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COMPARISON OF NAVEL AND LAMINAR STOMATA MORPHOLOGY AND STOMATAL CONDUCTANCE IN THE WHITE WATER LILY, *NYMPHAEA ODORATA* (NYMPHAEACEAE)

An Undergraduate Honor Thesis submitted in partial fulfillment of the

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by

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To: Dr. Steven Oberbauer, Chairperson Department of

Biological Sciences

This Undergraduate Honors Thesis in Biological Sciences, written by Brianna Almeida entitled "Comparison of Navel and Laminar Stomata Morphology and Stomatal Conductance in the White Water Lily, *Nymphaea odorata* (Nymphaeaceae)", is submitted to you in partial fulfillment of the requirements for Undergraduate Honors in Biological Sciences. The Biological Sciences Undergraduate Honors Committee and the candidate's research supervisor have read this thesis. We recommend that it be approved.

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This thesis by Brianna Almeida is approved.

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ABSTRACT

The white water lily, *Nymphaea odorata,* is an indicator species for the slough communities of the Everglades. Similar to other emergent aquatic plants, it has a gas flow-through system characterized by a pressure gradient that is controlled by differences in temperature and humidity between the inside and outside of the leaf. This flow-through system has not been completely described in *N. odorata*, but stomatal control may contribute to this system. The purpose of this study was to quantify stomatal size and density in the navel and lamina of *N. odorata* and determine if these morphological traits contribute to regulation of the flow-through system. Stomatal density, stomatal size, leaf area, and navel area were measured, in plants contained in high and low water treatments; stomatal measurements were made using compound microscopy and image processing was done with ImageJ software. Stomatal conductance, transpiration and leaf temperature were measured using a steady-state porometer in order to assess differences in gas exchange in different parts of the leaf and with different leaf ages. This study found that there is a large difference in navel and laminar stomata size with navel stomata being 2.4 times larger. There is also a difference in navel and laminar stomata density with laminar stomata being 10.44 times denser. Navel area forms a linear relationship with lamina area but only in the high water treatment. Porometer measurements showed that there were no differences in stomatal conductance, transpiration nor leaf temperature between the navel and lamina nor between old and new leaves. This study demonstrated that although navel and laminar stomata differ morphologically in *N. odorata*, they do not differ in stomatal conductance.

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INTRODUCTION

The Everglades has a unique ridge and slough landscape, with ridges and sloughs differing in vegetation. Historically, ridges and sloughs also differed in water depth and period of inundation (Miao and Zhou 2012). Ridges are slightly elevated above the slough as a result of peat accumulation and are dominated by sawgrass exceeding the water surface by one to two meters. Sloughs, in contrast, are dominated by aquatic species that rarely grow above the water surface. Ridges and sloughs are inundated for most of the year with water levels decreasing during the dry season (McVoy et al. 2011). However, since the 1800s, Everglades hydrology has been altered through drainage and building of canal systems, thereby changing the ridge and slough communities as well, with sawgrass growing into sloughs (LoGalbo et al. 2012). There are efforts to restore the Everglades to pre-drainage conditions through the Comprehensive Everglades Restoration Plan (CERP), which includes restoring the historic hydrology, improving water quality and providing more water storage (LoGalbo et al. 2012). In the past, the Everglades landscape stored water from Lake Okeechobee slowly flowing through marshes, but the canal system has altered this flow. High nutrient runoff from farms into the Everglades also threatens to alter the ecology of the historically nutrient-poor ecosystem (Perry 2004). Understanding the ecological needs and life histories of native vegetation could help restore the slough communities of the Everglades.

One native slough resident is the white water lily, *Nymphaea odorata*. Sediment cores from the slough communities show *N. odorata* pollen and seeds present in the pre-drainage era, but there has been a decline of *N. odorata* populations in the slough communities since the beginning of the 20th century, as a result of the dryer conditions resulting from drainage. This species is an indicator for Everglades slough communities and its presence can be used to determine whether an area has hydrology similar to pre-drainage conditions (LoGalbo et al. 2012). *Nymphaea odorata* is also efficient in storing phosphorous and nitrogen. Therefore, enhancing *N. odorata* populations in the Everglades could assist in removing excess nutrients (Miao and Zhou 2012).

Many aquatic plants with submerged parts must persist in anoxic soils for long periods of time. Therefore they require a system to transport oxygen to their submerged tissues (Dacey 1980). *Nymphaea odorata* uses a gas flow-through system in which oxygen flows in from young emergent leaves or stems through the rhizome and out older leaves or stems, aerating the submerged parts (roots and rhizomes) along the way. Waste gases, such as carbon dioxide or methane, flow out from older leaves or stems (Sorrell et al. 1997). *Nymphaea odorata* uses thermo-osmotic pressure gradients to establish this flow (Grosse and Bauch, 1991). In these flow-through systems, a pressure gradient is maintained within the plants' tissues via a difference in temperature and humidity between the inside and outside of the leaves. (Matthews and Seymour 2014). This pressurized system releases some oxygen into the surrounding soil altering soil chemistry by oxidizing them (Grosse and Tiebel 1996). A similar flow-through system occurs in another floating-leaved aquatic, the sacred lotus, *Nelumbo nucifera.* In lotus however, influx and efflux of gases occur in the same leaf. In *Nelumbo*, influx occurs through a specialized central plate (navel) while efflux of gases occurs in the rest of the lamina. In the navel of *N. nucifera*, stomata are 3 times larger than those present in the lamina (Vogel 2004). These specialized stomata regulate the gas flux of *N. nucifera* by altering rate of air flow (Matthews and Seymour 2014). While members of the family Nymphaeaceae do not contain the same specialized central plate that *N. nucifera* does, similar differences in stomatal size and density between the center (navel) and rest of the lamina have been found in the yellow water lily, *Nuphar lutea* (Schroeder et al., 1985).

Although an unusual region can be seen on the upper surface of the lamina where the petiole attaches below in *N. odorata*, occurrence of a navel has not been recognized in this species. It is unknown whether and how stomatal density varies across the leaf surface in *N. odorata*, and whether that variation contributes to the flow-through system. The purpose of this study was to quantify stomatal size and density in the navel and lamina of *N. odorata* and determine if these morphological/anatomical traits contribute to gas exchange regulation and flow-through conduction as shown by Vogel (2004) in *N. nucifera*. Variation in stomatal size between the navel and lamina may be indicative of conductance and transpiration functions that contribute to the flow-through system. In *N. odorata*, petioles increase in length with increasing water depth but petiolar area does not increase proportionally to water depth. (Richards et al. 2012). Biomass allocation also varies with water depth, and deeper plants have long petioles and fewer, larger laminae than those in shallower water (Richards et al. 2011). Therefore, measurements from leaves grown in low and high water conditions may determine whether and how stomatal characteristics and the flow-through system vary with water depth. Navel area was also examined to determine how this varies with lamina area or water depth.

MATERIALS AND METHODS

Nymphaea odorata plants were collected for a previous study, primarily from 25°47′8.881″N, 80°41′16.431″W in Florida Water Conservation Area 3A in southern Florida during July through October of 2005. Plants used in this study were the offspring of those originally collected since the original plants produced seeds that germinated and established in the mesocosms (Richards and Cao 2012). The plants have been grown in 3410 L outdoor mesocosms, made out of polypropylene cattle tanks with an inner diameter of 2.2 m and a depth of 1 m on the Florida International University Modesto Maidique campus. In September of 2015 plants from the mesocosms were planted in individual pots (23 cm deep and 25cm in diameter) filled with commercial compost; pots were positioned on the bottom of two of the mesocosm tanks. The high water treatment (HWT) mesocosm was maintained at a water level of approximately 90 cm and contained 10 pots with plants and the low water treatment (LWT) was kept at approximately 60 cm and contained 9 pots with plants.

Leaves of known ages were harvested from both HWT and LWT, by cutting at the attachment between the lamina and the petiole, from March until November 2016. Leaves were marked with a unique identifier weekly, to determine leaf age. Leaves were selected from both treatments based on ability to achieve picture clarity to measure leaf morphology, as some leaves became too damaged with age. Images were taken with a digital camera of the entire harvested lamina and a ruler in order to measure lamina area. Images were taken of the stomata under a compound light microscope (Leitz Dialux 20, Leica Microsystems, Wetzler, Germany) at 10, 16 and 40x magnification, to acquire measurements of stomatal size in the navel and lamina, stomatal density in the navel and lamina with three stomata sampled per navel and per lamina. Inner and outer navel area were determined using a dissecting microscope (Wild M3C, Leica, Heerbrugg, Switzerland) at 6.4, 10, 16, 25, and 40x magnification. For all magnifications, images of a ruler or micrometer were taken to account for actual size using ImageJ (Rasband 2016). Navel area was determined by taking the difference between outer and inner navel area to determine how navel area varied with lamina area (Figure 1, area of B - area of C). Navel area excluded the core of the area where the petiole attached (Figure 1, C). Stomatal measurements were also taken in the lamina center along the middle vein of the leaf (Figure 1, D) and in the lamina to the right of the vein (Fig. 1 A) in order to standardize location of measurements; areas and stomatal sizes were analyzed, using ImageJ image analysis software (Rasband 2016). Stomatal size was determined by measuring the length along the longest part of the stomata from the edge of each guard cell. Stomatal density was determined by measuring an area of each region of the leaf and counting how many stomata were present in that area. Measurements were taken on 20 leaves per water-level treatment within 2 hours after harvesting. Averages and standard deviations of stomatal size and density were calculated for the high and low treatments and for the navel and lamina.

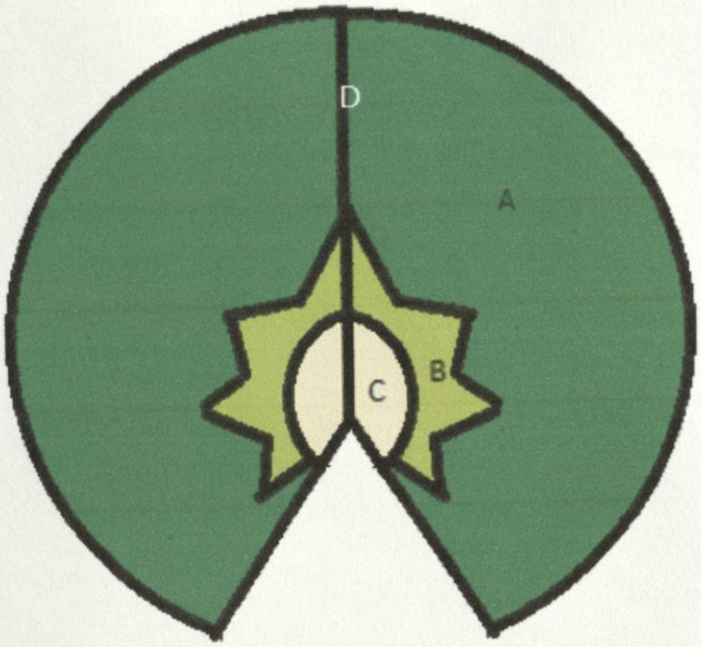

Figure 1: Diagram of *N.odorata* laminar view from above (adaxial surface of leaf); letters indicate location of different areas of the lamina (A) Lamina area; (B) Navel area; (C) Core area; which does not contain stomata, and (D) central midvein of lamina.

A steady state porometer (LI-1600m, LICOR, Inc, Lincoln, NE) was used to measure stomatal conductance, leaf temperature and transpiration in the navel and lamina. Porometer measurements were taken between 10 and 11 A.M on one old and one new leaf per plant in the high water treatment for a total of 10 plants.

Two sample *t*-tests assuming unequal variances were used to make comparisons between: HWT and LWT stomata size, HWT and LWT stomatal density, navel and laminar stomata size, navel and laminar stomata density, right and center stomata size, and right and center stomata density. Two sample *t*-tests were also used to compare HWT navel and HWT lamina, LWT navel and LWT lamina, center navel and center lamina, and right navel and right laminar stomatal measurements. Lamina area and whole leaf area were compared to navel area and outliers were removed. Two sample *t*-tests were also performed to compare navel and laminar porometer measurements. Paired *t*-tests were performed to compare porometer measurements between new and old leaves. All statistical analyses were performed in Microsoft Excel with α=0.05.

RESULTS

Laminae of *N.odorata* are heart shaped with a star-shaped pattern, the navel, in the center of the lamina (Figure 1). The very center (core) of the navel, where the leaf's petiole attaches, contained no stomata. The area of the lamina outside the navel and core includes the rest of the lamina (Figure 1). The average area of all leaves was 223.16 ± 74.29 cm^2 (Table 1). The average area of the leaves in the HWT was 254.72 ± 74.57 cm^2, while the mean area for leaves in the LWT was 191.61± 60.6 cm^2 (Table 1). These areas were significantly different (Table 1) with leaves in the HWT ~1.32 times larger than the LWT. Despite these differences in overall area, navel area was not significantly different between the two treatments (Table 1). Navel area and total lamina area had a linear relationship but only in the HWT (Figure 2). The low water treatment showed a very weak linear relationship between these two measurements (Figure 2). Navel area, including the core, was significantly different between the two treatments (Table 1). The average of the HWT navel + core area was 4.65 ± 1.11 cm^2, and the average of the LWT was 3.51± 0.98 cm^2 (Table 1). Lamina area was also significantly different (Table 1). The average HWT lamina area was 252.60 ± 73.52 cm^2 and the LWT lamina area was 189.88± 60.57 cm^2 (Table 1). The navel accounted for, on average, 0.92±0.49% of the total area in all leaves, 0.810 ± 0.38% in the HWT, and 1.033±0.581% in the LWT (Table 1). Overall, leaf and navel area varied between the two treatments.

Stomata of *N. odorata* were found only on the upper surface of the leaf (epistomatic). The stomata had two kidney-bean-shaped guard cells with anomocytic (irregularly surrounded) accessory cells (Figure 3A). In the navel, stomata often had a ring of accessory cells surrounding the guard cells (Figure 3B). The average navel stomata size was 0.051 ± 0.006 mm and the average laminar stomata size was 0.021± 0.003 mm (Table 2). Navel stomata size was 2.42 times greater than laminar stomata and that difference was significant (Table 2). Stomatal size differed only in the laminar stomata between the two treatments (Table 2)

Stomatal density differed between the lamina and navel, being greater in the lamina than in the navel. The average density of stomata in the navel was 48.91±16.11 stomata/mm². In the lamina the average density was 511.05 ± 116.94 s/mm² (Table 3). Thus, laminar stomatal density was 10.44 times greater than navel stomatal density and significantly differed in both the HWT and LWT (Table 3). Stomatal density also differed in location on the leaf. Center laminar stomatal density was significantly different from the stomatal density on the right of the leaf (Table 3). The average center laminar density was 474.55 ± 83.32 s/mm² and the right laminar density was 558.19 ± 121.89 s/mm² (Table 3). The navel stomata density also was significantly different between the center and right of the leaf (Table 3).

Average stomatal conductance in the navel was 305.95 ± 150.34 mmol $m^{-2}s^{-1}$ and was 325.05 ± 143.11 mmol $m^{-2}s^{-1}$ in the lamina. Stomatal conductance did not vary significantly between the navel and lamina in both old and new leaves (Tables 4, 5). Average transpiration was 8.19 ± 4.77 mmol $m^{-2}s^{-1}$ in the navel and 10.46 ± 8.25 mmol $m^{-2}s^{-1}$ in the lamina. Average temperature in the navel was 28.74 ± 2.10 °C and in the lamina was 29.33 ± 2.42 °C. There was no significant difference in transpiration or leaf temperature between the navel and lamina nor between old or new leaves (Tables 4, 5)

Table 1: Lamina area measurements for *N. odorata* plants growing in a high water treatment (HWT) and low water treatment (LWT); data include the percent the navel comprises of total leaf area. Data area means ± standard deviation of 20 laminae per water level.

	All Leaves (cm^2)	**HWT (cm^2)**	**LWT (cm^2)**	***p*-values**	***t***	***df***
Leaf Area	223.16 ± 74.29	254.72 ± 74.57	191.61 ± 60.6	0.005	2.93	36
Navel Area	1.92 ± 1.11	2.12 ± 1.53	1.73 ± 0.35	0.287	1.09	21
Navel+ Core Area	4.07 ± 1.18	4.65 ± 1.11	3.51 ± 0.98	0.001	3.44	37
Lamina Area	221.24 ± 73.68	252.60 ± 73.52	189.88 ± 60.57	0.005	2.94	37
% of Total Area with Navel	0.921 ± 0.498	0.810 ± 0.380	1.033 ± 0.003	0.1591	-1.44	33

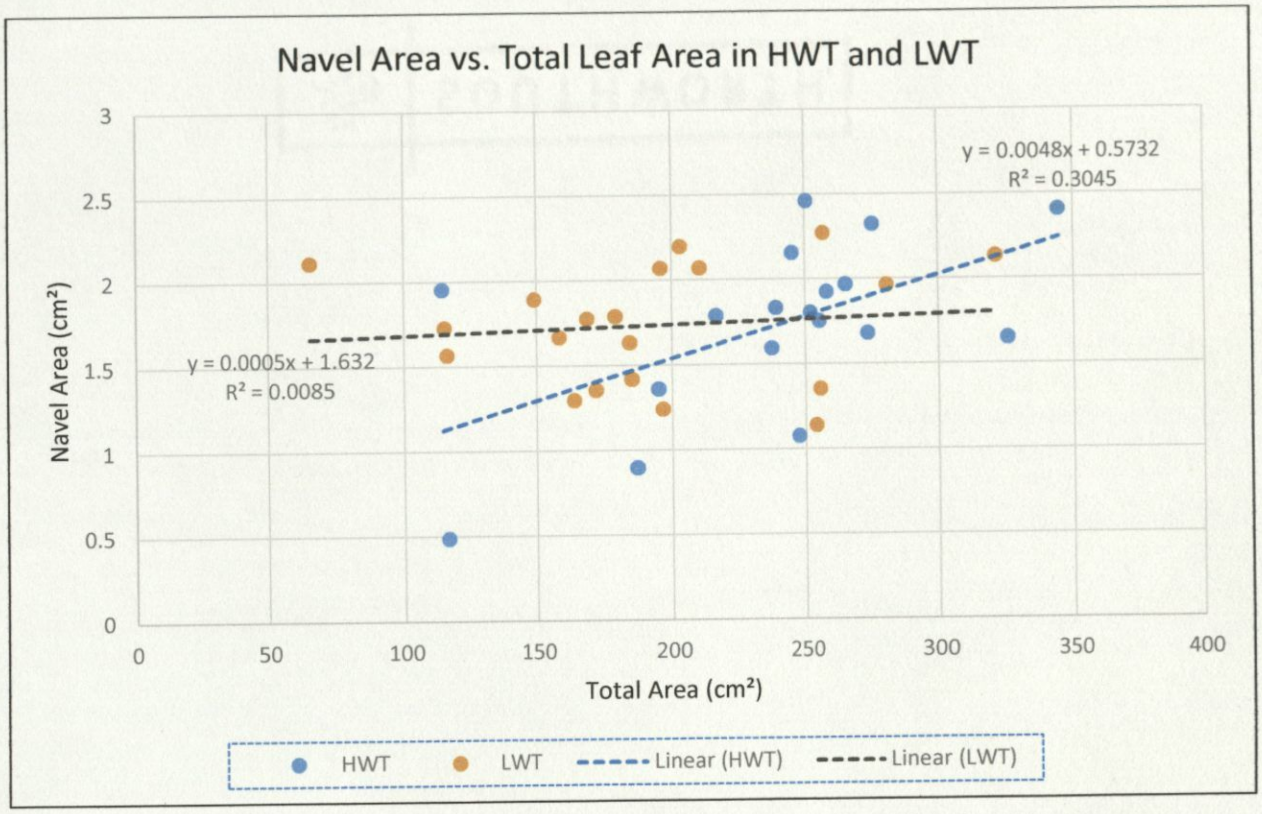

Figure 2: Navel area plotted against total leaf area for both LWT and HWT including a linear best fit line for both.

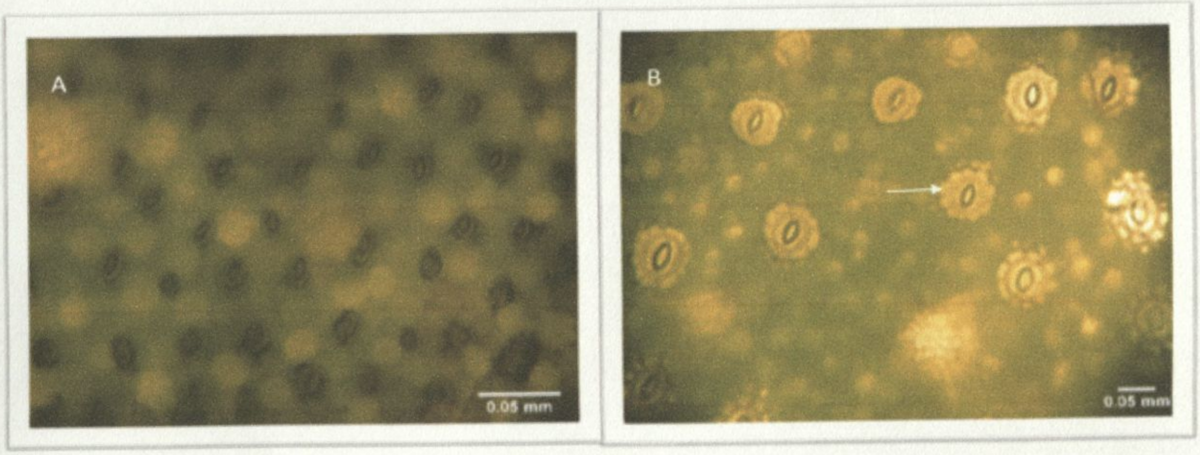

Figure 3: (A) Laminar stomata at 16x magnification. (B) Navel stomata at 16x magnification. The white arrow indicates the accessory cells surrounding the guard cells.

Table 2: Stomata size measurements for *N.odorata* plants growing in a high water treatment (HWT) and low water treatment (LWT). Data are means ± standard deviation. *p*- values were determined for two sample t-test between HWT and LWT and between measurements taken in the center and right of the lamina.

	All Leaves (mm)	**HWT (mm)**	**LWT (mm)**	**p-values**	**t**	**df**
Navel Stomata Size	0.051 ±0.006	0.051 ± 0.006	0.052 ± 0.007	0.189	1.32	237
Laminar Stomata Size	0.021 ± 0.003	0.021 ± 0.002	0.020 ± 0.003	0.04	2.06	238
		Center (mm)	**Right (mm)**	**p- values**	**t**	**df**
Navel Stomata Size	x	0.054 ± 0.006	0.049 ± 0.006	4.8E-09	-6.07	236
Laminar Stomata Size	x	0.022 ± 0.003	0.020 ± 0.003	1.68E-10	-6.68	235

Table 3: Stomata density measurements for *N. odorata* growing in a high water treatment (HWT) and low water treatment (LWT). Data are means ± standard deviation; *p*-values were determined for two sample *t*-tests between HWT and LWT and between measurements taken in the center and right of the lamina.

	All Leaves (s/mm²)	**HWT (s/mm²)**	**LWT (s/mm²)**	**p-values**	**t**	**df**
Navel Stomata Density	48.91 ± 16.11	46.33 ± 15.19	51.79 ± 17.06	0.134	-1.51	77
Laminar Stomata Density	511.05 ± 116.94	527.88 ± 117.14	504.87 ± 106.74	0.361	0.918	77
		Center (s/mm²)	**Right (s/mm²)**	**p-values**	**t**	**df**
Navel Stomata Density	x	42.26 ± 11.28	55.86 ± 17.76	2.18E-30	-32.51	40
Laminar Stomata Density	x	474.56 ± 83.32	558.19 ± 121.89	5.79E-27	-25.79	41

	All Leaves	Old	New	*p*-values	*t*	*df*
Navel	305.95 ± 150.34	335.9 ± 182.67	279 ± 111.14	0.254	-1.219	9
Lamina	325.05 ± 143.11	294.9 ± 121.0	355.2 ± 163.03	0.137	-1.630	9

Transpiration (mmol $m^{-2}s^{-1}$)						
	All Leaves	**Old**	**New**	***p*-values**	***t***	***df***
Navel	8.19 ± 4.77	9.26 ± 6.15	7.13 ± 2.77	0.165	1.508	9
Lamina	10.46 ± 8.25	11.40 ± 11.48	9.53 ± 3.18	0.588	0.561	9

Leaf Temperature (°C)						
	All Leaves	**Old**	**New**	***p*-values**	***t***	***df***
Navel	28.74 ± 2.10	28.35 ± 1.73	29.13 ± 2.45	0.374	-0.934	9
Lamina	29.33 ± 2.42	28.96 ± 2.90	29.7 ± 1.90	0.477	-0.740	9

Table 4: Porometer measurements for the navel and laminar regions in young and old floating leaves of *N. odorata* paired *t*-test statistics comparing measurements in the two leaf ages

A	***p*-value**	***t***	***df***
Stom. Cond.	0.562	0.591	16
Trans.	0.611	-0.520	14
Leaf Temp.	0.577	-0.569	15

B	***p*-value**	***t***	***df***
Stom. Cond.	0.222	-1.260	16
Trans.	0.089	-1.790	18
Leaf Temp.	0.569	-0.581	17

Table 5: A) A statistical summary of two-tailed *t*-tests comparing porometer measurements between the navel and lamina within old floating leaves of *N.odorata*. B) A statistical summary of two-tailed *t*-tests comparing porometer measurements between the navel and lamina within new leaves of *N.odorata*.

DISCUSSION

The findings strongly suggest that navel and laminar stomatal size do vary in *N. odorata* (Table 2). These results are similar to those demonstrated by Vogel (2004). As shown in Table 3, stomatal density also varies between the navel and lamina as in *N. odorata.* According to Matthews and Seymour (2014), the laminar stomatal density was 400 stomata/mm^2 and navel stomatal density was 60 s/mm^2 for *N. nucifera*. The results exhibit a similar finding in *N. odorata* with laminar stomata being denser and smaller than those found in the navel (Table 3). Matthews and Seymour (2014) found that since navel stomata were less dense but larger they were able to control efflux from the gas canals of *N. nucifera* during the day.

Table 3 demonstrates that stomatal density also varies between different parts of the leaf with a lower stomatal density along the middle vein of the leaf. This suggests that gases may travel in a specific way through different veins or sections of the leaf. The results indicate no variation in stomatal density between the HWT and LWT, suggesting that water depth does not affect stomatal density. Laminar stomatal size did vary between the two treatments, while navel stomatal size did not. This may be related to the differences in lamina area between the two treatments.

Table 1 demonstrates that the leaves in the HWT have a larger area per leaf than those in the LWT. These results are in agreement with those found by Richards et al. (2011). Figure 2 showed a linear relationship between navel size and total leaf size in the HWT. This relationship is not present in the LWT. Since navel area is not significantly different between the two treatments, this may indicate that navel area has a minimum size and so cannot vary greatly in the small lamina of the LWT, but it can vary in larger laminae, thus a linear relationship is present because of the larger leaf size that HWT plants are able to achieve.

Table 4 and 5 reveal that there is no difference in stomatal conductance, transpiration nor leaf temperature between the navel and lamina nor between old and young leaves. Therefore unlike *N. nucifera*, in *N. odorata* the physiology of the navel and laminar stomata does not vary. It also indicates that while new and old leaves contribute to the flow-through system differently, the differences in stomatal size and density between the different regions and leaf ages do not contribute to this system. This may be because the flow-through system works strictly between leaves as it does in *Nuphar lutea,* which is in the same order as *N. odorata* (Grosse 1996). The distance in phylogeny between *N. nucifera* and *N. odorata* may explain that although they have similar structures and gas flow-through system, they may be dealing with the same environment in different ways (AGP II 2003). Another contrast to *N. odorata* is found in the aquatic emergent, the cattail, *Typha latifolia* in which older portions of its leaves had higher stomatal conductance. This was explained by the difference in sunlight that hit different portions of the leaf (Knapp and Yavitt 1999). Since *N. odorata* leaves are flat on the surface of the water and in the full sun, the whole leaf experiences the same amount of sunlight, which could explain why stomatal conductance did not vary between leaf regions.

Further research may include characterizing the direction of gas flow and determining if any gas canals are present through the leaf between the navel and laminar stomata. Gas canals have been previously found in *N. nucifera* with navel stomata controlling the direction of airflow through these canals within the plant. It has also been found that some aquatic plants, specifically isoetids, are able to change the pressure in their tissues to absorb carbon dioxide from sediments (Matthews and Seymour 2014). Isoetids are submersed aquatics that absorb carbon dioxide and release oxygen. They also thrive in oligotrophic conditions, similar to *N. odorata* (Freeman and Urban 2012). Using pressure, they are able to transport CO_2 to their leaves for photosynthesis (Matthews and Seymour 2014).

Aquatic plants have been known to alter the rhizosphere around them through their gas exchange systems. This occurs in submerged aquatics that leak oxygen from their roots into the soil, which oxidizes the sediment (Freeman and Urban 2011). Oxygen leakage from a relative of *N. odorata, Nymphaea capensis*, has created oxygenated zones that altered the redox potential within anoxic soils (Grosse 1996). Therefore, determining whether *N. odorata* has similar physiological functions to *N. nucifera* could determine whether it is able to transport CO_2 out of sediments. If *N. odorata* is capable of absorption of carbon dioxide, this may alter the biogeochemical cycles in the rhizosphere of *N. odorata* thereby affecting landscape level processes such as nutrient uptake and carbon storage.

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