University of North Carolina Asheville
Journal of Undergraduate Research
Asheville, North Carolina
May 2017

Reproductive Effort and Output in Two Species of *Sarracenia* (Pitcher Plant) and Their Hybrids

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Abstract

Sarracenia jonesii (Wherry) (mountain sweet pitcher plant) and *Sarracenia purpurea* var. *montana* (mountain purple pitcher plant) are two morphologically distinct species of carnivorous plant endemic to the Appalachian region of North and South Carolina. Like other Sarraceniaceae, these species are known to hybridize in sympatry, but fitness differences among the taxa remain uncharacterized. In this study, we examined reproductive effort and reproductive output of *S. purpurea* var. *montana* in sites where it is the only member of its genus, and in sites where it co-occurs and hybridizes with *S. jonesii*. In summer 2015, ovaries, anthers, and later seeds were collected from 8 western North Carolina sites: 7 with only *S. purpurea* var. *montana*, and 1 with both parental species and their hybrids. All ovules were enumerated with light microscopy under 10X magnification, and a subset of pollen grains were counted in a hemocytometer under 40X magnification. Seeds were tested for viability using tetrazolium, or stratified and then germinated in a controlled climate growth chamber. Data were transformed to ensure normality, then analyzed using ANOVA with Tukey's Studentized Range tests (ovule and pollen counts) or Kruskal-Wallis tests followed by Dunn's tests (seed counts, seed viability, and seed germination). While seed production varied significantly among sites, viability and germination did not. Ovule counts varied significantly among parent species and hybrids, with hybrids producing more ovules than *S. jonesii*. Pollen counts also varied significantly, with *S. purpurea* var. *montana* producing more pollen than *S. jonesii*. However, seed counts, viability, and germination did not vary significantly among parental species or hybrids. Future experiments will examine the genetic composition of hybrid plants, to determine their generation. Abiotic and biotic reasons for differences in seed production among sites but not species are also being explored.

1. Introduction

Life history theories suggest that plants experience resource allocation trade-offs between reproduction and growth[17]. Ecological and physiological mechanisms are what ultimately drive these trade-offs, though the exact mechanisms by which they act remain unknown[17]. Effects of resource allocation on reproduction can be measured as reproductive effort[17], where plants require mineral nutrient stores to produce a floral apparatus and other supporting reproductive structures[24]. Rather than measuring seed output, measuring total reproductive effort has become more realistic for measuring the resources committed to producing the estimates of reproductive outputs[24]. Life history frameworks for reproductive output in plants is largely based off the concept of clutch size in birds[29]. Clutch size (eggs/clutch) is best equated to the number of ovules per plant (ovules/ovary). Compared to birds, plants have a highly variable reproductive output, several pollination and fertilization obstacles blocking seed production, and are more likely to abort fruits or seeds[29].

Reproductive effort measures the proportion of bulk energy committed by an organism to its reproduction[10]. Conventionally, reproductive effort is estimated by the ratio of reproductive dry mass to total dry mass[4]. However, other studies suggest measuring reproductive effort by quantitative pollen and ovule proportion[14]. Increased reproductive effort may result in lower life expectancy due to high energetic expenditures and drained reserves[4].

Reproductive effort is often correlated with an organism's reproductive output, or successful genetic contribution to future generations[10]. Reproductive output defines plant fitness through successful seed set and, later, seed viability and germination[19].

Plant size[16] and resource availability[19] affect reproductive effort by constraining pollen and ovule production. Typically, larger floral production is positively correlated with plant size, but it remains hard to discern the relative influence of genetics and environmental causation on plant size[16]. Germination rates in pollen or growth rates of the pollen tube at fertilization may be dependent on resource storage within plants[13]. One major discrepancy for reproductive output in plants is the presence or absence of a pollinator. Specific pollinators of *S. purpurea* and *S. jonesii* are not well known, however one study suggests species within the bumblebee genus (*Bombus*) and families within the sarcophagid fly[19]. Low pollinator visitation and inbreeding depression can potentially result in low reproductive output[19].

Plants tend toward a functional gender, where even monecious individuals differ from one another in their relative production of pollen or ovules[14]. This tendency provides an ideal measurement for gender strategies among parent plants, but does not aid in the gender identities of future generations[14]. Higher environmental resource availability is often positively correlated with ovule production, where flowers blooming earlier are more likely to be female-biased. Flowers that bloom late or plants that grow in dense populations are more likely to be male-biased flowers due to lower levels of resource availability[16].

Plants can exhibit pollen limitation, where reproduction is constrained either by pollen quality or quantity[1]. Pollen quality, a constituent of reproductive effort, may be limited by inbreeding depression, in which self-fertilization or outcrossing negatively affects seed production[1]. Excess production of ovules may be a tactic to compensate for the varieties of pollen types placed on stigmas, or may correlate with floral phenotypic traits[1]. The contribution of pollen quantity to reproductive output is determined by the number of grains that reach the stigma then fertilize ovules[1]. This process is highly dependent on pollinator activity[23].

The wetland-inhabiting plant family Sarraceniaceae is comprised of three genera of carnivorous pitcher plants throughout North and South America: *Darlingtonia*, *Heliamphora*, and *Sarracenia*[6]. All three genera live in nutrient poor habitats, where their tubular leaves produce an insect attractant nectar to lure, capture, and digest insects as supplemental nutrients[31]. The genus of interest for this study, *Sarracenia*, utilizes a waxy leaf surface and downward pointing hairs to trap its prey[31]. Other forms of resource availability are crucial for these pitcher plants, where photosynthesis from its modified leaves and nutrient uptake from the substrate gives *Sarracenia* the mineral nutrients to become reproductively viable[19].

Members within the genus *Sarracenia* (Sarraceniaceae) are found throughout eastern North America[6] and can often be distinguished by their pitcher and flower morphology[7]. *Sarracenia jonesii* (mountain sweet pitcher plant) and *Sarracenia purpurea* var. *montana* (mountain purple pitcher plant) are two morphologically different pitcher plant species endemic to the Appalachian mountain regions of North and South Carolina. Both species are rosette-forming, carnivorous plants that live in low-nutrient bog, swamp, or wetland areas[19].

Sarracenia purpurea pitcher plants have a large distribution throughout eastern North America[5], with multiple subspecies and varieties exhibiting phenotypic and genetic differences[21]. This study focused on *Sarracenia purpurea* var. *montana* (mountain purple pitcher plant), a short, low-lying pitcher with a flower that grows as high, or higher than its modified leaves. *S. purpurea* var. *montana* not only differs by its geographic region, but its morphologically stouter modified leaves, where the distal hood tips tend to curve inwards rather than flare outwards like its close relatives[21]. *Sarracenia purpurea* pitcher flowers vary in color from light pink to dark red or maroon[28]. In this species, resource availability from prey capture is positively correlated with seed set, and seed production might be limited by pollen availability[19].

Sarracenia jonesii, the mountain sweet pitcher plant, has tall, slender pitchers that range in height from 21 to 73 cm[9]. This species produces a fragrant flower (typically red or maroon) that grows higher than its modified leaves[15]. Though specific flower characteristics for the mountain sweet pitcher plant are unavailable, our sites contained *S. jonesii* flowers that appear both red and yellow in color. Due to *S. jonesii* populations being small and scattered within the Appalachian regions of North and South Carolina, and this species' vulnerability to habitat disruption, it was listed as federally endangered in 1988[9].

The conservation status of *Sarracenia purpurea* var. *montana* is currently under review as an Endangered or Threatened species in the southern Appalachia[26]. Mountain purple pitcher plants are a vulnerable variety because their sphagnous seepage bog habitats are disappearing due to human development and water table disruption. In addition, bog drainage has allowed other shrubs and trees to grow into bog areas and outcompete *S. purpurea* for light and water supply[21].

Current threats to *Sarracenia jonesii* and *Sarracenia purpurea* var. *montana* include habitat fragmentation and degradation from human impact. Human disturbances further reduce population densities, causing species to have difficulty attracting pollinators; this, in turn, indirectly reduces reproductive performance and creates a further decline in population density over time[31]. Reproductive success in plants is likely the net product of the processes of reproductive effort and reproductive output[31].

The purpose of this study was to examine the reproductive effort and output of *Sarracenia purpurea* var. *montana* in sites where it is the only member of its genus, and in sites where it co-occurs with *Sarracenia jonesii*. In the latter, we also quantified reproductive effort and output in *purpurea* x *jonesii* hybrids. We hypothesized that the reproductive output of *S. purpurea* and *S. jonesii* were lower than reproductive effort at site SF, where a hybrid plant co-occurred. We expected this would be due to competitive resource availability and pollen mismatch costs by the hybrid plants. We also expected that the hybrid plant would become equally as successful, if not more successful, at seed production in comparison to the parental plant types.

Note: Proper subscript citations will be added in the final draft.

2. Methods

2.1. Field Collection at Site SF

All hybrid flowers and no more than 10% of all parental flowers (*Sarracenia jonesii* and *Sarracenia purpurea* var. *montana*) were collected from site SF in June 2015. Male and female parts were separated and stored in collection tubes. Stamens were stored in 1.5 mL microcentrifuge tubes of 10% ethanol, and ovules were stored in 15 mL tubes of 100% glycerol. Ovules were collected from 61 individuals' flowers (30 *S. purpurea* var. *montana*, 18 *S. jonesii*, and 13 hybrid), and pollen was collected from 63 individuals' flowers (30 *S. purpurea*, 20 *S. jonesii*, and 13 hybrid) for pollen counts. Ten percent of all remaining flowers were then bagged post-pollination for seed collection.

2.2. Field Collection across Other Sites

No more than 10% of *Sarracenia purpurea* var. *montana* flowers were bagged post-pollination for seed collection from sites CM, CP, DB, DF, HC, MB, and RL. Four of these sites contained only *S. purpurea* var. *montana*, two sites contained both *S. purpurea* and *S. jonesii*, and two sites contained both parental species and their hybrid. *S. purpurea* var. *montana* seeds were collected from these sites, but pollen and ovule numbers were not measured as the sites were of conservation interest.

Table 1. Summary of sites listed by county.

County			
Henderson	Transylvania	Jackson	Macon
MB	CM	DB	RL
	CP		HC
	SF		
	DF		

2.3. Ovule Counts

Glycerol-preserved ovaries were removed from solution and examined under a Meiji dissecting microscope at 10X total magnification. Forceps were used to separate ovules from each ovary's wall and central axis, then distribute them throughout a gridded Petri dish. All ovules in each ovary were enumerated.

2.4. Pollen Counts

Jewelers' forceps were used to probe and disturb each antheridial head in the microcentrifuge tube for pollen removal. Each 1.5 mL microcentrifuge tube was individually centrifuged for 1 min at 13,3000 xg to settle pollen grains. A micropipette was quickly used to remove excess ethanol, reducing the tube volume to 0.1 mL (100 uL). The tube was vortexed for 1 min to re-suspend pollen grains. Ten microliters of the vortexed pollen grain sample was immediately pipetted onto a standard hemocytometer with Neubauer rulings, and pollen grains were counted under an Olympus CH30 compound microscope at 40X. Ten percent of the total hemocytometer area was counted.

2.5. Flower Color

Ten total flowers from *Sarracenia jonesii* were collected from site SF based on flower color (five red and five yellow). Flowers were dissected into male and female parts and stored in the same manner as all other flowers collected at site SF for ovule and pollen counts. Seed count was not compared between flower morphs.

2.6. Tests of Seed Viability

Viability of 10% of seeds from each flower was assessed using a tetrazolium assay[3]. Field-collected seeds were placed in Petri dishes and soaked in distilled, deionized water for up to 48 h. After soaking, seed coats were split open using forceps. Each seed was covered with a solution of 1% triphenyl tetrazolium chloride (TTC), and Petri dishes were stored in a cool, dark space for 24 - 48 h. Seed color was assessed under a Leica dissecting microscope at 10X total magnification; viable seed embryos were stained pink, while inviable seeds did not change color. The number of viable seeds per flower was recorded.

2.7. Seed Germination

Ten percent of seeds from each flower (1% of the population's seeds) were dry-stratified in the dark at 4 °C for 30 days, then stratified in moist soil in the dark at 4 °C for 60 days. Seeds were then sown in a controlled growth chamber in pots of sterile 1:1 peat:sand, and pots were put in trays of standing distilled deionized water. Trays were kept in a Conviron Growth Chamber at 25 °C, with a 17:7 hours of light:dark cycle (light = 300 umol/m^2/s) for 90 days at 75% relative humidity, and monitored weekly for 8 weeks to determine percent germination.

2.8. Statistical Analyses

Data were analyzed in SAS 9.4 and RStudio 3.1.0[20]. Homogeneity of variance for each data set was tested using Bartlett's tests; all samples met this assumption. Normality was tested with Kolmagorov-Smirnov Goodness of Fit tests. Pollen counts and pollen:ovule ratios were then normalized via log transformation, and ovule counts were normalized by removing a single statistical outlier. SAS PROC GLM, followed by a Tukey Studentized range test, was used to test the relationship between plant type (*S. purpurea* var. *montana*, *S. jonesii*, or hybrid) and ovule production, pollen production, and pollen:ovule ratio. Total seed number across all sites and at SF, percent viable seeds, and percent germination were not distributed normally, so Kruskal-Wallis tests followed by Dunn's *post hoc* tests (Zar 2010) were used in analyses.

3. Results

3.1. Ovule and Pollen Counts at SF

Ovule counts varied by plant type (ANOVA; df = 59, F=9.00, $P \leq 0.05$). A Tukey studentized range test ($P \leq 0.05$) revealed hybrids had significantly more ovules than *S. jonesii* individuals. Pollen counts also varied across plant type (ANOVA; df=62, F=3.97, $P \leq 0.05$), with *S. jonesii* and *S. purpurea* var. *montana* differing significantly (Tukey studentized range test, $P \leq 0.05$; Figure 2). Pollen and ovule ratios did not vary among plant type (ANOVA; df= 60, F= 2.490, P=0.092).

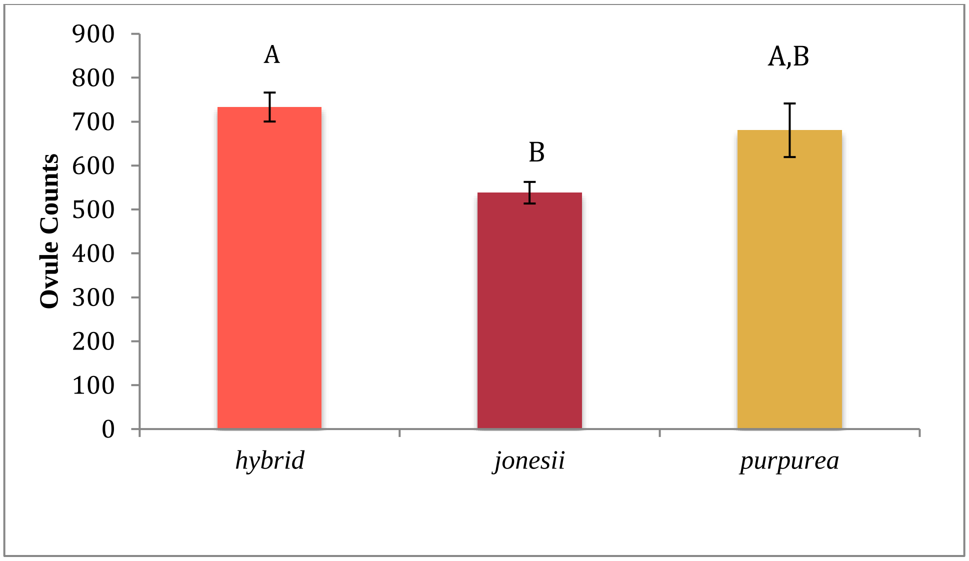

Figure 1. Ovule count by plant type at site SF.

Figure 1 Mean (± standard error) ovule counts by plant type at site SF. (df = 59, F=9.00, P ≤ 0.0003). A Tukey Standardized test revealed differences among plant type, indicated by the letters above bars.

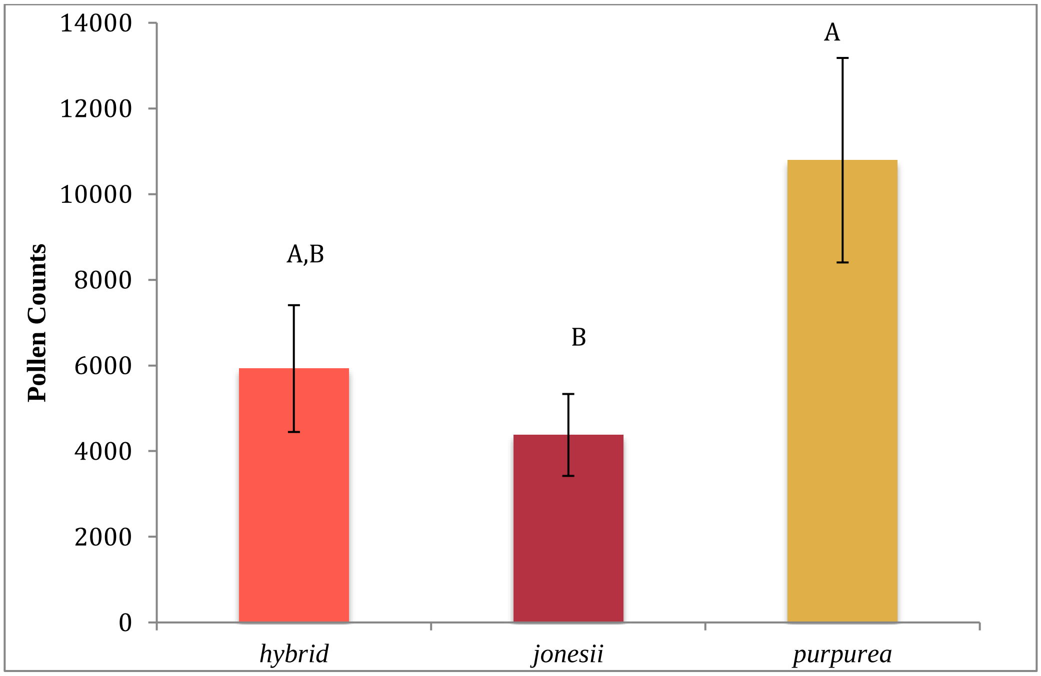

Figure 2. Pollen counts by plant type at site SF.

Figure 2 Mean (± standard error) pollen counts differ by plant type at site SF (df=62, F=3.97, P ≤ 0.0196). A Tukey Standardized test revealed differences among plant type, indicated by the letters above bars.

3.2. Seed Counts, Percent Viable, and Percent Germinated

Kruskal-Wallis results showed a significant difference in seed production across all sites (df = 7, χ^2= 35.03, P= 0.0001). Sites CM and HC produced the greatest amount of seeds, where sites DF and RL produced the least amount of seeds. However, there was no significant difference in percent viability (df=6, χ^2= 12.2238, P= 0.0572) or germination (df=6, χ^2= 4.8232, P= 0.5667) across sites. χ^2

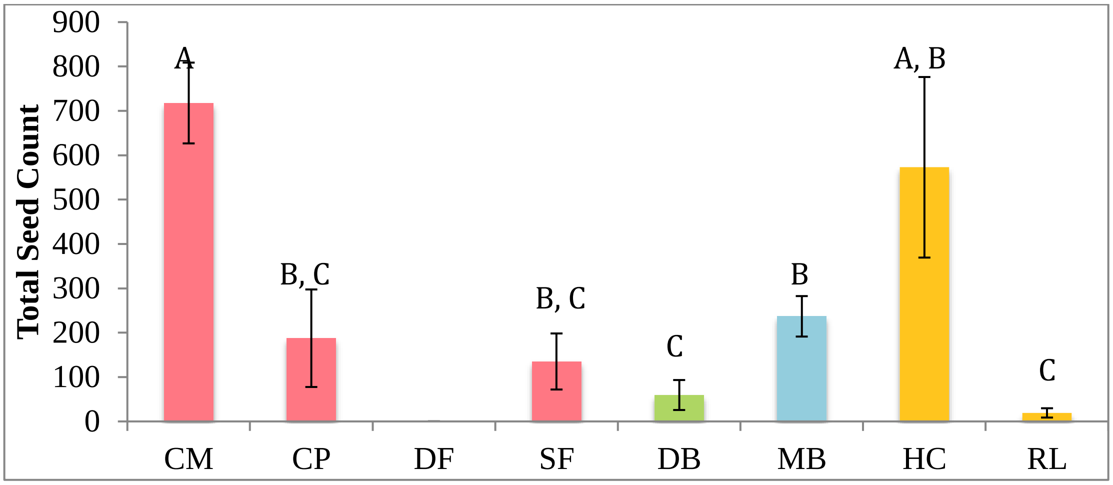

Figure 3. Total seed count across all sites.

Figure 3 Total seed count means (± standard error) across all *Sarracenia* sites (df = 7, χ^2= 35.03, P= 0.0001). A Dunn's *post hoc* test revealed differences among plant type, indicated by the letters above bars.

At SF, Kruskal-Wallis results showed no significant effect of plant type (*S. purpurea* var. *montana*, *S. jonesii*, or hybrid) on seed number (df = 2, χ^2= 2.2718, P= 0.3211). However, there were significant differences among types in percent viability (df = 2, χ^2= 11.1317, P=0.0038; Figure 4) and percent germination (df = 2, χ^2= 15.5726, P=0.0004; Figure 5). In addition, ovule counts (df=9, F=0.370, P= 0.5593), pollen counts (df= 9, F= 0.06, P= 0.8190), and ovule to pollen ratios (df=9, F= 0.24, P= 0.6375) did not vary significantly by flower color.

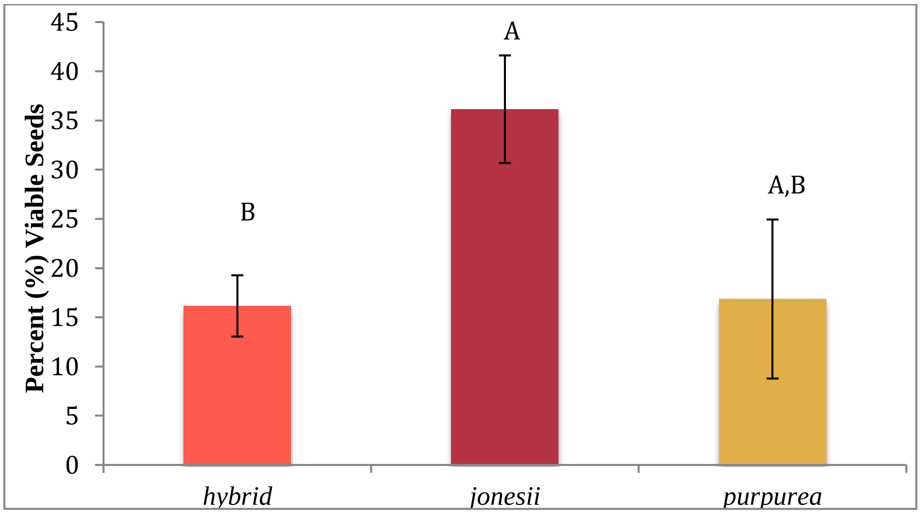

Figure 4. Percent seeds viable by plant type at site SF.

Figure 4 Mean (± standard error) percent of seeds viable by *Sarracenia* type at site SF (df = 2, χ^2= 11.1317, P=0.003826). A Dunn's *post hoc* test revealed differences among plant type, indicated by letters above bars.

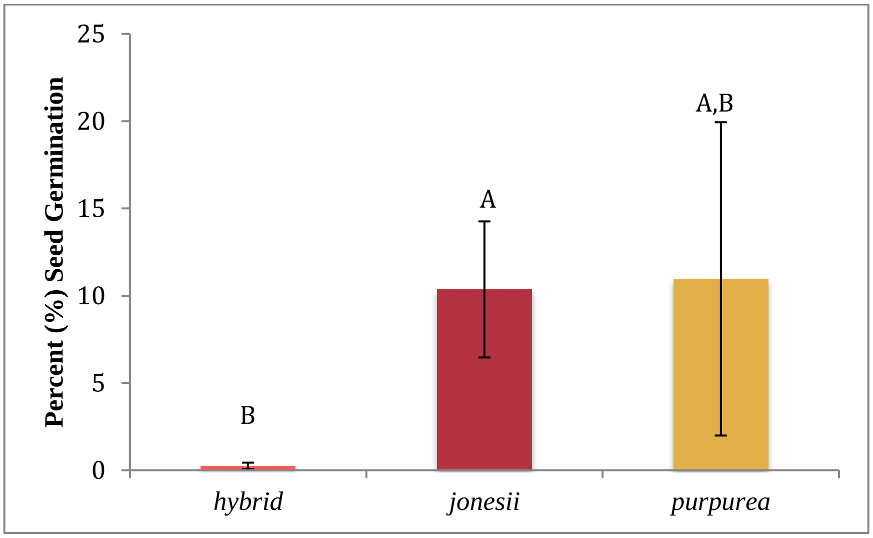

Figure 5. Percent seed germination by plant type at site SF.

Figure 5 Mean (± standard error) percent seed germination by plant type at site SF (df = 2, χ^2= 15.5726, P=0.0004154). A Dunn's *post hoc* test revealed differences among plant type, indicated by the letters above bars.

4. Discussion

4.1. Comparing Ovule, Pollen and Seed Counts at SF

Significant variation among pollen counts, ovule counts, percent viable, and percent germinated seeds were seen between the two parent species and their hybrid. Reproductive effort by *Sarracenia jonesii* overall was low compared to *Sarracenia purpurea* var. *montana* and hybrids. Low reproductive efforts could be due to competition for resources with *S. purpurea* and hybrid plants; comparing *S. jonesii* reproductive output in sites with versus without its congener could give further insight into this relationship. Though *Sarracenia jonesii* had low reproductive effort, it contained the highest percent viable seed set. These results suggest that though the reproductive efforts were low, *S. jonesii* success could be due to the use of resources to grow pollen tubes during fertilization[13], or through evolutionary bet hedging[11]. Though pollen counts were the lowest for *S. jonesii*, it could produce the best pollen quality[1] or pollen accessibility[12] to create more successfully viable seed sets. *Sarracenia jonesii* is known to produce a fragrant flower[15], which often leads to increased pollinator visitation and better pollination quality[8].

Sarracenia purpurea var. *montana* had higher pollen production than *S. jonesii* or hybrids, and a greater ovule count than *S. jonesii*. High pollen counts suggest several possible causes. One hypothesis suggests that plants tend to be more male-biased in areas with lower levels of resource availability, implying that denser populations and individuals that bloom later produce more pollen[16]. *Sarracenia purpurea* var. *montana* produced fewer seeds than *S. jonesii*, though this was not significant. However, significance was seen in *S. purpurea* var *montana* having greater seed germination than *S. jonesii*. Higher percent germination may relate to a fitness-based trait, combining resource allocation, stress tolerance, and germination timing to play a significant role in plant germination success[22].

Hybrid pollen counts trend for intermediate pollen production between *S. jonesii* and *S. purpurea* var *montana*, and the hybrids contained the highest ovule counts (only significantly differed from *S. jonesii*). Female-bias plants are hypothesized to occur when resources are readily available. Hybrid may be better at acquiring resources, or bloom earlier than other species in its surroundings[16]. High production of ovules is also predicted to compensate for interactions with other pollen types or poor quality pollen grains[1]. However, the hybrid's percent viable seeds and percent germinated seeds were significantly lower than *S. jonesii* and *S. purpurea* var. *montana*, and hybrid seed count was also low (though non significant). Results suggest that even though ovule production in hybrids were high and pollen production was intermediary, pollen quality or limitation[1] lead to lower levels of seed production and germination overall. *Sarracenia jonesii* and *S. purpurea* var. *montana* may have the upper hand due to successful use of evolutionary bet hedging[11]. Reproductive output results found here suggests that hybrid individuals will not pose a demographic threat to the two parental species. However, reproductive effort data suggest that hybrids have the potential to become more successful over time, and that small numbers of germinating hybrid seeds could result in significant introgression.

4.2. Seed Count across all Sites

There was a significant variation in seed count across sites for *S. purpurea* (P= 0.0001). Previous studies show that resource availability plays a significant role for seed set production in pitcher plant species. In *S. purpurea*, specifically, the majority of seed set is reliant on resource availability by prey capture[19]. A combination of prey capture, photosynthetic ability, and nutrient absorption from the soil are all likely factors of higher seed counts at some site than others. Sex allocation for resource availability may result from causes like phenotypic plasticity, natural selection, or genetic drift and gene flow[16]. The results for seed viability across all sites were nearly significant (P= 0.0572). Significance in seed viability would signify that some sites are limited by resources or access to pollen[12].

5. Conclusion

Other studies on carnivorous plants have shown that there is a somatic cost to reproduction, but that cost is lower than reproductive effort. Possible mechanisms to compensate for carnivorous plant reproductive investments include: a reproductive switch on when availability of resources passes threshold level in the environment, an adjustment in mineral nutrient foraging, or gaining an accumulation of resources until the plant has stored enough nutrients for reproductive investment[25]. Hybridization between two plant species often leads to a more genetically diverse individual, increasing hybrid fitness by heterosis or habitat competition. Over time, the hybrids can become invasive, leading to the extinction of parent species and affecting the colonization of other native plant and animals in the same shared habitat[2].

Next steps in relation to this study of co-occurring *Sarracenia* plants include testing for pollen viability and quality to predict successful reproductive output[1], and recording pollinator type and visitation rate to measure pollinator preference and pollinator effect on pollen quality[8]. Furthermore, flower color is a trait that often signifies a reward for pollinators[18]. A study comparing flower color to pollen and ovule counts with a larger sample size than the one presented in this study (red n= 5, yellow n= 5) may allude to a pollinator preference, increasing the reproductive output for one flower color trait over another[18]. Another study may include genetic analysis of each plant species to give further insight to inbreeding depression and reproductive outcrossing[1], as well as determining degrees of hybridization[2]. Lastly, future studies many include a demographic survey for the ratio of hybrid to parental species that exist at site SF to predict competition intensity by hybrids.

In conclusion, the presence of a hybrid plant may show varying effects for the future generations of these two endemic pitcher species. Small populations of *Sarracenia purpurea* var. *montana* and *Sarracenia jonesii* throughout the Appalachian mountains may become even more vulnerable if the presence of a hybrid plant outcompetes the reproductive effort and output of these endemic species, affecting the overall success of future generations of *Sarracenia jonesii* and *Sarracenia purpurea* var. *montana*. Assessing connections among reproductive effort, reproductive output, and performance will allow conservation recommendations to be made.

6. Acknowledgements

We are grateful to Miranda Satterfield, for field collection and data entry. Alexandra Gates, who provided help with seed counting and data entry. Elise Powell, for field collection and related studies to the *Sarracenia* at these sites. Funding for this project was provided by the United States Fish and Wildlife Service.

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