Small Bugs, Big Appetites: Experimental Quantification of Duckweed-Herbivore Interactions

by

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The role of herbivores in driving the structure of freshwater macrophyte communities remains poorly understood in comparison with terrestrial ecosystems. For instance, although duckweed (subfamily Lemnoideae) are globally distributed, can be ecologically dominant, and are of growing economic importance, their interactions with herbivores remain understudied. It remains unknown if herbivores could strongly impact duckweed's rapid population growth and how they could influence species composition of macrophyte communities. We here test whether the water-lily aphid (*Rhopalosiphum nymphaeae*) exhibits a preference between species of duckweed and how duckweed and aphids reciprocally affect each other's performance. Our two-way choice experiments reveal that aphids display preference for *Spirodela polyrhiza* > *Landoltia punctata* = *Lemna minor* >> *Wolffia brasiliensis*. By evaluating the growth of aphid populations on each duckweed species we found that preference may be adaptive in certain ecological conditions when high growth rate is advantageous. Quantifying the population growth rate of duckweed in the presence and absence of aphids revealed differential tolerance of herbivory across duckweed species. These results suggest that a single herbivore could have a significant impact on duckweed populations and species composition in nature. We pursued this possibility with a manipulative field experiment. Using exclosures in natural ponds, we manipulated the presence of herbivores. We found that herbivory impacts species composition of duckweed communities in a complex manner. Species are differently affected in ambient herbivory and herbivore addition treatments, and there is considerable variation in herbivore effect between sites due to difference in local herbivore communities. After finding interspecific variation in duckweed response to herbivory, we tested the possibility of intraspecific variation in plant defenses against herbivores. We found that resistance and tolerance vary between duckweed genotypes, and herbivory could be a potential driver of duckweed evolution. We here highlight the importance of quantifying the plant-herbivore interactions in aquatic ecosystems in the lab and the field.

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Preface

This undergraduate thesis was only possible due to the support of many people. This includes my family, who have given me the privilege of so many opportunities and allowed me to conduct my defense from my home during the COVID-19 pandemic. I also could not have done this without the help of the Turcotte Lab, especially Joshua Armstrong, David Conover, and Ashley Morris for their foundational work in the lab and Lillian Leak and Julie Everett for experimental and emotional support. I also thank my thesis advisor and mentor, Martin Turcotte, for giving me so many opportunities and especially for believing in me. His dog, Huxley, also deserves a thanks for convincing me to join his lab in the first place and being adorable. My committee was also invaluable during this process, and even through the stress of the pandemic took the time to give me invaluable feedback and encourage me. I would also like to thank the Office of Undergraduate Research, the James V. Harrison Fund, the University of Pittsburgh Honors College, the Pymatuning Lab of Ecology, Leasure K. Darbaker Prize in Botany, Norman H. Horowitz Award, and the Barry M. Goldwater National Scholarship for funding these projects and giving me the opportunity to research during my undergraduate career. I finally want to thank everyone who took the time to come to my virtual defense during the pandemic and listen to me talk about bugs and duckweed.

1.0 Introduction to Aquatic Herbivory and Duckweed

The consumption of plants by herbivores fundamentally structures ecosystems (Schmitz 2008) and impacts agricultural production (Hidding et al. 2016, Holland et al. 1992). Although there have been extensive studies that quantify herbivory in terrestrial plants (summarized in Turcotte et al. 2014a, Turcotte et al. 2014b), much less attention has been devoted to aquatic plants. Herbivory in aquatic ecosystems had been dismissed as having little to no effect in regulating vascular plant abundance historically, until Lodge (1991) showed the importance of this interaction (O'Hare et al. 2018, Wood et al. 2017). Since then, there has been growing interest in quantifying herbivory in aquatic macrophytes and evidence is emerging that herbivores can significantly impact macrophyte biomass resulting in community and ecosystem effects (Bakker et al. 2016, Bolser and Hay 1998, Carlsson et al. 2004, Jacobsen and Sand-Jensen 1992, Reeves and Lorch 2012, Wood et al. 2017).

Interactions between aquatic plants and their herbivores may differ from terrestrial counterparts due certain properties of aquatic ecosystems (Lodge et al. 1998). First, Cyr and Pace (1993) showed that there is significantly higher primary production and mass-specific herbivory rates in aquatic in comparison to terrestrial ecosystems. Median herbivory in marine ecosystems (40-44% biomass removal) and freshwater ecosystems (44-48%) are both considerably higher than in terrestrial ecosystems (4-8%) (Bakker et al. 2016, Turcotte et al. 2014a). Second, freshwater ecosystems have diverse and complex landscapes with a variety of submerged, emergent, and floating plants. Although there are generalist herbivores, some growth forms are inaccessible to certain herbivore species (Carlsson & Lacoursierre 2005, Gaevskaya 1969). The stark differences between these macrophyte groups could influence freshwater plant-herbivore interactions. Third, certain floating freshwater macrophytes specifically reproduce clonally with very short generation times in comparison to rooted macrophytes, and also have higher total phenolic content, secondary compounds used for defense or UV-B protection (Smolders et al. 2000). Rapid reproduction and high chemical defenses, at least in total phenolic content, may have important implications for their ability to resist and/or tolerate herbivory. The high abundance of certain floating clonal macrophyte species would suggest strong selection on herbivores to exploit them. Although the extent of research on freshwater herbivory is growing, experimental quantification of the effects of herbivory on macrophyte-herbivore interactions and for instance on whether herbivores might structure macrophyte communities is lacking (O'Hare et al. 2018).

A prominent group of freshwater macrophytes that have received little empirical attention are duckweeds (Landolt 1986), which are among the fastest growing and most productive higher plants (Laird and Barks 2018, Zeigler et al. 2015). This subfamily of 37 species are found in lentic and slow-moving freshwater systems worldwide and have the ability to tolerate a wide range of conditions (Ziegler et al. 2015). Due to their high reproductive rate, they are able to blanket entire water bodies, causing wide ecological impact (Driscoll et al. 2016, Tezanos Pinto and O'Farrell 2014,). They are an emerging experimental evolutionary-ecology model system (Hart et al. 2019, Laird and Barks 2018, Xu et al. 2019). Their ability to reproduce and reach high biomass quickly also makes duckweed a candidate for applications such as bioremediation, agricultural feed, and biofuel production (Hassan 1992, Laird and Barks 2018). Though duckweed is important economically and ecologically, there is a surprising lack of information and experimental quantification of their interactions with herbivores.

In this thesis we evaluate the interactions of duckweed with herbivores to shed light on this gap in literature. We conducted three studies to better understand this interaction. We evaluated the preference, performance, and impact of the water-lily aphid on multiple species of duckweed. We then quantified the effect of herbivory on duckweed species composition in the field. Finally, we quantified the genetic variation in resistance and tolerance of the duckweed species *L. minor*.

2.0 Preference, performance, and impact of the water-lily aphid on multiple species of duckweed

2.1 Introduction

2.1.1 Herbivore preference and performance

An effective way of gaining insight into plant-herbivore interactions is through testing the preference-performance hypothesis, which states that there will be a positive correlation between the species an herbivore selects as an oviposition site and the performance of its offspring on that host. Preference, if adaptive, should be consistent with performance, but herbivorous insects often show a preference for the host which is not best for their offspring (Gripenberg et al. 2010). In this case, preference may still be adaptive if for example the best host species is fewer abundant (optimal foraging) or if a poorer quality host plant has less predators (enemy-free space; Clark et al. 2011). Polyphagous insects could be more likely to show negative correlation than oligophagous insects as was shown by Gripenberg et al. (2010), and this could be due to insect neural capacity when faced with many choices at a time. Preference could be driven by a variety of factors including host plant quality and previous experience through natal or maternal host. Cornelissen et al.'s (2008) meta-analysis showed that herbivores prefer larger vigorous plants which may be driven by a greater production of visual or olfactory feeding cues or that larger host provide more resource for offspring (Liu et al. 2005). Importantly, an adult insect's preference may be bias towards hosts on which they developed as larvae, but there is more evidence for adult preference being influenced by conditioning from early adult life rather than preimaginal conditioning (Barron 2001). Maternal effects could also play a role in herbivore preference and performance by affecting offspring phenotype (Cahenzli and Erhardt 2013).

While there have been many studies supporting the preference and performance hypothesis in terrestrial systems (Clark et al. 2011, Gripenberg et al. 2010) they remain less common in freshwater systems limiting our understanding of the generality of the hypothesis. Studies using emergent macrophytes find support for the preference-performance hypothesis using coleopterans and lepidopterans choosing among water-lilies and water chestnut (Ding and Blossey 2009, Dorn et al. 2001, Solarz and Newman 2001). Solarz and Newman (2001) also found support for this hypothesis and that the host plant species on which the weevil is reared is important in determining preference among water milfoils.

Interactions between herbivores and rapidly clonally reproducing taxa such as duckweed may differ from those with other macrophytes or terrestrial plants for few important reasons. Herbivore preference among duckweed species is hypothesized to be weaker than in other macrophyte growth forms or ecosystems because there is relatively little morphological differentiation among species. A duckweed individual’s shoot is a single frond (leaf-like structure) and they have simple roots if any and few physical defenses (Landolt 1986). On the other hand, preference and host performance may differ strongly because of know differences in total phenolic content (Smolders et al. 2000).

2.1.2 Plant tolerance

The performance-preference hypothesis focuses on herbivore fitness, but herbivore choice also has implications for host fitness as well as species composition of plant communities. Strong preference for certain host plant species may drive changes in host community composition (Carlsson and Lacoursierre 2005, Kempel et al. 2015). In addition, changes in community composition may be driven by differences in the host's ability to tolerate herbivore damage. Variation in macrophyte species tolerance and resistance to herbivory has been shown in freshwater ecosystems (Hidding et al. 2010). The rapid generation times of aquatic taxa such as duckweed may provide them with ability to tolerate more herbivore damage and fast population recovery due to density-dependent population growth (Hart et al. 2019). Tolerance may differ among duckweed species due to known differences in growth rates as well as body size which may lower the per capita impact of herbivory.

Beyond descriptive studies of herbivores associated with duckweed [Scotland (1940)], experimental studies of duckweed herbivory remain rare. The duckweed species *Spirodela polyrhiza* was used as a treatment when evaluating freshwater turtle digestive performance (Bjorndal and Bolten 1993). Heide et al. (2006) used *Lemna minor* and China-mark moth larvae (*Cataclysta lemnata*) to show that global warming may decrease grazing pressure by herbivores. Mariani et al. (2020) used this same herbivore to show that they feed without preference on *L. minor* and *Lemna minuta*. Carlsson and Lacoursiere (2005) found that the herbivorous golden apple snail (*Pomacea canaliculata*) reduced plant biomass of three aquatic macrophyte species including the duckweed *L. minor* significantly, consuming all of the duckweed more quickly than the other two hosts tested. Mansor and Buckingham (1989) found the leaf-mining duckweed shore fly (*Lemnaphila scotlandae*) shows adaptive preference for *L. minor* over other species of duckweed and floating aquatic macrophytes. Research on the common herbivore *Rhopalosiphum nymphaeae* remains limited (Storey 2007).

Thus, to provide insight into freshwater herbivore interactions we test the interactions between four species of duckweed and a common generalist herbivore the water-lily aphid (*R. nymphaeae)*. We conducted three separate experiments in growth chambers to quantify 1) aphid preference and the impact of natal host using two-way choice trials, 2) aphid performance by quantifying population growth on each duckweed species, and finally 3) duckweed tolerance by quantifying duckweed population growth rate with and without aphid herbivory.

2.2 Materials and Methods

2.2.1 Study System

We selected the water-lily aphid, a globally distributed herbivore that feeds on 12 known genera of aquatic plants and numerous terrestrial plants and has been proposed as a biological control of aquatic weeds (Center et al. 2002, Oraze and Grigarick 1992). These aphids reproduce parthenogenetically and through live birth on aquatic macrophytes as their secondary hosts (Hance et al. 1994). In Western Pennsylvania, USA, we have observed aphids feeding on various duckweed species in many locations. Given that duckweed communities are often composed of multiple species or genera (Pers. Obs.) and that aphids can easily walk on the water surface implies that they may have the ability to select their hosts.

We established aphid colonies in September 2017 from a single aphid individual reproducing clonally collected from a duckweed community composed of a mixture *Spirodela polyrhiza, L. minor, W. brasiliensis* from Twin Lakes Park, Westmoreland County, Pennsylvania, USA (40.323383333, -79.472383333). The initial colony was split and grown on three species of duckweed to account for maternal and natal experience (henceforth referred to as “natal host”) five weeks before experiments. Aphids could not survive on *W. brasiliensis*. These colonies were kept in a growth chambers at 23.5 C, 50% humidity, 50 µmol/m^2/s light and 16:8 light/dark cycle.

Monospecific colonies of four duckweed species were established from a single individual (i.e., a frond, Figure 1). *Lemna minor, Spirodela polyrhiza*, and *Wolffia brasiliensis* were collected from Twin Lakes Park. Whereas *Landoltia punctata* was collected from Panhandle Trail, Allegheny County, Pennsylvania, USA (40.394216667 -80.135966667). All duckweed was originally collected in the summer of 2017 and colonies maintained in laboratory conditions. Colonies were kept at room temperature under 24-hour lighting preceding all experiments and maintained on 50% concentration growth media free of any herbivores (Kuehdorf et al. 2014).

2.2.2 Aphid Preference

To quantify aphid preference, we conducted two-way choice trials. Trials consisted of every possible two-way combination of the four duckweed species. These species differ greatly in morphology and size (Figure 1). To avoid confounding size differences between species we used approximately equal surface area of each duckweed species. To do so we placed duckweed in 6 well plates so there was a single dense but not overlapping layer of duckweed floating on the surface. The area of each species of duckweed was approximately 7.01 cm^2, representing approximately 38, 62, 100, and 841 individuals of *S. polyrhiza, L. minor, L. punctat*a and *W. brasiliensis* respectively. The two species in each trial were then transferred into a 59.1 mL jar with an area of 22.82 cm^2 filled with 40 mL of 25% concentration growth media and mixed to allow the aphid in the trial equal access to both species.

Each choice trial was conducted with a single 3rd instar aphid. Aphids in their 3rd instar were used because aphids are relatively immobile when younger and thus limit their ability to select host species. The aphid was placed onto a small (0.5 cm^2) floating plastic platform placed in the middle of the duckweed mat, as to not bias their initial choices. We conducted each choice trial accounting for natal host as a variable by using aphids from colonies that were previously grown on *L. punctata, L. minor,* or *S. polyrhiza* in a factorial design. Each choice trial was replicated 10 times. In total we conducted 180 trials. Trials were conducted under 20 µmol/m^2/s of 16:8 lighting at room temperature. Experimental jar position was block randomized at the beginning of the experiment. Each aphid and duckweed were tested only once.

Preference was determined by observing if the aphid's stylet was inserted into the duckweed. If the aphid was not feeding no choice was recorded. Aphid choice was recorded at different times to account for gustatory cues after feeding potentially changing preference if initial olfactory cues determine the first choice. We recorded choice at 5 minutes, 10 minutes, 1 hour, 4 hours, 24 hours, and 4 days. If the aphid died or crawled out of the jar, no choice was recorded.

Generalized linear mixed models were fit to the binomial choice trial data that was logit transformed using the R package "lme4" (Bates et al. 2015). Full models included natal host, time, and their interaction as fixed effects, with experimental jar as a random effect and an autoregressive correlation error structure to account for repeated measures. We tested the importance of natal host by comparing model AIC values using the "bbmle" package (Bolker and R Development Team 2017). Models that did not specify natal host were compared to those that had all three natal hosts or only the two species in the trial. All preference analyses were conducted on each pair of species separately. If the model with natal host had the best fit, analyses were run on the trial data separated by natal host. If time was not significant, then analyses were run data from a single time point at 24 hours.

2.2.3 Aphid Performance

We tested aphid performance on three species of duckweed (*L. punctata, L. minor,* and *S. polyrhiza*) by quantifying aphid population growth rate over 42 days. Under experimental conditions aphids generation time (birth to first reproduction) is approximately 10.2 days (Hance et al. 1994). As before we used 7.01 cm^2 of duckweed area for each species of duckweed. A single species of duckweed was placed into a 236.6 mL jar with an area of 43.74 cm^2 filled with 175 mL of 50% concentration growth media. It was replenished with 30 mL of 0.25 diluted media every other week. In each jar, we placed five 3rd instar aphids. We began with a low ratio of aphids to duckweed to ensure aphids were not limited by hosts and thus the only variable determining their performance was the species of duckweed. We tested the importance of natal host by having separate treatments for aphids grown on each species of duckweed. Each treatment was replicated 8 times and conducted in a growth chambers at 23.5°C, 50% humidity, 16:8 lighting. Jars were covered with fine mesh. Jar position was block randomized by replicate at the beginning of the experiment and re-randomized weekly.

We quantified population size twice per week. Because these aphids reproduce clonally, counting population size over time is a direct measure of fitness at the population level. We then parameterized population growth models and estimated growth rate and carrying capacity. Exponential and logistic population growth models were fit to time-series data using R package “nlme” (Pinheiro et al. 2018). Models were compared using AIC values using R package “bbmle” and likelihood ratio tests (Bolker and R Development Team 2017). Duckweed species was included as a fixed effect and individual experimental jar as a random effect and an autoregressive correlation error structure to account for repeated measures. To test the significance of aphid growth rate (r) and carrying capacity (K) differences among host species, we compared models that allowed these parameters to vary or not among treatments using AIC and likelihood ratio test. Significance of natal experience effects was tested by adding natal host as a fixed effect with and without the interaction in the population growth model and evaluating model fit with and without natal host using likelihood ratio test.

2.2.4 Duckweed Tolerance

We tested the tolerance of three species of duckweed (*L. punctata, L. minor,* and *S. polyrhiza*) to aphid herbivory by quantifying duckweed population growth in the presence and absence of aphids over multiple generations. Under experimental conditions duckweed generation time (birth to first reproduction) is under one week. Ten individuals of a single species of duckweed were placed into a 236.6 mL jar filled with 175 mL of 25% concentration growth media and replenished with 30 mL of 25% concentration media every other week. We added five 3rd instar aphids in the treatment with aphids. Control treatments received no aphids. All aphids were from a colony grown on *S. polyrhiza*. Each treatment was replicated 8 times, and the experiment was conducted in growth chambers at 23.5 C, 50% humidity, 16:8 lighting, and were covered with fine mesh. Experimental jar position was block randomized by replication at the beginning of the experiment and weekly.

Duckweed population size was quantified and population dry weight measured after 32 days of growth (~ five generations of duckweed). Because duckweed reproduces clonally, quantifying individuals is a direct measure of fitness at the population level. Aphid impact on each species of duckweed was tested using two factor ANOVA models on count or biomass data. Fixed effect were aphid presence, duckweed species, and their interaction. Tolerance of each species was calculated as the final abundance of duckweed with herbivory divided by the final abundance without (Strauss and Agrawal 1999). We randomly pair replicates to calculate tolerance values.

2.3 Results

2.3.1 Aphid Preference

Our results showed that aphids have preference for specific duckweed species (Figure 2) that were generally consistent over four days (Supplemental Figure S1). The only choice trials in which time was a significant predictor were *S. polyrhiza* versus *W. brasiliensis* and *L. minor* v. *W. brasiliensis*. This is because *W. brasiliensis* was only rarely selected in the early timepoints and never beyond 24 hours. Given this we proceeded with the analyses using only data from the 24-hour time point. Natal host had some impact on preference and will be discussed when it improved model fit.

Aphids preferred *S. polyrhiza* over *L. punctata* and *L. minor* (which are preferred equally) and almost never selected *W. brasiliensis* (Figures 1 & 2). *Spirodela polyrhiza* was preferred over all other species (Table 1). Natal hosts improved model fit in the *S. polyrhiza v. L. punctata* trial (model comparison, ΔAIC=14.3), in that *S. polyrhiza* is only preferred when *S. polyrhiza* is the host (100%, p=0). Aphid preference for *S. polyrhiza* was non-significant when the natal host was *L. punctata* (67%, *p*=0.32) or *L. minor* (60%, *p*=0.53). In the *L. punctata* v. *L. minor* trial, aphids showed no significant preference between the species (*p*=0.58). Although the model with natal host identity of all potential hosts fit better (model comparison, *ΔAIC*=17.9), no specific natal trial was significant (Table 1). *Wolffia brasiliensis* was very rarely selected as a host and thus never preferred. Only a few aphids even attempted to feed on *W. brasiliensis* but seemed unable to insert their stylet (Pers. Obs).

2.3.2 Aphid Performance

Aphid performance depended on duckweed species identity (Figure 3). The logistic growth models fit aphid population dynamic data better than the exponential models (model comparison, *ΔAIC*=1043.1; LRT, *p*<0.0001). Model comparisons showed intrinsic per capita growth rate (*r*) (model comparison, *ΔAIC*=88.4, LRT, *p*<0.0001) and carrying capacity (*K*) (model comparison, *ΔAIC*=99.3, LRT, *p*<0.0001) to be significantly different among host species. Natal host did not significantly impact aphid performance on any species (LRT, *p*<0.0001).

Aphids reached the highest abundance at the end of the experiment on *L. punctata*. However, the models show that aphids have the highest intrinsic per capita growth rate on *S. polyrhiza* and the highest potential carrying capacity on *L. minor* (Table 2).

2.3.3 Duckweed Tolerance

All duckweed species were severely negatively affected by the presence of aphids but to different extents (Figure 4). For both duckweed abundance and dry biomass, duckweed species, aphid presence, and their interaction were significant (LRT, *p*<0.001). To better visualize and interpret the interaction we calculated duckweed tolerance to herbivory (Figure 4). *Spriodela polyrhiza's* fitness was the least impacted by aphids, followed by *L. minor* and *L. punctata*. *S.*

polyrhiza maintained 64.5% and 42.2% fitness in abundance and biomass respectively. *L. minor* could maintained 49.3% abundance 29.6% biomass. *L. punctata* was affected the most with tolerance of 29.1% and 29% in abundance and biomass respectively. Our results show that *S. polyrhiza* is the most tolerant in both abundance and biomass.

2.4 Discussion

Our results show that water-lily aphids have clear preferences for certain duckweed species. Aphid population growth rate is maximized on one species but reaches a higher carrying capacity on another. Finally, duckweed species vary significantly in their tolerance to aphid damage. We here discuss these results in light of other studies and discuss possible mechanisms and implications for macrophyte communities.

2.4.1 Aphid Preference

Many aphids species show preference for plant species based on visual cues such as color (Archetti and Leather 2005), olfactory cues (Hori 1999), gustatory cues (Powell et al. 2006), and natal/maternal experience (Barro et al. 1995, Nikolakakis et al. 2003). Our results show water-lily aphids have strong preferences for different co-occurring species within the duckweed family. They prefer *S. polyrhiza* over *L. punctata* and *L. minor* (which are preferred equally) and strongly disfavor *W. brasiliensis*. Our results show that natal and/or maternal experience plays a minimal role in preference of water-lily aphids as they only alter species preference in one trial. When grown on *S. polyrhiza* aphids more strongly select *S. polyrhiza* over *L. punctata*. Increased preference for natal host is consistent with Solarz and Newman (2001) that find there to be an effect of natal experience in aquatic herbivores. Our preference result of *S. polyrhiza* being the most preferred is similar to Storey (2007), except that they found *L. punctata* was preferred over *L. minor*, and *W. brasiliensis* was not tested.

Our study was not meant to identify the mechanism of preference but one obvious corollary is individual size which strongly matches preference in our data (Figure 1). This is consistent with the hypothesis that herbivore preference is dependent on plant size and vigor (Cornelissen et al. 2008, Price 1991). It is also possible that larger duckweed species produce stronger olfactory or visual cues. Here, we control for that possibility at the population level by using the same total area of duckweed for all species but aphids may still prefer larger individual fronds.

Other preference cues however are possible as is suggested by Storey's (2007) slightly different results. One is root number which also correlates with size as these four species have multiple, two, one, and no roots for *S. polyrhiza, L. punctata, L. minor, and W. brasiliensis* respectively. Other morphological cues could include vasculature and phloem content. Given that aphids are phloem feeders they may prefer species with more phloem or greater vasculature. *S. polyrhiza* is the largest species and has multiple roots which means it should have higher phloem content (Segovia and Brown 1978). *Lemna minor* is larger than *L. punctata*, but they could be preferred equally due to *L. punctata* having multiple roots, making up for its smaller size. Aphids seem incapable of properly feeding on *W. brasiliensis.* This is most likely due to the fact that this duckweed is smaller than the aphid. Aphids seem unable to simultaneously hold onto this free-floating macrophyte while inserting their stylet (Pers. Obs.). We hypothesize that the small size of Wolffia species may be adaptive against piercing and sucking herbivores.

In addition to morphological traits, preference may be driven nutrient composition, chemical and physical defenses, and olfactory cues. Appenroth et al. (2018) found differences in nutrient content in the duckweed genus *Wolffia* but differences among species evaluated here are unknown. In terms of chemical defenses, Smolders et al. (2000) measured total phenolic concentration in *S. polyrhiza* and *L. minor*, and showed that *S. polyrhiza* had four times the defenses of *L. minor*. *L. punctata or W. brasiliensis* was not measured in this study. This should result in *S. polyrhiza* being less preferred by aphids, but the function of these compounds could be variable and not affect piercing and sucking herbivores. Many other secondary chemicals remain to be quantified among duckweed species especially those in the salicylic acid pathway that are known to affect aphids should be investigated (Cao et al. 2016). Thus, aphids preferentially feed on certain duckweed species suggesting they could impact species composition. This preference may also be adaptive.

2.4.2 Aphid Performance

Similarly to preference, variation in aphid performance on different species of host plant can be caused by variation in amino acid composition of phloem sap (Sandstrom and Pettersson 1994), presence of secondary chemicals, and local adaptation to hosts (Nikolakakis et al. 2003). We found that aphids perform differently on each species of duckweed, and that natal and maternal experience have little effect on performance. Which host maximizes aphid fitness depends on the measure of performance. Intrinsic per capita growth rate (r) was highest on *S. polyrhiza*. This could be due to this species having the largest size and most roots. It could also be because multiple aphids can feed on one individual *S. polyrhiza*, however in our experiment we controlled for total area of each species at the population level. Yet, as time goes on, the health of *S. polyrhiza* could decrease substantially and cause the population of aphids to also decrease in performance. In contrast, aphids have the lowest intrinsic per capita growth on *L. minor* but have a much higher carrying capacity (about three times that of *S. polyrhiza*). Whether this difference is a consequence of changes in the health status of *S. polyrhiza* or due to difference in host plant growth rate remains untested. Insight on the nutrient composition and plant defenses of duckweed species could shed light on why aphids perform so differently on each species. How these results compare to other macrophyte herbivore data is unknown because most studies simply quantify herbivore performance at a single time point.

2.4.3 Implications for the Performance-Preference Hypothesis

Whether our results support the preference-performance hypothesis is contingent on the ecological context of the host species. Other aquatic herbivory studies support it, with Solarz and Newman (2001) finding that natal host was a significant factor in the preference of the milfoil weevil (*Euhrychiopsis lecontei Dietz*), unlike our study in which natal host had a minor effect. This could be due to the weevil's life cycle being far longer and incurring more effect of natal host, or the relative specialization of the weevil on the genus *Myriophyllum* compared to our generalist herbivore which may be under more diffuse selection from its host. These studies all measured insect performance using offspring survival, offspring size, and developmental time (Ding & Blossey 2009, Dorn et al. 2001, Solarz & Newman 2001). However, we are using a species with multiple generations and asexual reproduction, so our fitness measure of population growth rate is directly linked to fitness and provides population dynamic data. Our results support the preference-performance hypothesis when looking at intrinsic per capita growth rate. However, if our metric is carrying capacity, aphids show negatively correlated preference and performance. The relevancy of each measure is dependent on ecological conditions. Jacobsen and Sand-Jensen (1992) found that the effect of invertebrate herbivory on freshwater macrophytes in natural communities is significant, but that there is strong seasonal variation in these dynamics. Our experiment is limited in being confined to the lab, and natural conditions such as seasonal changes in duckweed population size could influence aphid-duckweed dynamics. In early summer, when duckweed population size increases rapidly in eutrophic ponds, aphid growth rate (*r*) may be the best measure of fitness as there should be a plenty of resource available for consumption, and aphids could disperse onto different duckweed host species before reaching carrying capacity on a single species. However later in the season, duckweed population growth slows or is in decline. At this time, aphid ability to maintain high density (*K*) may be a better measure of fitness. Thus, time of year could also be a significant determinant of which measure is more appropriate.

2.4.4 Duckweed Tolerance

To better understand the impact of herbivores on macrophyte communities it is critical to quantify the fitness on host species (Hidding et al. 2010, Kempel et al. 2015). Herbivores can have large and species-specific effects on their host plants (Carlsson and Lacoursiere 2005). We show addition of five water-lily aphids to 10 duckweed can reduce population abundance by as much as 70% within 32 days (Figure 4), more than the average 47.3% shown in the aquatic herbivory meta-analysis by Wood et al. (2017).

Differences in tolerance between duckweed species could be driven by a number of factors. The fastest reproducing duckweed species may be more tolerant to damage by being able to compensate for losses and re-grow in abundance very quickly. Faster growing species are also predicted by the Resource Availability Hypothesis to be less well defended but more tolerant of herbivory (Endara and Coley 2011). Alternatively, the initial exponential nature of duckweed population growth may imply that damage gets compounded through time making more susceptible species less tolerant even if they grow faster without herbivory. Our results suggest the latter. *S. polyrhiza* has the slowest growth rate and is much more tolerant of aphid damage than the faster growing species (Figure 4). These results however could also be caused by body size. Since *S. polyrhiza* is large and can support multiple aphid individuals at a time, it could suffer a lower per capita impact and potentially tolerate more aphids. It is also possible that the ability to tolerate aphids could be adaptive, since *S. polyrhiza* is also the species most preferred by aphids (Agrawal 2007).

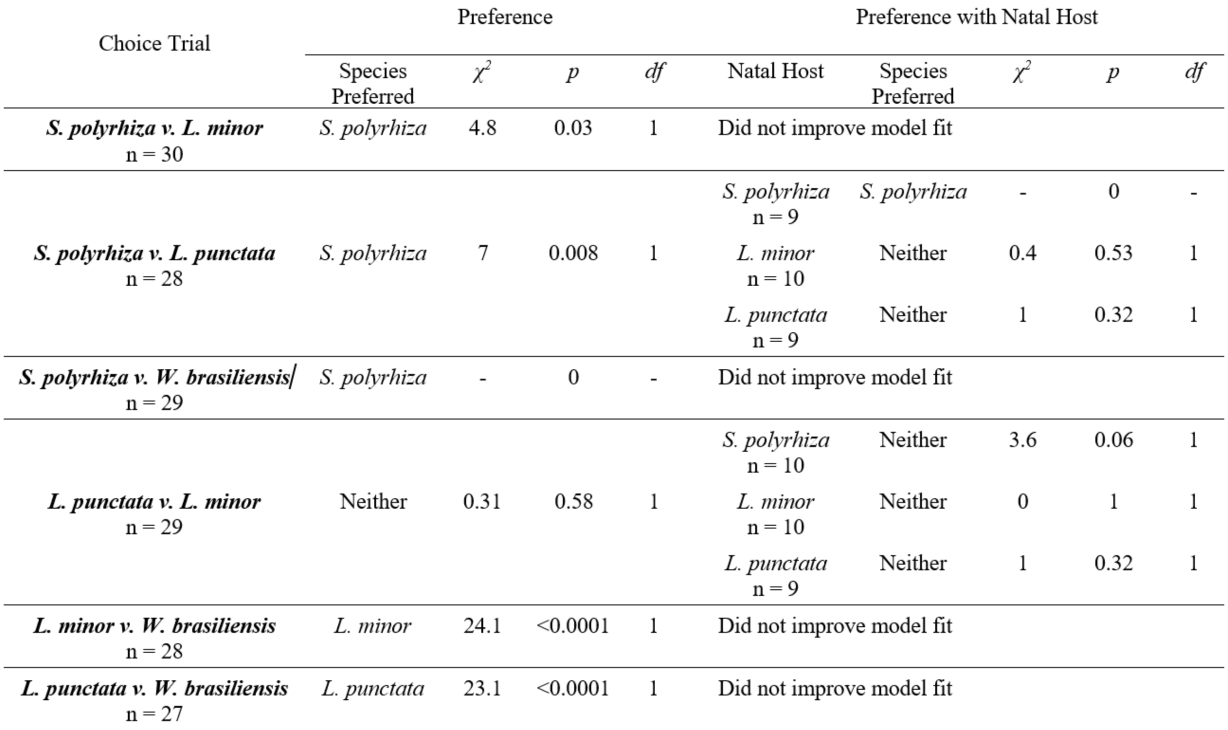
Our study also provides data concerning the important potential trade-off between resistance and tolerance to herbivory which has important implications for understanding the ecology and evolution of plant-herbivore interactions (Agrawal 2007). As with the performance-preference hypothesis our results depend on which measure of resistance we use. Our most tolerant species is *S. polyrhiza*. The tradeoff is supported if we consider aphid intrinsic growth rate (r) as our measure of resistance, which implies that *S. polyrhiza* is the least resistant species and *L. minor* is the most. However, it is not supported if we consider aphid carrying capacity or total phenolic concentration (Smolders et al. 2000). *S. polyrhiza* may have evolved higher aphid resistance as predicted by the phenolic content, which is supported partially by our data in terms of aphid carrying capacity being the lowest on this species (Smolders et al. 2000). *L. minor* is predicted to have lower resistance to herbivores by phenolic content, and this is partially supported by aphid carrying capacity being highest on this species. *L. punctata* has the lowest tolerance to aphids, and has intermediate resistance when looking at both measures of aphid performance. Our results show that under long-term, resource limited conditions, *S. polyrhiza* could have both highest resistance and tolerance, *L. minor* lowest resistance and intermediate tolerance, and *L. punctata* intermediate resistance and lowest tolerance. In short-term, resource rich conditions, *S. polyrhiza* could maintain high tolerance and low resistance, *L. minor* high resistance and intermediate tolerance, and *L. punctata* low tolerance and intermediate resistance. Tradeoffs for resistance and tolerance are present in both conditions, and the differences in strategies between seasons could indicate the maintenance of both strategies in these species (Núñez-Farfán et al. 2007).

2.4.5 Conclusions

This study provides critical information concerning plant-herbivore interactions in aquatic ecosystems. We here begin to address the dearth in work on herbivory on duckweeds, as a widespread group of common aquatic macrophytes with large ecological impacts and great economic potential. The knowledge that aphids preferentially feed on some species over others and affect duckweed species differently shows that duckweed community species composition could be driven by aphids, as is demonstrated by Kempel et al. (2015). We also affirm the results of Stenberg and Stenberg (2012), that herbivory on floating aquatic plants could cause increased light penetration into ponds resulting in widespread impacts. While understanding the exact relationship between aphids and duckweeds is difficult due to the added complications of natal and maternal effects and population response variables, it is clear that aphids affect duckweeds significantly and are reciprocally affected by duckweed species. Parsing apart this relationship would be benefited by evaluating the mechanisms by which this plant and herbivore interact, such as phloem nutrient content and secondary metabolites. It would also be beneficial to include multiple duckweed and aphid genotypes to account for intraspecific variation in these traits as well as the possibility of the selection of a specific co-adapted aphid genotype. If a single herbivore species can reduce biomass by at least 57.8%, the multitudes of herbivores that feed on duckweed in nature could have an even larger effect on duckweed populations. Field studies may be required to firmly address this issue. The effects of herbivores could lead to ecosystem-wide impacts due to the importance of duckweeds in freshwater ecosystems. Determining the role herbivory plays in duckweed biomass and abundance regulation is essential to ensuring efficiency in duckweed production. This study highlights the need for more work on duckweed herbivory in the literature as well as general herbivory work in aquatic ecosystems.

2.5 Tables and Figures

Table 1: GLMM results of preference of water-lily aphids between four species of duckweed in two-way choice trials



Choice Trial	Preference				Preference with Natal Host				
	Species Preferred	χ^2	*p*	*df*	Natal Host	Species Preferred	χ^2	*p*	*df*
S. polyrhiza* v. *L. minor n = 30	*S. polyrhiza*	4.8	0.03	1	Did not improve model fit				
S. polyrhiza* v. *L. punctata n = 28	*S. polyrhiza*	7	0.008	1	*S. polyrhiza* n = 9	*S. polyrhiza*	-	0	-
					L. minor n = 10	Neither	0.4	0.53	1
					L. punctata n = 9	Neither	1	0.32	1
S. polyrhiza* v. *W. brasiliensis n = 29	*S. polyrhiza*	-	0	-	Did not improve model fit				
L. punctata* v. *L. minor n = 29	Neither	0.31	0.58	1	*S. polyrhiza* n = 10	Neither	3.6	0.06	1
					L. minor n = 10	Neither	0	1	1
					L. punctata n = 9	Neither	1	0.32	1
L. minor* v. *W. brasiliensis n = 28	*L. minor*	24.1	<0.0001	1	Did not improve model fit				
L. punctata* v. *W. brasiliensis n = 27	*L. punctata*	23.1	<0.0001	1	Did not improve model fit				

Table 2: Parameters of logistic models for aphid performance on three species of duckweed

Duckweed Species	Intrinsic per capita Growth Rate $p < 0.0001$				Carrying Capacity $p < 0.0001$			
	r	SE	df	t	K	SE	df	t
Spirodela polyrhiza	0.213	0.0137	764	37.44	101.83	20.34	764	12.49
Lemna minor	0.0954	0.0128	764	22.20	335.19	51.48	764	24.90
Landoltia punctata	0.1767	0.0053	764	33.16	203.78	9.36	764	21.78

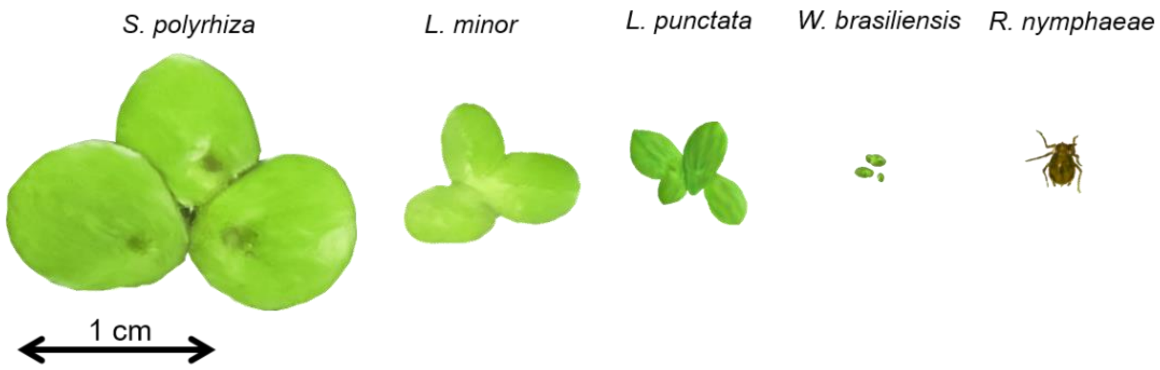


Figure 1: Top down view of relative size of four species of duckweed. Each frond (leaf like structure) is an individual that grows in clusters before separating. All fronds within a cluster are of the same genotype. In addition, we present the water-lily aphid (*Rhopalosiphum nymphaeae)*

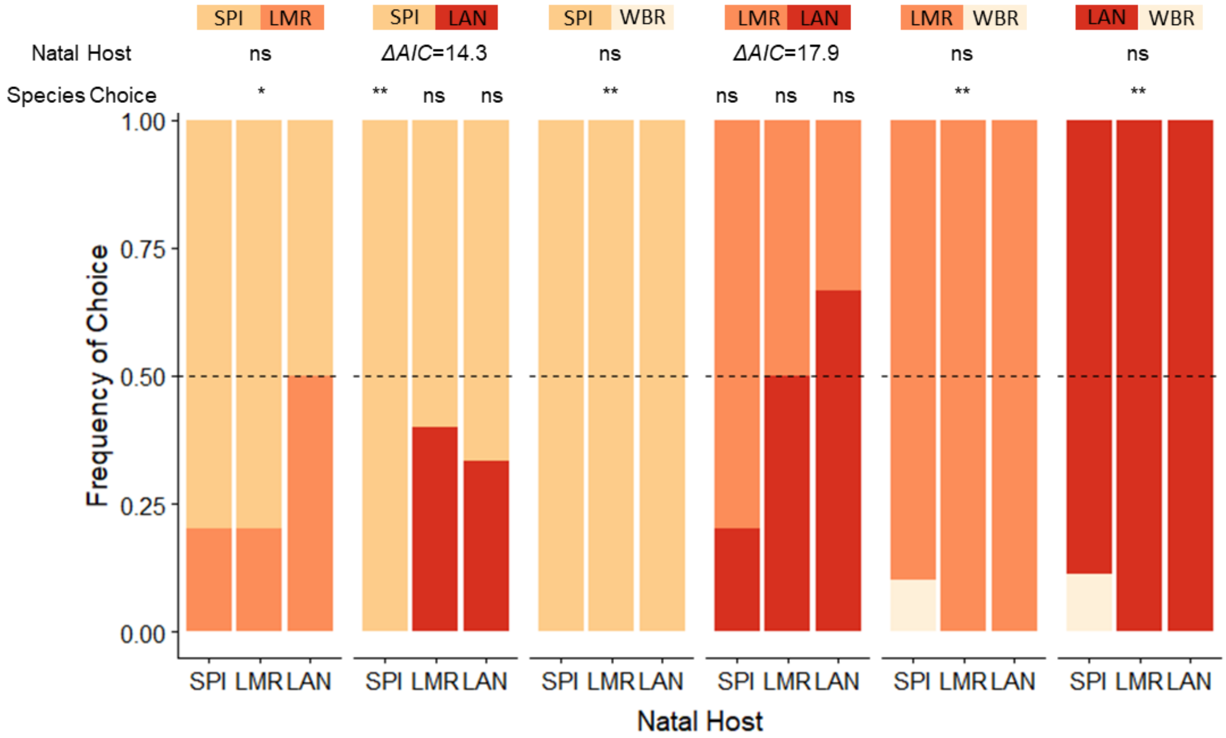


Figure 2: Preference of aphids among four species of duckweed (SPI: *Spirodela polyrhiza*, LMR: *Lemna minor*, LAN: *Landoltia punctata*, WBR: *Wolffia brasiliensis*) in two-way choice trials at 24 hours. When a model with natal host had the best fit, analyses were conducted using natal hosts separately. The statistical analyses (shown in table above bars) test the null hypothesis of equal preference, denoted by the dotted line at 0.50. Significant preference for one species over another is denoted * indicating p<0.05 and ** indicating p<0.0001.

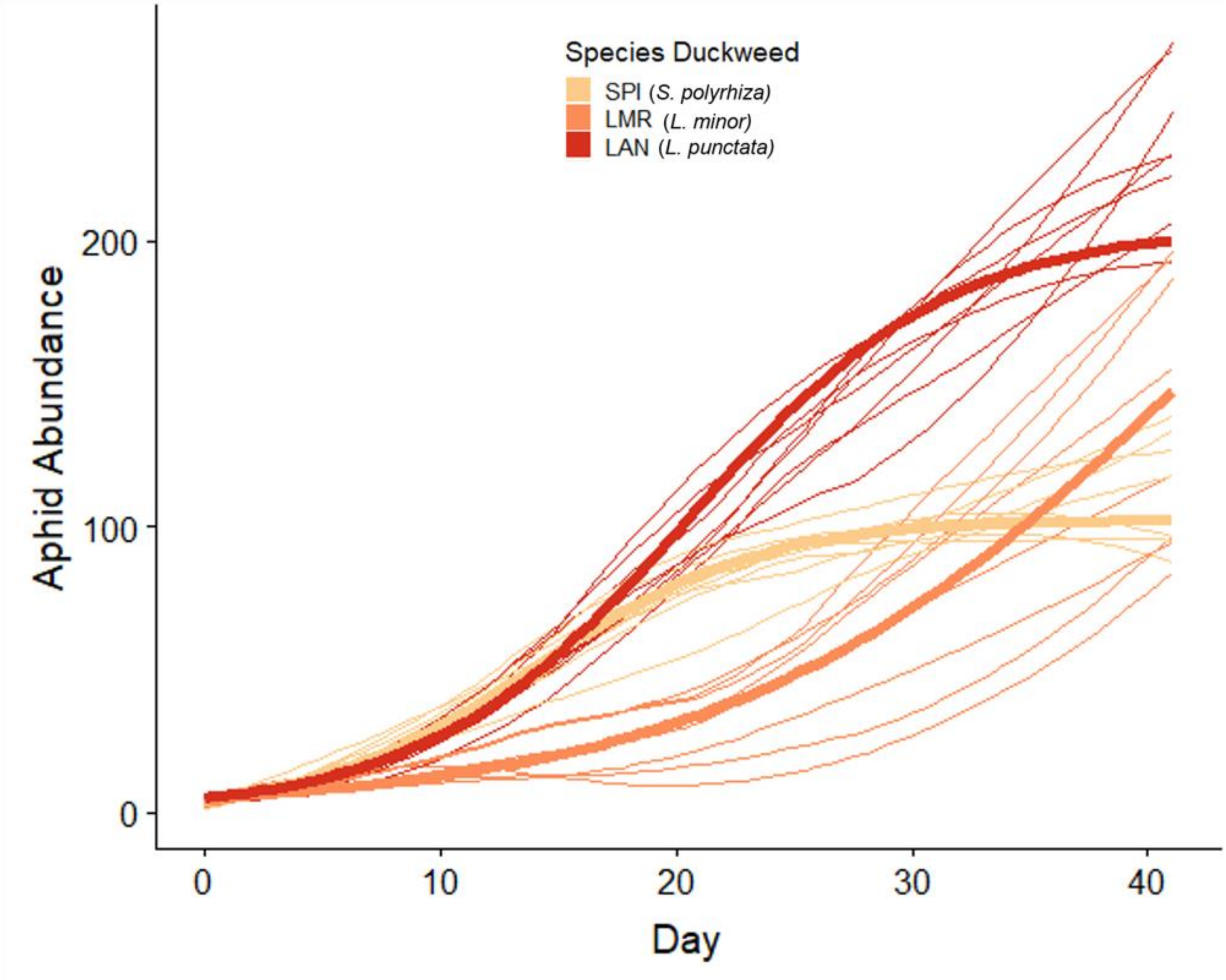


Figure 3: Performance of water-lily aphids on three species of duckweed over 42 days. Each thin lines is one of eight replicates illustrated with a spline fit. The bold lines represent the best logistic model fits.

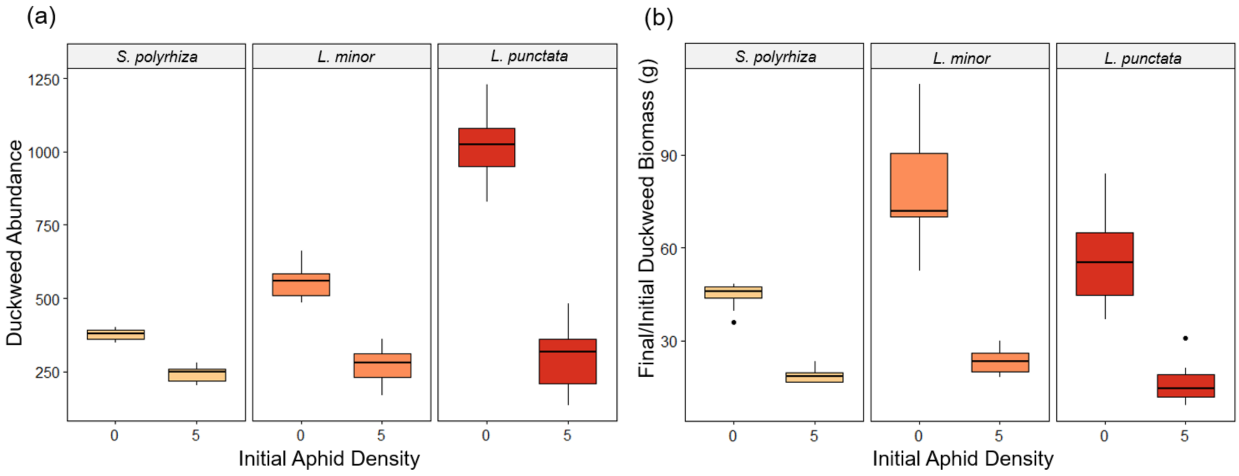


Figure 4: Performance of duckweed populations (initiated with 10 individuals) with and without aphids after 32 days. (a) Final duckweed abundance. (b) Total duckweed dry biomass

3.0 Impact of herbivory on duckweed biodiversity in natural pond ecosystems

3.1 Introduction

Herbivory can significantly impact species interactions and alter species composition of plant communities (Chesson 2000). Abiotic factors such as resource availability and their relation to interspecific competition have been thoroughly studied in terms of their role in structuring plant communities (Kardol et al. 2013, Pavoine et al. 2011, Thuiller et al. 2004, Weiher et al. 1998) but top-down biotic factors such as herbivory are less well understood (Alberti et al. 2017, Carson and Root 1999, Kempel et al. 2015). There is evidence that predator interactions with a prey community alters competitive interactions between prey (Bonsall and Hassell 1997, Morris et al. 2004). A generalist predator acting equally on different prey species should result in analogous effects to limitation by a resource, i.e. the species with the highest tolerance of predation will drive the other species extinct, lowering diversity of the community (Chesson 2000). However, herbivores could hold a stabilizing role strengthening species coexistence by specializing on competitively dominant prey species reducing their density directly or by maximizing distance between reproductive adults and lowering fitness (Chesson 2000; Janzen-Connell hypothesis, Comita et al. 2014). Generalist herbivores could also be a stabilizing mechanism when they have weak preference and propensity for prey switching (Chesson 2000, Murdoch 1969). A diverse community of herbivores can also consume plant species differently, which could both increase and decrease plant community diversity (Ritchie and Olff 1999).

Herbivores can drive plant evolution of different mechanisms. One of these to avoid being preferred by herbivores using various visual, olfactory, or gustatory cues, as strong preference for certain host plant species may drive changes in host species relative abundance (Carlsson and Lacoursierre 2005). Herbivores can also drive the evolution of plant defensive strategies (Agrawal 2007). Plant defense strategies can be in the form of resistance that reduces damage by reducing fitness of the herbivore using secondary metabolites or attracting herbivore predators. This strategy has implications for preference and performance of herbivores and can have costs, both of which factor into how plant species perform relative to each other (Agrawal 2007, Muola et al. 2010). Another strategy is tolerance of herbivores, the mitigation of fitness loss due to herbivory. Tolerance can sometimes occur through overcompensatory growth in high resource environments, and ability to tolerate herbivory can give some plant species advantage over others (Hawkes and Sullivan 2001, Kempel et al. 2015, Strauss and Agrawal 1999).

An approach to quantify the effect of herbivores on plant community structure that has been used extensively in terrestrial systems is herbivore exclusion experiments. Although there is a lot of work in terrestrial systems (Olff and Ritchie 1998), there is much less work in aquatic systems. In grasslands, there seems to be variety in the effect of herbivores on plant diversity, with the trend being to promote diversity (Olff and Ritchie 1998). The few studies that address this question in aquatic ecosystems follow a similar pattern to those in terrestrial systems of showing variable results, indicating that this pattern might not be easily generalizable (Bakker et al. 2016). When evaluating beaver herbivory, Law et al. (2014) found that effects on emergent macrophyte diversity were negligible in the short term and positive in the long term. Waterfowl herbivory has been quantified in this sense multiple times, with conflicting results that show they increase and reduce evenness of submerged macrophytes (Bakker et al. 2016). As for the effect of herbivory on community composition in floating macrophytes, Center et al. (2005) showed that specialist herbivores and nutrient availability can alter competitive dynamics between two species of floating macrophytes and this leads to less stability in coexistence. Tipping et al. (2009) found that that herbivory on *Salvinia minima* had no effect on its competition with the duckweed species *Spirodela polyrhiza,* but due to *S. minima*'s extirpation in herbivory treatments there was a reduction in diversity. While these studies evaluate prevalent aquatic macrophytes, there is a lack of study of the effect of herbivory on community composition of common and abundant Lemnaceae family of macrophyte; the duckweeds.

Subramanian (2.0, 2020) demonstrated differential tolerance and resistance of duckweed species to the water-lily aphid (*Rhopalosiphum nymphaeae*), as well as strong preference for some duckweed species over others. They found that duckweed might adhere to the resource availability hypothesis. According to the resource availability hypothesis, those species that grow faster should have lower investment in defenses, while slower growing species should have more defenses to herbivores (Endara and Coley 2011). *Lemna minor* is faster growing than *Spirodela polyrhiza* in the absence of herbivores and is less defended in phenolic content (Smolders et al. 2000) and resistance to aphids (measured as aphid exponential growth rate) (Endara and Coley 2011, Subramanian 2.0 2020)). From these results we thus hypothesize that *L. minor* should be affected more by herbivores than *S. polyrhiza,* resulting in species composition being skewed toward *S. polyrhiza* in the face of herbivory in nature.

These results suggest that a single herbivore could have a significant impact on duckweed populations and species composition in nature yet this remains to our knowledge never experimentally tested. In fact, although herbivores are common *Lemna minor* is much more common than *Spirodela polyrhiza* in both number of sites and density within sites (Pers Obs, Armitage and Jones 2019).We pursued this possibility with a manipulative field experiment to answer: 1) How do invertebrate herbivores affect diversity of duckweed communities in nature?

and 2) Are these impacts consistent across sites? Using exclosures in natural ponds containing two species of duckweed, *S. polyrhiza* and *L. minor*, we manipulated the presence of herbivores and included addition treatments of two common herbivores, the duckweed weevil (*Tanysphyrus lemnae*) and the water-lily aphid. We here highlight the importance of quantifying the plant-herbivore interactions in aquatic ecosystems in the field.

3.2 Materials and Methods

3.2.1 Study System

Monospecific colonies of the duckweed species were established from a single individual (i.e., a frond). A single genotype of each species was used to avoid rapid evolutionary dynamics confounding our results. *Lemna minor* and *Spirodela polyrhiza* were collected from Pymatuning State Park, Pennsylvania, USA. All duckweed was originally collected in the summer of 2017 and colonies maintained in laboratory conditions. Colonies were kept at room temperature on windowsills for a week preceding the experiment and maintained on 50% concentration growth media free of any herbivores (Kuehdorf et al. 2014).

We established aphid colonies in September 2017 from a single aphid individual reproducing clonally collected from a duckweed community composed of a mixture *S. polyrhiza, L. minor, W. brasiliensis* and from Twin Lakes Park Westmoreland County, Pennsylvania, USA. These colonies were kept at room temperature on windowsills for a week preceding the experiment and were grown on *Spirodela polyrhiza* from Twin Lakes Park.

Weevils were collected the day preceding experiment setup from the site Shelter Nine, Pymatuning State Park, Pennsylvania, USA. Weevils were kept on *S. polyrhiza* and *L. minor* collected from this site until placement on experimental duckweed.

3.2.2 Experimental and Statistical Methods

We received permission from the Department of Conservation and Natural Resources to conduct the experiment in Pymatuning State Park. The four sites chosen already had almost total duckweed cover on the surface of the water, and also had variable duckweed species composition. The four sites are Shelter Nine (41.506991, -80.457792), Spillway Close (41.630392, -80.441723), Spillway Far (41.632432, -80.439675), and Stuart's Bay (41.645797, -80.431482) (Figure 5). All sites had *L. minor* and *S. polyrhiza* in varying amounts, and Stuart's Bay had a small amount of *Wolffia brasiliensis*, a tiny species of duckweed that aphid herbivores cannot consume to our knowledge (Figure 6).

We constructed floating chambers in which we added two common species of duckweed that frequently co-occur, *L. minor* and *S. polyrhiza*, with equal amounts of initial surface area (60 fronds of *S. polyrhiza* and 90 fronds of *L. minor*) to ensure that population biomass would not be a factor in herbivore feeding. Chambers (Figure 7) were constructed from plastic, gallon sized, square containers 26x12.5 cm, clear, PET square gripped wide mouth jars (product code 0070-08), SKS Bottle and Packaging, USA) with white lids. Using a hot knife, 15x8 cm openings were cut out from three sides, and two holes for a threaded rod were cut in the fourth side, and perforations were melted along the edges of the openings for glue adherence. A 7.5x7.5 square was cut out from the plastic lid. We hot glued various meshes over the openings we cut out to manipulate herbivore presence using Gorilla Glue ® All-Temperature hot glue (Joann Fabrics, USA). White casa chiffon fabric (product code 15079494, Joann Fabrics, USA) was used as our fine mesh with holes less than 1 mm, and white petticoat netting fabric (product code 1825165, Joann Fabrics, USA) with holes around 6.35 mm was used as our more open mesh. We glued fine mesh onto all lids regardless of herbivore treatment. The containers were attached to a 13x13 cm square piece of polystyrene foam (product code 304090, Lowe's, USA) to allow them to float evenly. 0.635 cm plastic threaded rods, hex nuts, and washers (U.S. Plastic Corp, USA) were used to attach four containers to the styrofoam block. The whole chamber was grounded in the pond with a garden stake through the middle of the styrofoam block.

Four treatments were included in this experiment: ambient herbivory, herbivore exclusion, and two herbivore additions. The ambient herbivory treatment is the quantification of natural herbivory. We used the open mesh that had wide enough holes (~6.35 mm) to allow most aquatic, walking and flying invertebrate herbivores in to feed on the duckweed. The herbivore exclusion treatment used a very fine mesh that excluded all herbivores in the field. We then had two herbivore addition treatments, which consisted of the fine mesh and the addition of herbivores. One treatment included five initial weevils (*T. lemnae*) and the other 15 initial aphids (*R. nymphaeae*). Quantity of herbivores were determined approximately based on observation of the number of weevils and aphids found on a 156.25 cm^2 area of duckweed in the field to reflect the area of the chambers. Each treatment was replicated four times in each site.

Exclosures in all sites were set up on July 2, 2019. Duckweed population size was quantified after 21-24 days. Because duckweed reproduces clonally, quantifying individuals is a direct measure of fitness. Duckweed counts were converted into relative growth rate by dividing the final abundances by the initial abundance of each species. Herbivore impact on each species of duckweed was tested using three-way factorial ANOVA models on abundance data using R.

Fixed effects were herbivore treatment, site, duckweed species, and their interaction. Analyses were also run with only ambient herbivory and exclusion treatments to isolate the effect of natural herbivory.

In addition, we evaluated herbivore composition in the site and in the ambient treatments. Herbivore samples were collected from each site during the experiment as well as from the ambient herbivory treatment at the end of the experiment and preserved in 70% ethanol. Quantification of herbivores in each site is not yet complete. Water chemistry assays were conducted on four samples of water from each exclosure location on the last day of the experiment by Pennsylvania State University Agricultural Analytical Services Laboratory.

3.3 Results

Relative growth rate of duckweed differed both among sites and among treatments (Figure 8). Data from Shelter Nine was excluded from analysis and discussion due to duckweed death resulting from a large drying event that grounded our chambers in this pond (Figure 8, Shelter Nine).

Spirodela polyrhiza and *L. minor* varied in performance across sites and between ambient herbivory and herbivore exclusion treatments. When models were run excluding herbivore addition treatments, site, herbivore treatment, duckweed species, and their interaction were significant (ANOVA, $p= 0.007$) (Table 3). In the full analysis including the addition treatments, site, herbivore treatment, and duckweed species were independently significant but their interaction was not (treatment: $p<0.001$, site: $p<0.001$, duckweed species: $p<0.001$, interaction: $p=0.08$) (Table 4).

We found spatial variation in duckweed performance and some evidence of interactions with experimental treatment. In our full analysis, we saw a few general results of site and duckweed species. Duckweed growing in Stuart's Bay had significantly higher relative growth rate than both Spillway Close (Tukey HSD, $p<0.001$) and Spillway Far (Tukey HSD, $p<0.001$). Spillway Close had the next highest growth rate, significantly higher than Spillway Far (Tukey HSD, $p=0.02$). The species that across sites and treatment performed the best was *L. minor* (Tukey HSD, $p=0.002$). Water chemistry analysis of each site revealed a variable amount of phosphorus in each site. Nitrogen (NO3) was below detection limit, <0.02 mg/L, for all sites. However phosphorus content in each site was different. Stuart's Bay had the highest phosphorus concentrations at an average of 0.26 ± 0.008 mg/L, Spillway Close had an average of 0.035 ± 0.004 mg/L, and Spillway Far had the lowest phosphorus concentration that was below detection limit (<0.025 mg/L). Herbivore composition varied between sites. Through observations of damage and herbivore presence in ambient herbivory treatments, we determined what herbivores were present in each site (Table 5). The only differences between sites is that duckweed moths were present in Spillway Far and water-lily aphids were present in Stuart's Bay.

Models with only 2 treatments (ambient herbivory treatment and exclusion treatment) did have a significant interaction between site, herbivore treatment, and species duckweed (Table 4). Ambient herbivory treatments worked and the mesh allowed both crawling and flying aquatic invertebrate duckweed herbivores in to feed on the duckweed. Relative growth rate of duckweed was significantly lower in ambient herbivory treatments than exclusion across all sites (Tukey HSD, $p<0.001$). Effect of ambient herbivory on duckweed growth rate varied between sites (Figure 8).

Relative growth rate of each duckweed species was not significantly different in specific treatments in Spillway Close and Stuart's Bay, but *L. minor* was significantly more successful in the exclusion treatment compared to *L. minor* in the ambient herbivory treatment (Tukey HSD, *p*=0.002). *L. minor* was more successful than *S. polyrhiza* in the exclusion treatment (Tukey HSD, *p*=0.07) The rank order of species changed in this site with the presence of natural herbivory, with *L. minor* having a higher growth rate in the exclusion treatment and *S. polyrhiza* having a higher growth rate in the ambient herbivory treatment. In the ambient herbivory treatment in Spillway Far, the two species of duckweed were at similar relative growth rates (Tukey HSD, *p*=0.993).

Models including addition treatments did not have significant interaction between site, herbivory treatment, and/or species duckweed (Table 4). Duckweed growth in addition treatments was significantly negatively impacted by the herbivores compared to the exclusion treatments (Tukey HSD, weevil addition: *p*=0.02, aphid addition: *p*=0.03). Addition treatments did not significantly differ from the ambient herbivory treatment (Tukey HSD, weevil addition: *p*=0.22, aphid addition: *p*=0.17), and both had very similar effects on duckweed growth (Tukey HSD, *p*=0.999).

3.4 Discussion

Our field experiments shows that duckweed community composition is influenced by herbivores, but that the effect of herbivory is influenced by local conditions. Here we discuss the role of herbivory in maintaining diversity in freshwater ecosystems.

Our results are notable due to the spatial variation in duckweed performance with and without herbivory. The overall growth rate of duckweed was the highest in Stuart's Bay, then

Spillway Close, then lowest in Spillway Far. A general pattern seen across treatments and sites is that *L. minor* has higher overall relative growth rate compared to *S. polyrhiza*, but the extent of its advantage depended on site and presence of natural herbivory. This could be a result of differences in biotic and abiotic factors between these sites, although all sites were within 1.5 miles of each other. An abiotic factor that could be a driver of this difference is nutrient composition of each pond, Stuart's Bay had much high phosphorus. The variable effects on relative growth rate could also be attributed to the difference in natural herbivore composition (Table 5).

Nutrient levels have also been previously shown to affect the outcome of herbivory on competitive interactions between freshwater macrophytes (Center et al. 2005). The significant site by site variation was driven by the results in Spillway Far, which had significant differences in *L. minor* relative growth rate between ambient herbivory and exclusion treatments, changing the rank order of species relative growth rate. Spillway Close and Stuart's Bay did not have this significant difference in growth rates. Spillway Far had the lowest phosphorus concentration, which is a limiting nutrient in aquatic ecosystems (Correll 1999). This is a potential reason why we see *L. minor* perform much better than *S. polyrhiza* in the exclusion treatment of Spillway Far, but not in the other sites which are richer in phosphorus and yield higher duckweed growth rates. *L. minor* could be a better competitor for phosphorus uptake, and the phosphorus-poor environment of Spillway Far could have selected for *L. minor* over *S. polyrhiza* when herbivory was absent. Natural duckweed species composition in each site reflects this pattern, with *L. minor* dominating both Stuart's Bay and Spillway Close. However Spillway Far has very few *L. minor* in its natural community, while our exclusion treatment showed it to outcompete *S. polyrhiza* when herbivores were not present. This leads us to the effect of ambient herbivory on duckweed species composition.

While *L. minor* performed very well when herbivores were not present in Spillway Far, this species is affected significantly by ambient herbivory. This change in *L. minor* growth rate is further evidence that herbivory might be altering competitive interactions between prey species in this system (Chesson 2000, Morris et al. 2004). However this effect was not seen in the other sites. In some terrestrial plant groups, it has been demonstrated that resource availability can influence tolerance and compensation for herbivore damage, with those plants in higher resources generally having more ability to compensate (Hawkes and Sullivan 2001). Stuart's Bay and Spillway Close were both higher nutrient environments than Spillway Far in terms of phosphorus as a limiting nutrient, and this could have resulted in compensation for the herbivore damage, reducing the effects we would see of herbivory on duckweed growth rate. Effects on growth rate are suggested to be more quantifiable in resource-limited environments, such as the reduction in fitness seen in the nutrient-poor Spillway Far (Züst and Agrawal 2017).

This result is also dependent on herbivore community composition. In Spillway Far, there was a high concentration of duckweed moths, and duckweed moths were not found at any of the other sites. Visible damage on *L. minor* was almost all from chewing insect such as the duckweed moth. This specific herbivore, which chews large amounts of duckweed and creates cases during its larval stage, could be the driver of the significant decrease in *L. minor*. The other sites were very similar in herbivore community composition, with Stuart's Bay having a few aphids near the end of the experiment. This could be part of why the results in those sites were similar.

The effects of herbivore community can be understood better by looking at herbivore addition treatments. There was no significant interaction with site, treatment, and species duckweed when herbivore additions were included. Aphids and weevils affect duckweed growth negatively to the same extent, and this effect does not interact with species of duckweed. This lack of significance could indicate that they have similar effects on duckweed species and that they don't show a strong preference for one species over another, which is not supported by our previous results in the lab (Subramanian 2.0 2020). We do see that *S. polyrhiza* growth rate was affected more than *L. minor* compared to the exclusion treatments, but this experiment may have lacked power to detect a significant effect. It could also be a result of herbivore density in the addition treatments being too low, or that the experiment was not run for enough time to see these effects in the field (Law et al. 2014). Our results also show the effect of herbivore additions on duckweed growth does not significantly differ from that of natural herbivory. While there isn't a significant interaction, rank order in the ambient herbivory treatment of Spillway Far is different from that of the addition treatments, while it is similar in Stuart's Bay and Spillway Close. This spatial variation in difference between addition and ambient herbivory treatments reflects what would be expected by the variation in herbivore community between sites.

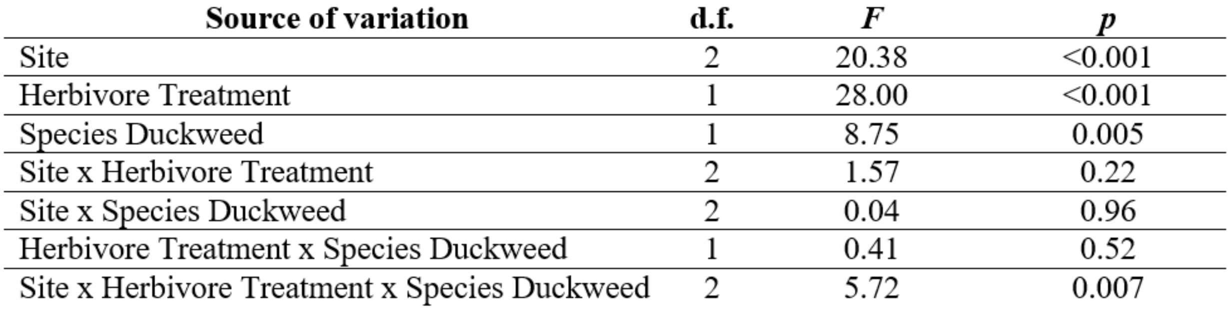
Spillway Far did follow the predictions of the resource availability hypothesis and our previous study, that *L. minor*'s high growth rate is advantageous in no-herbivore environments but its allocation to defenses and tolerance is lower as a result (Endara and Coley 2011, Subramanian 2.0 2020)). However alternatively to our hypothesis, this did not result in *S. polyrhiza* overtaking *L. minor* in the short time span of our study and lowering diversity of the community as is predicted by Chesson (2000). *L. minor* went from almost significantly outcompeting *S. polyrhiza* when herbivores were excluded to significantly dropping to a similar relative growth rate to *S. polyrhiza*. This indicates that herbivory in this system could be acting as a stabilizing force in duckweed community diversity, which supports the long term results obtained by Law et al. (2014) and Hidding et al. (2010) in aquatic ecosystems. Since the ambient herbivory treatment acted as a measure of natural herbivory with all insect herbivores in the field that would enter our mesh, this could be a result of herbivores consuming species differently, resulting in a balancing effect on coexistence (Ritchie and Olff 1999). Due to the presence of duckweed moths in this site, it is also possible that this herbivore acted as a stabilizing mechanism by specializing on the more abundant *L. minor* as proposed by Chesson (2000). However this is counter to what has been previously demonstrated in floating macrophytes by Center et al. (2005), who showed that herbivory could lead to less stable coexistence and domination of the less competitive plant. This could be due length of time of our study, and it is uncertain whether a longer-term study would have shown a more extreme effect of herbivory on *L. minor*, which led to reduced diversity.

3.4.1 Conclusions

This study provides insight into the role of herbivory on plant community composition in freshwater ecosystems. We here show that studying these effects requires testing multiple sites to get a full scope of herbivore community and nutrient composition, which could cause large variation in this interaction. Herbivory could be acting as a stabilizing mechanism for duckweed community coexistence under certain conditions. Future work would be benefitted by testing species separately to understand effects of competition and continuing this experiment for more time and increasing replications for more power. It would also be beneficial to include multiple duckweed and aphid genotypes to account for intraspecific variation in these traits, and to include more study sites to try and uncover a larger pattern of herbivory. The effects of herbivores could lead to ecosystem-wide impacts due to the importance of duckweeds in freshwater ecosystems. This study highlights the need for more studies of herbivory in aquatic ecosystems and their role in community composition.

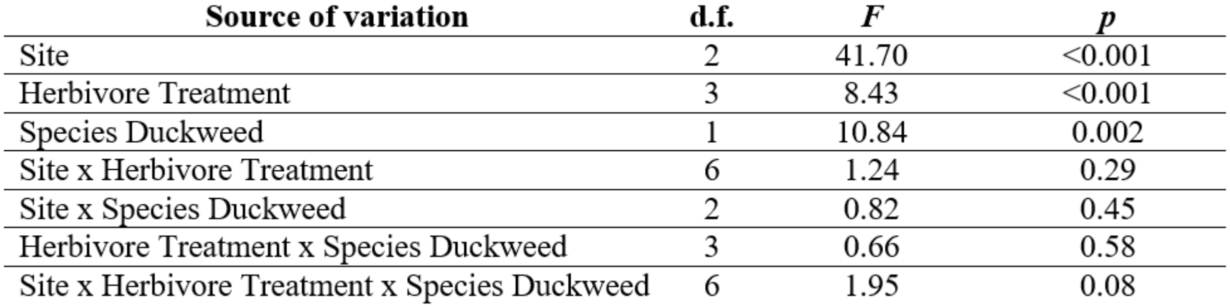
3.5 Tables and Figures

Table 3: ANOVA results of effect of site, herbivore treatment (ambient herbivory and herbivore exclusion only), species identity, and their interactions on duckweed relative growth rate in the field.



Source of variation	**d.f.**	***F***	***p***
Site	2	20.38	<0.001
Herbivore Treatment	1	28.00	<0.001
Species Duckweed	1	8.75	0.005
Site x Herbivore Treatment	2	1.57	0.22
Site x Species Duckweed	2	0.04	0.96
Herbivore Treatment x Species Duckweed	1	0.41	0.52
Site x Herbivore Treatment x Species Duckweed	2	5.72	0.007

Table 4: ANOVA results of effect of site, herbivore treatment (ambient herbivory, herbivore exclusion, weevil addition, and aphid addition), species identity, and their interactions on duckweed relative growth rate in the field.



Source of variation	**d.f.**	***F***	***p***
Site	2	41.70	<0.001
Herbivore Treatment	3	8.43	<0.001
Species Duckweed	1	10.84	0.002
Site x Herbivore Treatment	6	1.24	0.29
Site x Species Duckweed	2	0.82	0.45
Herbivore Treatment x Species Duckweed	3	0.66	0.58
Site x Herbivore Treatment x Species Duckweed	6	1.95	0.08

Table 5: Herbivore community composition at each experimental site immediately adjacent to the experimental chambers

Spillway Close	**Spillway Far**	**Stuart's Bay**
Duckweed Weevil (*Tanysphyrus lemnae*)	Duckweed Weevil (*Tanysphyrus lemnae*)	Duckweed Weevil (*Tanysphyrus lemnae*)
Duckweed Fly (*Lemnaphila scotlandae)*	Duckweed Fly (*Lemnaphila scotlandae)*	Duckweed Fly (*Lemnaphila scotlandae)*
Springtail (*Podura aquatica*)	Springtail (*Podura aquatica*)	Springtail (*Podura aquatica*)
	Duckweed moth (*Elophila spp.*)	Water-lily aphid (*Rhopalosiphum nymphaeae*)

Figure 5: Locations of field experiment set up in Pymatuning State Park, Pennsylvania, USA.

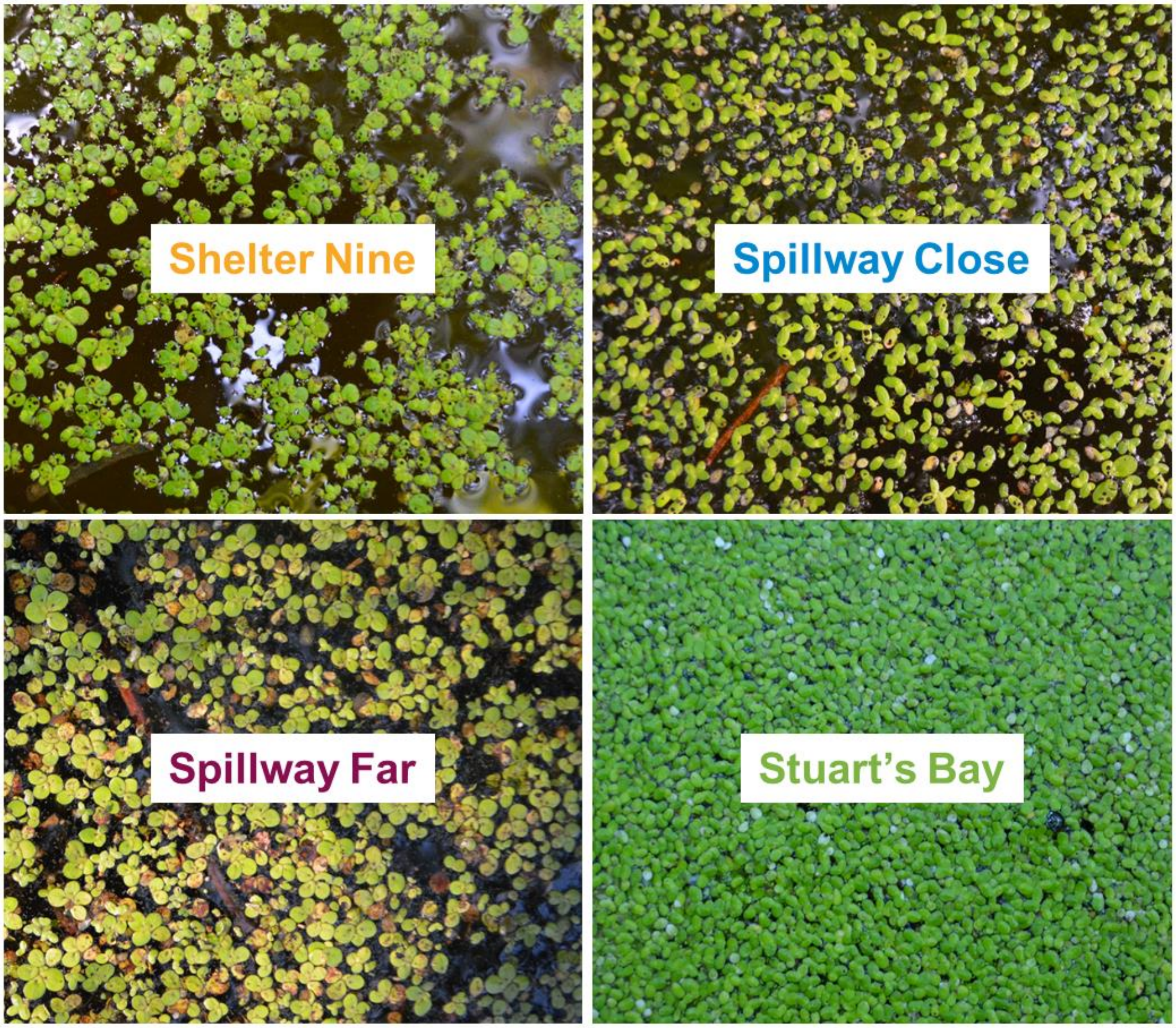


Figure 6: Natural species distribution of duckweed species at four field sites in Pymatuning State Park, Pennsylvania, USA (Figure 5)

Figure 7: Chambers constructed for field experiment to contain duckweed and manipulate herbivore presence using mesh. These photos are from field site Shelter Nine.

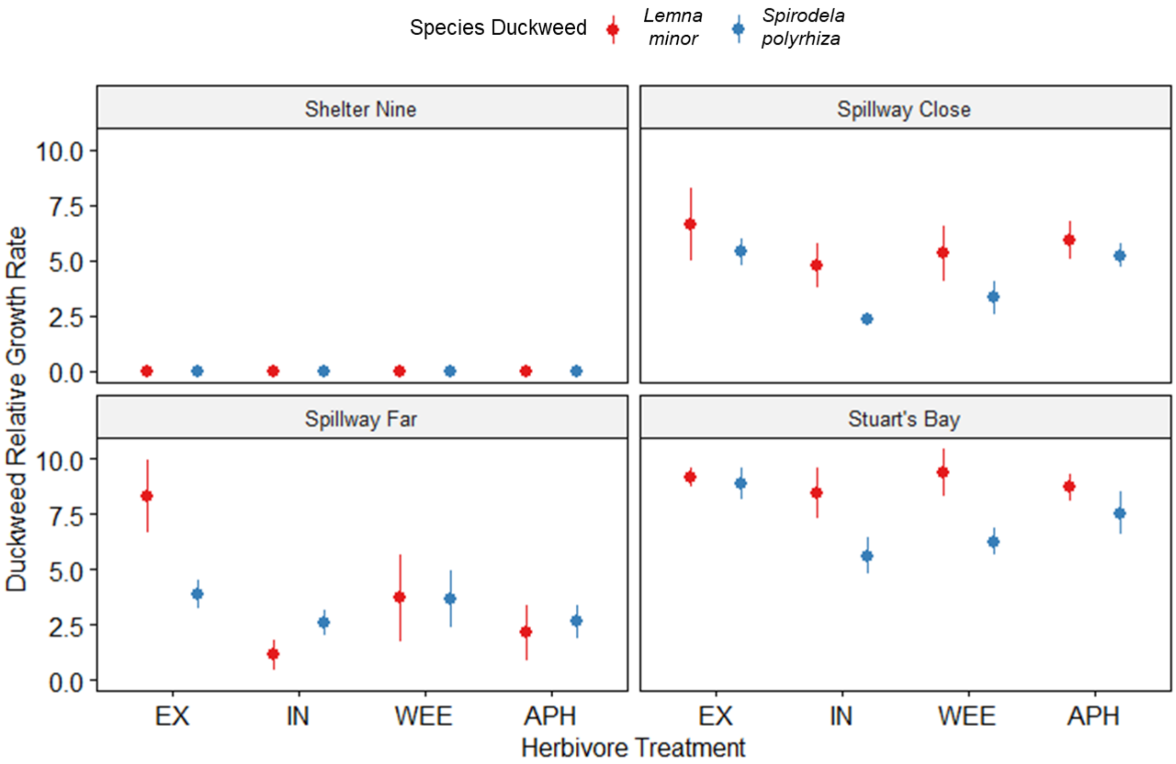


Figure 8: Duckweed relative growth rate in four different herbivory treatments after 21-24 days in four field sites. Herbivory treatments include herbivore exclusion (EX), ambient herbivory (IN), weevil addition (WEE), and aphid addition (APH).

4.0 Genetic variation in duckweed resistance and tolerance

4.1 Introduction

Over millions of years, plants evolved defenses against herbivores, forcing herbivores to evolve adaptations to avoid those defenses, resulting in an arms race (Ehrlich and Raven 1964). This plant-herbivore coevolution has led to species diversification resulting in most of the macroscopic biodiversity on the planet (Futuyma and Agrawal 2009). Herbivores impose selection on plant traits to adapt to the negative effects of damage (Agrawal 2005, Turley et al 2013). Plants have evolved two general strategies against herbivores: traits that reduce damage (resistance) and traits that reduce fitness impacts of herbivore damage (tolerance) (Muola et al. 2010, Züst and Agrawal 2017).

Resistance as a defense strategy reduces the damage of herbivory, and the measures used to understand how plants use to achieve this goal can vary. Resistance has been measured through the analysis of plant secondary metabolites, inverse of herbivory, life-history traits, morphological traits, and attracting herbivore predators (Carmona et al. 2011, Johnson 2008, Stevens et al. 2007, Wang et al. 2012, Zangerl and Berenbaum 2005, Züst and Agrawal 2017). Plant tolerance to herbivory has been measured by comparing plants damaged by herbivory to undamaged plants, to quantify how herbivory impacts the fitness of plants (Strauss and Agrawal 1999). Plants can mediate negative fitness effects through rapid growth, compensatory growth after damage, or by size which would reduce per capita damage. (Hawkes and Sullivan 2001). It is thought that resistance and tolerance tradeoff with each other due to their costs and redundancy in maintaining plant fitness (Fineblum and Rausher 1995, Strauss and Agrawal 1999). However evidence for resistance-tolerance tradeoffs are mixed to weak (Leimu and Koricheva 2006, Mauricio et al. 1997, Núñez-Farfán et al. 2007). Intraspecific variation in resistance and tolerance traits could have implications for evolution of plant populations and for their herbivores.

Although we know tolerance and resistance can evolve it remains unclear how quickly this can occur. A few field experiments manipulating the presence of herbivores over multiple plant generations suggest that rapid contemporary evolution of resistance and tolerance is possible (Agrawal et al. 2012, Didiano et al. 2014, Jogesh et al. 2014), but others show that plant defense strategies remain unchanged, and growth rate is the only trait that revolves in response to herbivory (Turley et al. 2013). To determine the potential for evolutionary change, determining genetic variation in resistance or tolerance is a critical prerequisite. Intraspecific variation not only affects evolutionary potential but also affects ecological interactions directly, which can have community and ecosystem-wide effects (Bolnick et al. 2011, Des Roche et al. 2018).

In terrestrial systems there is evidence of genetic variation in resistance and tolerance. It has been determined that different genotypes of plants can vary in resistance and influence herbivore preference and performance in terrestrial systems (Horner and Abrahamson 1992, Johnson 2008, Karban 1987, Wang et al. 2012). Tolerance also varies within plant species in terrestrial systems (Muola et al. 2010, Stevens et al. 2007). While the prospect of genetic variation in resistance and tolerance in plant populations has been tested in terrestrial herbivores, it has received much less attention in freshwater systems. Due to the dismissal of herbivory as a significant remover of producer biomass in aquatic systems until Cyr and Pace (1991), it could have been assumed that herbivory was not acting as a strong selection pressure on aquatic plants. However it has since been established that herbivory in aquatic ecosystems is higher than in terrestrial systems, indicating an even stronger selection pressure for defense against herbivory

(Bakker et al. 2016). Hughes and Stachowicz (2004) found evidence that genetic variation promotes resistance to herbivory in marine ecosystems, but this possibility has not been explored in freshwater ecosystems to our knowledge.

Herbivores are prevalent in freshwater ecosystems and feed with large, varying effects on floating macrophytes (Carlsson and Lacoursierre 2005, Subramanian 2.0 2020, Wood et al. 2017). Intraspecific differences in resistance and tolerance could be comparable or even stronger than the interspecific differences present in freshwater ecosystems (Des Roches et al. 2018). Floating macrophytes are known for their rapid reproduction and large population sizes, which would lead us to believe they can evolve rapidly to local herbivores maintaining genetic variation for resistance and tolerance. In addition, genotypic variation in intrinsic growth rate can be large (Ziegler et al. 2015) which may suggest large differences in tolerance.

The floating macrophyte duckweed has variable genetic diversity in natural populations, with some species having higher intraspecific variation than others (Ho et al. 2019, Xu et al. 2015, 2019) It is unknown how herbivory would select for resistance and tolerance within species in freshwater ecosystems, and whether these strategies would be independently selected for or redundant, as has been explored in terrestrial systems (Leimu and Koricheva 2006, Núñez-Farfán et al. 2007).

In this study, we will determine whether there is genetic variation within the duckweed species *Lemna minor* for resistance and tolerance to the water-lily aphid (*Rhopalosiphum nymphaeae*). Previous research has shown that aphids have a strong preference between species of duckweed and affect duckweed significantly, reducing *L. minor*’s abundance by 50.7% and biomass by 70.4%. Our previous study only included one genotype, not accounting for the clonal variation that could exist within a species of duckweed in defensive traits. Aphids could be acting as a strong selective pressure on duckweed, as aphids are a common herbivore on this common species of duckweed. We here conduct an experiment that manipulates the presence of herbivory on different genotypes of *L. minor* and quantify the resistance and tolerance of these traits using population parameters.

4.2 Materials and Methods

4.2.1 Study System

Monospecific clonal colonies of *Lemna minor* were established from a single individual (i.e., a frond). *Lemna minor* genotypes were collected from 14 different locations for this experiment (Table 6). *Lemna minor* DNA was extracted using a modified CTAB procedure and genotyped using microsatellite primers by J. Armstrong.

All duckweed was originally collected in the summer of 2017 and colonies maintained in laboratory conditions. Colonies were kept at room temperature under 24 hour lighting for 3 weeks preceding the experiment and maintained on 50% concentration growth media free of any herbivores (Kuehdorf et al. 2014).

We established water-lily aphid (*Rhopalosiphum nymphaeae*) colonies in September 2017 from a single aphid individual reproducing clonally collected from a duckweed community from Twin Lakes Park Westmoreland County, Pennsylvania, USA. Under experimental conditions aphids generation time (birth to first reproduction) is approximately 10.2 days (Hance et al. 1994). These colonies were kept on the duckweed species *Spirodela polyrhiza* to avoid maternal effects on a specific genotype of *L. minor* in a growth chambers at 23.5 C, 50% humidity, 50 µmol/m^2/s and 16:8 light/dark cycle.

4.2.2 Experimental and Statistical Methods

Genotypes were selected based on a few factors, including location of collection. 14 different collection locations were used all throughout Western PA to allow for potentially different herbivore selection pressure. Genotypes with varying growth rate, size, and specific leaf area were also chosen (unpublished data).

We measured tolerance and resistance to herbivory by quantifying duckweed population growth in the presence and absence of aphids over multiple generations. Under experimental conditions duckweed doubling time ranges from 1.34 to 4.54 days (Ziegler et al. 2015). Eight individuals of a single genotype were placed in an 236.6 mL jar with an area of 43.74 cm^2 filled with 175 mL of 50% concentration growth media. It was replenished with 30 mL of 25% concentration media every other week. We added eight 3rd instar aphids in the treatment with aphids. Control treatments received no aphids. Each treatment was replicated four times, and the experiment was conducted in growth chambers at 23.5 C, 50% humidity, 16:8 lighting and were covered with fine mesh. Experimental jar position was block randomized by replication at the beginning of the experiment and weekly.

Some experimental jars were contaminated with algae through the experiment. Level of algae was visually quantified in three levels, none, low, and high, and algae was added as a fixed factor to linear mixed models.

Duckweed and aphid population size was quantified and duckweed population dry weight measured after 15 days. Final population abundance and biomass are measures of fitness that integrate both survival and reproduction over 2-3 generations for both species.

Duckweed resistance was quantified as aphid fitness on duckweed genotypes (final abundance at day 15). Resistance was tested using a linear mixed model using the package "lme4" and fitting aphid abundance data with algae as a fixed factor and duckweed genotype as a random factor (Bates et al. 2015). Because duckweed reproduce clonally we can quantify genetic variation as variation explained by the random effect of genotypes (Via and Shaw 1996). Then by doing V_G/V_T we estimated broad sense heritability (h^2) of resistance. We tested for the statistical significance of clonal variation with model comparison using AIC. *P*-values were obtained using the "lmerTest" package which evaluated models using the Satterthwaite method (Luke 2017, Kuznetsova et al. 2017).

Tolerance of each clone was calculated as the final abundance of duckweed with herbivory divided by the final abundance without herbivory. A linear mixed model was fit to tolerance values with a fixed factor of algae and a random factor of duckweed genotype. Then by doing V_G/V_T we estimated broad sense heritability (h^2) of tolerance.

To test relationship between resistance and tolerance, a linear model with algae and genotype as fixed effects was fit to the aphid abundance data and to the tolerance calculations. Means of resistance and tolerance of each duckweed genotype were obtained using the package "emmeans" (Lenth 2020). Correlation of these resistance and tolerance were tested using Pearson correlation.

Effect of herbivory on duckweed genotype abundance was tested by fitting an ANOVA model to final duckweed abundance data with aphid presence, genotype, algae, and their interaction as fixed effects.

4.3 Results

Duckweed performance in the presence of herbivory, resistance, and tolerance varied between genotypes (Figures 9, 10, 11, 12). Algae contamination was an insignificant predictor except in the cases of resistance (ANOVA, $p<0.001$) and tolerance (ANOVA, $p<0.001$). Jars with heavy algae contamination were removed from subsequent analyses but we kept none and low. Statistics suggest that algae is an insignificant predictor all models after heavily contaminated jars were removed.

Duckweed resistance to aphids varied between genotypes ($\Delta AIC=10.4$) (Figure 9). Duckweed genotypes ranged from having an average of 7 final aphids to 66.5 final aphids. Broad sense heritability (h^2) of resistance was 0.298.

Tolerance to herbivory varied with duckweed genotype ($\Delta AIC=5$). Tolerance ranged from being able to maintain 21% abundance to 59% abundance when damaged (Figure 10). Broad sense heritability (h^2) of resistance was 0.229.

While there was a strong trend between tolerance and resistance, the correlation was not significant ($r=0.5$, $p=0.08$).

Duckweed performance was significantly affected by the interaction of aphid presence and duckweed genotype (ANOVA, $p=0.02$) (Figures 11, 12).

4.4 Discussion

Our results show that *L. minor* genotypes have variable resistance and tolerance to aphids. We also find there is not a clear tradeoff between resistance and tolerance. Duckweed abundance is affected differently by aphids between genotypes.

4.4.1 Resistance

Resistance measured through herbivore performance has been shown in terrestrial plants to differ by genotype (Karban 1987, Muola et al. 2010, Wang et al. 2012). Our results confirm this in duckweed, with some genotypes having significantly higher resistance than others (Figure 9). It has been demonstrated that there can be genotypic variation in secondary metabolites within a species, and the differential performance of aphids on each genotype could indicate that, although we did not quantify phenolics in this study (Stevens et al. 2007, Wang et al. 2012). Phenolics have been quantified in this species, but was not specific in how many genotypes of *L. minor* were used (Smolders et al. 2000).

Another possible reason for this difference in resistance could be life history of the different genotypes, or morphological differences such as frond size and growth rate of each genotype (Carmona et al. 2011, Wang et al. 2012). These genotypes were all collected from different locations, and this could mean that they were exposed to different levels and types of herbivory. This could have resulted in the development of different defenses, as was found by Wang et al. (2012), or in the development of different morphological traits that are specialized to certain guilds of herbivores (Carmona et al. 2011). Those clones that had the lowest and highest resistance were also the fastest and slowest growing clones in terms of final abundance, respectively (Figure 12).

This follows the predictions of the resource availability hypothesis, implying that the clone with the fastest growth rate invests less in defenses than the clone with the slowest growth rate (Endara and Coley 2011).

4.4.2 Tolerance

Tolerance can also be variable between genotypes of a plant, and this is showcased in terrestrial studies (Muola et al. 2010; Rowntree et al. 2010; Stevens et al. 2007; Tiffin & Rausher, 1999). Muola et al. (2010) found that artificial damage yielded differences in tolerance between genotypes, but not damage by an herbivore. Our results show tolerance is significantly different between duckweed genotypes (Figure 10). This indicates that fitness of genotypes is affected to a different extent by herbivory. Surprisingly, this is does not seem to be due to compensatory growth as the duckweed genotype with highest tolerance did not have the highest abundance (Figure 12). It could however be due to body size differences between genotypes. This data has not been processed as of yet, but duckweed can vary greatly in size between genotypes, and those that are larger could have lower per capita effects than smaller genotypes.

4.4.3 Resistance and Tolerance Trade-off

It has been proposed that resistance and tolerance could have tradeoffs with each other due to conferring similar fitness benefits and therefore thus selection would favor specializing on only one or the other and avoiding the costs of investing in both (Núñez-Farfán et al. 2007, Strauss and Agrawal 1999). It has also been demonstrated that there might not be tradeoffs between these strategies and that herbivory could select for the maintenance of both of these strategies (Leimu and Koricheva 2006, Muola et al. 2010, Núñez-Farfán et al. 2007). Our study shows that there is not a clear tradeoff between resistance and tolerance, and that there could be a variety of strategies in allocation to resistance and tolerance. We found an almost significant negative correlation between resistance and tolerance, implying that there could be a tradeoff of one strategy over another. The clearest example of this is the genotype with the highest resistance and also had the lowest tolerance. This could indicate that there are costs to being more tolerant or more resistant to herbivores in freshwater ecosystems, and that investing in one over another is advantageous. However the insignificance of this correlation implies that this is not a clear trend, and there might be variation in selection pressures between sample locations resulting in strong tradeoffs in some genotypes and not others. There is the possibility that tradeoffs could be more apparent under strong resource limitation or heavier herbivory (Leimu and Koricheva 2006).

4.4.4 Duckweed genotype by herbivory interaction

Herbivory can drive rapid evolution in resistance and tolerance (Agrawal et al. 2012, Didiano et al. 2014, Jogesh et al. 2014), as well as plant growth rate (Turley et al. 2013). We find that duckweed fitness is differently affected between genotypes by herbivory (Figure 11, 12). This result suggests that the presence of aphid herbivory can change the evolutionary trajectory of *L. minor* populations. Herbivory had negative effect on the population growth rate of all genotypes. While the lowest performing and highest performing genotypes maintained their rank in the presence of herbivory, the rank order of other genotypes changed (Figure 12). This is due in part to different extents of negative effects on fitness, tolerance, on genotypes, but is also dependent on the population growth rate of each genotype. The genotype that is the highest rank when herbivory is present is not the most tolerant or most resistant, but the one that reaches the highest abundance with and without herbivory. This is analogous to growth rate-defense tradeoffs, although in this study system growth rate is also fitness (Züst and Agrawal 2017). Genetic variance accounts for more than 22% of variance in both resistance and tolerance, indicating that selection on these traits could cause phenotypic evolution over time, but that these traits are largely influenced by environmental factors as well. These results demonstrate that there could be local adaptation to herbivory occurring in *L. minor*, as was demonstrated in a terrestrial system by Wang et al. (2012). The variety in responses to herbivory could be a mechanism of maintaining genetic diversity in this species.

4.4.5 Conclusions

Our study demonstrates that duckweed populations have the potential to evolve in the characters of resistance and tolerance, and herbivory can select for some genotypes over others in abundance. *Lemna minor* shows genetic variability in resistance and tolerance to aphids, and there could be a tradeoff between these two strategies under certain conditions. Our results also show that duckweed evolution could be altered by herbivory in terms of abundance as has been shown to occur in terrestrial systems (Agrawal et al. 2012, Didiano et al. 2014, Jogesh et al. 2014). There is a high degree of genetic variation in these traits between water bodies within Western Pennsylvania, and the variety in herbivory pressure between locations could be maintaining diversity in resistance and tolerance strategies in this species. Future work should include the possibility of aphid variation in effect on duckweed and response to duckweed resistance, and test genetic variation in the herbivore as well. Evolution in these traits could be demonstrated using experimental evolution as well. There could also be a different effect of herbivory in the field due to nutrient limitation and the large variety of herbivore selective pressure (Leimu and Koricheva

2006). This study suggests that genetic variation could hold a large role in aquatic plant-herbivore interactions, and should be explored more in freshwater ecosystems.

4.5 Tables and Figures

Table 6: Collection locations of duckweed genotypes

Genotype Identity	Location	GPS Coordinates
LM.00013	Lake Julia, Buhl Farms Park, Sharpsville, Mercer County, PA	41.2478, -80.4843
LM.00027	Cross Creek County Park, Avella, Washington County, PA	40.2585, -80.4002
LM.00031	Deer Lakes Park, Tarentum, Allegheny County, PA	40.6207, -79.8280
LM.00038	Deer Lakes Park, Tarentum, Allegheny County, PA	40.6207, -79.8280
LM.00039	Brady’s Run Park, Beaver Falls, Beaver County, PA	40.7307, -80.359
LM.00044	Deer Lakes Park, Tarentum, Allegheny County, PA	40.6220, -79.8243
LM.00077	Eighty Four Fishing Ponds, Eighty Four, Washington County, PA	40.1784, -80.1151
LM.00096	Mingo Creek County Park, Washington County, PA	40.2667, -80.0375
LM.00121	Northmoreland Park, Westmoreland County, PA	40.5751, -79.6148
LM.00139	Phelp’s Road, Crawford County, PA	41.6750, -80.5127
LM.00142	Panhandle Trail, Allegheny County, PA	40.3942, -80.1359
LM.00145	Peter’s Lake Park, Canonsburg, Washington County, PA	40.2566, -80.1069
LM.00172	Stuart’s Bay, Crawford County, PA	41.6454, -80.4308
LM.00191	Schenely Park Westinghouse Pond, Pittsburgh, Allegheny County, PA	40.4393, -79.9428
LM.00214	Twin Lakes Park, Westmoreland County, PA	40.3233, -79.4723

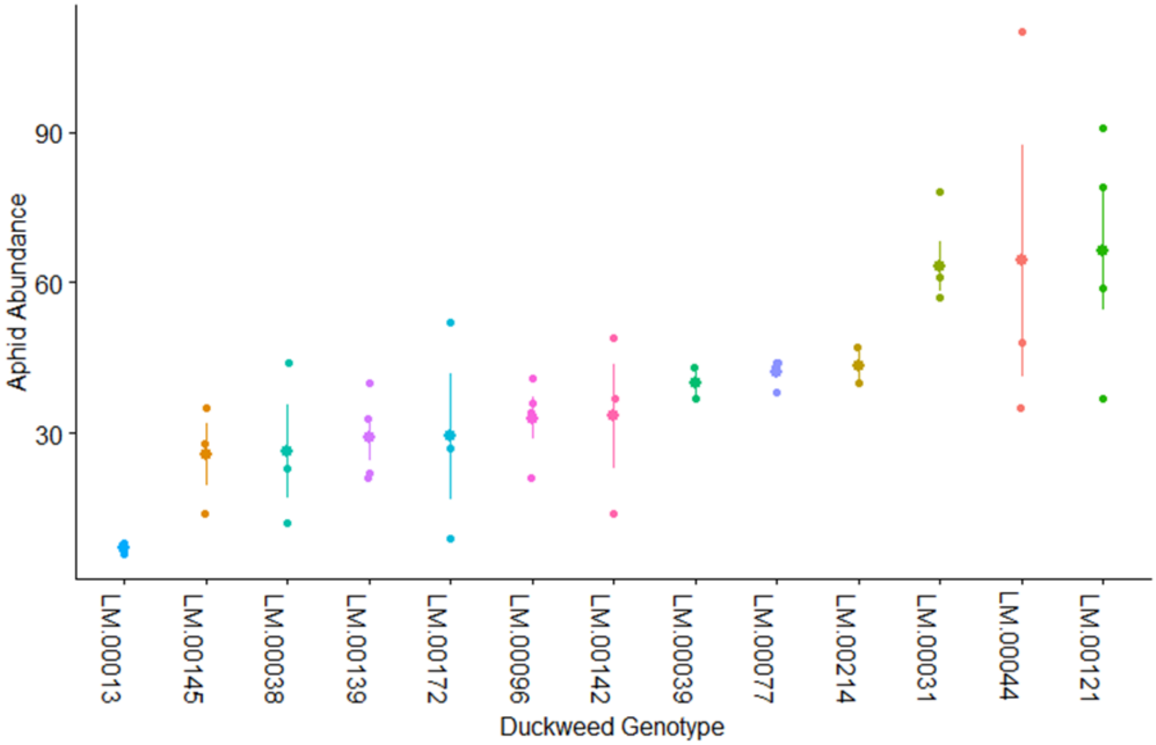


Figure 9: Duckweed genotypic variability in resistance shown by final aphid abundance with standard error on duckweed genotypes after 15 days. Genotypes with lower aphid abundance are more resistant to aphids.

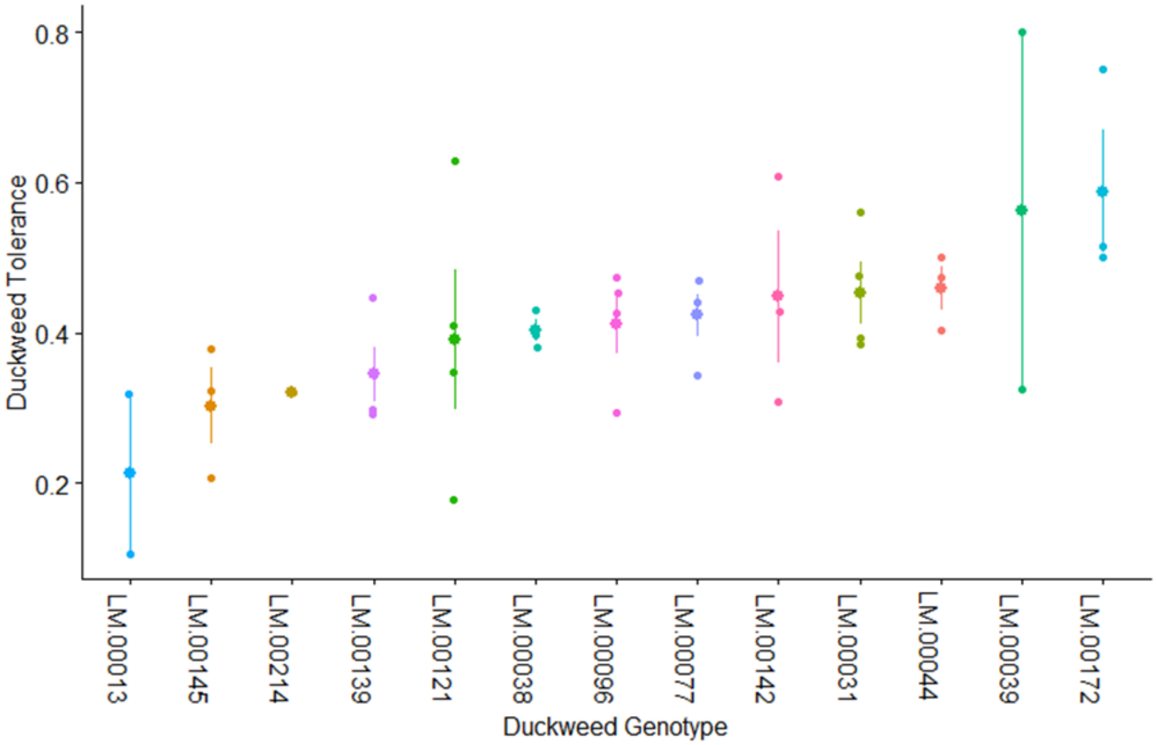


Figure 10: Duckweed genetic variability in tolerance to aphids after 15 days. Tolerance is the final number of duckweed with aphids divided by the number without.

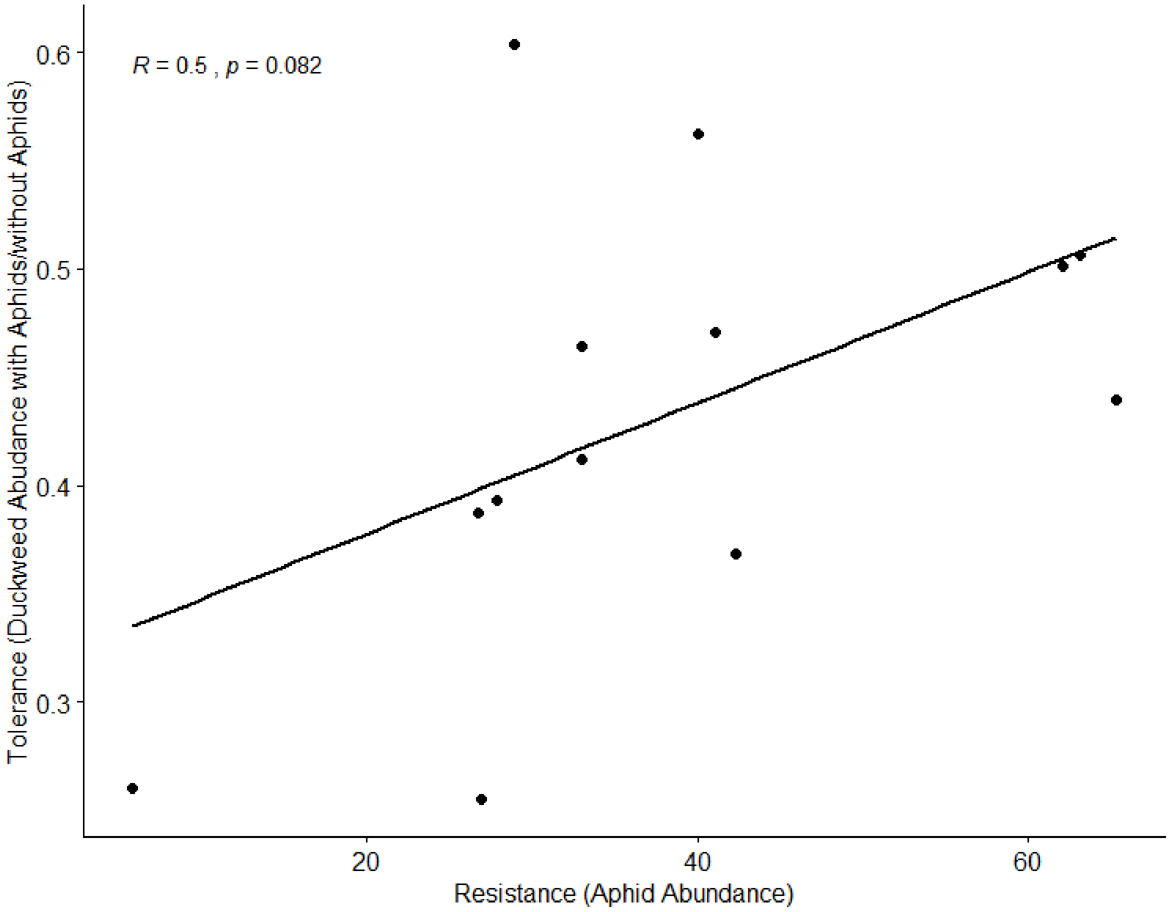


Figure 11: Correlation between genotypic means of resistance and tolerance. Higher aphid abundance indicates lower resistance of the genotype, therefore r is -0.5.

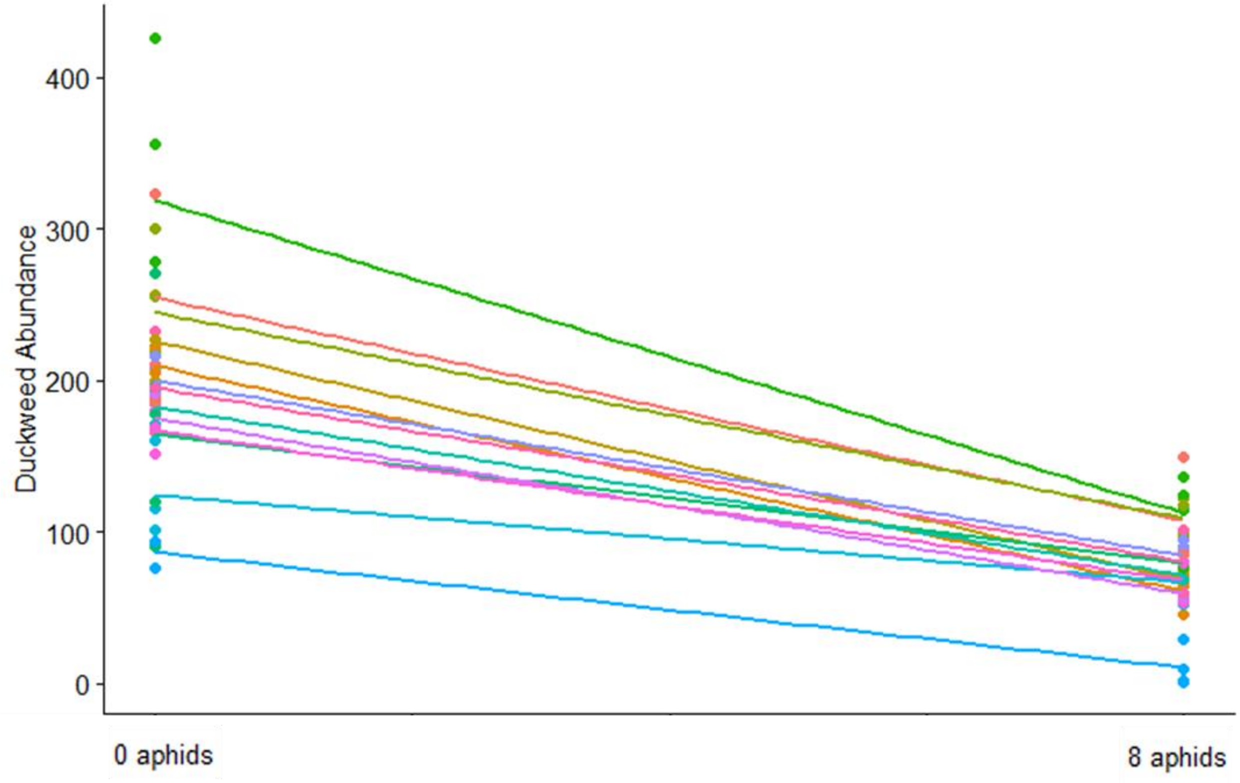


Figure 12: **Duckweed genotype by herbivory interaction.** **Abundance of duckweed after 15 days in the presence and absence of herbivory**

5.0 Concluding Remarks

This set of studies explored the interaction of duckweed and its herbivores through multiple experiments.

We evaluated the preference, performance, and impact of water-lily aphids on multiple duckweed species. Our results show water-lily aphids have strong preferences for different co-occurring species of within the duckweed family and could impact species composition. This preference may also be adaptive. We found that aphids perform differently on each species of duckweed, and that natal and maternal experience have little effect on performance. Which host maximizes aphid fitness depends on the measure of performance. Our results support the preference-performance hypothesis when looking at intrinsic per capita growth rate. However, if our metric is carrying capacity, aphids show negatively correlated preference and performance. We also show addition of water-lily aphids to duckweed can reduce population abundance by as much as 70% within 32 days, affirming that the effect of herbivory in aquatic systems can be extreme. We also show some support for the resistance-tolerance tradeoff between duckweed species. In short-term, resource rich conditions, *S. polyrhiza* could maintain high tolerance and low resistance, *L. minor* high resistance and intermediate tolerance, and *L. punctata* low tolerance and intermediate resistance. Tradeoffs for resistance and tolerance are present in both conditions, and the differences in strategies between seasons could indicate the maintenance of both strategies in these species.

We then quantified how duckweed diversity is impacted by herbivory between sites in nature. Our field experiments shows that duckweed community composition is influenced by herbivores, but that the effect of herbivory is influenced by local conditions. We found that

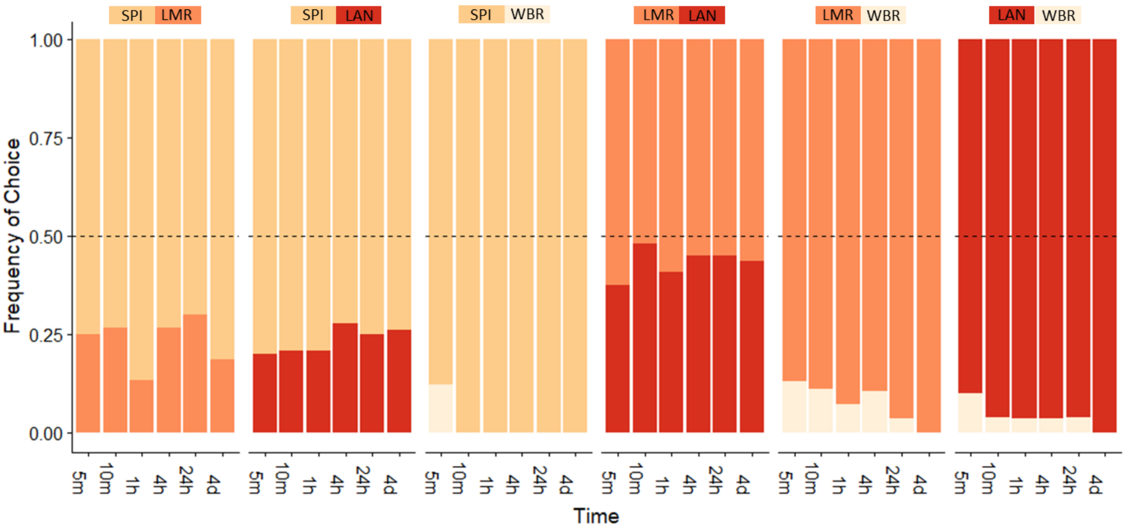
Spillway Far showed significant effects of ambient herbivory on species composition, but Spillway Close and Stuart's Bay did not have this significant change. Stuart's Bay and Spillway Close were both higher nutrient environments than Spillway Far which could have resulted in compensation for the herbivore damage. Our results also show the effect of herbivore additions on duckweed growth does not significantly differ from that of natural herbivory. We determined that *L. minor* went from almost significantly outcompeting *S. polyrhiza* when herbivores were excluded to significantly dropping to a similar relative growth rate to *S. polyrhiza*, which could indicate that herbivory in this system could be acting as a stabilizing force in duckweed community diversity.

We finally tested the genetic variation in resistance and tolerance of *L. minor*. Our results showed that some genotypes have significantly higher resistance than others. These genotypes were all collected from different locations, and this could mean that they were exposed to different levels and types of herbivory, leading to the development of different defenses. Those clones that had the lowest and highest resistance were also the fastest and slowest growing clones in terms of final abundance, respectively, showing support for the resource availability hypothesis. Our results show tolerance is significantly different between duckweed genotypes, but this is does not seem to be due to compensatory growth as the duckweed genotype with highest tolerance did not have the highest abundance. We found an almost significant negative correlation between resistance and tolerance, implying that there could be a tradeoff of one strategy over another. However the insignificance of this correlation implies that this is not a clear trend, and there might be variation in selection pressures between sample locations resulting in strong tradeoffs in some genotypes and not others. These results suggests that the presence of aphid herbivory can change the evolutionary trajectory of *L. minor* populations.

There is a high degree of genetic variation in these traits between water bodies within Western Pennsylvania, and the variety in herbivory pressure between locations could be maintaining diversity in resistance and tolerance strategies in this species through local adaptation.

Our results in this group of studies show that duckweed is affected by herbivory in a myriad of ways, and that duckweed-herbivore dynamics are influenced by the surrounding environment as well as their direct interaction. We have found interspecific and intraspecific differences in duckweed response to herbivory in both the lab and the field. We also found that herbivory in aquatic systems can have huge effects on floating macrophtyes on both the population and community level. The prevalence of these macrophytes in freshwater ecosystems implies that this interaction can subsequently have large effects on the ecosystem. With this system we have the opportunity to study the way plants and herbivores interact on a physically small scale, but with an effect size that is vast. Future work should include the possibility of intraspecific variation in herbivore populations, as well as the study of multiple herbivores of duckweed. Experimental evolution of duckweed to herbivory should also be explored. This set of studies highlights the need for further exploration of duckweed-herbivore interactions and general herbivory work in freshwater ecosystems. As we can see here, although one might see a body of water covered with small plants, this does not mean that they are not affected by small bugs- with big appetites.

Supplementary Figures



Supplementary Figure 1: **Preference of water-lily aphids between four species of duckweed in two-way choice trials over five time points.**

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