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A Comparative Analysis of the Relative Water Content of the Pollen of Early Diverging Angiosperms

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Honors Thesis

by

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Abstract:

The pollen of most angiosperms goes through a process of dehydration before anthesis, the opening of the flower (Heslop-Harrison 1979a). During dispersal, further dehydration often occurs (Heslop-Harrison 1979b). Dehydrated pollen comes in two degrees: hydrated (also termed desiccation-sensitive or recalcitrant) at greater than 30% water content by mass and dehydrated (also termed desiccation-tolerant or orthodox) at less than 30% water by mass (Franchi et al 2002, 2011). Most species tend to undergo some degree of dehydration, or developmental arrest, before anther opening (Franchi et al 2002). Angiosperms are known to have much faster reproductive processes than other seed plants, which may be in part due to a novel ability to germinate pollen rapidly. The degree to which mature pollen is hydrated is directly correlated to germination speed, and with little being known about the pollen germination process of early diverging angiosperm lineages other than their ability to rapidly do so, the degree of hydration of a species is of particular interest. As such, this study is meant to shed light on the hydration status of pollen within early divergent angiosperm lineages. Five early-diverging angiosperms were selected in order to encompass a wide range of ecological strategies. The pollen of the woody terrestrial *Liriodendron tulipifera* and the aquatic *Nymphaea odorata* were found to be dehydrated, whereas that of the woody semi-aquatic *Hedyosmum brasiliense* and the herbaceous aquatics *Nuphar advena* and *Brasenia shreberi* were found to have hydrated pollen. These data reveal a remarkable diversity of pollen strategies among ancient angiosperms.

Introduction:

It has been known for some time that the pollen of most seed plants goes through a process of dehydration before anthesis, the time of pollen dispersal (Heslop-Harrison 1979a). A number of flowering plants, however, have fully hydrated pollen at anthesis. The transition between being desiccation-resistant (partially dehydrated pollen with less than 30% water content by mass) or desiccation-tolerant (partially hydrated pollen with greater than 30% water content by mass) is assumed to be an easy one as the trait is often variable within families (Franchi et al 2002). Dehydrated pollen grains can rehydrate through the absorption of stigmatic fluid (present in many angiosperms) upon landing on the stigma (Heslop-Harrison 1979a). Generally a species has pollen grains well below or well above the 30% mark, although high variance has been observed (Franchi et al 2002 and 2011). This appears to be the case even though multiple genes influence the trait (Franchi et al 2002). The pollen grains of desiccation-tolerant pollen are often found to be larger than those of desiccation-tolerant pollen, which is most likely due to a function of the ratio of volume to surface area (Franchi 2011). As the pollen grain increases in size, its volume to surface area ratio increases due to volume increasing at a cubic rate and area increasing at a squared rate. This allows for a smaller amount of water loss through the pollen cell wall, as there is a proportionally greater amount of internal water to surface area as the cell increases in size.

Both desiccation-sensitive and desiccation-tolerant strategies appear to incur both selective benefits and hindrances to the pollen grain. Desiccation-tolerant pollen grains tend to be smaller and significantly lighter than desiccation-sensitive pollen grains (Franchi 2011). Small and light pollen grains are especially important for plant species which rely on abiotic pollination (wind, water). The relatively small mass of the pollen grains allows for a large number of pollen grains to be produced, which is essential for strategies involving random mass dispersal. The small mass of the pollen grains also allows the pollen to better move within the environment and cover greater distances when they are carried by the wind and water. Another advantage incurred by desiccation-tolerant pollen is its ability to

survive for a long period of time after anthesis. After dehydration, the pollen grain is kept in a state of low metabolic activity, and as such is able to stay alive for an extended amount of time. Such dormancy extends the window of pollen viability. The main downside to having desiccation-tolerant pollen is that due to the low metabolic activity of the pollen grain caused by its dehydrated state, the pollen grain must first rehydrate before it can restart metabolic processes and then germinate. The downside of being slow to germinate is that pollen grains that germinate faster can deliver sperm to the ovary earlier than those that germinate slower, which means that their genes will be passed on preferentially. This is due to the fact that a pollen grain must be metabolically active in order to begin the formation of a pollen tube, the structure that grows out of the pollen grain and transports the sperm to the ovary. Since hydrated pollen is already metabolically active when it reaches the stigma, it is better able to deliver its sperm to the ovary than that of dehydrated pollen. Due to this, all things being equal, it is more advantageous to germinate quickly than slowly as it increases the pollen grains' ability to compete with other pollen grains. The benefits to desiccation-sensitive pollen are its quick germination time, while its downsides are its susceptibility to dehydration and early death of the pollen grain and thus a more narrow pollination window (Franchi et al 2002, Carlson and Swanson 2009).

Additional factors need to be taken into account when examining pollen hydration. For instance, some plants utilize pollen as a food reward for insects (Franchi et al 2002). Pollen thus utilized is generally of the desiccation-tolerant sort as it is more nutritionally dense than desiccation sensitive pollen due to the smaller proportion of water (Franchi et al 2002). While in a dormant, desiccated state, the pollen also does not use up as much of its energy reserves as it would were it in an active hydrated state. This allows for more energy to be available to consumers of the pollen grain per unit mass when the pollen is dehydrated, making it a more attractive food source.

Several techniques have been employed in examining pollen hydration status (pollen water content). One common technique that is utilized is to dry out pollen in an oven to establish a dry weight for a sample of pollen which is then measured against the fresh weight of the sample taken before it

was placed in the oven (Franchi et al 2002). Another technique used is to measure pollen shape with a high powered microscope. (Payne 1972, Payne 1981). By assuming an even ovoid shape, a rough volumetric measurement can be ascertained by measuring along the two axes of the sample. The changes in volume after being dried in an oven are used to determine the hydration status of the fresh pollen. A third method produces qualitative results but not quantitative ones. In many species of early diverging angiosperms with dehydrated pollen, a deep furrow is present along the long axis that is visible under the microscope relating directly to the aperture of a monosulcate pollen grain. This furrow occurs through the collapsing of the pollen grain as its volume is reduced while it maintains a constant surface area. The pollen of dehydrated species is also often found to have an oblate spherical shape, most commonly an ovoid (Franchi et al 2002). This occurs from the fully hydrated spherical pollen having its volume reduced, with one of the axes of the pollen not reducing in length while the other does. The presence of such traits in pollen strongly suggests the dehydrated trait is present.

Due to the novel trait of rapid pollen germination found in angiosperms and the correlation between relative water content and germination speed, the status of early diverging angiosperm pollen with respect to its relative water content is of great interest. The reason for this is that it may shed light on a novel strategy used by early diverging angiosperms to outcompete other seed plants, and may shed new light on the way that the angiosperms came to dominance.

Methods:

In order to determine the hydration status of the pollen grains two protocols were used. In the first protocol, a sample of fresh pollen was weighed on a microbalance immediately after collection and is thereafter placed in an oven set to 105 degrees Celsius to dehydrate. After 72 hours, the pollen was then reweighed to determine the dry weight of the pollen. The difference between the two measurements when divided by the initial weight and multiplied by 100 gives the percent mass lost in the oven, from which the relative water content in the fresh pollen is inferred. In order to weigh the

pollen, individual strips of weigh paper are first weighed and then pollen is place on them. The pollen and weigh paper are then taken to a Mettler Toldeo microbalance with error of 0.1 µg at thirty second intervals for fifteen minutes in order to find an average value for the mass. The weight of the paper is then subtracted from this number.

The benefits to the weight method are that you are actually measuring the true fresh pollen water content by weight as opposed to measuring a trait that is correlated with water content. This means that if the weight measurements are accurate, then the data will accurately convey the hydration status of the pollen grain. The downsides for the weight method are that the measurements have to be very exact to be meaningful and the data is very susceptible to disturbances. This method is also more time intensive as only one sample can be gathered and processed at a time near the balance, which is especially difficult with species like *Brasenia shreberi* that have very limited flowering times.

The second method involves measuring changes in fresh pollen volume after drying or hydrating in water to infer fresh pollen water content (Figure 1). Pollen is collected and dehydrated it in a similar fashion to the first protocol, but instead of measuring the mass of the pollen, the volume of the pollen is measured under a high powered microscope. The pollen is collected in three tubes, one filled with immersion oil, one filled with water, and one empty to hold the dry sample. The empty tube is placed in an oven at 105 degrees Celsius for 72 hours to give the dehydrated volume of the pollen. The mixture of oil and pollen (the purpose of which is to prevent dehydration of the pollen) in the other tube is transferred to a microscope slide and will give the fresh volume of the pollen. Pictures of the pollen in oil are taken through the microscope using Zeiss AxioVision 4.1 software to determine the dimensions of the pollen grain. By assuming that the pollen is symmetrical and ovoid, with respect to its two equatorial axes, the following formula was used to calculate pollen volume:

$$V = 4\pi a^2 b/3,$$

where a is the radius of the short axis and b is the radius of the long axis (Williams 2012).

Pictures and measurements of the pollen are taken and compared to those of the fresh pollen.

The difference in volumetric measurements is representative of a loss of volume upon dehydration, which I hypothesize is correlated with a loss in water mass (Figure 1). This would mean that more desiccation sensitive pollen will result in a greater degree of volume loss than desiccation-tolerant pollen. The volumetric analysis was also performed on pollen grains that had been placed in water for 15 minutes or 6 hours to determine the maximum size of the pollen grains upon undergoing hydration. The experiment predicts that the pollen placed in water will absorb water until it is fully hydrated, giving the maximum volume of the pollen grain, with dehydrated pollen grains absorbing more water than hydrated pollen grains, and thereby gaining more volume as shown in figure 1.

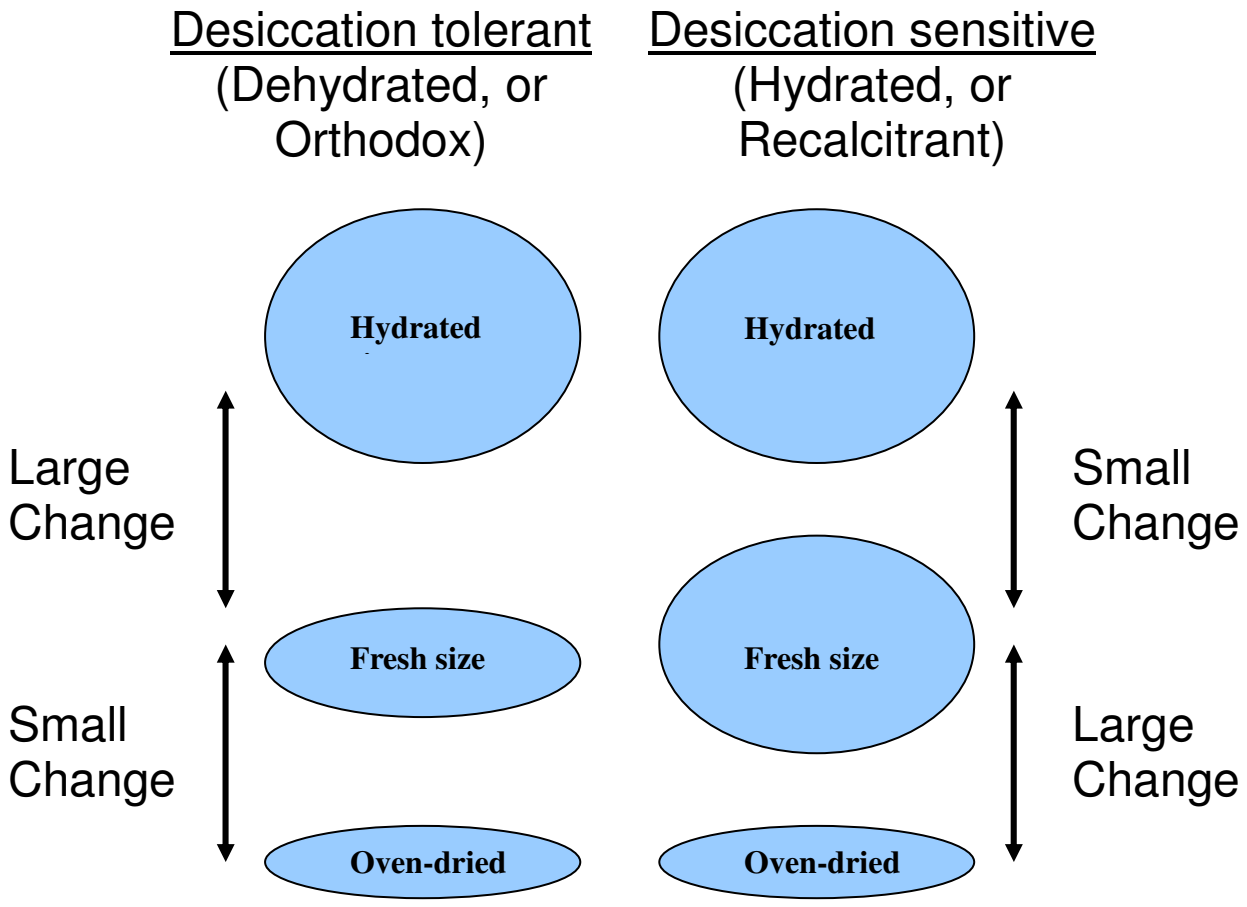

Figure 1. Qualitative changes in fresh pollen grain size after either hydration or oven drying. The hydration status of fresh pollen is inferred from the pattern of size changes. The dessication tolerant pattern is shown in left column and the dessication sensitive pattern on the right.

The benefits to the volumetric analysis are related to the downsides to the mass analysis. The most critical benefit comes from the accuracy of the readings. Because the pollen grains themselves are being observed and measured, the volume that is measured for each pollen grain is very accurate for species with uniformly shaped pollen grains (some species such as *Nymphaea oderata* have irregularly shaped pollen, with *Nymphaea* having pollen that is somewhat mushroom like shaped). To compensate for these irregularities, care was taken to only measure the transverse, ovular, axis that is in the same plane as the aperture of zonosulcate pollen. An additional benefit is that multiple samples can be gathered at a time, and the samples do not have to be rushed to the balance. An additional benefit to the volumetric analysis is that the maximum pollen hydration level can be determined by measuring the size of the pollen grain after it has been placed in a vial of water, whereas mass-based protocols are incapable of doing such as measuring the fully hydrated pollen is not possible with such a method. The main downside is that the technique gives a qualitative analysis rather than a quantitative analysis. This is because a greater volume loss in one species over another is due to that species being more hydrated than the other, but the exact amount to which it is more hydrated is not precisely determinable because of variation in the thickness of the pollen walls and characteristics of the aperture. Possible reasons for this come from the fact that water has a different density than the dry matter left over, and as such does not correspond in a 1:1 relationship between volume and mass lost. Another possible reason is that structures in the pollen grain itself resist size compression, upon desiccation or expansion from hydration, and may replace some of the vacated areas with air or other things.

For the purpose of this investigation, a qualitative analysis was more important than a quantitative analysis, because the goal of the experiment was to determine whether or not the mature pollen each species is hydrated or dehydrated, not the exact water content. Because a much broader sample of species is needed, a field method such as this one is desired. This combined with difficulties encountered when attempting the mass measurement method, led to the adopting of the volumetric analysis method.

The species:

Five early diverging angiosperm species were studied: *Nuphar advena* Ait. and *Nymphaea odorata* Ait. (Nymphaeaceae)*, Brasenia schreberi* J.F.Gmel. (Cabombaceae)*, Liriodendron tulipifera* L. (Magnoliaceae)*,* and *Hedyosmum brasiliense* Miq. (Chloranthaceae) *H. brasiliense* is found in the Mata Atlantica rainforest biome of Brazil, where it is a pioneer species occurring along stream margins at forest edges (Lorenzi 2009). It is dioecious and wind-pollinated. *N. advena* and *N. odorata* are water lilies endemic to eastern North America (Flora of North America 2012). *L.* tulipifera is a deciduous tree endemic to eastern North America. (Flora of North America 2012) *B. shreberi* is a water lily with a worldwide distribution and is found in generally warm conditions from temperate to tropical zones and is wind pollinated (Flora of North America 2012). The *Liriodendron* flowers were sampled on April 6 and 20, 2012 from the campus of the University of Tennessee. The *Nuphar,* sampled between April 20 and April 30, 2012, were grown in an outdoor pond at the University of Tennessee, and the other three species were grown in the Hesler greenhouse at the University of Tennessee, and were also sampled between April 20 and April 30, 2012. The source of the *Nuphar* is near Sparta, TN (35° 55’ 11’’ N, 85° 20’ 41’’ W) (Abercrombie et al 2011). The source of the *Nymphaea* was at Monterey Lake, Putnam County, TN, USA (36° 06’, 85° 14’ E) (Williams et al 2010). The source of the *Brasenia* was also Monterey Lake, Putnam County, TN, USA (36° 06’, 85° 14’ E) (Taylor and Williams 2009). Of these, the only species with mass data are *Nuphar* and *Nymphaea*, and only hydrated and fresh volume was gathered for *Hedyosmum* due to an interruption in its flowering. Hydrated volume was not collected for *Nuphar*.

Results:

Based on predictions from Figure 1, dehydrated species will experience a relatively small decrease in volume when dried in the oven, while showing a large increase in size when hydrated in a vial of water. The opposite effect is seen for hydrated species. Of the species studied, *Liriodendron, Nuphar* and *Nymphaea* were found to be dehydrated, and *Brasenia* and *Hedyosmum* were found to be hydrated.

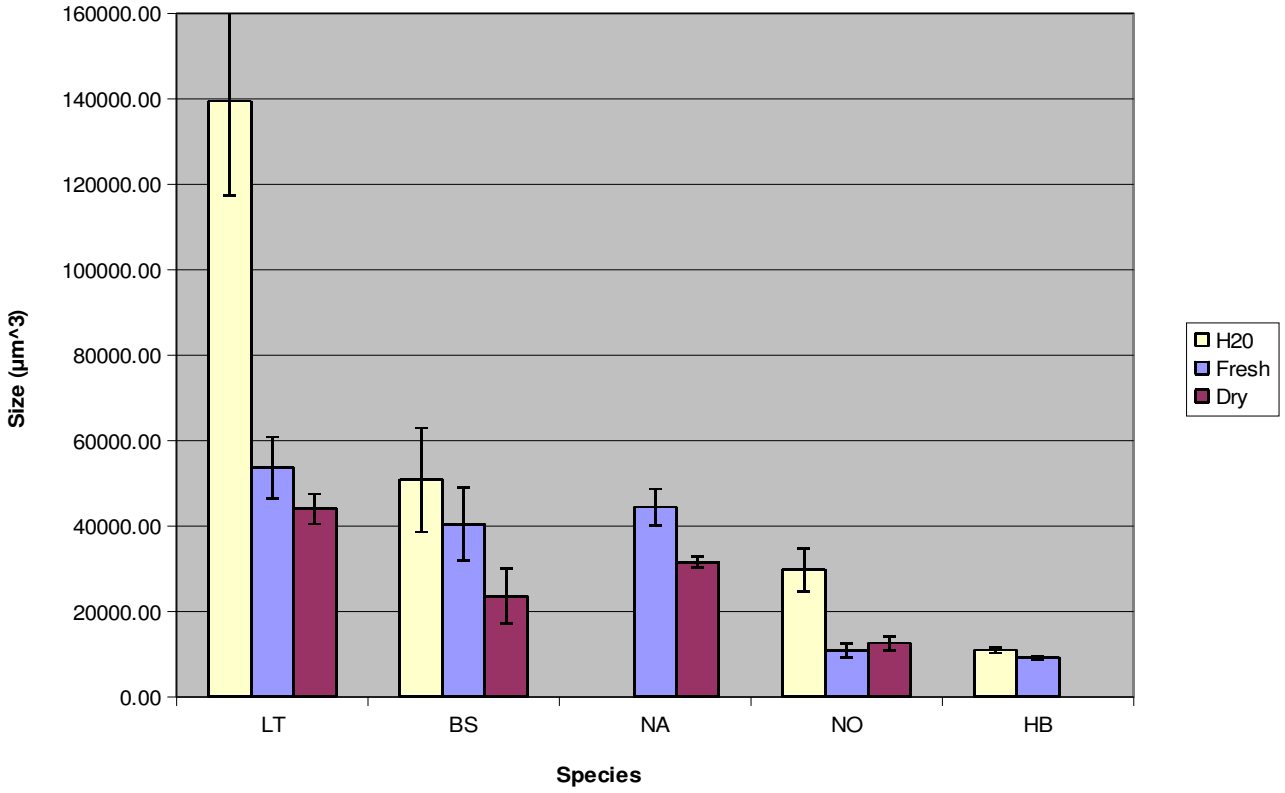

Figure 2. Volumes (μm^3) of pollen grains from *Liriodendron tulipifera, Brasenia shreberi, Nuphar advena, Nymphaea odorata, Hedyosmum brasiliense*. Error bars are standard deviations. H20 refers to hydrated size, whereas dry refers to oven-dried size.

The terrestrial tree *Liriodendron* (LT) lost approximately 12% of its volume upon desiccation, and the water lily *Nymphaea* gained approximately 16% of its volume upon desiccation. The water lily *Nuphar* (NA) lost approximately 29% of its volume upon desiccation, the water lily *Brasenia* (BS) lost

approximately 42% of its volume upon desiccation, Upon being placed in water and allowed to fully hydrate *Liriodendron* pollen increased in volume by 160%, *Nymphaea* increased by approximately 174%, *Hedyosmum* (a semi-aquatic shrub) gained approximately 19% volume, and *Brasenia* increased by approximately 26%. The underlying data can be found in Appendix A.

The pollen of *Nuphar* decreased in mass by approximately 45% after a 72 hour treatment in the oven. However, the weighing paper initially used to hold the pollen while it was drying in the oven lost six percent of its mass after being in the oven for 72 hours, which greatly overshadowed the mass lost by the pollen grains due to the disparity in mass between the pollen sample and the paper holding it. When this is accounted for, the *Nuphar* pollen shows a 23 % loss in mass.

Discussion:

The findings of the study were in line with some expectations, but not others. One expectation of the study was that these early-diverging lineages would have dehydrated pollen as hydrated pollen is a derived trait not generally present in gymnosperms. As these plants represent the most ancient lineages of angiosperms, it was uncertain if by the time of their divergence hydrated pollen had arisen as a novel adaptation. Another expectation that the data did not support was that wind pollinated plants (such as *Hedyosmum* and *Brasenia*) would be dehydrated, but it was found that they were in fact hydrated. A strict delineation between strategies observed by terrestrial versus aquatic plants was not universally observed either. Overall, what was found was a great degree in diversity among the early-diverging angiosperms studied.

Pollen from plants that lived in aquatic environments tended to have a greater volume loss upon drying, and as such, a higher water content, whereas plants that lived in more terrestrial environments tended to have a lower volume loss, which was interpreted as corresponding to a lower water content. Overall, the data within each species was consistent with regards to the percent volume lost in each sample. The difference in observed data between BS12-11/12 and BS12-13/14/15 with respect to their

dry volume is of particular interest. The first set experienced a thirty percent loss in volume while the last set experienced a sixty percent loss in volume. The last set was also left in oven longer than the first set, one week as apposed to three days, as it was hypothesized that after three days complete dehydration should have occurred. The second set's volume loss more closely corresponds to the data gained by other researchers who performed mass loss experiments, and as such, is more likely the correct value (Taylor and Williams 2009). The difference in values most likely comes from a collapse in cellular structure as the first set retained the same length as the fresh samples, while the second set was reduced in both width and length. The most likely explanation was that some structure in the pollen grain was forcibly holding the rigidity of the cell structure in the first set and had collapsed after continued exposure to the heat. This forcible holding of the cell shape most likely exaggerated the size of the contents inside and the cell itself.

The qualitative analysis of the data showed that pollen of *Brasenia* and *Hedyosmum* were both likely hydrated at the time of pollen dispersal with respect to their pollen, while *Liriodendron, Nuphar* and *Nymphaea* pollen were dehydrated. This is consistent with expectations as while *Brasenia* utilizes wind for pollination (Taylor and Williams 2009), its populations are much more clustered, and as such, the pollen does not have to travel as far as would normally be true of a wind pollinated plant such as *Hedyosmum*. *Liriodendron* was found to have dehydrated pollen which is consistent with it being a terrestrial insect-pollinated species. *Nymphaea* pollen was shown to have little change in its volume when dehydrated with some samples losing volume and some gaining it. Several possible conclusions can be drawn from this. One possible explanation is that its exine and membrane are either permeable to the immersion oil in which it was placed, while its plasma membrane was not, causing oil build up between the exine and the membrane. Another would be that the boiling away of the water in the pollen grain expanded the cell wall, which did not subsequently retract.

The maximum hydration analysis further confirmed the results. The dehydrated species *Liriodendron and Nymphaea* showed massive increases in pollen size after being allowed to hydrate in

water. A greater than two-fold increase was seen in *Liriodendron* and *Nymphaea.* This is in contrast with the relatively small increase in volume of the hydrated species *Brasenia*, which showed a 29% increase in size. *Hedyosmum* also showed a very small increase in size at 19%, and as such, can be assumed to be hydrated as well. The spherical shape of *Hedyosmum* fresh pollen is also indicative of a hydrated species. The mass loss of *Nuphar* indicates that the pollen could be dehydrated, but due to the high variance in the data and low sample size this should be further investigated. The somewhat high rate of volume loss at 29% also makes it seem unclear as to the hydration status of *Nuphar*. The shape of *Nuphar* is in alignment with dehydrated pollen.

The final results indicate that the pollen of *Liriodendron, Nuphar* and *Nymphaea* (a woody terrestrial and two herbaceous aquatic species respectively) are both dehydrated, whereas the pollen of *Brasenia,* (both aquatic species) and *Hedyosmum* (a semi-aquatic species) are hydrated. The data regarding *Nuphar* should be further investigated due to high variance and low sample counts. This is interesting as it has aquatic plants as both hydrated and dehydrated as well as terrestrial plants as hydrated and dehydrated. Plausible explanations for each strategy for the particular species can be identified. *Liriodendron* is insect-pollinated, and as such, is more likely to use dehydrated pollen due to it being a better food source for insects and for its ability to survive the possibly long amount of time the insect takes from getting from its flower to another plants. *Nymphaea* is likewise pollinated by insects, and shares the same reasons for why dehydrated pollen would be desirable as *Liriodendron.* *Hedyosmum* and *Brasenia* are both wind pollinated, and as such would normally be expected to have dehydrated pollen. However, in *Hedyosmum's* case the environment in which it is found could be an explanation. *Hedyosmum* is endemic to the tropics of Brazil, which are extremely humid. As such, the hydrated pollen does not run a significant risk of dying in the air due to rapid desiccation, and as such, can gain the benefits of rapid germination afforded to hydrated pollen without the downside of their sensitivity to rapid desiccation. *Brasenia*, as was discussed earlier, tends to be located in dense populations, and as such, does not need its pollen to survive for very long. It is also wind pollinated,

but the pollen is likely to land in the water of the pond it is growing in if it does not find a flower to land on very quickly, and as such, it does not need to consider the longevity of its pollen grains offers no benefit as they will either quickly reach a stigma or quickly land in the water. *Nuphar* is likely governed by similar factors (such as insect pollination and selfing) as *Brasenia* and *Nymphaea.*

Further Research:

Further extension of the research presented in this report would include further mass analyses, energy reserve patterns, and a widening of the species of interest. A refining of the techniques used would also improve the data, specifically with regard to processing time after collection. Specifically, gaining dry volumetric data on *Hedyosmum* and maximum hydration volumetric data on *Nuphar* would greatly increase the usefulness of the findings as it would complete the set of data for the given species.

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Apendix A: Pollen Volumetric Data. Values are given in cubic micrometers and percentages.

Liriodendron Tulipifera	Fresh	Dry	H20	15 Min	% Volume Lost	% Volume Gained
LT12-1	68815.39		114625.30			66.57%
LT12-2	52649.88		148208.70			181.50%
LT12-3	65619.91		127194.70			93.84%
LT12-4	51731		131787.40			154.76%
LT12-5	46672.85		126794.70			171.67%
LT12-6	55412.26		181860.70			228.20%
LT12-7	50538.55		145355.50			187.61%
LT12-11	44488.35	38906.17			12.55%	
LT12-12	51132.24	44641.04			12.69%	
LT12-13	49147.41	42824.30			12.87%	
LT12-14	52339.77	45734.61			12.62%	
LT12-15	55061.93	48227.62			12.41%	
Brasenia schreberi						
BS12-1	34597.99		63528.68			83.62%
BS12-2	36395.56		45143.43			24.04%
BS12-3	27095.97		36729.25			35.55%
BS12-4	34885.41		63516.28	110336.80		82.07%
BS12-5	32689.3		45164.45	66384.89		38.16%
BS12-11	42212.76	29383.47			30.39%	
BS12-12	45216.83	31730.65			29.83%	
BS12-13	49717.9	19281.60			61.22%	
BS12-14	52861.42	18400.27			65.19%	
BS12-15	48834.98	19003.11			61.09%	
Nuphar advena						
NA12-11	42799.17	31098.71			27.34%	
NA12-12	48480.19	33295.53			31.32%	
NA12-13	43204.68	30852.75			28.59%	
NA12-14	38474.21	29971.2			22.10%	
NA12-15	48956.74	32626.17			33.36%	
Nymphaea odorata						
NO12-1	9279.95		29527.80			218.19%
NO12-2	8729.65		37761.68			332.57%
NO12-5	10419.63		23635.21			126.83%
NO12-6	9555.062		29962.97	26259.39		213.58%
NO12-7	9712.152		27789.69	27217.35		186.13%
NO12-11	11160.37	13686.15			-22.63%	
NO12-12	12896.94	14202.19			-10.12%	
NO12-13	13077.37	11722.36			10.36%	
NO12-14	12944.29	10621.06			17.95%	

Hedyosmum brasiliensis			
HB12-1	8878.687	10219.599	15.10%
HB12-2	9526.572	11261.759	18.21%
HB12-3	9202.113	11280.606	22.59%