Autopolyploidy provides a mechanism for the evolution of tricellular pollen in *Galax urceolata*

A Thesis Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

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December 2025

ABSTRACT

Polyploidy has played an important role in the diversification of flowering plants. In general, the initial phenotypic effects of whole genome duplication (WGD) occur at the cell level - increased cell size and longer cell cycles - and result in larger and delayed development of organs. However, rapid evolution of the rate and duration of the cell cycle can occur during early stages of polyploid evolution. The male gametophyte of seed plants is highly reduced, developing as a single-celled organism within the pollen grain. In most flowering plants, pollen is dispersed as in a sexually immature bicellular state, but remarkably, 30% of angiosperms have evolved to disperse pollen that has reached sexual maturity, having already formed its two sperm cells, the tricellular state. Little is known about the mechanisms that underline unidirectional evolutionary transitions from bicellular to tricellular pollen, because species are rarely stably polymorphic for pollen at both stages.

Galax urceolata is a mixed ploidy species, containing single-cytotype populations of diploids ($2n = 2x = 12$) and autotetraploids ($4x = 24$). To investigate the role of polyploidy in pollen evolution I examined the development of pollen in twelve single cytotype populations. Flow cytometry was used to confirm ploidy levels. Conventional methods of staining and microscopy were used to calculate the proportion of bicellular and tricellular pollen present in flowers at four stages of maturation. Temperature accumulation is summed from 18 days of data prior to each collection using the nearest weather stations.

Diploid populations produced a mix of both bicellular and tricellular pollen in flowers of all ages (mean = 23%), whereas flowers of autotetraploid anthers contained a high proportion of tricellular pollen at all floral stages (mean = 93%). Pollen of both cytotypes is dehydrated at dispersal and maintained > 65% viability for three days after collection. Temperature accumulation was similar for both 2*x* and 4*x* plants, and bud growth rates were similar. All indicators of the acceleration, not prolongation, of pollen development in autotetraploids. By duplicating gene dosage, WGD provides a mechanism for evolving faster biosynthetic rates, despite initial disadvantages of larger cells and slower cell cycles.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Section One Introduction

One major mechanistic contributor to the diversification of angiosperm species is changes in ploidy level (Soltis et al., 2009; Wood et al., 2009; Otto & Whitton, 2000). Polyploid cells arise from whole genome multiplication events after incorrect segregation of chromosome sets in mitosis or meiosis (Stebbins, 1971), and polyploid organisms are defined by having at least one extra set of chromosomes compared to their progenitors (Wood et al., 2009). Most angiosperm polyploids are the result of whole genome duplications (WGDs), as shown by the fact that most are even numbered polyploids (Carta et al., 2020). Ancient WGDs have occurred in all major seed plant lineages (Jaio et al., 2011), and polyploidy was also common in many extinct ancient angiosperms, based on their larger leaf guard cell sizes (Goldblatt, 1980; Masterson, 1994). Larger cell sizes are perhaps the most conspicuous phenotypic consequence of WGD (Doyle & Coate, 2019). Polyploids, with their larger cell sizes, are most often associated with prolonged cell cycles and reduced developmental rates (Bennett, 1971; Levin, 1983). Most research on errors in cell cycles associated with ploidy have focused on comparing closely related species that have long since established and evolved. To truly understand the effects that ploidy has on cellular development, more studies focused on comparison between individuals or populations within species is needed (Spoelhoff et al., 2023)

Polyploids are defined by their mode of origin, with two commonly recognized types. Allopolyploids are formed when two different species hybridize followed by stabilization of meiosis in the hybrid by WGD (Stebbins, 1947; Soltis et al., 2016; Tossi et al., 2022). Allopolyploids have high heterozygosity, which is maintained by disomic segregation of each of the original maternal and paternal chromosome sets. The other types of polyploids are, autopolyploids, which arise from intraspecific WGD events within a single species (Stebbins,

1947; Meudt et al., 2021). Autopolyploids may or may not have high heterozygosity, but parental chromosome sets initially display multisomic segregation. Initial formation of polyploids in angiosperms is most often linked to sexual polyploidization, in which WGD occurs from errors in meiosis, and the resulting 2n gametes produce polyploid zygotes (Ramsey & Schemske, 2002; De Storme and Geleen, 2013). Errors of meiosis are thought to arise more frequently in male than in female meiosis, though the mechanism for higher mutation rates in pollen than in ovules is debated (Williams, 2021).

Male gametophytes or pollen grains are single-celled organisms that produce two sperm cells within their cytoplasm. Inside the protective anther there will be four genetically distinct haploid (1n) microspores produced through two meiotic phases beginning with one diploid (2*n*) sporophyte cell (Esau, 1977). First division restitution are errors that occur during the first phase of meiosis resulting in the production of two unreduced 2*n* microspores (Younis et al., 2014). Similarly, the failure of sister chromatids to separate during meiosis II is a second division restitution that produces two unreduced 2*n* microspores (Younis et al., 2014). For microspores to become pollen grains they must complete two rounds of mitosis (Halbritter et al., 2018). During mitosis I the microspore nucleus divides to produce a vegetative nucleus and generative nucleus. After asymmetric cytokinesis, the microspore becomes subdivided into a large vegetative cell and a small generative cell, commonly referred to as the “bicellular stage” of male gametophyte development. At this point, the generative cell detaches from the spore wall to become enclosed within the vegetative cell cytoplasm. The generative cell completes the final stage of cell division with the development of two sperm cells. Varying degrees of programmed dehydration will occur as the anther reaches maturity in both bicellular and tricellular pollen (Nepi et al.,

2001). Desiccation is critical for survival outside of the anther and reproductive success in many angiosperm species.

However, pollen is not sexually mature until after the second mitosis, in which the free generative cell divides once more to form the two sperm cells needed for double fertilization. Pollen in which sperm have formed has reached the "tricellular stage." The dispersal of pollen grains can occur either before (bicellular pollen) or after sexual maturity (tricellular pollen) is reached. Pollen dispersal ends when the stigma of a flower is reached, completing the process of pollination. Upon pollination, pollen germinates, and the single-celled vegetative cell grows a pollen tube towards the ovule and female gametophyte. The pollen tube transports the two sperm to the egg and central cell of the female gametophyte. If pollen is dispersed in a bicellular state, then mitosis II will occur within the pollen tube as it grows towards the female gametophyte within female tissues of the ovule and carpel.

The state of sexual maturity of pollen at the time of dispersal has many consequences for viability, longevity and its competitive ability upon pollination (**Table 1**). When bicellular or sexually immature pollen is dispersed, it is usually in a dehydrated and dormant state, as opposed to sexually mature tricellular pollen that is more often in a relatively hydrated state and much more metabolically active when released (Williams & Brown, 2018). Tricellular pollen grains can be shorter-lived in comparison to bicellular pollen grains, but much more competitive once they reach the stigma (Hoekstra & Bruinsma, 1975; Mulcahy & Mulcahy, 1983). The germination of bicellular pollen is delayed compared to terminally differentiated tricellular pollen because it has to both re-hydrate on the stigma and continue its sexual maturation to form two sperm within the growing pollen tube.

Table 1: Pollen dispersal states associated with sexually immature bicellular pollen and sexually mature tricellular pollen in angiosperms.

Trait	Bicellular	Tricellular
Water content	Dehydrated	Hydrated
Metabolic state	Dormant	Active
Longevity	Long-Lived	Short-Lived
Germination speed	Slower	Faster

Male gametophytes are sexually immature at the time of pollen dispersal in all gymnosperms and 70% of angiosperms, whereas tricellular (sexually mature) pollen is unique to angiosperms and has originated repeatedly in many independent lineages (Brewbaker, 1967; Williams et al., 2014). The 30% of angiosperms that disperse tricellular pollen comprise some of the most speciose lineages, such as grasses and asters (Brewbaker, 1967), and many annuals with rapid reproduction and short life cycles (Reese & Williams, 2019). “Reversals” from tricellular to bicellular pollen are rare and such secondarily formed bicellular lineages are never speciose in flowering plant linaeges (Williams et al., 2014). Given the largely uni-directional and widespread shifts from bicellular to tricellular pollen, and the association of tricellular pollen with rapid reproduction, a mechanism for how such shifts occur has long been sought (Williams, 2014). Species, or even closely related species, with variation in states of pollen sexual maturity would be needed for such studies. Yet, as long-ago noted by Schürhoff (1926) and Brewbaker

(1967), many taxa are characterized by either the bicellular-only or tricellular-only state at the level of genus, family or higher levels. Intraspecific variation is often ignored because of typological approaches to taxonomy, but also because in many cases polymorphism consists of minor variants explained by excessive heat or collection of pollen from senescent flowers (Williams et al., 2014).

Given the widespread occurrence of polyploidy in angiosperms and the direct effects of WGD on the cell cycle, polyploidy might play a role in facilitating change or maintaining stasis in pollen sexual maturity states. There are many naturally occurring mixed ploidy species (Porturas et al., 2019; Bomblies, 2020) that would allow comparisons of developmental rates of pollen with respect to ploidy level at early stages of polyploid evolution. For example, a transition from bicellular to tricellular pollen ultimately involves either accelerating the male gametophyte cell cycle to reach the tricellular stage before pollen dehydration occurs, or the duration of pollen development must be extended, such as by later maturation of the anther (Williams et al., 2014; Williams and Reese, 2019). As noted above, WGD is generally thought to slow or extend development, whereas after WGD, polyploids may have higher metabolic rates, enabling faster development (Williams and Oliveira, 2020).

The naturally occurring autopolyploid, *Galax urceolata* (Poir.) Brummitt (herein *Galax*), is ideal for studying the early stages of polyploid evolution both within species and populations. *Galax* is the sole member of its genus and one of fifteen species in the, Diapensiaceae (order Ericales). There are naturally occurring diploid ($2n = 2x = 12$), triploid ($3n = 3x = 18$), and tetraploid ($2n = 4x = 24$) cytotypes throughout its range (Nesom, 1983; Servick 2015). *Galax* was among the first taxa considered to have stable intraspecific, tetraploid populations, which was determined on the basis of a doubling of chromosome numbers and because differences in morphology were mostly

due to larger organs and parts (Baldwin, 1941). Recent research on *Galax* has focused on ecological interactions that shape the reproductive and population dynamics of cytotypes (Barringer & Galloway, 2017; Gaynor et al., 2025).

A recent study on *Galax* (Williams et al., 2025) found that pollen production was 85% higher in tetraploid relative to diploid populations. Since flowering times were similar or even earlier in tetraploids at the same elevation (Barringer & Galloway, 2017), it was concluded that higher pollen numbers must have evolved in tetraploids by accelerating the cell cycles of pollen precursor cells in the anther. During that study it also became apparent that the anthers of tetraploid flowers commonly contained tricellular pollen grains whereas those of diploids contained few, suggesting that the faster cell cycles that produced pollen might also extend to the male gametophyte cell cycle. In this study, I asked four questions: 1) Do tetraploids differ from diploids in the proportion of tricellular pollen grains at four sequential floral developmental stages? 2) Does temperature or elevation interact with ploidy to explain any differences? 3) Do floral bud growth rates differ between 2*x* and 4*x* plants? 4) Does the water content, viability, and longevity of pollen grains differ between 2*x* and 4*x* plants at the time of pollen dispersal?

Section Two Methods and Materials

Study Species and Sampling- *Galax* grows from Virginia to northern Georgia and can be found in dense patches along the forest floor. This perennial endemic of the Appalachian Mountains spreads rhizomatously and have evergreen leaves that turn red in the fall. *Galax* populations begin flowering in late May through mid-July. The racemose inflorescence produced by *Galax* consists of small androgynous flowers with acropetal development. Each flower has five sepals and five semi-fused white petals with a fully fused androecium consisting of five anthers.

A total of six diploid (2*x*) and six tetraploid (4*x*) populations of single cytotype were studied in the southeastern Appalachian Mountains, from 5/14/25 to 6/22/25 (**Table 2**). Populations were chosen based on previous work indicating only a single cytotype present per population (Servick et al., 2015; Williams et al., 2025). Cytotypes were verified by flow cytometry (below). Elevations varied from 340-1,463 m in diploid populations, and 882-1,676 m among tetraploid populations. A range of 15-31 diploid and 10-31 tetraploid plants were sampled along a transect with a minimum of 3-5 meters between each plant. Generally, 50-75% of flowers per inflorescence had opened at the time of collection. Buds and flowers were sampled at eight stages of development along each inflorescence (**Figure 1**). Samples were fixed in an FAA solution at collection, then replaced with 70% EtOH after 36 h and stored permanently at 4^{o}C. Temperature was recorded at each site during the time of collection with a Kestrel 3000 Pocket Weather Meter. Over a three-day period spent in the field, temperature was recorded at each time of sampling approximately five to ten inches away from each. The time of day for the recorded temperature ranged from late morning to early afternoon. The maximum temperature over three days was compared to the maximum temperature at the nearest weather station for that same day. The difference between the field max and the weather station max was used as a correction

Table 2. Populations of Galax urceolata examined in the study. The majority of sites can be found in North Carolina, along the southeast region of the Appalachian Mts. The Location of population 18 will remain anonymous due to high activity of pouching suspected in the area. Putative cytotype was confirmed through flow cytometry methods (Servick et al., 2015, Williams et al., 2025). Weather data was taken from the nearest weather station to each site. BLR, near Blue Ridge Parkway; GSMNP, Great Smoky Mountain National Park.

Pop #	Population Id	Putative Cytotype	Lat/Long	Elevation	*N* (plants)	Julian dates	Weather Station
7	Woodfin Valley, BLR	2*x*	35.45, -83.09	1250	15	165-167	Waynesville 1E
11	James Lake A, NC	2*x*	35.76, -81.92	340	29	139-141	Morgantown
12	James Lake B, NC	2*x*	35.76, -81.87	340	31	139-141	Morgantown
14	Dupont, TN	2*x*	35.82, -83.68	427	26	135-137	Sevierville
17	Mt. Jefferson, NC	2*x*	36.40, -81.46	1402	26	160-162	West Jefferson
18	GSMNP[a]	2*x*		1463	26	167-169	Mt. Leconte
5	Boone Fork, BLR	4*x*	36.12, -81.77	1190	10	154-156	Boone 1 SE
8	Art loeb Tr., BLR	4*x*	35.30, -82.88	1670	20	170-172	Pisgah Forest 3NE
9	Mt. Pisgah Picnic Area, BLR	4*x*	35.41, -82.75	1509	31	170-172	Waynesville 1E
10	Tanawha Tr., BLR	4*x*	36.08, -81.82	1292	31	154-156	Boone 1 SE
15	South Mt., NC	4*x*	35.60, -81.63	882	20	143-145	Morgantown
19	John Rock Overlook, BLR	4*x*	35.31, -82.86	1676	23	172-174	Brevard

[a] Location withheld.

Figure 1. *Galax* inflorescence showing floral buds (stages 1-6) and open flowers (stages 6-8).

factor. Field observations revealed an approximately 12-14 day flowering period for diploid and tetraploid populations, and gametophyte development of other species is also known to occur over a period of 2-3 weeks (Bennett, 1977; Endress, 1977; Williams et al., 1991). To calculate heat accumulation during development, the correction factor was applied to the mid-point of the min/max temperatures recorded from meteorological data for each of a total of eighteen days prior to and including the day flowers were sampled for bicellular and tricellular pollen. In this way, each sampled population was associated with a single estimate of heating degree days (*HDD*). *HDD* = sum of (min/max midpoints minus correction factor) over 18 days prior to and including the day collection.

Flow Cytometry- Ploidy levels were inferred using flow cytometry (FCM) on fresh (< 2-day-old leaves) for populations 14, 15, 18, and 19. The remaining populations were previously verified as single-cytotype (Williams et al., 2025). *Pisum sativum cv. Ctirad* was used as the standard for comparison of peaks. We co-chopped 0.25 cm^2 of fresh *Galax* leaf material from each of three individuals with 0.5 cm^2 of the *Pisum* standard for each tube. If two sample G 1 /G 0 peaks appeared for the same tube, leaves were rerun individually. Samples were chopped in 1 mL of ice-cold Otto I buffer and filtered through a 35 µm mesh. Tubes were centrifuged at 150g for 5

min., the supernatant removed, and 100 µL of ice-cold Otto I buffer added. Tubes were incubated at room temperature for ~ 30 minutes with occasional shaking before adding 0.8 mL of staining solution, prepared with 2 mL Propidium Iodide/Rnase (BD Pharmingen item # 550825), 20 mL PBS supplemented with 0.2% (w/v) bovine serum albumin, Ph 7.4 (BD Pharmingen stain buffer, item # 554657), and 4 µL β-mercaptoethanol, filtered through a 0.2 µm filter.

Data were analyzed using FCS Express 7 (De Novo Software 2024, Pasadena, CA). To estimate C-values in picograms (pg), we used the formula (Temsch et al., 2022): Sample 2C- value = Standard 2C-value × (Sample G 1 /G 0 fluorescence peak (RFUs)/Standard G 1 /G 0 fluorescence peak (RFUs), where RFUs are relative fluorescence intensity units. Using the most recent estimation of genome size for the *Pisum* standard, 7.75 pg (Nix et al., 2024), peaks near a value of 2.29 pg were interpreted as diploids and 4.71 pg as tetraploids (Williams et al., 2025). Individuals with intermediate peaks were excluded from the analysis.

Bicellular and Tricellular Pollen Counts- *Galax* pollen from developmental stages 5-8 (**Figure 2**) were used to assess the stage of male gametophyte development within pollen grains, bicellular or tricellular stages, in *Galax* flowers. The pollen from fixed anthers was placed onto a slide and crushed in 20µL of Acetocarmine stain and 10% glycerin, before being heated at 58°C for 5min. A total of 35 pollen grains from one anther per flower from 20 individual plants in each population was photographed at 400x magnification with a Zeiss Axiocam camera and Zeiss Axioplan light microscope,. To avoid biases in counting we prepared both diploid and tetraploid samples at the same time, while alternating slide viewing between 2*x* and 4*x* plants. Pollen was considered bicellular when a long, ovoid generative cell was present, whereas in tricellular pollen, there were two conspicuous but smaller spheroid sperm cells (**Figure 2**).

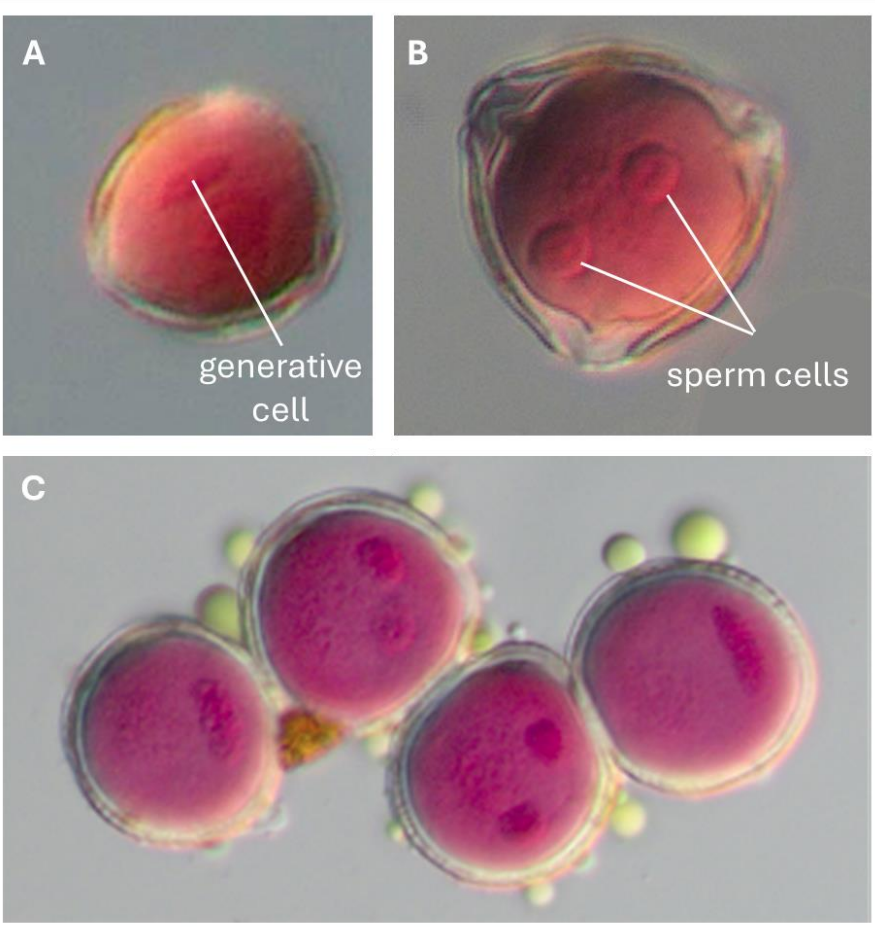

Figure 2. *Galax* pollen stained with acetocarmine. A) immature bicellular pollen with the generative cell, B) sexually mature tricellular pollen with two sperm cells, and C) pollen at both stages within the same anther. Pollen exines exude abundant pollenkitt, visible as greenish oil.

Pollen Hydration- To gauge if 1x pollen from diploids and 2*x* pollen from tetraploids differs in water content at the time of dispersal, we sampled pollen from mature (stage 7) flowers from five individuals per population in the field. At the time of collection, pollen was removed from a single anther with a toothpick and placed in either: 1) immersion oil, or 2) water (**Figure 3**). *Fresh pollen* in oil was placed on a slide under a coverslip for later viewing. *Fully hydrated* pollen samples were allowed to swell in water for approximately three hours prior to viewing on a slide under a coverslip in water. Twenty pollen grains per flower were photographed at 400x magnification under a light microscope. Pollen diameter was measured along the longest (*L*) and shortest (S) axes from the inner intine wall (Figure 3). To calculate volume, we used the formula: $V = (\pi \times L^2 \times S)/6$.

A hydration index for pollen of each flower was constructed, as per Nepi et al. (2001):

HI = mean volume of fresh pollen divided by mean volume of fully hydrated pollen.

Because pollen shrinks upon dehydration, and most pollen is dispersed in a dehydrated state (Williams & Brown, 2018), pollen that is small relative to its fully hydrated state is considered to be dehydrated (Figure 3). A hydration index of < 0.5 can be interpreted as indicative of a relatively dehydrated state (Nepi et al., 2001).

Pollen Longevity- To assess pollen longevity, the conjoined androecium of five anthers on mature flowers was placed in a petri dish at room temperature and one anther was removed at five sequential time points, 0, 10, 24, 36 and 72 hours after collection where they were placed in a 0.2mL tube. A total 20µL of Alexander's solution (Westerman et al., 2024) was pipetted on to a microscope slide with a coverslip to assess the proportion of pollen grains that had non-stained cytoplasm or that were small or deformed (**Figure 4**). Individuals with pollen viability < 30%

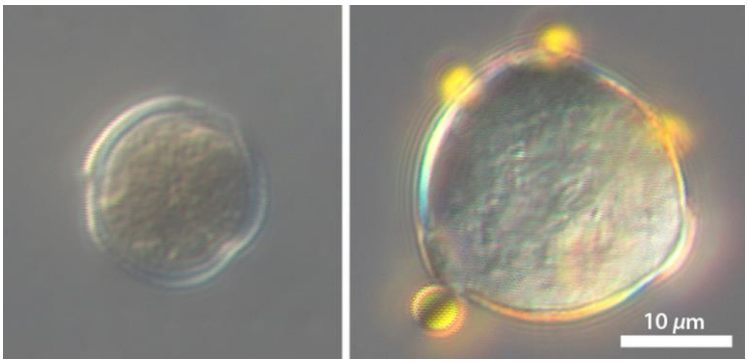

Figure 3. *Galax* pollen collected from the field. Photo on the left of fresh pollen placed directly into immersion oil, and on the right of pollen from the same flower placed directly into water. The scale bar equals 10 μm for both images. The volume of the pollen grain on left is 35% the volume of that on right.

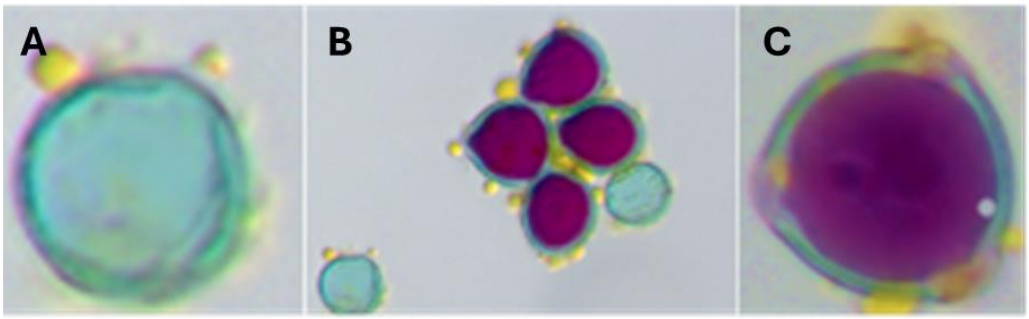

Figure 4. Pollen viability as scored using Alexander's staining. A) Dead pollen inferred from lack of uptake of stain. B) Viable and inviable pollen. C) Viable pollen inferred from uptake of stain into cytoplasm.

were excluded from the analysis. To measure longevity, the decay in pollen viability of diploids and tetraploids over time was assessed using light microscopy (Westermann et al., 2024). At each time point, an anther was crushed in Alexander's solution in a 0.2mL tube and 20uL of the solution with pollen was then pipetted onto a slide for viewing under the microscope. All photographs were taken with a Zeiss Axiocam camera with a Zeiss Axioplan light microscope, and image analyses were done using Zeiss Axiovision v. 8.2 software.

Bud Growth Rates- Inflorescences were photographed alongside a ruler for scale. To detect any differences in bud growth rates between diploids (2*x*) and tetraploids (4*x*), we chose a representative subset of five individual inflorescences from each population. Buds were sampled at 5 equally spaced intervals, from below the inflorescence apex to the oldest and largest bud, just before its opening (Figure 1). These were photographed with a stereoscope at 60x magnification, and bud length and width were measured. To detect differences in growth rate, we regressed $\log_{10}$ bud size on developmental stage (1-6) to test for a difference in slopes between cytotypes.

Statistical Analyses- To test if the proportion of tricellular pollen differs among cytotypes at four sequential developmental stages, we used a mixed model ANOVA with repeated measures. The generalized linear mixed model with a binomial distribution and logit link function will account for the fixed and random effects while allowing us to detect if there are any differences in the mean proportion of tricellular pollen both within and between cytotypes. Specifically, we want to know how variation in the proportion of pollen at the tricellular stage is explained by our main fixed effects, ploidy (2*x* vs. 4*x*), floral stage (5-8), and the interaction between ploidy and floral stage. Because elevation and HDD might affect the speed or duration of pollen development, both were included as covariates in the full model with plant and population, nested within

ploidy as random effects. Reduced models were analyzed in a stepwise manner to determine which reduced model had the lowest AIC score. All analyses were performed in JMP v. 16.0 (SAS Institute Inc., 2021).

An ANCOVA with least squares estimation was used to determine if the hydration index of pollen differed between 2*x* and 4*x* populations. The response variable for the model, *HI*, was normally distributed for both ploidies and no transformation were used. Because *HI (fresh pollen volume divided by fully hydrated volume)* varies with pollen volume, the volume of fresh pollen was used as a covariate.

A mixed model ANOVA with least squares estimation was used to determine if pollen viability differed with ploidy or time since dispersal (longevity). Transformation of the response variable was not needed. Six tetraploid plants from this and all other analyses were removed, due to < 3 % pollen viability at time zero.

Comparison of floral bud size distributions was analyzed with two-way ANOVA with standard least squares estimation. Here we asked if ploidy (2*x* vs. 4*x*), stage (1-6), or the potential effect of one on the other (ploidy*stage) could explain any differences in bud growth rates between diploid and tetraploid plants.

Section Three Results

Populations of the two cytotypes did not differ in elevation. The mean {95% confidence interval (CI)} elevations of diploids and tetraploids were 963 m {597, 1328} and 1381 m {987, 1776} (P = 0.11; N = 6 populations/cytotype). Temperature accumulation during flower and pollen development also did not differ between the cytotypes, with mean *HDDs* of 2*x* and 4*x* populations of 433 ^oC {383, 483} and 441 ^oC {387, 495}, respectively (P = 0.82; N = 6 populations/cytotype).

Bicellular and Tricellular Pollen Counts- The proportion of tricellular pollen found in anthers at four floral developmental stages 5-8 was significantly higher in 4*x* than in 2*x* individuals (P <0.0001; **Figure 5**; **Table 3**). Floral stage had minimal, but positive, influence on that result (P = 0.0005). The probability of reaching the tricellular stage increased similarly in both cytotypes, as indicated by the lack of an interaction effect (Table 3). The proportion of tricellular pollen increased from 19% to 27% in unopened to senescent flowers of diploids, and similarly from 92% to 95% in tetraploids (Figure 5).

Although temperature (HDD) and elevation were included in the full model (Appendix Table A1), they were dropped from the final model since their inclusion degraded the models performance. HDD had a positive effect (P = 0.05), and elevation a negative (P = 0.0005), on the proportion of tricellular pollen (see Full model in Table A1 of appendix). Variation among populations in tricellular pollen production accounted for 6.3% of the variance (**Appendix Figure 1; Appendix Table A2**).

Pollen Hydration- The *HI* index was strongly positively associated with initial (fresh) pollen size (**Figure 6**; **Table 4**). After dropping the non-significant interaction effect (P = 0.19) and accounting for the positive effect of pollen size, 4*x* plants had a slightly lower *HI* than 2*x* plants

Table 3. Repeated measures ANOVA on the proportion of tricellular pollen by ploidy and floral developmental stage (reduced model, $R^2_{adj.} = 0.89$).

Main effects	*DF*	*F* ratio	Prob > *F*
Ploidy	1	742.3113	< 0.0001
Stage	3	5.9774	0.0005
Ploidy*Stage	3	0.4543	0.7144

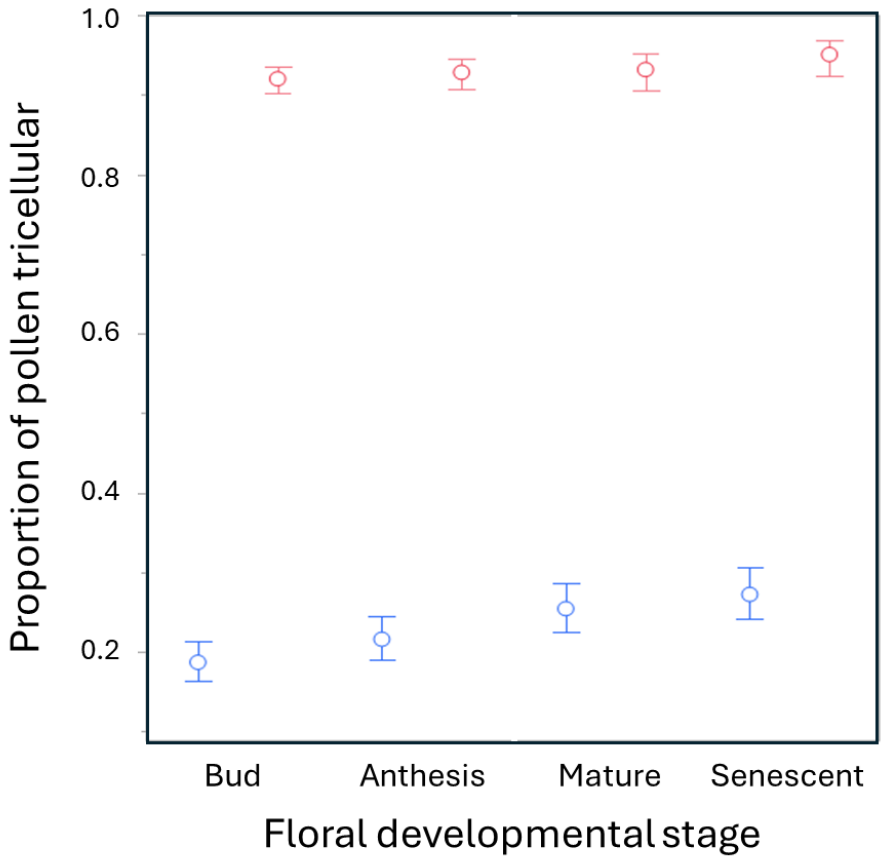

Figure 5: The mean proportion of pollen per anther that had reached the tricellular stage across four sequential floral stages. Diploid populations in blue, tetraploid in red. Open circles are untransformed means with 95% confidence intervals indicated by the bars.

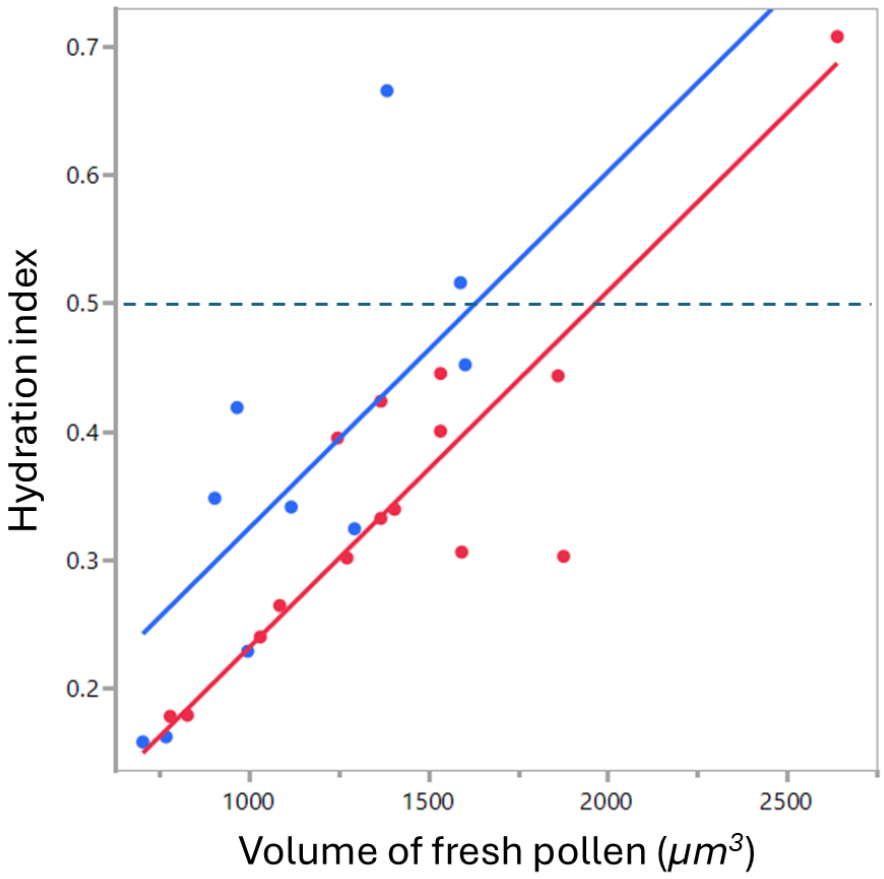

Figure 6. Pollen hydration indices for diploid and tetraploid *Galax urceolata* plants. Diploids in blue, tetraploids in red. Values of less than 0.5, indicated by a horizontal dashed line, represent the threshold for dehydrated pollen. Regression lines from ANCOVA with fresh pollen volume as the covariate.

Table 4. ANCOVA results of analysis of hydration index of 2*x* and 4*x* plants. Fresh volume of pollen at the time of dispersal was used as a covariate (reduced model, $R^2_{adj.} = 0.62$).

Main effects	*DF*		*F* ratio	Prob > *F*
Ploidy	1	0.045786	6.1348	0.0214
Fresh Volume	1	0.305415	40.9222	<0.0001

(*P* = 0.02). The mean *HI*s "bolded", within their 95% confidence intervals, were {0.270, **0.318,** 0.365} and {0.352, **0.411,** 0.469}. for 2*x* and 4*x* plants, respectively. For any given fresh pollen size, 4*x* plants had a 9% lower *HI* than diploids. All but three plants dispersed pollen with an *HI* index < 0.5.

Pollen Longevity- The ANOVA analysis did not find evidence for significant differences in the longevity of pollen from 2*x* and 4*x* populations (**Figure 7**; **Table 5**). Both cytotypes had viabilities of approximately 88% in fully open flowers, but 2*x* pollen from the tetraploids lost viability faster than diploids, as can be seen in the significant interaction effect *(P* = 0.02; Table 5). Over three days tetraploids lost 7.1 % viability per day, whereas diploid viability is decreased by only 2.6 % per day.

Bud Growth Rates- Buds at six floral developmental stages were sequentially larger towards the base of the inflorescences, such that the slope of $\log_{10}$-transformed bud sizes was linear for both cytotypes (**Figure 8**; **Table 6**). The lack of an interaction effect indicates the log slopes were similar *(P* = 0.52), and therefore that progression in bud sizes at sequential stages followed the same growth trajectory within 2*x* and 4*x* inflorescences. At each floral developmental stage, tetraploid buds were 51% larger than diploid buds.

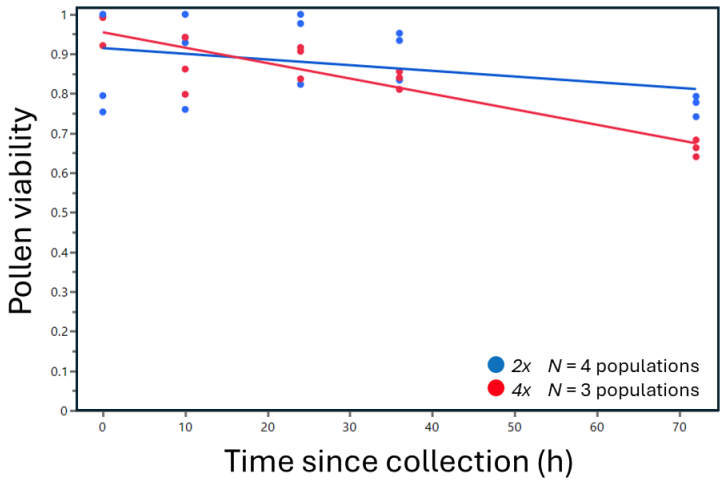

Figure 7. Pollen longevity in diploid and tetraploid populations of *Galax urceolata.*

Table 5. ANOVA results of analysis of longevity and viability in 2*x* and 4*x* plants by ploidy and time (reduced model, $R^2_{adj.} = 0.53$).

Main effects	*DF*	*F* ratio	Prob > *F*
Ploidy	1	0.0008	0.9295
Time	1	19.1855	<0.0001
Ploidy*Time	1	5.7013	0.0188

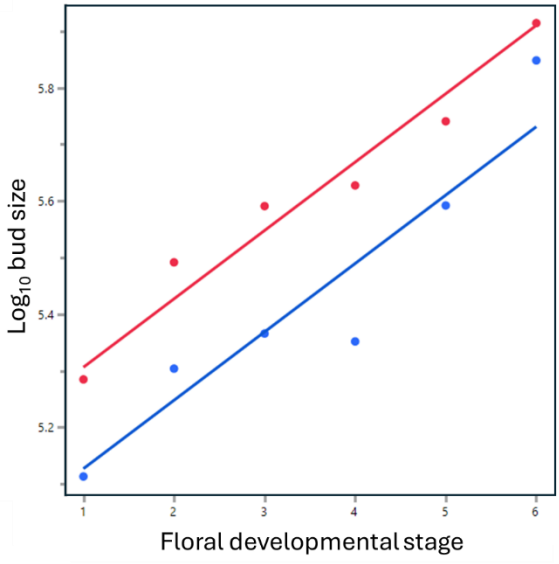

Figure 8: Bud sizes at six sequential stages. Stage 1 buds are near apex, with progressively basal buds up to stage 6 buds that are just opened flowers.

Table 6. Two-way ANOVA analysis of bud sizes by ploidy and floral developmental stage (reduced model, $R^2_{adj.} = 0.91$).1

Main effects	*DF*	*F* ratio	Prob > *F*
Ploidy	1	47.2307	< 0.0001
Stage	1	159.137	<0.0001
Ploidy*Stage	1	0.4559	0.5186

Section Four Discussion

The main result of this study is that six separate autopolyploid populations have converged on the production of pollen that reaches a sexually mature tricellular state before pollen dehydration and presentation. Tricellular pollen is also regularly produced in diploid progenitor populations, but in much smaller quantities. Importantly, both temperature during development and floral developmental stages did not affect the differences in pollen sexual maturity between cytotypes. These results suggest that WGD could provide a heritable mechanism for shifting male gametophyte development to earlier sexual maturation before pollen is dispersed.

Pollen of tetraploid plants is 33% larger than pollen of diploid plants (Williams et al., 2025), and larger pollen often has higher water content at the time of its dispersal, as inferred from positive hydration index-size correlations (Franchi et al., 2002; Williams & Brown, 2018). Larger pollen has a smaller surface to volume ratio, and therefore reduced water loss should allow higher water retention. If polyploidy delays flower development and maturation, then well hydrated pollen would have more time in the anther to proceed from the bicellular to the tricellular stage of development. Prolonged development would be especially likely if *Galax* pollen was typically dispersed in a hydrated state (Williams & Brown, 2018). Thus, we supposed initially that pollen of the tetraploids would be hydrated, or at least more hydrated, than pollen of the diploids, and that longer anther development and higher water content of 2x pollen would enable more pollen to reach the tricellular stage before pollen dispersal.

The pollen of most angiosperms undergoes programmed dehydration in the anther and is in a relatively dehydrated (dormant) state at the time of pollen presentation and dispersal (Firon et al., 2012; Pacini & Dolferous, 2019). But pollen of both 2*x* and 4*x* cytotypes of *Galax* had hydration indices that were consistent with their dehydration in the anther. Therefore, to reach the terminal

tricellular state before dehydration occurs, such pollen would need to develop faster (Williams & Reese, 2019). Interestingly, for any single hydration index, 2*x* pollen was about 30% larger in volume than 1*x* pollen, or about the same as the measured effect of autopolyploidy on pollen volume (Williams et al., 2025). Thus, polyploidy did not affect water content directly, it affected water content through it effect on enlarging pollen.

Variation in pollen water content within cytotypes might explain variation in pollen sexual maturity. The hydration index measures the magnitude of pollen size change after imbibition, and an *HI* of < 50% correlates with water content by weight of < 30% (Nepi et al. 2001). *Annona squamosa* pollen has a relatively high water content of 60 %, as measured by weight. As one of many species that disperses its pollen in a hydrated state, variation in the degree of hydration might explain why it disperses both bicellular and tricellular pollen. In *A. squamosa* tricellular pollen was found only in older flowers (Lora et al., 2009), and the tricellular stage of development in *A. squamosa* was first reached > 9h after flower opening. In contrast, the high frequencies of tricellular, dehydrated pollen in *Galax* were reached at least a day before flower opening. Fewer than 10% of angiosperm species sampled to date have both tricellular and dehydrated pollen (eg. *Arabidopsis*, Moon et al., 2020; see Williams and Brown 2018), since it is difficult to reach a later stage of pollen development without complementary changes in the timing or duration of anther growth and ontogeny at the same time (Williams et al., 2014).

Programmed dehydration of pollen in both cytotypes is also reflected by their relatively high longevity (Dafni & Firmage, 2000). An inverse relationship is thought to exist between pollen water content and longevity. *Galax* pollen had initially high levels of viability in both 1*x* and 2*x* pollen (above 88%, in both this study and in Williams et al., 2025). A common measure of pollen longevity is the time required for pollen viability to become reduced to 50% (Kumar,

1995). We found viability of both cytotypes was still above 65% after three days. Thus, by any measure, both had fairly high pollen longevity (Dafni & Firmage, 2000). Pollen released from tetraploid plants had a slightly greater loss of viability after three days than pollen of diploids, which suggests that the larger 2*x* pollen loses water faster than 1*x* pollen. The smaller surface-to-volume ratio of a larger sphere would suggest the opposite result – slower water loss in the larger sphere - but it is also possible that polyploids have a different pollen wall composition and/or thickness (Corneillie et al., 2019).

Pollen of tetraploid *Galax* is 33% larger than that of diploids (Williams et al., 2025), which means that development must either occur faster or for a longer duration to proceed beyond what we infer is an ancestrally bicellular stage. All reports for species in Diapensiaceae and its familial relatives, Styraceae, Simplocaceae, and Theaceae, find pollen in these families is bicellular (8 genera; Williams et al., 2014). Traditional hypotheses consider polyploidy to be accompanied by slower rates of development, due to increases in chromosome numbers and cell and organ sizes ("gigas effects"; Doyle & Coate 2019). Bud sizes (L x W) were 51% larger in 4x plants, and floral organs were 34-35% longer (Williams et al., 2025). Yet, there was no evidence for differences in the rate of floral bud growth between the diploid and tetraploid *Galax* populations. Since flowering times are similar when *Galax* cytotypes are sampled at the same elevation (Barringer & Galloway, 2017), anthers appear to have a similar amount of time in which to develop pollen grains. Williams et al. (2025) found that the larger *Galax* tetraploid flowers produced 85% more pollen than their smaller diploid progenitors. Since both cytotypes have comparable flowering times the increased numbers of larger pollen grains suggest that more cell divisions occurred in the developing 2*x* pollen in the same amount of time.

In this study, the similarity in bud growth rates, flowering times, developmental arrest by dehydration in the anther, and increased pollen numbers all suggest that the tetraploid plants have evolved accelerated pollen development. The origin of tricellular pollen in 4*x* plants involves acceleration of pollen cell cycles, not prolongation of anther and pollen development before programmed pollen dehydration and pollen presentation and dispersal, as occurs in *Annona* and other Annonnaceae. In *Galax*, the timing of programmed pollen dehydration imposes a deadline for completion of development, consistent with our finding that there was very little increase in the frequency of tricellular pollen in anthers after flower opening in older flowers. Polyploidy provides a mechanism for cell cycle acceleration in that WGD provides doubled gene template dosage, conserving the stoichiometry of biosynthetic pathways, which can enable increased metabolic rates to evolve.

Bennett (1971) and Bennet and Smith (1972) found that polyploid *species* have shorter durations of meiosis and pollen maturation, which suggested that evolution after WGD can result in faster biosynthetic processes, relative to diploid species. Pollen gene expression begins at the bicellular stage of development (Nelms & Walbott, 2022), but after WGD pollen genes have diploid, not haploid, expression. *2n* pollen heterosis might contribute to "pollen vigor" (Williams, 2021). Pollen tube growth rates (PTGRs) have long been used as a proxy for pollen metabolic vigor. In intraspecific comparisons of 1*x* and 2*x* PTGRs, 2*x* pollen was always slower than or similar in growth rate to 1*x* pollen (Reese & Williams, 2019; Westermann et al., 2024). However, two comparative analyses found that polyploid species have evolved faster PTGRs than species of the diploid base chromosome number of their genus (Reese & Williams, 2019; Williams & Oliveira, 2020). Thus, if WGD makes it harder to reach later stages of development within the same time frame as that of diploid progenitors, then polyploidy must enable faster biosynthetic

rates to evolve after WGD, compensating and often exceeding the performance rates of their progenitors.

Although we have discussed the evolution of monomorphic pollen from a polymorphic predecessor here, an equally interesting question is why were *Galax* diploid populations all polymorphic for bi- and tri-cellular pollen in the first place? Having both types of pollen cell state at dispersal has been noted in some early-diverging families of flowering plants but limited records exist due to typological biases in considering only the bicellular or tricellular state as characteristic of a species. Yet, Brewbaker (1967) reported a number of unrelated cases of intraspecific polymorphism, whereas others have found polymorphism to be phylogenetically localized. For example, Gan et al. (2025) included 89 families in their study and found 16 species of Annonaceae release both bicellular and tricellular pollen. Intraspecific polymorphism might be caused by environmental conditions such as fluctuating temperatures. Perhaps *Galax* 4*x* populations are responding to higher temperatures differently than *2x* populations, resulting in earlier maturation of pollen grains. Diploid and tetraploid *Galax* cytotypes seem to occupy very similar niches, including the same potential pollinator assemblages (Johnson et al., 2003; Barringer and Galloway 2017; Gaynor et al., 2018). Our finding of similar *HDDs* among cytotypes suggests that despite elevational variation, floral development in both cytotypes is tracking temperature. The phenotypic effects of polyploidy are more important than environmental variation in determining the speed and duration of pollen development. Dehydrated pollen, such as bicellular pollen, tends to have greater longevity because dehydration triggers desiccation tolerance mechanisms and slows down metabolic activity. In contrast, hydrated pollen remains metabolically active, making them ready for rapid germination. But continued metabolic activity uses up stored reserves during presentation, dispersal, and

germination, with the result that tricellular pollen is much shorter-lived. For example, pollen from *Arabidopsis* was found to be dehydrated at dispersal while remaining viable outside of the anther for up to 3 days, compared to many grass pollens which have high water contents at dispersal and remain viable for less than 30 minutes once dispersed from the anther (Williams 2012). The results of this study are somewhat contradictory in that we have variation within a single species of bicellular pollen that is more hydrated and longer lived, compared to tricellular pollen which was more dehydrated and shorter lived than bicellular pollen. Our results suggest that in *Galax* diploids, as in many other species, pollen undergoes dehydration late in its cell cycle, such that dehydrated, bicellular pollen is near, or in, prophase of mitosis II at the time of dehydration and dispersal. Some pollen has even gone through mitosis II to form sperm, before undergoing developmental arrest with dehydration. Since polyploidy did not alter either floral bud and presumably anther growth rates or the fact of programmed dehydration and given that producing larger pollen grains requires more work in the same amount of time, the data suggests that tricellular pollen has selective value, or at least that some deterministic process is driving the transition to high frequency of tricellular pollen production in *Galax.*

Section Five Conclusion

Few examples of intraspecific variation in pollen sexual maturity exist. The tricellular state is a later stage of development than the bicellular state, and yet in large-scale phylogenies, the tricellular state is most often a derived state. Polyploidy is thought to prolong development, but faster biosynthetic rates may evolve rapidly due to duplication of genes within chemical and signaling pathways. In *Galax* tetraploids, anthers produce both larger and more pollen without extending developmental times, and the same appears to be the case for the male gametophyte – acceleration of polyploid gametophytic cell cycle has resulted in early sexual maturation of pollen. Given widespread ancient and current WGDs in angiosperms, polyploidy may have often provided a pathway to faster reproductive development and shorter life cycles, before diploidization has obscured that history.

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Appendix

Table A1. ANOVA (full model) analysis on proportion of pollen per flower at the tricellular stage ($R^2_{adj.}$ = 0.89).

Main effects	*DF*	*F* ratio	Prob > *F*
ploidy	1	242.3957	**< 0.0001**
stage	3	7.1372	**0.0001**
ploidy*stage	3	0.3531	0.7869
hdd	1	3.9704	**0.0476**
hdd*ploidy	1	0.4376	0.509
elevation	1	12.6448	**0.0005**
elevation*ploidy	1	6.5026	**0.0114**

Table A2. Variation among populations (nested within ploidy level) in the proportion of pollen tricellular.

Random Effect	Var Ratio	Var Component	95% Lower	95% Upper	Wald p-value	Pct of Total
Pop[ploidy]	0.0668986	0.048365	-0.02912	0.125846	0.2212	6.27
Residual		0.722955	0.646374	0.814074		93.73
Total		0.771319	0.670248	0.897239		100

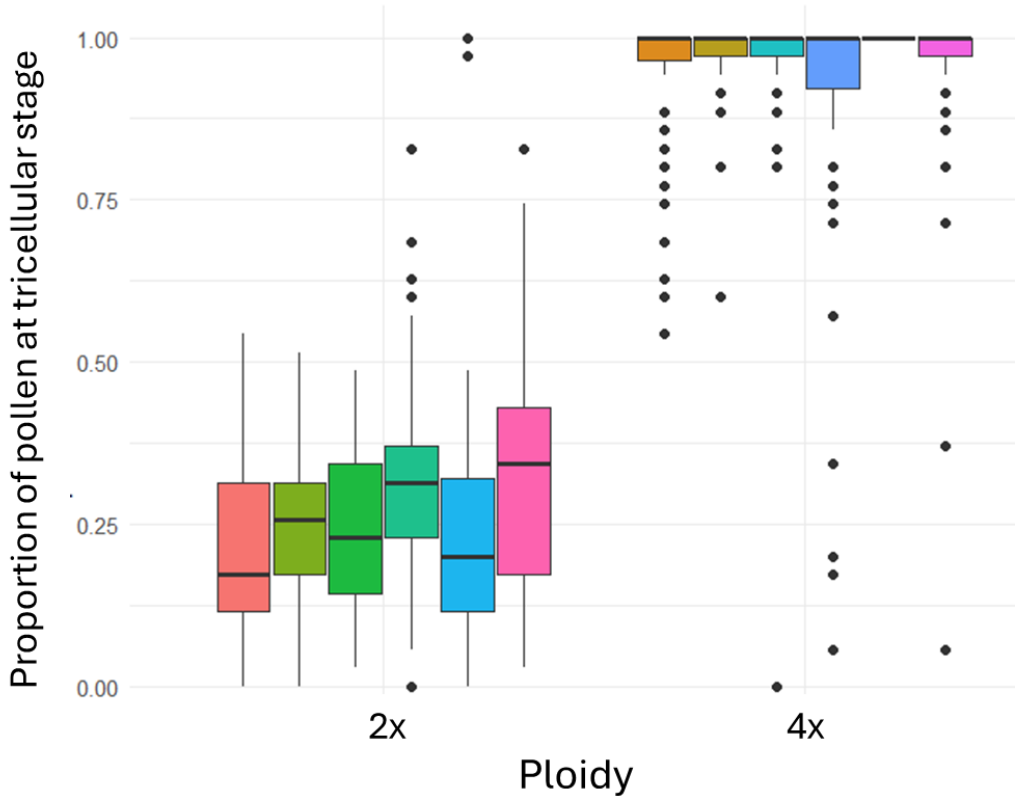

Figure A1. Variation among populations in tricellular pollen production. (means across four sequential stages)

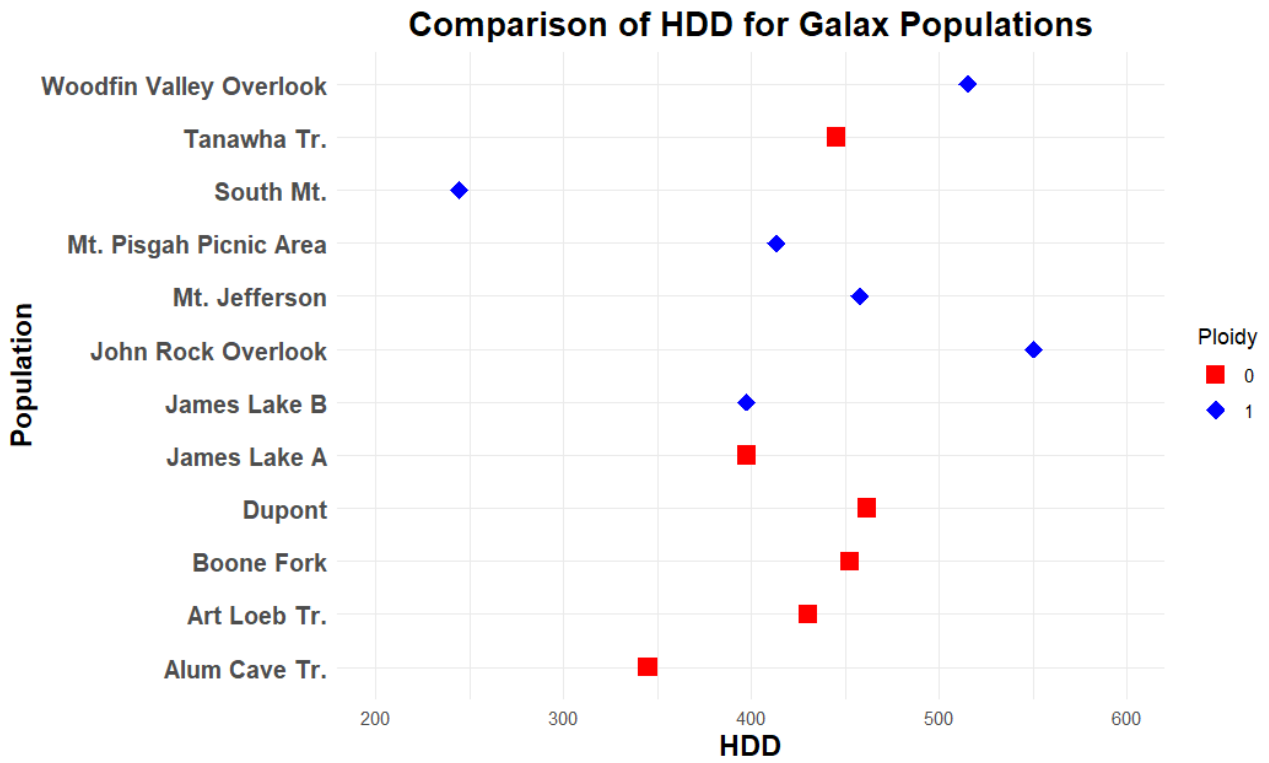

Figure A2. Comparison of *HDD* (Heating Degree Days) between diploid and tetraploid populations sampled. Red squares represent diploid populations (0). Blue diamond's represent tetraploid populations (1).

Vita

Following a twelve-year run as a veterinarian assistant/technician, Tonika Birmley began her academic career in 2016. After completing the Associates of Science program in 2018 at Pellissippi State community College, she enrolled at the University of Tennessee, Knoxville (UTK) in 2019. During her time as an undergraduate she worked as a laboratory technician in the Williams lab at UTK and graduated in the summer of 2023 with a Bachelors of Biological Sciences. The research interests of both Tonika and the Williams lab aligned with a focus on questions about flowering plant reproductive evolution. Tonika entered the Ecology and Evolutionary Biology program at UTK in August 2023 and received a Master of Biological Science in August 2025. Her experience as a student teaching assistant has lasted for four semesters, focusing on Introduction to Plant Biology courses at UTK. In her free time, Tonika enjoys volunteering with local organizations like Native Plant Rescue Squad and the Naturalists Club. With over 4,500 in grant money as a graduate student, Tonika was able to present several posters and travel to major botany conferences as a speaker during her time at UTK.