

Life history and physiology of iteroparous reproduction in *Neoregelia tigrina* (Bromeliaceae)

A Senior Thesis Presented to

The Faculty of the Department of Organismal Biology & Ecology, Colorado College

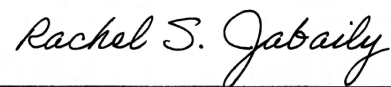
By

Emma Fetterly

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Approved by:



Dr. Rachel Jabaily, Primary Thesis Advisor



Dr. Shane Heschel, Secondary Thesis Advisor

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Abstract

Plant life history is of critical importance to population dynamics and species distribution patterns and can be associated with distinct ecophysiological responses. This study examines the physiology of one iteroparous bromeliad *Neoregelia tigrina* (Ruschi) Ruschi to address questions of reproductive allocation and functional responses at various stages of development. We seek to address how flowering and growth of vegetative ramets, a form of iteroparity, affects the functional responses of the maternal rosette. This study measured photosystem efficiency (PE), chlorophyll content (CCI), stomatal density, leaf area, leaf mass, specific leaf area (SLA), longest leaf length and foliar sucrose concentration of similarly aged mature plants in a greenhouse setting at three stages of development. The shift from pre-flowering to flowering was found to be associated with a decline in PE and stomatal density and with an increase in sucrose. This is likely a water conservation response to flowering and an observed shift in photosynthetic potential to a strategy of higher gas exchange in post-flowering plants. With a higher intensity of vegetative ramets we found a marginally lower SLA, higher leaf area and higher stomatal density, supporting evidence for the plants' shift from high photosynthetic potential early in development to higher gas exchange. The plants produced an increasing concentration of photosynthate throughout flowering and the growth of ramets, indicating that there is not a significant tradeoff between sexual and asexual reproduction in this species although additional study is needed to clarify source-sink relationships between ramets, the inflorescence and the vegetative body.

Section I: Life history evolution

The evolution of life history strategy and its effect on species diversification and stress tolerance is an important consideration in both plant evolutionary biology and ecophysiology (Schaffer and Schaffer, 1977; Stearns, 1992). Life history strategies are classically understood as a distinct binary of iteroparity and semelparity, however a more nuanced view that defines a multidimensional continuum within each strategy is beginning to emerge (Hughes, 2017; Jabaily et al. *in prep*; Salguero-Gómez et al., 2016). Most organisms will attempt sexual reproduction several times in their lives, a life history strategy of iteroparity (or polycarpy). Semelparous, or monocarpic organisms will attempt only one sexual reproductive event in their lifetime. For many perennial organisms this event is so energetically costly that resources for continued growth and tissue maintenance are exhausted, leading the individual to senescence (Young, 1990). Semelparity has evolved convergently in a wide array of long-lived angiosperm lineages from an iteroparous ancestral state (Young, 1990). There is often a suite of characteristics associated with the evolution of semelparity in longer lived species which include a rosette body plan, low adult survival, early senescence, increased time between reproductive episodes, high population growth rate and high juvenile survival (Che-Castaldo and Inouye, 2011; Clauss and Venable, 2000; Young, 1984; 1990; Young and Augspurger, 1991). Semelparity has been interpreted as a strategy to increase fecundity in severe habitats as plants will invest all potential reproductive effort into one event rather than the continuous maintenance of vegetative structures in stressful conditions (Young, 1990). However, semelparity is also found in stable, predictable environments that favor slow growth with low frequencies of disturbance events. The mechanisms of selection for a certain life history are not entirely understood and could be a result of abiotic or biotic selective pressures and a plant's functional responses and ability to

tolerate environmental stress (Rocha et al., 2005; Young, 1990; Young and Augspurger, 1991). The focus of this study is the physiological tradeoffs that may exist in resource allocation patterns of sexual and vegetative reproductive modes within iteroparous taxa and if this could contribute to selection pressures for a particular life history state.

Within the study of life history evolution of plants, particularly in consideration of longer-lived taxa, three major theories of semelparous evolution have been identified and modeled (Young and Augspurger, 1991). The first is the bet-hedging model, which predicts that in a variable and unpredictable environment there will be selection for iteroparity, as a single reproductive event is far too risky (Clausen and Venable, 2000; Young and Augspurger, 1991). However, many longer lived semelparous plants are endemic to unpredictable and severe habitats, including drought prone areas with ephemeral precipitation (Young and Augspurger, 1991). The semelparous *Lobelia telekii* was found to occur more frequently in drier habitats and had a lower mortality in drier conditions than the iteroparous *Lobelia keniensis* (Young, 1984). The bet-hedging theory does model some plant systems but is not representative of all semelparous taxa, especially those found in unstable and severe habitats.

The demographic model is the second approach to understanding semelparous evolution. In this model, semelparity is most likely to occur in environments where future reproductive events are unlikely, especially when adult survival is low (Young and Augspurger, 1991). When tested against long term data, Young (1990) found the demographic model to represent the high elevation African *Lobelia* system, which includes both semelparous and iteroparous taxa in the same community, with variation in microhabitat. The semelparous *Lobelia telekii* inhabits a highly variable environment with low soil moisture. Consequently, *L. telekii* demonstrate low adult survivorship when compared to the iteroparous *Lobelia keniensis* and produce much larger

inflorescences with a higher seed count. For this system, long term demographic data more accurately explains the variations in life history adaptation. The benefit of long-term sampling is seen more clearly in the *Frasera speciosa* system, where masting, or synchronous flowering, is critical to the reproductive strategies of the semelparous taxa (Che-Castaldo and Inouye, 2011). As relatively few studies are able to achieve comparable long-term sampling periods, this model is often unable to offer the scope of a comparative analysis. More data is needed both in demographic and experimental studies to further inform our understanding of a demographic approach and the systems it may represent.

The third model is known as the reproductive effort model (Schaffer and Rosenzweig, 1977; Young, 1990; Young and Augspurger, 1991). This model predicts that with an increasing reproductive effort through the increased production of sexual reproductive tissues there will be a positive and accelerating association with reproductive success in semelparous taxa only. The plant will invest most, if not all, of its available resources into one major reproductive event yielding high fecundity. After the reproductive episode the plant will have exhausted its resources for maintaining vegetative structures and it will begin to senesce. This “Big Bang” strategy can be predicted by a disproportionately large reproductive effort which may include the size of the inflorescence, viable seeds by weight or by analysis of the post reproductive half-life of an individual (Schaffer and Schaffer, 1977). The selective mechanism of this model has not been fully explained. Many studies point to the influence of pollinator preference for large inflorescences and the effect of masting to encourage seed predator satiation (Borchert and DeFalco, 2016; Che-Castaldo and Inouye, 2011; Janzen, 1976; Keeley and Bond, 1999; Kelly, 1994; Kelly and Sork, 2002). Rocha et al. (2005) found that pollinators select for larger inflorescences in *Agave*, especially in *Agave striata*, a semelparous variety. This pattern was

found earlier by Schaffer and Schaffer (1977; 1979) who suggest that with an interspecies comparison between iteroparous and semelparous taxa within *Yucca* and *Agave* bee pollinators are able to categorize the inflorescences by size and preferentially visit stalks with more flowers and nectar, especially in times of high resource availability. The pollinator selectivity hypothesis (Schaffer and Schaffer, 1977) would explain selection for a larger reproductive effort at the expense of all available photosynthate. Jabaily and Sytsma (2013) have postulated that this pollinator selection for larger inflorescence and larger, longer-lived rosettes may explain evolution towards semelparity in high elevation species of *Puya* (Bromeliaceae).

Within taxa that contain both iteroparous and semelparous life histories lies the complicating factor of asexual, or clonal, reproduction. Life history and reproductive allocation studies do not often account for the potential cost or benefits of asexual reproduction even though it is typically a significant portion of the total plant biomass. Although ramet production does not require the large diversion of resources of a terminal inflorescence, there does exist a tradeoff between diverting energy to asexual reproduction and potentially delaying sexual reproduction by extending the juvenile stage in *Aechmea magdalenae* (Bromeliaceae) (Villegas, 2001). Iteroparous and clonally reproducing taxa of *Yucca* typically produce smaller rosettes with faster growth rates and lower surface areas than semelparous relatives (Huxman and Loik, 1997). They have also been shown to produce fewer and less viable seeds than semelparous *Yucca* plants but reproduce more often through the process of asexual reproduction and have more opportunities to flower (Huxman and Loik, 1997). Within Poaceae, a rosette-forming monocot family related to Bromeliaceae and *Yucca*, asexual reproduction is of particular importance to life history strategy due to the high intensity of ramet production involved with population persistence and survival. Ott and Hartnett (2011) studied the effects of vegetative

growth and sexual reproduction in *Andropogon gerardii* (Poaceae) to determine if tradeoffs exist at multiple levels (ramet or whole plant). This study found that flowering ramets had increased ramet recruitment and more mature buds than non-flowering individuals and found no measurable negative effects of sexual reproduction on ramet recruitment. This may indicate that a minimum size threshold must be met before initiation of both ramets and flowers and once that size is achieved there are low relative costs to both sexual and vegetative reproduction. Reekie (1991) found a comparable result in *Agropyron repens* (Poaceae), where no significant tradeoffs were found between seed production and rhizome production. However, in high light and high nitrogen environments there was an increased cost of sexual production and less rhizomes were produced. The relative costs of reproductive modes are highly dependent on the suit of resources available and in optimal conditions the cost of asexual reproduction relative to flowering is typically very low (Reekie, 1991). The mechanism of this relationship has been explored by Lord (1998) and hypothesized to be a result of increased photosynthate produced from ramet leaves offsetting the cost of ramet growth.

Ramet production has been shown to depend on various environmental factors such as high moisture, high light and seasonality (Villegas, 2001). This strategy can be an important tool for population maintenance and growth, particularly in the outer reaches of a habitat range (Augspurger, 1985; De Steven, 1989; Garcia-Franco and Rico-Gray, 1995; Villegas, 2001). It allows for greater flexibility, as vegetative reproduction has been shown to have an exploratory nature and the maternal rosette has the ability to abort or sever the daughter ramets if they are not sustainable (Villegas, 2001; Young, 1984). Additionally, if the maternal rosette dies then axillary ramets still have the potential to grow and sexually reproduce in some cases, continuing the genet's survival (Cook, 1983). Axillary ramets are a critical mechanism for the continued growth

and survival of the genet after a monocarpic maternal rosette produces a terminal inflorescence, thus representing an alternative to the binary theory of semelparity and iteroparity.

This study will explore asexual reproduction in the context of plants with a rosette body form in which ramets emerge from initiated axillary meristems of the maternal rosette. True semelparity will be hereon defined as a maternal rosette that *never* produces ramets. An intermediate classification coined by Jabaily and Sytsma (2013) of semi-semelparity describes maternal rosettes that have a greatly diminished ability to produce ramets and only after a sexual reproductive event is achieved. Iteroparity in this context will be defined as a monocarpic maternal rosette accompanied by axillary ramets. The maternal rosette will have a single attempt at sexual reproduction through the conversion of the apical meristem to an inflorescence, but the genet will have repeated opportunities for sexual reproduction through axillary ramets. Tradeoffs between reproductive effort and eventual reproductive success are central in our understanding of life history evolution, especially when considering sustained successful asexual reproduction as the point at which selection pressures move a system towards iteroparity.

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Section II: The physiology of iteroparous reproduction in *Neoregelia tigrina* (Bromeliaceae)

Introduction

Selection for certain life histories can be a result of varying strategies for resource acquisition and use in different environments (Stearns, 1992). Life history and energetic investment are closely linked, resulting in a unique reproductive allocation schedule that depends on the functional responses of a plant to available resources and environmental stressors (Wenk and Falster, 2015). Reproductive allocation refers specifically to the fraction of surplus energy diverted to reproduction in a given period, often a result of suites of functional traits working to increase carbon assimilation and photosynthate (Wenk and Falster, 2015; Wenk et al., 2018). Functional traits can be informative measures of a plant's physiology and resource acquisition strategies (Reich et al., 2003). Measures of photosynthetic potential such as photosystem efficiency (PE), chlorophyll content (CCI) and stomatal conductance (g) can be important indicators of a plant's ability to assimilate carbon and the rate at which they will be able to grow and reproduce (Reich et al., 2003; Wenk et al., 2018). Life history modeling points to a clear association of physiology and life history evolution, yet there have been few studies that have examined both aspects within one system. It is difficult to incorporate the larger sampling, genetic and developmental pieces needed for macro evolution-centered analyses into *in-situ* physiology experiments and few studies are able to investigate both areas (Reich et al., 2003). The aim of this study is to investigate questions of life history tradeoffs between sexual and asexual reproduction with a physiological approach based in functional trait analyses.

Functional traits can vary depending on a plant's resource acquisition needs, often in response to environmental conditions and the demands of maintaining photosynthate production to support continued growth. The efficiency with which photons are captured by leaves for light

reactions, or photosystem efficiency, can be a limiting factor in photosynthate production along with foliar chlorophyll concentration (Heschel et al., 2014). Stomatal density and leaf area can also be limiting traits in gas exchange and therefore potential carbon assimilation rates (Marler, 2011a, 2011b). These traits also depend on the plant's water stress condition, as the plant loses water to transpire and assimilate carbon. Water stress may manifest in other traits, such as an increase in water-storing hydrenchyma tissue that may decrease the specific leaf area to mass ratio and increase succulence (Larcher, 2003). The careful balance between drought tolerance, carbon assimilation and maximizing photosynthetic potential is especially relevant in canopy-bound epiphytes, as they have limited access to water, light and nutrients for growth (Benzing, 1980; 2000). Many taxa in the Bromeliaceae family are epiphytic and show unique morphological and physiological strategies to support growth and distinct life history strategies.

Bromeliaceae is a family of particular interest in the field of life history biology and ecophysiology (Benzing 2000; Givnish et al. 2014; Males 2016; Males and Griffiths 2018; Palma-Silva et al. 2016; Villegas 2001; Jabaily et al. *in prep.*). Bromeliads represent a large and diverse Neotropical family that contains 54 genera and over 3,500 species. Semelparity in bromeliads is widely represented across genera and follows similar patterns to semelparity evolution in *Agave*, *Yucca* and *Lobelia* systems, with long-lived rosette forming plants that exhibit large terminal inflorescences. A common feature in iteroparous bromeliads is the ability to clonally propagate and produce several axillary ramets or "pups" from a mother rosette (Benzing, 1980; 2000). Within the family there are multiple axes or levels of asexual iteroparous reproduction depending on the timing of the maternal inflorescence relative to the ramet production, ramet growth rate, anatomical origin of ramets, ramet to mother connectivity and ramet number (Jabaily et al. *in prep.*). Due to the existence of a diverse array of asexual

reproduction it has been theorized that many pathways exist along the evolution from iteroparity to semelparity as a result of potential physiological tradeoffs from the production of ramets relative to sexual reproduction.

The morphology of most members of the Bromeliaceae family has considerable impact on the reproductive strategies and life histories of taxa due to its limitations on growth form and inflorescence type. Bromeliads share a single body plan, which is often found in other monocots, and consists of a very short stem with spirally arranged and tightly associated leaves (Benzing, 2000). The overlapping leaf bases in many bromeliads form a tank center that has the capabilities to hold and absorb water through specialized trichomes on the leaf surface. At each node of the stem lies an axillary meristem that has the capabilities to initiate and produce an axillary ramet (Benzing, 1980). In most cases of truly semelparous bromeliads, the axillary buds remain dormant due to the dominance of the shoot apical meristem (SAM) (Benzing, 1980). The root systems of most bromeliads are fibrous, adventitious and exist for structural support (Benzing, 2000). In epiphytic species roots are highly thigmotropic, growing against tree bark irrespective of gravity and serve as a point of fixation in the tree canopy. Most tank bromeliads rely on trichomes and not root systems to absorb nutrients and water (Benzing, 1980; 2000). The vegetative body is primarily photosynthetic and continues to grow until it reaches the critical size at which reproduction or clonal production can occur (Augsburger, 1985).

The inflorescence of most bromeliads emerges from the SAM as a series of brightly colored bracts ending in a terminal inflorescence of several to many flowers (Benzing, 2000). Bromeliads can be pollinated by a wide variety of bees, hummingbirds, or bats depending on the habitat, or can self in certain cases (Aguilar-Rodriguez et al., 2019a; 2019b; Benzing, 1980, 2000; Martinelli, 1995; Palma-Silva et al., 2015; Restrepo-Chica and Argenis Bonilla-Gomez,

2017; Sayago et al., 2018). The reproductive habit and population dynamics of iteroparous bromeliads have been relatively well studied in a demographic and population genetics context, however few physiological studies have examined the effects of both sexual and asexual reproduction (Barbará et al., 2009; Cascante-Marín et al., 2006; Loh et al., 2015; Manhaes et al. 2020; Sgorbati et al., 2004; Soares et al., 2018; Villegas, 2001; Zanella et al., 2011). Although ecophysiological studies are limited in wild bromeliads, the common pineapple, *Ananas comosus* (Bromeliaceae), has been the subject of many horticulture studies due to its global agro-economic importance. Marler (2011a, 2011b) found no change in leaf transpiration at different stages of flowering and ramet production in *A. comosus* and no variance in biomass partitioning between flowering and vegetative plants, indicating no significant tradeoffs between sexual and asexual reproduction. Additional studies that reflect the diversity of physiological strategies for iteroparous bromeliads are needed to assess the role of ramet growth in life history strategy.

This study examines the functional traits of one iteroparous species of Bromeliaceae, *Neoregelia tigrina* (Ruschi) Ruschi, at three developmental stages along a continuum of ramet growth and flowering to identify patterns of variation in functional traits. The categories of development evaluated in this study include pre-flowering with minimal young ramets, flowering with minimal young ramets and flowering with developed, stolon attached ramets. We measure both physiological and morphological parameters such as photosystem efficiency, chlorophyll content, stomatal density, leaf area, longest leaf length and sucrose content. Our goal is to identify patterns of functional trait variation and potential tradeoffs of flowering and ramet production to inform our understanding of the physiological mechanisms of life history strategy in Bromeliaceae. The questions we seek to address are: 1) Do functional traits vary with flowering in *Neoregelia tigrina*? 2) Does functional trait variation exist with different stages of

ramet growth? 3) Is there evidence for potential tradeoffs between sexual and asexual reproduction in *N. tigrina*? We hypothesize based on the repeated convergent evolution of semelparity from iteroparous lineages that there is some physiologic cost to the maternal rosette in the production of ramets, but also that the effect on selection pressures will likely depend on environmental factors and the many axes of iteroparity in Bromeliaceae.

Methods

Study Organism

Neoregelia tigrina (Ruschi) Ruschi is a CAM epiphytic bromeliad (Bromeliaceae) endemic to the Atlantic Rainforest of Southeastern Brazil. The CAM metabolism of *N. tigrina* was established through a pilot study of nocturnal stomatal conductance (E. Fetterly and M.S. Heschel, unpublished data; $\bar{x} \pm \text{SE}$, 23:00-1:00: $58.74 \text{ mmol/m}^2\text{s}^{-1} \pm 2.53$; 15:00: $0 \text{ mmol/m}^2\text{s}^{-1} \pm 0$). Plants grow 25 - 40 cm tall in a dense sub-ovoid rosette with spiraling leaves that form a central water tank (Ruschi, 1954). Rosettes grow in shade conditions within the tree canopy in a montane ombrophylous forest ecosystem, 700 – 1200 m altitude (Martinelli, 1995; Salguero-Gómez et al., 2016). Average precipitation for this region is 2,000 – 2,500 mm/year and average relative humidity is 68%. Temperatures range from 13°C to 25 °C, peaking in November through March (INMET, 2019). *N. tigrina* has an iteroparous life history and reproduces sexually via outcrossing and asexually through clonal offshoots also referred to as “pups” (Benzing, 1980; Martinelli, 1995). Corollas are a pale violet and develop in the central tank while fully submerged underwater, emerging above the water level at anthesis. It is hypothesized that *N. tigrina* is pollinated by hummingbirds similar to other taxa within genus *Neoregelia*, however no pollination study has been conducted for the species (Martinelli, 1995).

Experimental Design

The study population of 45 *N. tigrina* adult plants were individually selected, purchased, and shipped from Tropiflora, Inc., a major horticultural company specializing in bromeliads in Sarasota, FL, in early June of 2019. This population was originally germinated, likely from a single seed capsule, in Colombia and transported to Tropiflora, Inc. as seedlings early in development, although the precise date of planting and conditions are unknown. Within the population of siblings grown from seed, three categories of development were identified: pre-inflorescence with small ramets (Category 1, $n = 15$), flowering with small ramets (Category 2, $n = 15$) and flowering with large, stolon attached ramets (Category 3, $n = 15$); plants in these three categories should be approximately the same age (Figure 1).

Data was collected in the Colorado College greenhouse from June 2019 – September 2019. Relative humidity was maintained at 50-55% with temperatures ranging from 15-24°C. Upon arrival to the CC greenhouse plants were individually potted in 4-inch pots with soil, top dressed with potting soil and treated with neem oil for fungi and insects. Plants were individually fertilized depending on color morphology, with lighter leaved plants ($n = 21$) receiving 2x the fertilizer than darker leaved plants ($n = 24$). A randomized complete block design was implemented. Plants were arranged on one greenhouse bench into three blocks, each block containing 5 plants from each category. Plants were randomly selected and randomly positioned in the block. All plants were top watered regularly to maintain a full water tank and fertilized June through July. Full leaf expansion was estimated to take 4-5 weeks (E. Fetterly, personal observation) and thus the microenvironment of the greenhouse likely influenced developing leaves throughout the course of data collection.

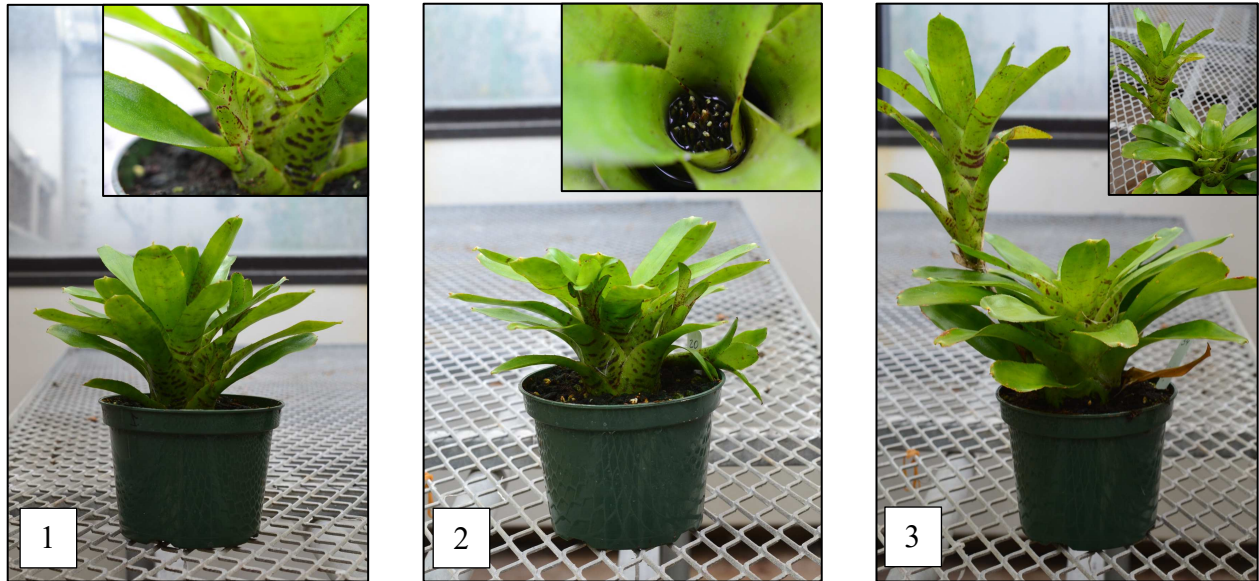


Figure 1. Developmental categories in study population of *Neoregelia tigrina*. Category 1: pre-flowering with minimal ramets. Category 2: flowering with minimal ramets. Category 3: flowering with large, stolon connected ramets.

Photosystem Efficiency

Photosystem efficiency measures the red light absorbed by leaves and produces an index of photosynthetic potential. Percent photosystem efficiency (PE) was measured on randomly selected 3rd and 4th whorl fully expanded leaves using a Plant Photosynthesis Monitoring BV fluorometer (EARS, NL). The device was placed centrally on the adaxial side of the leaf, approximately 2 cm from the leaf tip. A black background was placed on the abaxial side of the leaf to prevent light interference. The fluorometer projects red light onto the leaf section and the value is recorded as the ratio of variable to maximal fluorescence (F_v/F_m). This measurement was repeated for five days, randomly selecting a 3rd and 4th whorl leaf on each plant per day. All measurements were taken between 10:00 hr and 15:00 hr.

Chlorophyll Index

Chlorophyll content provides a relative measurement of foliar chlorophyll concentration and can give an estimate of photosynthetic potential. Chlorophyll content index (CCI) was

measured on randomly selected 3rd and 4th whorl recently expanded leaves using a SPAD meter. (CCM 200 plus, Opti-Sciences Inc., NH). The same 3rd and 4th whorl leaves used to measure CCI were used to measure percent photosystem efficiency. The SPAD meter was clamped centrally to the adaxial side of the leaf, approximately 2 cm from the leaf tip taking care to completely cover the sensor region with the leaf section. All measurements were taken between 10:00 hr and 15:00 hr.

Stomatal Density

Stomatal density was examined as an estimate of foliar gas exchange potential. Increased foliar gas exchange rates can correlate with increased carbon assimilation. A 4th whorl fully expanded leaf was selected randomly from each plant and removed at the leaf base.

Nitrocellulose varnish was applied to the abaxial side of the leaf and allowed to dry under a fan for 10 minutes. The varnish was carefully removed in two sections using forceps, sampling from the edges of the leaf to produce sections approximately 1 cm x 2 cm. The sections were placed on a slide and stained with one drop of 10x concentrate Methylene Blue and a cover slip was applied. Slides were inspected with a compound light microscope (Swift Optical, CA) at 10x magnification and stomata within the field of view were counted. Area of the field of view was calculated using the formula: $\text{Field Size} = \text{Field Number (fn)} / \text{Objective MA magnification (Mo)}$ (Nikon Corp., Tokyo, Japan). Area calculations were used to determine stomatal density (density = number of stomata / area of field of view) of each count. Two counts were conducted from each peel and two peels were sampled from each plant.

Sucrose Concentration

Sucrose concentrations were obtained from a 4th whorl recently expanded leaf that was frozen at -80 °C. Tissue was removed from the freezer and horizontally sectioned into 1 mm

ribbons starting 2 cm from the leaf tip. The leaf base and leaf tip were not used due to their higher concentrations of fibrous tissue. The sectioned tissues were each massed to 0.25 grams and placed in a 1.7 mL PowerBead tube with 2.38mm metal bead (Qiagen corp., Venlo, NL) and 400 microL of ultrapure water. The tubes were frozen at -20°C overnight and thawed. The tubes were placed in a Powerlyer 24 (MoBio Laboratories, Venlo, NL) for 4 minutes at 1700 rpm. The tubes were centrifuged for 10 minutes at 2500 rpm and 150 microL of supernatant was extracted with a micropipette. The supernatant was diluted with 40 microL of ultrapure water to bring the total volume to 190 microL. The solution was frozen and thawed in small batches for BRIX index quantification with an ABT-32 portable refractometer (VEE GEE scientific, IL). All 190 microL were used for each quantification, which was determined as the minimal amount to completely cover the sensor region of the refractometer.

Longest Leaf Length, Leaf Number, and SLA

Plants were visually inspected for a potential longest leaf and then each potential leaf was measured using a flexible ruler from the leaf base to the tip of the leaf to determine longest leaf length in cm (LLL). Leaf number was determined by counting fully expanded leaves from a visual inspection. Specific leaf area (SLA = Leaf area / leaf mass) was determined using the mass and area of a randomly selected 4th whorl leaf cut at the leaf base. The 4th whorl leaf was the same leaf used for stomatal density and sucrose analyses. Note: fresh weight of leaves was used in SLA calculations.

Statistical Analysis

Statistical analyses were conducted in JMP (version 5.1.2., SAS Institute). Data were inspected for normality and log base 10 transformations were conducted on CCI, stomatal density, LLL and leaf number data to normalize data. Analysis of variance (ANOVA) was

conducted with generalized least square linear models to test for differences in traits between categories. Category was considered a fixed factor and block a random factor in the analysis (mixed model). Interaction with block was excluded from this analysis. Residual distributions were examined for normality. Planned contrasts between categories (t - test) were conducted for traits that had a significant effect of category ($P < 0.05$).

Results

Photosynthetic capacity

The shift in pre-flowering (Category 1) to flowering plants (Category 2) was found to be associated with significant variation in percent photosystem efficiency (PE) and sucrose, with nonsignificant trends found in chlorophyll content index (CCI). Category had a significant effect on PE (Table 1). PE in Category 1 was marginally higher than Category 2 ($P < 0.10$) and significantly higher than Category 3 ($P < 0.001$) (Figure 2). PE decreased from Category 1 to Category 3 indicating a reduced efficiency as plants produced inflorescences and larger ramets. Category did not have a significant effect on chlorophyll content index but there was a marginal trend of decreasing CCI from Category 1 to Category 3 (Table 1, Figure 2). No significant block effects were detected for PE or CCI data.

There was a significant effect of category found in sucrose concentrations (Table 1). Category 1 had a significantly lower BRIX index than Category 2 and Category 3. Sucrose increases from Category 1 to 3 although there is not a significant difference between Categories 2 and 3. Categories 1 and 3 are significantly different in sucrose content ($P < 0.001$) and Categories 1 and 2 are marginally different ($P < 0.10$). There is an increase in sucrose content as plants increase their production of both inflorescences and ramets. No significant block effect was detected for sucrose concentration.

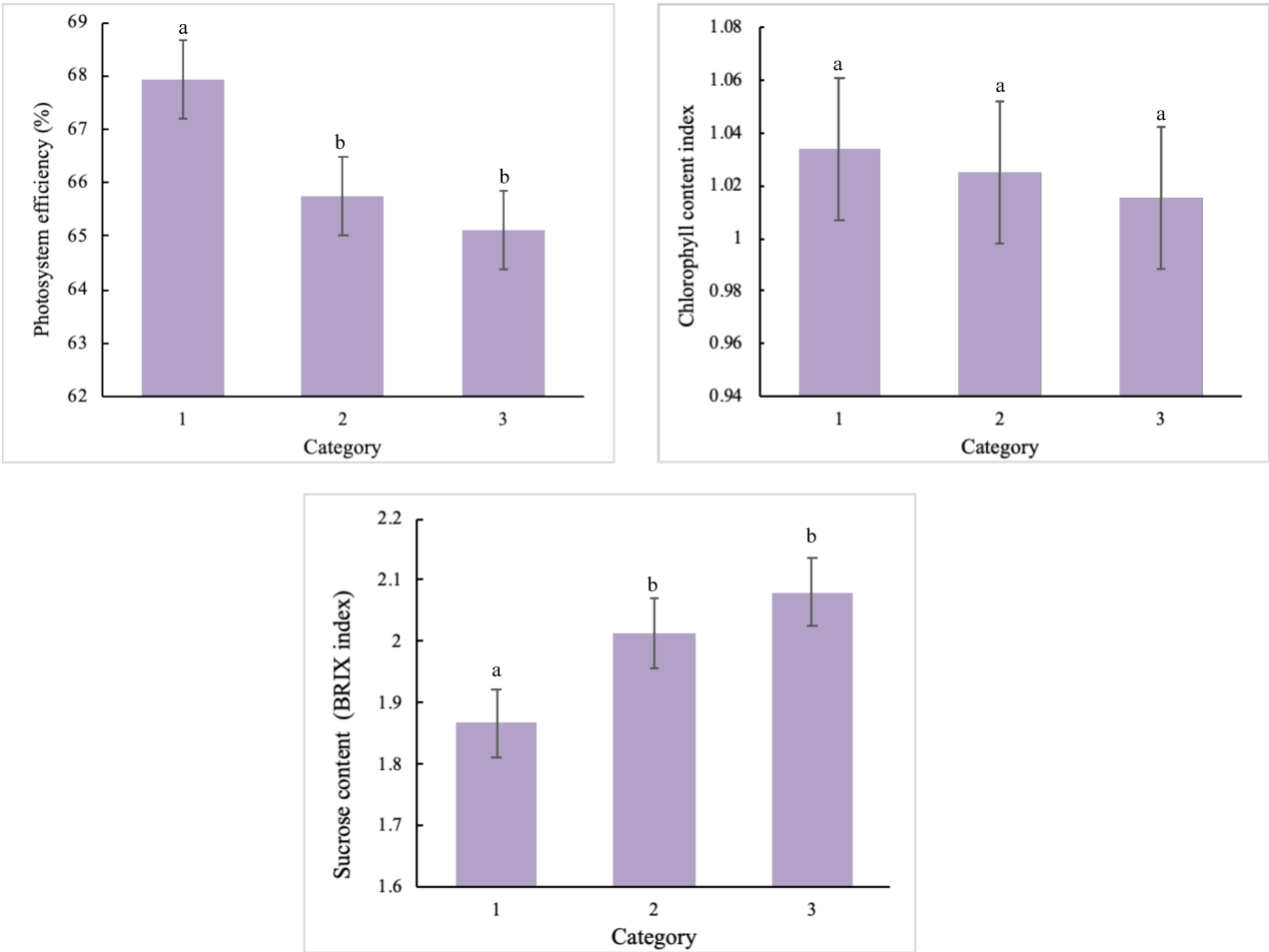


Figure 2. Photosystem efficiency (PE), chlorophyll content index (CCI) and sucrose content (BRIX index) measured for Categories 1 (pre flowering with minimal ramets), 2 (flowering with minimal ramets) and 3 (flowering with large ramets). LS means \pm SE shown. Shared letters indicate non-significant ($p > 0.05$) planned contrasts

Morphology

The life history transition from small ramets subtended by live leaves (Category 1 and 2) to developed, stolon-attached ramets (Category 3) was found to be associated with significant morphological changes in stomatal density, leaf area, SLA and leaf number with longest leaf length remaining consistent across categories. Category 2 showed significantly lower stomatal density than Categories 1 and 3 (Figure 3). The greatest difference in stomatal density is seen

between Categories 2 and 3 ($P < 0.001$), with Categories 1 and 2 also being significantly different ($P < 0.05$) (Fig. 3). A significant effect of developmental category on leaf number was found (Table 1). Category 1 had a significantly higher leaf number than Category 2 ($P < 0.05$) and Category 3 ($P < 0.001$) (Fig. 3). Leaf area increases across Categories 1-3 (Fig. 3) with Category 3 showing significantly higher leaf areas than Category 1 ($P < 0.001$) and Category 2 ($P < 0.05$) (Fig. 2). There was no significant effect of category found for longest leaf length (Table 1). No significant block effects were detected for stomatal density, longest leaf length, leaf number or leaf area (Table 1). Specific leaf area (SLA = Leaf area / leaf mass) was found to vary marginally with category (Fig. 2) and significantly with block (Table 1). Block C showed significantly higher SLA than Block A ($P < 0.05$) and Block B ($P < 0.0001$).

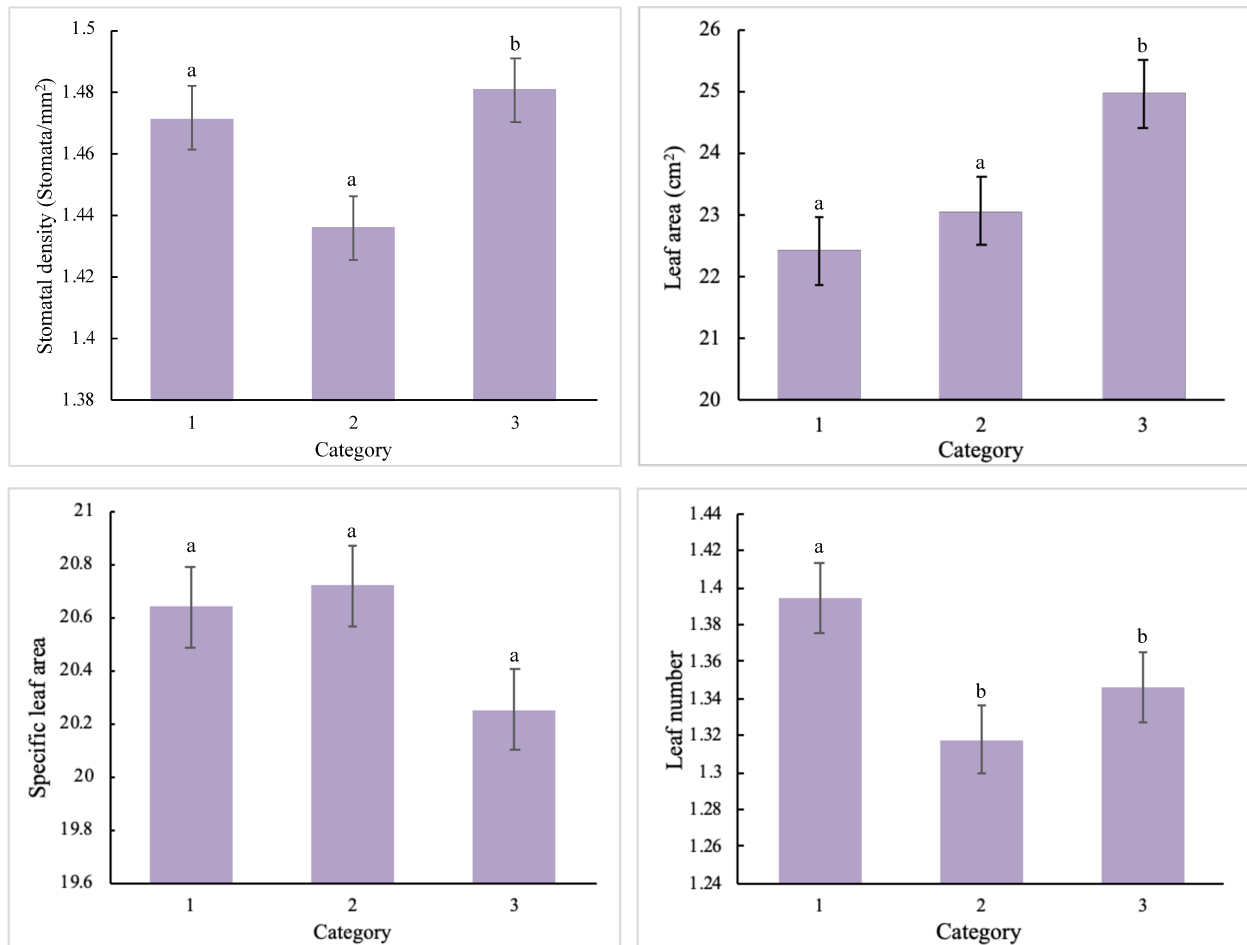


Figure 3. Stomatal density, leaf area, specific leaf area (SLA = Leaf area / leaf mass) and leaf number measured for Categories 1 (pre flowering with minimal ramets), 2 (flowering with minimal ramets) and 3 (flowering with large ramets). LS means \pm SE shown. Shared letters indicate non-significant ($p > 0.05$) planned contrasts

Table 1. Analysis of variance (ANOVA) for category and block on physiological and morphological traits. *F* statistics and *P* values are reported for photosystem efficiency (PE), chlorophyll content index (CCI), specific leaf area (SLA = leaf area/ leaf biomass), stomatal density, sucrose content (BRIX index), leaf number and longest leaf length (LLL).

	PE	CCI	SLA	Stomatal Density	BRIX Index	Leaf Number	LLL	Leaf area
Block	0.9173	0.1222	6.5706**	0.6672	0.6916	0.0823	0.7842	0.8440
Category	4.1505*	0.3471	2.6835+	5.3834**	3.8617*	4.3027*	0.7015	5.7647**

** $P < 0.01$; * $P < 0.05$; + $P < 0.10$

Overall Developmental Strategy Matrix: Morphology and Physiology

The life history stages of flowering and ramet development were found to associate with shifts in specific functional traits. Table 2 presents a qualitative summary of functional trait variation with the plant's life history stage of producing ramets, flowers or both ramet and flowers. Initial ramet growth pre-flowering is associated with higher PE and leaf number. Flowering is associated with higher sucrose, low stomatal density and lower levels of PE. When plants produce both flowers and ramets it is associated with high stomatal density, sucrose and leaf area with low SLA. CCI and LLL do not represent statistically significant trends.

Table 2. Development strategy matrix. *P* values are reported from Table 1 for photosystem efficiency (PE), chlorophyll content index (CCI), specific leaf area (SLA = leaf area/ leaf biomass), stomatal density, sucrose content (BRIX index), leaf number and longest leaf length (LLL). CCI and LLL data represent trends and are not statistically significant.

	PE*	CCI	SLA+	Stomatal Density**	BRIX Index*	Leaf Number*	LLL	Leaf Area**
Goal: Ramet	high	high	medium	medium	low	high	medium	low
Goal: Flower	medium	medium	high	low	medium	low	low	medium
Goal: Flower and ramet	low	low	low	high	high	medium	high	high

** $P < 0.01$; * $P < 0.05$; + $P < 0.10$ for significant effect of category.

Discussion

This study examines the functional responses of *Neoregelia tigrina* at three stages of development: pre-inflorescence with minimal young ramets (Category 1), post inflorescence with minimal young ramets (Category 2) and post-inflorescence with developed ramets (Category 3). Functional traits were measured to determine the resource acquisition strategies at each stage and establish how plants respond to meet the costs of sexual and asexual reproduction (Table 2). Flowering was found to be the life cycle stage at which the most significant changes were observed, including a decrease in photosystem efficiency, stomatal density and leaf number with increased sucrose concentrations (Table 2). This life history transition prompted a water conservation response beginning in Category 2 with decreased stomatal density and in Category 3 though a marginal decrease in SLA. Block was found to have a significant effect on SLA, with Block C showing significantly increased SLA compared to Blocks A and B. This may be due to the marginally increased sunlight that Block C received (E. Fetterly, personal observation), producing relatively larger, thinner leaves. Bromeliads have high plasticity to sun/shade responses, with high light environments typically producing leaves with a lower SLA and increased succulence (Benzing, 2002; Medina et al. 1993). This is likely due to the high variability of light conditions for epiphytes within the tree canopy depending on position and exposure. However, this trend is not supported in the significantly increased SLA found in the sunnier microclimate in Block C, which may be a result of a slightly drier environment reducing tank water levels and leaf succulence during sampling. Vegetative reproduction did not require a significant decrease in photosynthate levels or a large shift in photosynthetic potential and throughout vegetative ramet development plants continued to maximize photosynthate

production with higher gas exchange potentials that supported the growth and addition of biomass through ramets.

Functional trait variation with flowering

The shift from pre-flowering to post-flowering is the transition in which *N. tigrina* experienced the highest degree of functional trait variation and changes in photosynthetic capacity through both morphology and physiology. Categories 1 and 2 demonstrate the developmental transition from pre-inflorescence to post-inflorescence stages at the same level of minimal ramet production. Category 1 (pre-inflorescence) had significantly higher photosystem efficiency, increased stomatal density, decreased sucrose and increased leaf number than Category 2 (post-inflorescence).

The higher leaf number in plants pre-flowering reflects the maximum number of leaves produced before the plant is able to accumulate enough photosynthate and carbon to flower. It is expected to be highest in plants in the period directly prior to flowering due to the conversion of the shoot apical meristem to a terminal inflorescence, thus preventing any subsequent vegetative growth after flowering. Pre-flowering plants may still be producing vegetative tissue to reach a minimum size threshold for production of photosynthate to be used for future carbon sinks such as flowers or ramets. Consequently, plants that have produced an inflorescence are no longer able to produce new leaves and older leaves on the outer whorls may senesce resulting in flowering plants with lower leaf numbers than pre-flowering plants. The dead leaves retained by the plant, or marcescence, can help to create a humidity boundary layer which may reduce heat and drought stress during flowering (Males, 2016).

Flowers can be a significant carbon sink due to the high sucrose content of nectar and growth of specialized tissues, thus prior to anthesis the plant is likely to favor increasing

photosynthate production and higher carbon assimilation (Ordano and Ornelas, 2005). The higher photosystem efficiency in Category 1 is likely a strategy to increase photosynthate production as the plant is preparing to flower. Similarly, the higher stomatal density seen in Category 1 is a strategy to assimilate more carbon in preparation for allocation to sexual reproduction and potentially ramet growth. Although the inflorescence remains energetically costly to maintain after the initial bolting, stomatal density and photosystem efficiency significantly decline from Category 1 to Category 2. The decrease in stomatal density from pre-flowering plants to flowering plants may be a water conservation strategy, as the production and subsequent maintenance of flowers is one of the most water-intensive activities that occurs during development (Males, 2016; Ordano and Ornelas, 2005). Epiphytes are particularly sensitive to water stress considering their precarious placement in the canopy and dependence on rainfall to maintain an adequate tank water supply (Benzing, 2000). As the study population was well-watered throughout the sampling period this water conservation strategy may be the result of a genetic basis for water conservation during flowering. Photosystem efficiency may decline as a function of the shift in life history stage from the vegetative stage to flowering; the plant may be transitioning from a strategy of high photosystem efficiency to high gas exchange in order to maintain photosynthate production.

Additionally, there is the possibility that leaf age may be affecting the photosystem efficiency across all categories. The exact age and germination date of this population is unknown though seeds likely originated from the same capsule and thus the plants could differ slightly in age, with the more developed categories showing both a life history shift and subtle effects of leaf aging. Photosynthetic capacity can be influenced by leaf age, with a significant

decrease in photosynthetic potential found in older leaves (*Arabidopsis*; Bielczynski et al., 2017; *Oryza sativa*; Makino et al., 1983).

These data suggest that the production of an inflorescence has a significant effect on the functional responses of the plant as can be observed in the reduction in photosynthetic potential and water conservation strategies. While photosystem efficiency and stomatal density decrease in the flowering group, chlorophyll content is not significantly affected, potentially a strategy to maintain production of photosynthate during flowering as PE and stomatal density decrease. The relatively consistent chlorophyll content found in all three categories could assist the plant to mitigate tradeoffs of sexual reproduction and maintain consistent biomass and growth across all three categories of development. A comparative approach involving various iteroparous taxa and other inflorescence types within Bromeliaceae would be useful to corroborate these findings, as *N. tigrina* displays a unique, genus-specific sunken inflorescence that is atypical for the family which may influence the relative metabolic costs associated with functional strategies.

Functional trait variation with ramet growth

While the physiological effects of flowering are clearly demonstrated, the effect of ramet size and developmental stage are less explicit. Variation in functional response associated with increasing ramet development can be seen between Categories 2 and 3, as both groups have produced a maternal inflorescence, but each displays a different stage of ramet development. Category 3, containing the larger stolon connected ramets had marginally lower SLA, higher stomatal density and higher leaf area than the small ramet producing Category 2 (Figure 3).

With the presence of extended ramet growth there is an increase in overall gas exchange potential through greater leaf area and stomatal density, however there is not a significant difference in sucrose content or photosystem efficiency. There is a marginal trend for increasing

sucrose between Categories 2 and 3, pointing to evidence that the plant is able to maintain production of photosynthate while supporting the growth of larger ramets. The increase in gas exchange potential could be a result of the continued growth of ramets in conjunction with the inflorescence, creating additional carbon sinks as ramets accumulate biomass. Interestingly, the stomatal density in Category 3 is not significantly different to Category 1, supporting the hypothesis that plants might avoid water loss during the production of an inflorescence but then return to a state of high carbon assimilation after the initial conversion of the apical meristem to an inflorescence. Thus, it may be possible that the increase in stomatal density from Categories 2 to 3 is both a result of plastic adjustments to water loss and an increase in carbon assimilation to support the carbon sinks of both flowering and ramet growth.

Category 3 displays a marginally lower SLA, translating to thicker, more succulent leaves that might contain additional hydrenchyma tissue to serve as a water storage mechanism. By transitioning from a low stomatal density to a low specific leaf area the plant continues to conserve water while simultaneously increasing carbon gain. These functional trait patterns do reveal a significant shift between Categories 2 and 3, but the plant is able to transition without loss of photosynthetic capacity or flowering ability, therefore this study does not find evidence for a high cost associated with ramet production relative to that of flowering. Other unmeasured traits or manipulation of environmental conditions such as water or nutrient availability may display a greater cost to increased ramet growth (Villegas, 2001; Liu et al., 2009).

Evidence for tradeoffs

Without fitness estimates it is difficult to fully evaluate tradeoffs between sexual and asexual reproduction, however patterns of photosynthate levels and physiologic responses can be indicative of trends in reproductive allocation between these two modes. Sucrose content

increases across categories, initially due to high PE and stomatal densities in Category 1 that favor the accumulation of photosynthate for production of an inflorescence. As PE begins to decline in Category 2 plants shift to increasing stomatal density and leaf area in Category 3 as a strategy to increase carbon assimilation for larger ramets and flowers. Through this transition of strategies, the maternal rosette is able to maintain homeostasis of primary productivity for vegetative and sexual reproduction. These results support those of Zotz et al. (2002) who found a positive association between plant development and carbon assimilation in epiphytic bromeliad *Vriesea sanguinolenta*, a study that also points to the benefits of a broader sampling to include more fine-scale developmental categories in carbon source-sink analyses.

Biomass can also be used as a metric to evaluate tradeoffs in reproductive allocation, and although this study was unable to destructively sample for biomass, longest leaf length has been shown to tightly correlate with biomass in many species within Bromeliaceae (Augsburger, 1985; Villegas, 2001; Zotz et al., 2011). The longest leaf lengths are not significantly different between all three categories, which supports our hypothesis that there are not significant tradeoffs in the biomass of the maternal rosette regardless of ramet size or flowering status. Although not measured in this study, nutrient levels may play an important role in the existence of tradeoffs between sexual and asexual reproduction (Liu et al., 2009; Ott and Hartnett, 2011). Under conditions of high nutrient levels and high water availability the monoecious *Sagittaria pygmaea* was found to exhibit no tradeoffs between ramet production and sexual reproduction (Liu et al., 2009). Further study involving fitness parameters and biomass, as well as an experimental manipulation of nutrients, water availability and light conditions would give a more robust assessment of tradeoffs between asexual and sexual reproduction in this species.

Conclusion

This study examines the functional responses of *Neoregelia tigrina* at three stages of development: pre-inflorescence with minimal young ramets (Category 1), post inflorescence with minimal young ramets (Category 2) and post-inflorescence with developed ramets (Category 3). Functional traits were measured to determine the resource acquisition strategies at each stage and establish how plants respond to meet the costs of sexual and asexual reproduction. Flowering was found to be the life history stage at which the most significant changes to photosynthetic capacity were observed, including a decrease in photosystem efficiency, stomatal density and leaf number with increased sucrose concentrations. This life history transition prompted a water conservation response in Category 2 with decreased stomatal density and in Category 3 though a decrease in SLA. Vegetative reproduction did not require a significant cost to photosynthate levels or a large shift in photosynthetic potential, but plants continued to maximize photosynthate production with higher gas exchange potential that supported the growth and addition of biomass through vegetative ramets.

Life history strategy is of critical importance in the study of ecophysiology and adaptive radiation in Bromeliaceae and yet there have been few studies that have investigated questions of reproductive allocation and the physiologic effects of vegetative propagation. Here, flowering had a significant effect on functional traits but with a tendency for plants to maintain photosynthetic homeostasis. This study did not find conclusive evidence for a tradeoff between sexual and asexual reproduction, however in the consideration of the multidimensional space that is emerging to define iteroparity, *Neoregelia tigrina* may represent an extreme in which ramet production can readily occur under most conditions. This relationship is likely extremely taxon specific, especially if the maternal inflorescence represents a greater energetic cost, as many

bromeliads display extremely large and brightly colored bracts, petals and fruits. The evolution of semelparity in Bromeliaceae is still a question of environment, demography and reproductive effort, but the consideration of physiological resource acquisition strategies and life history should be subject to further study.

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