there are interruptions in mitosis. If the cell cycle is aborted after DNA synthesis, the resulting cell will have additional sets of chromosomes. Upon resuming mitosis, a second round of DNA synthesis will occur. The products will be two cells with additional sets of chromosomes. Autopolyploidy can also occur with unequal division of chromosomes in anaphase. In this case, one daughter cell would be haploid and the other would be triploid. However, this is much less common and therefore odd numbered polyploids are not common in nature (Klug et al. 2016).

In alloploidy, the genetic material is from different individuals, possibly even different species. Hybridization of species can result in sterile organisms due to the inability to pair chromosomes for division. This can cause additional DNA synthesis and creation of a second set of chromosomes. Following division, the daughter cells would contain multiple sets of chromosomes originating from both parental species. Due to the different sources of chromosomes, allopolyploids have increased heterozygosity and variation in phenotypes.

It is widely accepted that polyploids have different traits than diploids. However, the origin of these traits is highly debated. In a review by te Beest et al. 2011, two possible mechanisms are postulated. The first suggests that the novel traits are acquired purely by undergoing polyploidization. The second mechanism involves the ability of polyploids to express greater plasticity due to their increased genetic material. This could lead to adaptation of novel traits. In simpler terms, the first states that the state of

polyploidy itself gives novel traits whereas the second states that polyploidy provides the means for adaptation and expression of these novel traits.

Questions remain about how polyploid traits originated; however, most research agrees on a set of common impacts to plant traits. It has been supported in many studies that increases in genome size are correlated to larger anatomical features such as: larger cells and leaves (Liu et al. 2010; Mráz et al. 2014), greater leaf mass per unit area (Beaulieu et al. 2007), longer stomatal guard cells (Buggs et al. 2007; Manzaneda 2015), larger and fewer stomata (Maherali et al. 2009; Liu et al. 2010; Van Laere et al. 2010; Manzaneda et al. 2015), and larger vessel diameters (Maherali et al. 2009; Hao et al. 2012).

Many studies also found plants with higher ploidies had increased ability to survive drought, which seems to be a large contributor to their success in novel, usually dry, habitats. In order to perform better in arid environments, plants must have more efficient water use or a strategy to access more water in times of water stress. Such physiological changes may be due to altered leaf anatomy and physiology. Manzaeda et al. 2015 found polyploids to maintain higher integrated water use efficiency than diploids in water-stressed environments. This may be due to reductions in stomatal conductance without a change in photosynthesis rate. The stomata, or plant pores, are key to regulating physiological processes such as transpiration and carbon dioxide uptake. Thus, it is rational to attribute differences in gas exchange traits to anatomical changes.

Polyploids have been found to have leaf water relations traits that signify their ability to survive drought better than diploids. These traits and references are as follows: increased relative water content (RWC) in water stress (Maherali et al. 2009; Liu et al.

2010; Van Laere et al. 2010) and less negative water potential during drought compared to diploids (Maherali et al. 2009; Van Laere et al. 2010, but see Hao et al. 2012). Plant water moves according to a water energy gradient. This gradient has several parts including the uptake of water into the roots, flux of water through the vascular tissue to the leaf, the water within the leaf, and the humidity inside and outside the leaf. Water within the plant flows in the liquid phase; however, at the leaf cell-air barrier, water is lost to the air via vapor diffusion. When the water is lost, it pulls on all of the other water molecules within the plant causing a vacuum-like pressure. Thus, the leaf water potential, or measure of this gradient, is negative. As the plant gets more water stressed, the water potential becomes more negative. This allows for the continuation of a favorable gradient for water uptake, but comes at an expense.

Leaf water potential undergoes a diurnal curve. Early in the morning stomata open for carbon dioxide assimilation and concurrent transpiration occurs. Then they begin to close in the afternoon. Transpiration rates are at their maximum in the afternoon before stomatal closure and when the ambient humidity is at a minimum. Thus, fluxes in water are natural and are representative of a healthy plant. In drought stress, plants may keep stomata closed, resulting in a steady state water potential. Essentially, this means that the plant can't photosynthesize. Lack of photosynthesis over an extended amount of time could lead to starvation. If carbon stores become too low, it could even lead to death. Alternatively, leaves can synthesize solutes to lower their water potential, maintain flow of water from drying soil and continue to keep stomata open. However, the intensely negative water potential can cause cavitation, or breaks in the water column, in vascular tissue. Once cavitation occurs, that xylem tube is no longer

operable until refilled with fluid, and the plant must exert energy to do so. Too many breaks can lead to hydraulic failure and death.

Pressure-volume (PV) curves use the concurrent loss of water volume and decreasing water potential to ascertain how a plant is managing its water content and water energy gradient as it transpires. In doing so, multiple traits can be measured, such as turgor loss point (π_{tlp}), relative water content at turgor loss point (RWC_{tlp}), modulus of elasticity, capacitance at full turgor and capacitance at turgor loss. Each give insight into water management and physiological control in different, but related ways. Recently, π_{tlp} has received attention as a key metric for how plants manage drought stress (Bartlett et al. 2012; Meinzer et al. 2016). Turgor, or the rigidity of plant cells resulting from hydrostatic pressure, is essential for plant structure, but also physiological functions of a plant. Also, localized changes in guard cell turgor is necessary to control stomatal opening and closing. Once π_{tlp} is reached, normal physiological functions fail. The rate at which turgor is lost can depend on many factors including modulus of elasticity.

Modulus of elasticity is a measure of the rigidity of the cell walls. Increased flexibility, or low modulus of elasticity, allows the plant to maintain turgor as water is being lost by allowing the cell walls to flex. Oppositely, increasing rigidity, or high modulus of elasticity, decreases the volume of water that can be lost while the plant still maintains turgor. For example, a balloon wall is highly flexible can remain inflated in a wide range of air volumes. If the balloon was made out of something less flexible, like a plastic shopping bag, it would stay inflated across a very small range of air volumes. The ability of plant cells to flex may be an important factor in maintaining turgor and physiological function during drought.

Capacitance, is the change in RWC per change in water potential. RWC can be measured at any leaf hydration. One example is RWC_{tlp}, which is the relative water content at turgor loss. It is used to determine how hydrated a leaf is when it losses turgor. It is thought that cell hydration and high RWC may be more important that turgor, as dehydration has harsh adverse metabolic effects (Bartlett et al. 2012). The ability of a plant to continue to retain water, thus maintaining a high RWC_{tlp}, while still having a favorable gradient for water uptake is advantageous in drought conditions. High capacitance means that a lot of the plant's water volume is being transpired as water potential decreases. If capacitance is low, then water volume being taken up through the roots makes up the majority of the water being transpired, such that the plant maintains a fairly constant RWC while water potential decreases. Thus, high capacitance may offer increased drought tolerance.

This study will use blackberry plants, Genus *Rubus* Subgenus *Rubus,* as subjects. By using a closely related set of species, variance in traits due to gene variance will be minimized. Additionally, *Rubus* exist in a wide range of ploidies and I have easy access to them, making them excellent subjects for this study. Some previous studies using *Rubus* as subjects are McDowell 2002 and McNellis and Howard 2015. These studies concerned themselves with the physiology and did not consider ploidy.

McDowell 2002 focused on the differences between native and invasive *Rubus.* They used two invasive *Rubus, R. armeniacus* and *R. laciniatus*. Both of these are commonly found to be tetraploids. The native species used were *Rubus ursinus* and *Rubus leucodermis*. *R. ursinus* has commonly been found having ploidies ranging from hexaploid to dodecaploid, while *R. leucodermis* is commonly diploid. They found that invasive *Rubus* maintained an increased capability for photosynthesis compared to the

noninvasive *Rubus.* These findings suggest that invasive *Rubus* are able to stay competitive longer throughout the spring season that the natives (also see Caplan and Yeakley 2012). The increased competitiveness may be due to physiological differences correlated with increased ploidy.

McNellis and Howard 2015 were also unconcerned with ploidy. However one of the critiques from this paper was analyzing ploidy as a possible contributing factor to the physiological data they collected. They used two invasive *Rubus* species, *R. armeniacus* and *R. laciniatus*, and three native *Rubus*, *R. spectabilis, R. ursinus* and *R. parviflorus*. The authors found that there is variation in daytime and nighttime gas exchange between species. Daytime gas exchange was not correlated, or was very weakly correlated, with nighttime gas exchange. It is possible that daytime transpiration may have separate genetic controls than those of nighttime transpiration, even though they are usually highly correlated. *Rubus armeniacus* and *R. laciniatus* had lower rates of nighttime stomatal conductance and nighttime transpiration and higher rates of daytime stomatal conductance and daytime transpiration than those of *R. spectabilis* and *R. parviflorus*, showing divergence. Excluding *R. ursinus*, the physiology of the higher ploidy *R. armeniacus* and *R. laciniatus* agree with previous studies, like Buggs et al. 2007, in that polyploids often have higher daytime transpiration.

Although much has been discovered about polyploidization, there still seems to be a very large gap in the knowledge of the effect of ploidy on various plant traits. There is still much debate on whether polyploids gain advantages in water relations traits due purely to their increased genetic content or due to natural selection acting on their large amount of genetic material. Furthermore, studies disagree on some traits of polyploids, such as whether they have relatively less negative water potential in drought conditions

or not. More research is needed to determine whether generalizations of polyploid traits can be made across species, or if traits of polyploids are completely subject to species.

In this study, I will attempt to correlate polyploidy to various water relations traits including modulus of elasticity, abaxial stomatal density and length, RWC_{tlp}, π_{tlp}, capacitance and daytime and nighttime water potential. This will allow me to understand the plants' water management strategies. The traits I have chosen to measure are easily obtainable and give a general insight into the physiology of the plants. More importantly, it seems that the difference in physiological traits between diploids and polyploids is the greatest when plants are drought stressed. Perhaps this is due to different water management strategies that can be understood by the water relations traits I plan to study.

Most literature concerning physiology of polyploid plants agree that anatomical features are larger. Specifically, stomata are larger and less dense in polyploids than in diploids. Thus, I will first attempt to find a trend in stomatal size and density according to increasing ploidy. If this trend in anatomical traits does in fact exist in *Rubus*, I am interested in exploring if this translates to trends in physiological traits. As stomata set the thresholds of much of a plant's physiology, it seems that a change in stomatal size or density should induce a change in physiology.

Implications of this project extend beyond the scientific community into the public. Genetically modifying crop plants and produce is becoming more common and controversial in public policies and laws. Research in polyploidization of crops could replace genetically modified crops as well as allow for plants that have greater production at a lower costs. There is also a possibility that we could induce polyploidy to get plants to grow in different climates in the event that future climate changes inhibit

crop and produce production. The research conducted so far seems to suggest that polyploidization allows plants to tolerate drought better, so perhaps this could be used to allow plants to grow in harsher environments.

Methods

Experimental

All plants used were housed in the USDA National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon. They were located in outdoor screen houses and potted in either two or five gallon plastic pots. Fertilization occurred monthly with either organic bone and feather meal or 16-16-16 Osmocote. Plants were connected to a watering system that watered for two minutes three times per week. Leaf samples were chosen based on their health by appearance, maturity, and petiole length. Leaves with obvious blemishes or injuries were avoided as were those with petioles less than one centimeter in length. Ideally, the newest fully matured leaves were chosen; however, in instances where these leaves were injured, more mature leaves were chosen. For all measurements, leaves were cut with a razor blade, leaving as much petiole with the blade as possible. Immediately after being cut, they were placed in a ziplock bag and stored in a cooler with ice packs.

The experimental design consists of 29 *R. ursinus* accessions, some of which were *R. ursinus* hybrids. I used a large number of plants of this species because it has the largest range in ploidy. Twelve of these accessions were previously ploidy typed by other studies and had four different ploidies. The other 17 lacked ploidy documentation. I then wished to sample as many different ploidies as possible using species within the Genus

Rubus Subgenus *Rubus*. In order to control for species variations, I allowed only two samples per species per ploidy. Using accessions previously ploidy typed, I selected samples for the study. Initially, over twenty samples were selected based on their species and ploidy. Once examining the plants, only twenty were fit for the study. These twenty included seven ploidy states and thirteen species. The final design included 50 samples, consisting of nine ploidy states and thirteen species plus some *R. ursinus* hybrids.

Ploidy Typing

Flow cytometry was used to determine the ploidy of the accessions. All reagents, filters, and sample tubes used were from Sysmex (Görlitz, Germany). Leaf samples were collected about an hour before testing and were kept in petri dishes near an ice pack. In one instance, samples were collected in coin envelopes and frozen in order to be tested at a later date. Using the Sysmex Partec PA II (Görlitz, Germany), I tested at least three separate samples from each accession. Pea was used as the genome standard. Approximately 25 mm^2 of sample and standard tissue was minced using a razor in extraction buffer. This tissue and buffer solution was poured through a 20 micrometer filter into sample tubes and DAPI was used to illuminate the DNA content. The filter was removed and the sample tube was inserted into the flow cytometer. Genomic content data from the flow cytometer was analyzed with the genomic pea standard in excel to calculate the number of genome copies, or ploidy number.

Pressure-Volume (PV) Curves

Pressure-volume curves measure the water weight being lost and concurrent water potential in order to determine turgor loss point and relative water content, among other physiological measures. As leaves first begin losing water, their water potential will quickly drop. There is a point in water loss in which the leaf gets floppy. This is referred to as the turgor loss point. As they approach this point, their water potential will drop more slowly. After they have surpassed their turgor loss point, water potential will drop very slowly. This is visualized on a line graph in which the water potential measurement is used to form the y-variable and the leaf weight is used to form the x-variable (Supplemental Figure 1). The drying and remeasuring of leaf water potential and weight is repeated until the curve maintains a linear slope again, suggesting the leaf has surpassed its turgor loss point. Weights and water potentials were recorded in a pre-made spreadsheet titled "Pressure-Volume Curve Analysis Spreadsheet" created by L. Sack and J. Kok on prometheuswiki.org, which also provided the calculations for the PV measurements. This enabled me to view the curve as it was being formed. Once the curve had returned to a constant slope that contained at least three points, measurements were ceased. The leaves were placed in coin envelopes and then dried in a drying oven. Lastly, the dry weight was recorded for each leaf.

Water Potential Measurements

Nighttime water potential was measured at approximately 7:00 am, prior to civil twilight. Healthy leaves as described prior were taken and then pressure bombed in the PMS Pressure Chamber (PMS Instruments, Albany, OR). Daytime measures were made at approximately 1:00 pm. These leaves were then resealed in plastic bags and transported

to a lab in a cooler with ice packs. They were scanned at 200 DPI and images were analyzed in ImageJ (NIH, https://imagej.nih.gov/ij/download.html).

Stomatal Measurements

Leaf samples were collected in the same manner as in previous methods; using a razor blade and placed in ziplock bags. They were cut once more along the midrib. One half was used to make the abaxial impression while the other was used for the adaxial impression. 3rd Generation Affinity InFlex hydroactive impression material (Brookfield, CT) was pushed onto the leaves and left to dry. The leaf was removed and clear nail polish was painted on the dried impression material. Clear packing tape was used to pick up the nail polish from the impression and then was put on a microscope slide and labeled.

Next, slides were placed under a standard light microscope that was connected to a computer. I then viewed the slides at 400x and captured images of the slides using Photoshop CS3 (Adobe, https://adobe-photoshop-cs3-update.en.softonic.com). Under the same magnification, I captured a picture of a slide micrometer to calibrate the size of the images. The stomatal pictures were then used in Image J (NIH, https://imagej.nih.gov/ij/download.html) to obtain abaxial (underside of the leaf) stomatal length and density. The top side, or adaxial side, of leaves have few to no stomata, so for this study stomatal density and length refer to the abaxial side.

Data Analysis

Data analysis was done in SAS University Edition (SAS Institute Inc., Cary, NC, USA). Regression analyses were completed used the PROC REG procedure with ploidy as

the independent variable. The mixed model ANOVA used the PROC GLIMMIX procedure with ploidy as a fixed effect. At first, species was set as a random effect in the ANOVA model, however the G metric was found to be not positive definite; meaning that after controlling for the fixed effect in the model, there was no variance left to attribute to the random effect. Thus, the random effect of species was removed. The regression model treated ploidy as a continuous variable, whereas the ANOVA model treated it as a categorical variable. Categories were determined based on both sample size and logic based on polyploidy processes (Table 1). Outliers were removed based on the 1.5 x IQR (interquartile range) procedure.

Results

During the initial experimental design, two plants per species per ploidy were selected to be in the study. Since plants from the same species would likely have similar physiological measures, I limited the number of plants that would be of the same species and in the same ploidy category. In doing so, I hoped to minimize the effect of species while still maintaining an adequate sample size. This design was created prior to visualizing them; Thus, the design changed due to health and availability of the plants. The resulting categorization and sample size is shown in Table 1.

Table 1: Description of ploidy categories

Group Name	Biological Description	Ploidy	Plants sampled per ploidy	Total plants sampled per Group
None	No ploidy event (Diploid)	2n	8	8
One	One ploidy event	3n 4n	1 7	8
Two	Two ploidy events	5n 6n	2 5	7
TwoPlus	Two or more ploidy events	7n 8n	9 3	12
Many	Multiple ploidy events & high ploidy	10n	13	13

Next, all plants were ploidy typed using flow cytometry. With the exception of two plants, experimental ploidy values were within 2n away (± 2 chromosome sets) from recorded literature values (Table 2). I suspect that these two plants were of a ploidy that caused them to be confounded with the pea standard that was used. Due to the large disagreement between the experimental and literature ploidy, those plants more than 2n away were taken out of the study. Using the experimental values, the design was reevaluated and resulted in the final design shown in Table 1.

Table 2: Comparison of Literature and Experimental Ploidy values. Literature values and ID numbers are from the NCGR (National Clonal Germplasm Repository) Corvallis database. Asterisks indicate plants removed from the study due to ploidy typing results and dashes represent plants not previously ploidy typed.

NCGR ID Number	Species	Literature Ploidy	Experimental Ploidy
33	*Rubus grabowskii*	2	2
34	*Rubus ulmifolius*	4	4
42	*Rubus wahlbergii*	5	5
51	*Rubus hirtus*	4	4
54	*Rubus caucasicus*	4	4
56	*Rubus hirtus*	4	4
79	*Rubus ursinus*	-	8
137	*Rubus ursinus*	-	8
139	*Rubus ursinus*	-	7
197	*Rubus ursinus*	12	10
260	*Rubus trivialis*	2	2
356	*Rubus ursinus*	-	7
367	*Rubus ursinus*	-	7
413	*Rubus laciniatus*	4	4
418	*Rubus trivialis*	2	2
611	*Rubus ursinus**	12	4
615	*Rubus ursinus**	12	5
785	*Rubus canadensis*	2	2
804	*Rubus ursinus*	12	10
817	*Rubus canadensis*	2	2
818	*Rubus ulmifolius*	2	2
832	*Rubus trivialis*	2	2
1054	*Rubus sanctus*	2	2
1095	*Rubus slesvicensis*	6	6

Table 2: Comparison of Literature and Experimental Ploidy values. Literature values and ID numbers are from the NCGR (National Clonal Germplasm Repository) Corvallis database. Asterisks indicate plants removed from the study due to ploidy typing results and dashes represent plants not previously ploidy typed.

1140	*Rubus ursinus*	-	8
1596	*Rubus laciniatus*	4	4
1825	*Rubus flaggellarius*	-	5
2110	*Rubus flaggellarius*	-	4
2201	*Rubus trivialis*	3	3
2292	*Rubus ursinus*	-	10
2293	*Rubus ursinus*	-	10
2294	*Rubus ursinus*	-	10
2295	*Rubus ursinus*	-	10
2296	*Rubus ursinus*	-	10
2313	*Rubus ursinus*	-	10
2314	*Rubus ursinus*	-	10
2315	*Rubus ursinus*	-	10
2316	*Rubus ursinus*	-	10
2319	*Rubus ursinus*	-	10
2320	*Rubus ursinus*	-	10
2334	*Rubus corylifolius L. aggr.*	8	7
2345	*Rubus* hybrid blackberry	7	7
2363	*Rubus flaggellarius*	7	6
2575	*Rubus* hybrid blackberry	7	7
2577	*Rubus* hybrid blackberry	7	7
2603	*Rubus ursinus* hybrid	6	6

Table 2: Comparison of Literature and Experimental Ploidy values. Literature values and ID numbers are from the NCGR (National Clonal Germplasm Repository) Corvallis database. Asterisks indicate plants removed from the study due to ploidy typing results and dashes represent plants not previously ploidy typed.

2604	*Rubus ursinus* hybrid	6	6
2605	*Rubus ursinus* hybrid	6	6
2608	*Rubus ursinus*	8	7
2611	*Rubus* hybrid blackberry	7	7

Two anatomical and seven physiological traits were chosen to explore the relationship between ploidy and plant physiology. As mentioned previously, the scientific community is in agreement that increases in ploidy are correlated with increases in anatomical traits such as stomatal length and density. Thus, I chose to measure these traits to attempt to support this finding. Additionally, the scientific community agrees polyploid traits seem most different from diploid traits while experiencing drought conditions. For this reason, water relations traits were the physiological measures I focused on; specifically, those traits measurable through the use of PV curves.

As I began the process of data analysis, the nature of ploidy as a continual variable was put into question. Previous literature has treated it as a categorical model; however, I am not aware of a study on plant physiology and anatomy that has the extensive range of ploidies that are present in this study. Thus, I treated ploidy both as a categorical variable and as a continual variable using ANOVAs and linear regressions, respectively. Statistical results from both types of analyses are presented in Table 3. Both models were used to address the relationship between anatomical traits and ploidy.

Table 3: Statistical results from categorical ANOVAs and continuous linear regression analyses. Values in parentheses are degrees of freedom and asterisks denote significance (* p < 0.05, *** p < 0.001). Dashes denote traits that could not be fit with a linear regression model.

	Categorical Model	**Regression Model**
Trait Variable	F-value, P-value	R^2, P-value
Stomatal Density	**$7.68_{(4,31)}$, 0.0002*** **	**0.4657 , < 0.0001*** **
Stomatal Length	**$6.82_{(4,34)}$, 0.0004*** **	**0.3976 , < 0.0001*** **
Modulus of Elasticity	$1.68_{(4,36)}$, 0.1752	-
Capacitance at full turgor	$0.69_{(4,33)}$, 0.6026	-
Capacitance at turgor loss point	$1.09_{(4,36)}$, 0.3768	-
Relative Water Content	$0.67_{(4,34)}$, 0.6157	-
Turgor Loss Point	$1.05_{(4,36)}$, 0.3933	-
Daytime water potential	$1.30_{(4,35)}$, 0.2906	-
Nighttime water potential	**$2.95_{(4,36)}$, 0.0332***	-

I first used the ANOVA model to determine the relationship between ploidy and anatomical traits. I found that as ploidy increased, so too did stomatal length ($F_{4,34}$ = 6.82, P = 0.0004; Figure 1). The linear regression model also fit correctly and was significant (R^2 = 0.3976, P < 0.0001; Table 3). Ploidy and stomatal density were found to have a significant inverse relationship ($F_{4,31}$ = 7.68, P = 0.0002; Figure 1). Again, the regression model was also significant (R^2 = 0.4657, P < 0.0001; Table 3). Thus, I was able to support the conclusion that there are larger and fewer stomata with increasing ploidy among *Rubus* Subgenus *Rubus* species and hybrids.

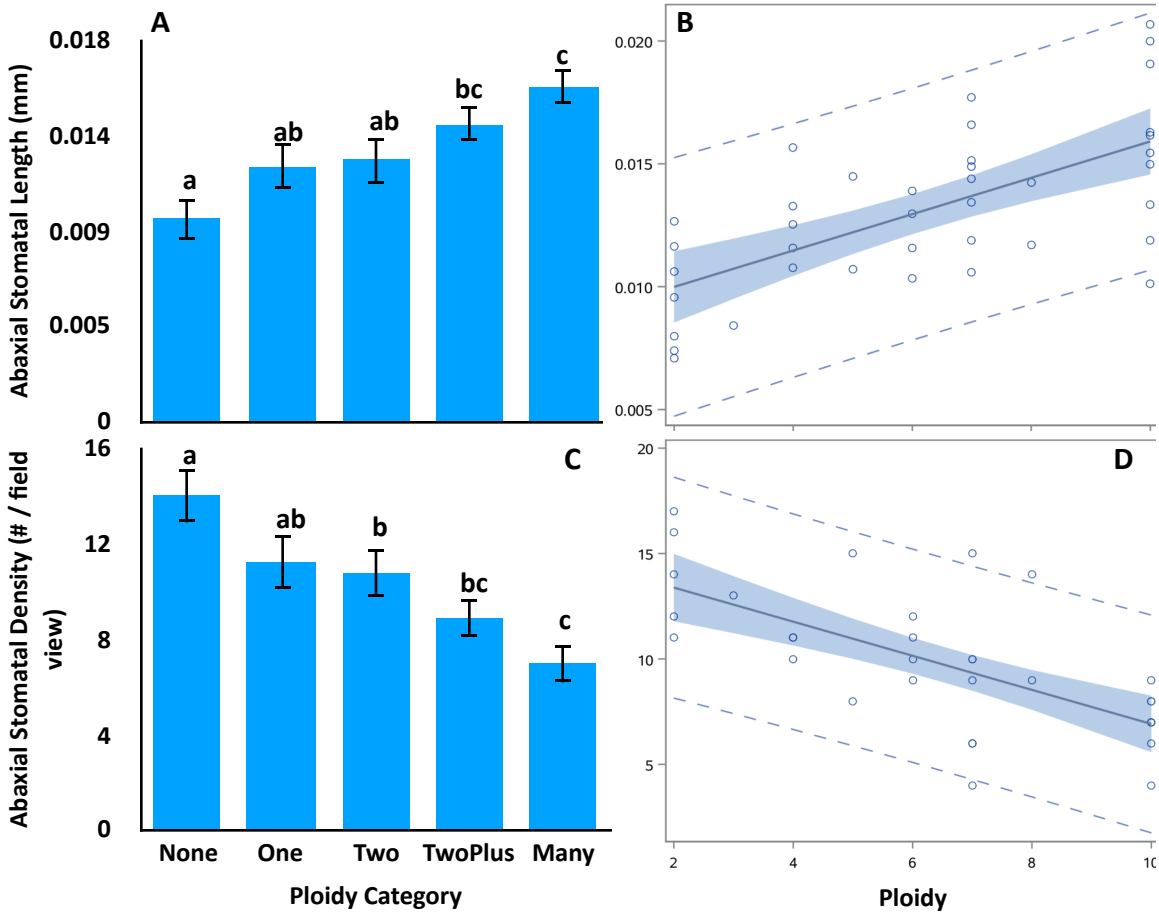


Figure 1: Abaxial stomatal length (A,B) and abaxial stomatal density (C,D) results from the two model types. On the left are categorical ANOVA means for each of the ploidy categories. Error bars represent one standard deviation and letters represent significant difference. (Ploidy category sample size: None, n = 8; One, n = 8; Two, n = 7; TwoPlus, n = 11; Many n = 13) On the right are linear regression analyses. The shaded area represents 95% confidence limit and the dashed line represents 95% prediction limit.

Next, I analyzed physiological data. Linear regression did not fit the physiological data, so only categorical ANOVAs were used. Nighttime water potential was the only physiological trait that had a significant relationship with ploidy ($F_{4,36} = 2.95$, $P = 0.0332$; Figure 2). Daytime water potential was not significant ($P > 0.05$), nor were any of the other physiological traits.

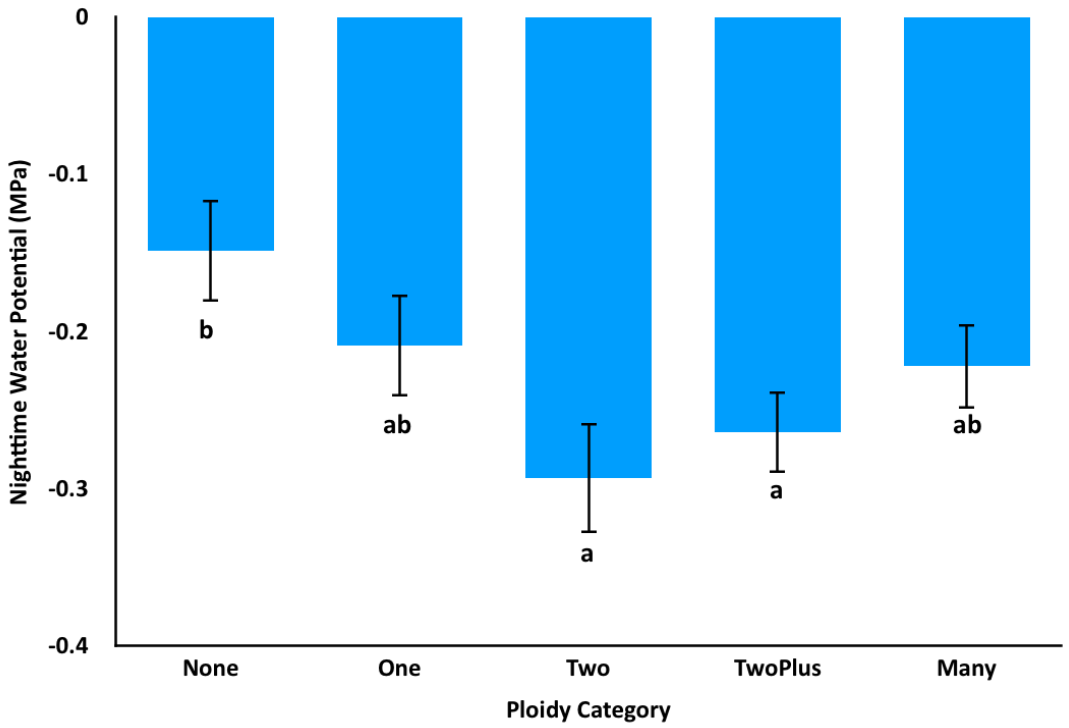


Figure 2: Bulk leaf nighttime water potential means for each of the ploidy categories. Error bars represent one standard deviation and letters represent significant difference. (Ploidy category sample size: None, n = 7; One, n = 7; Two, n = 6; TwoPlus, n = 11; Many n = 10).

From the P-V curves, I analyzed five different traits. These curves allow for the understanding of how plants manage water content and water energy state as they lose water via transpiration. None of the five traits analyzed were correlated with ploidy in either model. Comparing the significantly correlated stomatal length scatter plot to the rest of the scatter plots in Figure 3, it is easy to see that these other five traits are not strongly correlated in a liner fashion. However, these traits may fit a parabolic regression line or another functional regression line, which could be a future direction for this type of research.

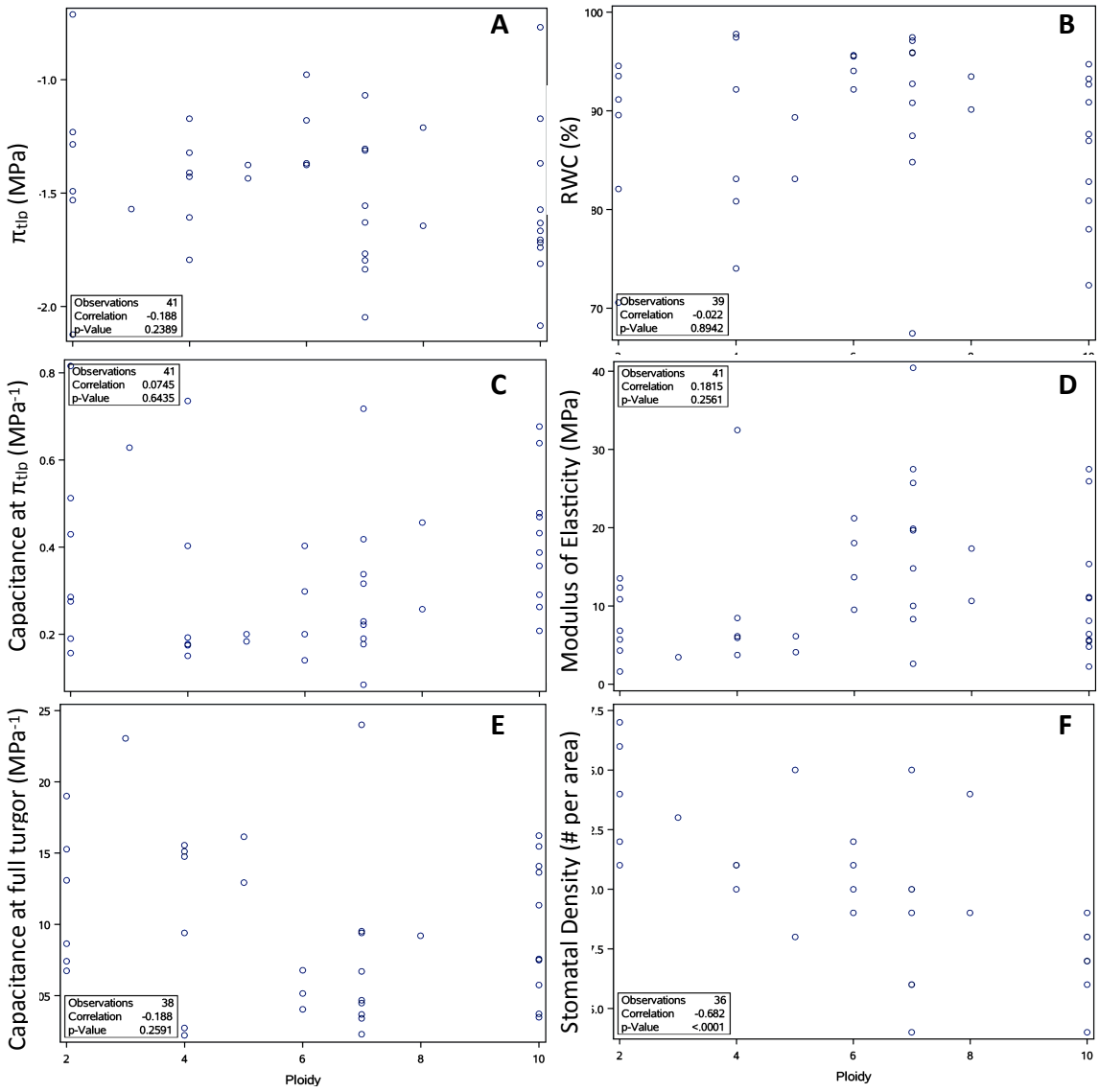


Figure 3: Correlation scatter plots showing relationship between ploidy and π_{tlp} (A), RWC_{tlp} (B), capacitance at turgor loss (C), modulus of elasticity (D), capacitance at full turgor (E) and stomatal density (F). Sample size, correlation coefficient and P-value is displayed on the plots. The stomatal density plot shows a significant correlation ($P < 0.0001$), but the rest do not ($P > 0.05$).

Discussion

The stomatal findings reported here are in agreement with previous findings (Maherali et al. 2009; Liu et al. 2010; Van Laere et al. 2010; Manzaneda et al. 2015). By the definition, polyploids have much more DNA content than diploids. This means that

the cells need to be larger to store all of the extra chromosomes. Developmentally, this means that there will be fewer cells overall, as the area of the tissue is conserved rather than the number of cells. Therefore, it makes sense that polyploids have larger and fewer stomata. They are larger due to increased DNA content and fewer due to physical constraints during development.

Despite this change in stomatal anatomy, there wasn't a corresponding change in physiological trait values, as I had expected. Stomata regulate the upper and lower thresholds for water loss. Any physiological control will be confined within these thresholds. Perhaps the change in stomatal anatomy didn't change the thresholds because increasing stomatal size was countered by decreasing stomatal density. Alternatively, the thresholds could have changed, but the plant may regulate stomata of different size and density to maintain the same physiological optimum for water loss. This could explain the lack significance in daytime leaf water potential.

Bulk leaf nighttime water potential varied significantly with ploidy (Figure 2). Nighttime water potential may be driven by drying soils. However, it is unlikely that dry soil confounded the findings, because the plants were well watered prior to and during measurements. More likely is change in nighttime leaf water potential is due to altered nighttime conductance. Since conductance is a measure of how easily water can move through stomata, one would expect a strong correlation between water potential and conductance if stomatal control was in fact the driving factor.

Stomatal control of transpiration acts to maintain internal water homeostasis as the ambient environment and soil are drying throughout the day (Meinzer et al. 2016). This control results in two strategies, being anisohydry and isohydry. Anisohydric plants allow their leaf water potential to decrease as soils dry. This decline is regulated through

the metabolism of compatible solutes to create a favorable water gradient that continues the uptake of water into the plant. In doing so, it is able to continue to photosynthesize. However, as its water potential continues to get more and more negative, the plant risks higher rates of cavitation that cause major problems for the movement of water through the plant. Alternatively, isohydric plants maintain a consistent minimum daytime leaf water potential as soils dry. In order to keep that level water potential, stomata must close, which puts a halt to photosynthesis. Where the anisohydric plant risks high rates of cavitation, the isohydric plant risks decreased photosynthetic abilities.

Since nighttime water potential, but not daytime water potential, had a significant relationship with ploidy, *Rubus* may be a highly isohydric species, as supported by Qiu et al. 2017. If all of the *Rubus* species in this study are in fact isohydric, then it is logical not to see any significant trends in daytime water traits, as they will be only indicative of the level water potential maintained by the plants once stomata close. The lack of significant PV traits suggests the improved performance of higher ploidy plants during drought may be due to anatomical changes, such as bigger and deeper roots, that help an isohydric plant avoid drought effects rather than tolerate or withstand dry conditions.

Future directions for this research should focus on gas exchange measurements to compliment the various water relations measurements done here. That would allow a better distinction between results being due to the physiological controls of the plant versus the environmental thresholds the plant is experiencing. Furthermore, hydraulic trade-offs are made in order to keep a plant functioning photosynthetically. Thus, carbon

assimilation during photosynthesis as well as nighttime leaf respiration could offer some interesting insights into how polyploids may differ from diploids.

In our changing climate, it will become more important to understand the water use and water strategies of plants. If drought tolerance or more specific water traits are increased in various wild polyploid plants, these plants may be utilized in place of current-day crops. Alternatively, polyploidy may be induced in certain lines of present-day crops to offer drought tolerance characteristics. Other polyploid characteristics such as larger anatomical features like fruits are already taken advantage of in genetically modified crops. Increased research may enable us to grow crops in increasingly dry climates, grow more produce per plant, and explore alternates to genetically modified crops. This area of research clearly has much to offer and much yet to be understood.

References

Bartlett, Megan K., Christine Scoffoni, and Lawren Sack. "The determinants of leaf turgor loss point and prediction of drought tolerance of species and biomes: a global meta-analysis." *Ecology Letters* 15.5 (2012): 393-405.

Beaulieu, Jeremy M., Ilia J. Leitch, and Charles A. Knight. "Genome size evolution in relation to leaf strategy and metabolic rates revisited." *Annals of Botany* 99.3 (2007): 495-505.

Buggs, Richard JA, and John R. Pannell. "Ecological differentiation and diploid superiority across a moving ploidy contact zone." *Evolution* 61.1 (2007): 125-140.

Caplan, Joshua S., and J. Alan Yeakley. "Water relations advantages for invasive Rubusáarmeniacus over two native ruderal congeners." *Plant ecology* 210.1 (2010): 169-179.

Hao, Guang-You, et al. "Polyploidy enhances the occupation of heterogeneous environments through hydraulic related trade-offs in Atriplex canescens (Chenopodiaceae)." *New Phytologist* 197.3 (2013): 970-978.

Klug, William S., et al. *Essentials of genetics*. No. 575.1 E78e. Pearson, 2016.

Liu, Siyu, et al. "In vitro induced tetraploid of Dendranthema nankingense (Nakai) Tzvel. shows an improved level of abiotic stress tolerance." *Scientia horticulturae* 127.3 (2011): 411-419.

Maherali, Hafiz, Alison E. Walden, and Brian C. Husband. "Genome duplication and the evolution of physiological responses to water stress." *New phytologist* 184.3 (2009): 721-731.

Manzaneda, Antonio J., et al. "Environmental aridity is associated with cytotype segregation and polyploidy occurrence in Brachypodium distachyon (Poaceae)." *New Phytologist* 193.3 (2012): 797-805.

Manzaneda, Antonio J., et al. "Natural variation, differentiation, and genetic trade-offs of ecophysiological traits in response to water limitation in Brachypodium distachyon and its descendent allotetraploid B. hybridum (Poaceae)." *Evolution* 69.10 (2015): 2689-2704.

McDowell, Susan CL. "Photosynthetic characteristics of invasive and noninvasive species of Rubus (Rosaceae)." *American Journal of Botany* 89.9 (2002): 1431-1438.

McNellis, Brandon, and Ava R. Howard. "Phylogenetic and ecological patterns in nighttime transpiration among five members of the genus Rubus co-occurring in western Oregon." *Ecology and evolution* 5.17 (2015): 3557-3569.

Meinzer, Frederick C., et al. "Mapping 'hydroscapes' along the iso-to anisohydric continuum of stomatal regulation of plant water status." *Ecology letters* 19.11 (2016): 1343-1352.

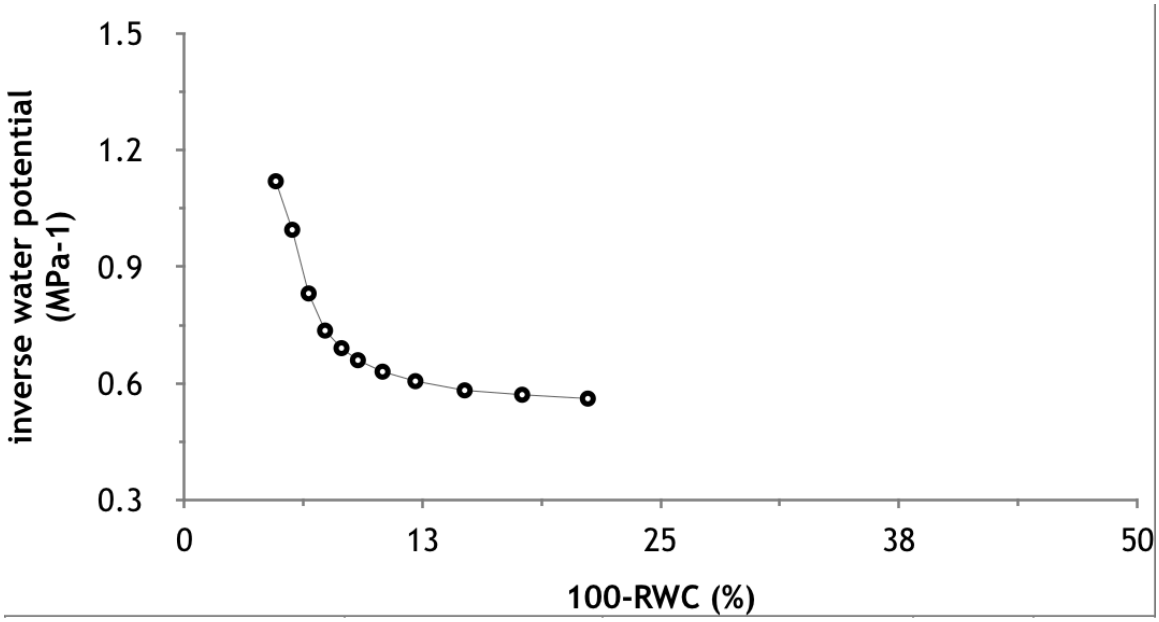
Mráz, Patrik, Elham Tarbush, and Heinz Müller-Schärer. "Drought tolerance and plasticity in the invasive knapweed Centaurea stoebe sl (Asteraceae): effect of populations stronger than those of cytotype and range." *Annals of botany* 114.2 (2014): 289-299.

Qiu, Changpeng, et al. "Persistent negative temperature response of mesophyll conductance in red raspberry (Rubus idaeus L.) leaves under both high and low vapour pressure deficits: a role for abscisic acid?." *Plant, cell & environment* 40.9 (2017): 1940-1959.

Te Beest, Mariska, et al. "The more the better? The role of polyploidy in facilitating plant invasions." *Annals of botany* 109.1 (2011): 19-45.

Van Laere, Katrijn, et al. "Influence of ploidy level on morphology, growth and drought susceptibility in Spathiphyllum wallisii." *Acta Physiologiae Plantarum* 33.4 (2011): 1149-1156.

Appendix



Supplemental Figure 1: Example pressure-volume curve that was created and used to derive many water relations measurements.