Comparative Reproductive Biology of Two Self-Compatible Bromeliaceae

A Senior Thesis Presented To

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Josh Felton

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Approved by:

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Dr. Rachel Jabaily

Primary Thesis Advisor

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Dr. Shane Heschel

Secondary Thesis Advisor

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Bromeliaceae taxonomy and diversity

Bromeliaceae Juss. is a diverse angiosperm family of over 3,000 species. Bromeliads have an extensive geographical range, with the northern tip of distribution found near the mid-Atlantic United States south to central Chile and Argentina. While the majority of species can be found in the Neotropics, one species is endemic to West Africa (*Pitcairnia feliciana* (A.Chev.) Harms & Mildbr.), the result of long-distance dispersal (Givnish et al., 2011). Bromeliaceae have diversified broadly in their vegetative and reproductive morphological traits. Some of these traits are the reason this family has been incredibly successful at adaptive radiation even while sharing the same ecological niche, as evolutionary forces have linked and driven the various traits together. The tank habit and epiphytism are two major traits that will be discussed heavily in future sections. The tank habit incorporates leaves with wide overlapping bases that allow for water to be collected in shoot. Adapted trichomes that absorb water and nutrients unite this trait to epiphytism as absorptive roots are not required. These mentioned traits led to the rise of lineages of epiphytes where the new ecological niche of the forest canopy and not being constrained to the ground led to the diversification of lineages. The linkage of these and other not mentioned traits have further helped the success of Bromeliaceae as a family in both arid and rain- and cloud-forests (Givnish et al., 2014).

There are eight subfamilies now recognized in Bromeliaceae and now I will focus on describing the relative number of species, distribution, defining floral and fruit traits and animal interactions. The two most species-rich subfamilies are Tillandsioideae and Bromelioideae while

Brocchiniodeae, Lindmanioideae, Hechtioideae, Navioideae, Pitcairnioideae, and Puyoideae vary in size and were reclassified into these subfamilies after the traditional Pitcairnioideae was found paraphyletic following molecular systematic characterization (Givnish et al., 2011). These new monophyletic subfamilies are also supported by their morphology. Brocchiniodeae contains one sole genus *Brocchinia* which includes 21 species (Butcher & Gouda 2021–). The monotypic subfamily is sister to all other bromeliads. *Brocchinia* is restricted to the Guyana highlands of northern South America, a wet region with tepuis and sand plains that are particularly infertile (Givnish et al., 1984). The literature often focuses on the unparalleled adaptative radiation that has occurred in the genus *Brocchinia*, producing numerous mechanisms of nutrient capture (carnivory, myrmecophytes, tank capture, nitrogen fixing, and terrestrial root absorption) (Gonzalez et al., 1991, Givnish et al., 2007). *Brocchinia* inflorescences can either be racemose, paniculate, or capitate, however, the petals and the whole floral structure are minute as insect pollination syndromes can be found in all species of *Brocchinia* (Givnish et al., 2007). Brocchinioideae is further defined by its capsular fruits and seeds having two taillike appendages. Lindmanioideae, with two genera restricted to similar regions as Brocchinioideae, has similar fruits and seeds to Brocchiniodeae. Literature on reproductive biology is lacking (Wolowski & Freitas, 2015), but insects are the pollinators for the small flowered genus *Lindmania* while the genus *Connellia* has larger flowers with brightly colored petals, suggesting putative avian pollinators (Givnish et. al 2007). Hechtioideae is formed by one genus, *Hechtia*, of which all but one of 75 species are dioecious (Morillo et al., 2014). The genus is most diverse in Mexico and is endemic to the Megamexico region, a biogeographical area that includes the Tamaulipan, The Sonoran and Chihuahuan Deserts and countries of Belize, Honduras, Guatemala, El Salvador, and the northern section of Nicaragua. (Rzedowski, 1991, Hernández-Cárdenas et al., 2020). Hechtioideae have fruits for capsules with seeds that can be winged to having slight protrusions. The seeds are wind dispersed (Romero-Soler et al., 2022). Puyoideae only has one genus, *Puya* which has had one of the largest genus-level radiations in Bromeliaceae, distributed across central and the western countries of South America, primarily in the Andes. This radiation has led to extreme variation in inflorescence structure and floral color as well, as hummingbirds are the putative primary pollinators (Jabaily

& Sytsma, 2010). *Puya* seeds have limited seed mobility from wings that surround the circumference of the seed that assist wind dispersal (Benzing, 2000). Insect-pollinated Navioideae is primarily distributed in the Guayana Shield, with only the genus *Cottendorfia* distributed in the Brazilian Shield. There are 5 genera in the subfamily and inflorescence type varies from paniculate to capitate. Navioideae also have capsular fruits, like the subfamilies above (Givnish et al., 2014).

The new Pitcairnoideae subfamily is the third largest in the family including 678 species now across only 6 genera. Flowers are often small and self-compatible. A notable exception in breeding system form is found in the genus *Pitcairnia* where species possess showy, tubular flowers that are commonly have a red corolla (Smith & Downs, 1986). *Pitcairnia* species have primarily have ornithophily pollination syndromes (Fumero-Cabán & Meléndez-Ackerman, 2007, Rios & Cascante-Marin, 2017) but entomophily as secondary pollinators or nectar thieves has also been noted. Work on *Dyckia* reports the genus also having a mixed pollinator system that includes insects and hummingbirds (Lenzi & Paggi, 2020).

Figure 1. Reproductive structures across the subfamilies *Bromelioideae and Tillandsioideae. (a) Wallisia cyanea* Barfuss & W.Till with a paddle-shaped inflorescence. The bracts are a bright pink, and the flowers are a violet hue. *(b) Guzmania blassii* Rauh. *(c) Vriesea ensiformis* (Vell.) Beer. *(d) Vriesea* × 'Marcella' inflorescence structure with dehisced capsules. *(e) Aechmea chantinii* 'Grey Ghost' inflorescence pre-anthesis. *(f) Vriesea rafaelii* Leme. *(g)* Unlabeled *Billbergia* sp. *(h) Neoregelia tigrina* (Ruschi) Ruschi. The inflorescence is sunk down into the tank, protecting the ovary. *(i) Aechmea recurvata (Klotzsch) L.B.Sm.*

Tillandsioideae is the largest subfamily in Bromeliaceae with 1527 species (Gouda et al. cont. updated) and as such, greatly varies reproductively in flowers, fruits, and seeds (Fig. 1a,c,d). Corolla shape varies across the nine genera in the subfamily from tubular to

campanulate (Barfuss et al., 2016). The variation in shape can be partially explained by the two key pollinators in the subfamily, hummingbirds who can access nectar that is deep in tubular flowers and bats which are less precise when feeding but do well with bell-shaped corollas that they can stick their head into. Stigma type also varies throughout the subfamily and has been used in taxonomic classification for various groups in the family (Souza et al., 2016). Stigma morphology ranges from simple types to convolute, coralliform, cupulate, tubo-laciniate or urceolate form (Barfuss et al., 2016). Seed appendage type can be grouped into three main types across the subfamily. The differences are due to where the plumose appendage is attached (apical or basal end) and differences in outer seed integument (Barfuss et al., 2016). The literature is lacking on the evolutionary advantages of the differences in seed appendage morphology, but the form has been useful as well with classifications within the subfamily. Since all fruits are capsular and seeds dispersed via the wind, aided by the plumose appendage, animal mediated dispersal is not involved in this subfamily.

Taxonomically, the subfamily has long been supported as a group, defined by having leaves with entire margins and capsule fruits with plumose seeds, but molecular systematics has allowed for the designation of many new genera from the broadly paraphyletic *Tillandsia* and *Vriesea*, amongst others (Barfuss et al., 2016). The new taxonomic revision by Barfuss et al. (2016) defines 11 new genera mainly from the splitting up of the genera *Tillandsia*, *Mezobromelia* and *Vriesea*. There are now 21 described genera in the subfamily now which are monophyletic and can be defined by morphology.

Epiphytism, the tank habit and plumose seeds occurred at the split of Tillandsioideae from the rest of Bromeliaceae, estimated at ca. 14–15.4 Mya. Diversification and the vast geographical rage of the subfamily is attributed to the combination of these traits. Coevolution with hummingbirds has led to over 1,000 species in the subfamily (Givnish et al., 2014). There are two contrasting hypotheses of the ancestral pollinator for the subfamily. Recent work utilized more species in their ancestral state reconstruction hypothesize bats rather than hummingbirds as the ancestral pollinator. Bats as the ancestral pollinator contrasts many neotropical studies that show a unidirectional bird to bad pollination evolution (Givnish et al., 2014, Aguilar-Rodriguez et al., 2019).

Bromelioideae is the second largest subfamily in Bromeliaceae with 33 genera and approximately 950 species that often have highly visual reproductive features including bracts, sepals, and fleshy fruits (Fig. 1b,c,e-i) (Givnish et al. 2011). Bromelioideae petals are almost always appendaged, and sepals can be asymmetric with one sepal lobe being larger than the others, providing a wing-like appearance to the flower. *Bromelia*, *Deinacanthon*, *Greigia*, *Ochagavia*, *Fascicularia* and *Fernseea* are the early diverging genera within the subfamily (Schulte et al., 2009), morphologically different from the rest of the Core Bromelioideae in that they are tankless and terrestrial or lithophytic (growing in or on rocks) (Sass & Specht, 2010). Symmetric sepals are restricted to earliest diverging and solely terrestrial lineages, in contrast asymmetric sepals are found in terrestrial genus *Ananas* and the primarily epiphytic Core Bromelioids (Schulte et al., 2009). This latter clade has diversified rapidly, sparked by the evolution of the tank habit, a synapomorphy for the Core Bromelioids. Fruits are indehiscent berries with seeds that once mature are unappendaged (Smith, 1988) and very gelatinous (Sajo et al., 2004). Bromelioideae fruits are thought to primarily be dispersed by animals (lizards, birds and bats), however there is a gap in the literature of field observations and experiments to confirm the exact dispersers (Benzing, 2000, Silva et al., 2020). The mucilaginous chalazal appendages of Bromelioideae seeds is the adaptation to helping seeds stick to substrate after fruit dispersal, crucial for the establishment of the seedling as epiphytes on a tree branch. The subfamily is found throughout the Neotropics and exists in all types of tropical biomes, from open sand dunes to humid montane cloud forests (Sass & Specht, 2010). The center of diversity of Core Bromelioids is located in the Brazilian Atlantic Forest (BAF), with subsequent establishment and diversification in northern South America, the Andes, and Central America (Schulte et al., 2005).

Eastern Brazil is now one of the main centers of diversity for Bromelioideae, but distribution can also be found throughout subtropical and tropical regions of the Americas (BFG, 2015). Lineages in this subfamily that have selected for CAM physiology are connected to higher speciation, while the tank habitat in Bromelioideae is connected to lower extinction rates for those groups. Not all members of the subfamily use CAM photosynthesis with many of the early diverging lineages in the subfamily distributed in mesic environments in the

North/Central Andes using C3 (Givnish et al. 2011, Silvestro et al., 2014). Diverse fruit and seed features throughout the subfamily can be attributed to ancestral state of the subfamily recruiting hummingbirds as pollinators. Compared to Tillandsioideae, entomophily is also more common in flowers that have generalist traits allowing bats and other vertebrates to act as secondary pollinators (Aguilar-Rodriguez et al., 2019). Bromelioideae often utilize different animals other than their putative pollinator to disperse their fruits (Silva et al., 2020).

Silva et al., (2020) hypothesizes that lizards are probable fruit dispersers for species distributed on rocky outcrops as they utilize bromeliads for food and water supply. Fruit dispersal by birds has been noted in species that have red, white, blue or brown fruits joined by colorful bracts for attraction as well. Bats have been noted as the main dispersers of fruit in the genus *Billbergia* Thunb. where the reflective surfaces of the fruits from scales on the pericarp help reflect dim light at night. Species in the genus *Neoregelia* L.B.Sm. have inflorescences that are congested so much that petals cannot fully open in some taxa. Short peduncles ensure that only as much of the flower that is needed for successful pollination is above the water tank (Santos-Silva et al., 2017) which is a key reproductive feature found in this genus to protect the ovary as a bud and when developing fruits from herbivory (Benzing, 2000). This nested inflorescence is one of several inflorescence types in tank forming bromeliads (Santos-Silva et al., 2017). Large, colorful, showy inflorescences that are a substantial investment in the species reproductive effort are more commonly found throughout the subfamily. The nested inflorescence type however reduces the cost of reproductive effort as it requires less biomass than non-nested inflorescence structures (Mantovani & Iglesias, 2009).

Key adaptive traits in Bromeliaceae

Morphological characters of reproductive structures had been key to taxonomic definitions across the family. Our understanding of the adaptive role of the various reproductive features and how these might be impacted by developmental or functional tradeoffs lags behind our understanding of vegetative features related to the key innovations for speciation in the family. This has limited our understanding of floral morphology which once

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better understood can provide evidence to interpret questions about the ecology and systematics of Bromeliaceae that have yet to be answered (Santa-Rosa et al., 2020).

High rates of species diversification in Bromeliaceae have occurred in the canopy of Neotropical forests as the canopy was a novel habitat where additional resources (food, shelter, light) were available to flora and fauna that could access the space (Marines-Macías et al., 2018). Frugivores bats are incredibly diverse in tropical canopies where they play important roles in pollination and seed dispersal (Kays & Allison, 2001, Kalko & Handley, 2001). Bromeliaceae tanks have been a space for diversification of other taxa in the canopy of Neotropical forests. Copelatinae diving beetles that are found in ephemeral small water bodies began to use bromeliad tanks at the same time of the origin of the tank habitat 12–23 Mya and have maintained a parallel diversification with epiphytic bromeliads since the origin of the tank habit (Balke et al., 2008). Fertile humid montane habitats repeatedly selected for the epiphytic habit observed in Orchidaceae and Bromeliaceae. (Acharya et al., 2011, Givnish et al., 2014). The epiphyte habit provided a new ecological niche with a large range of microsites where this diversification occurs without substrates as the source for water and nutrients (Schulte et al., 2009). The tank body plan has evolved three times in Bromelieaceae- in Bromelioideae, Tillandsioideae, and once in a species of *Brocchinia*. The tank habitat is a key adaptive feature in bromeliads because it evolved during the rise of epiphytism in the tillandsioids and bromelioids. Not all bromeliads that are epiphytes have a tank, such as *Pitcairnia heterophylla* (Lindl.) Beer whose ramets have long linear leaves that are shed before floral anthesis (Rios & Cascante-Marin, 2017). Climates with heavy rain and low evaporation rates favor the tank habit as the tank allows for a space for water running down trees to collect, providing nutrients from the rainwater to the bromeliad. Investment towards roots structures is then only necessary to secure the plant to a wall or branch, as epiphytic tank forming bromeliads also evolved absorptive leaf trichomes that can directly uptake the water and nutrients inside the tank.

Epiphytism has selected for several seed modifications to ensure seeds attach to twigs and branches in the canopy (Givnish et al., 2014, Silvestro et al., 2014, Chilpa-Galván et al., 2018, Silva et al., 2020). Subfamily Tillandsioideae have capsular fruits and wind dispersed seeds with an extensive attached plumose coma which helps the seed stay in the air for a

longer time, potentially increasing dispersal and helping attach to the branch if the coma is wet. Subfamily Bromelioideae has fleshy, animal dispersed fruits and species often have seeds with a mucilaginous coat that may act as a protective barrier while passing through the digestive tract of vertebrates. The coat may also help the seed stick to bark (Benzing, 2000).

A variety of niches in close geographic space are found in Neotropical montane habitats such as the Andes and BAF where considerable shifts in ecological conditions occur over short horizontal and vertical distances. CAM photosynthesis provides a water use efficiency advantage for the spatial and temporal shifts in conditions of light, temperature, and CO_2 experienced by bromeliads in these habitats. Organisms can efficiently react to outcomes of these interactions, allowing them to inhabit a wider variety of ecological niches (Lüttge, 2010, Silvestro et al., 2014). Working together, these bromeliad features of epiphytism, tanks to collect water and nutrients, absorptive leaf trichomes, avian pollination instead of entomophily, and CAM photosynthesis have promoted rapid speciation (Givnish et al., 2014). The subfamilies Tillandsioideae and Bromelioideae are the parallel cases of these major adaptations (epiphytism & tank habit) in the family and are the main reason these two subfamilies have the highest number of species that have been able to occupy new zones throughout the Neotropics.

Bromeliaceae life history variation

Life history is defined by the allocation of resources during growth and development which affect the timing and number of reproductive events before death. As growth and developmental time are both on their own axes, life history should be envisioned in a multidimensional space where taxa occupy various spaces due to different timing and amount of energy investments throughout their life cycle (Jabaily et al., 2021). The majority of Bromeliaceae reproduce sexually via terminal inflorescences which transform the shoot apical meristem to a terminal floral meristem, ending the parental rosette's ability to produce new vegetative growth (Jabaily et al., 2021). Sexual reproduction via terminal inflorescence is the major reproductive effort event and perhaps most important in Bromeliaceae evolution, but bromeliads can also asexually reproduce through initiation of axillary meristems to produce clonal ramets. Asexual reproduction is a life-history strategy to continue the parental genotype

(genet) after the parental rosette has switched to a terminal inflorescence, which may or may not be successful in producing seeds. This strategy provides continuity to the genet so that unsuccessful reproductive efforts are not the end of the line for the lineage. Clonal ramets, or genetically identical clones, have a lower risk of desiccation than seeds as they start as small versions of their genet, which is especially advantageous in an epiphytic habitat. Clonal ramets are an advantage for the family as they allow for an individual to forage easily for light, water, and mineral nutrients (Silvertown, 2008). This ability of both sexual and asexual reproduction can happen simultaneously, or at different development intervals in different species of Bromeliaceae, and there is no apparent parental ramet energetic tradeoff for initiating asexual reproduction (Jabaily et al., 2021). The high rate of adaptations coupled with the mixed reproductive system can benefit the family while facing competitive situations as noted with clonal reproduction in the genus *Opuntia* (Lenzi & Orth, 2012).

The other extreme of bromeliad life-history is semelparity where the individual has a single reproductive episode, does not produce any axillary ramets and dies after flowering. These semelparous episodes often exhaust all the energy accumulated over the plant's lifespan as deferring energy for future growth/reproduction is not required (Hughes & Simons, 2014). Semelparity has evolved repeatedly in few species across the Bromeliaceae.

Focal taxa *Vriesea* and *Billbergia*

We will focus on the genera *Vriesea* Lindl. and *Billbergia* Thunb. as they contain our focal study species *Vriesea rafaelii* Leme *and Billbergia brasiliensis* L.B.Sm. *Vriesea* is one of the largest genera in the Tillandsioideae subfamily. Together with *Alcantarea*, *Stigmadon,* and *Waltillia,* they form the subtribe Vrieseinae (Faria et al., 2021). Inside of this subtribe, the phylogenetic relationships are not fully resolved, as *Vriesea* is a paraphyletic group at the moment. Historically, there were two sections in *Vriesea* that separated species based on reproductive traits. Section *Vriesea* were species grouped together based on the presence of exserted stamen, red to yellow floral bracts and diurnal anthesis, suggesting hummingbird pollination (Gomes-da-Silva & Souza-Chies, 2018), while section *Xiphion* species were grouped together through brown and green floral bracts, inserted stamen and nocturnal anthesis.

Species in section *Xiphion* were thought to be bat or moth pollinated based on the reproductive characters. (Gomes-da-Silva et al., 2012). Recent work by Neves et al. (2020) investigated these pollination syndromes as species with mixed morphologies are known with phylogenetic relationships under resolved. They determined that species pollinated by hummingbirds have mostly red or yellow floral bracts (Fig. 1c) that are frequently equal or longer in length than the flower. Arrangement of flowers in the inflorescence can either be distichous (arranged in two opposite rows) or polystichous (arranged in more than three opposite rows). Hummingbird pollinated species have odorless flowers that open during the day. Corollas are commonly yellow with exserted stamens and a tubular shape that allows for hummingbirds to insert their beak and part of their head into the flower to reach the nectar. In contrast, bat-pollinated species have floral bracts that are not showy, and have a green, brown, or purple pigment (Fig. 1f). Flowers are arranged distichously, and compound inflorescences often are secund (flowers shifting to an angle of 90° towards one side of the inflorescence) at anthesis, allowing bats easy access to the campanulate flowers. Anthesis occurs at dusk/night with a garlic odor that helps attract the bats. Stamens do not extend outside the corolla, which are often a pale yellowish color that is almost transparent (Neves et al., 2020). Pollinator type in *Vriesea* is habitat dependent, with bat-pollinated taxa occurring in habitats that are less obstructed which make it easier for bats to echolocate the flowers as well as perceive the olfactory signals (Gonzalez-Terrazas et al., 2016). Conversely, hummingbird-pollinated species are found closer to the forest understory if distributed in the highlands or lowlands and are terrestrial if found in restingaas (coastal sand broadleaf forest of Eastern Brazil) (Neves et al., 2020). All things considered, the majority of *Vriesea* taxa have a hummingbird pollination syndrome (ca. 137 sp.) compared to around 90 taxa that present a bat pollination syndrome (Neves et al., 2020). Vegetatively, the genus is similar to other genera in the family with a basal leaf arrangement that features many axillary meristems that are able to initiate and produce clonal ramets, or genetically identical clones that allow for Bromeliaceae to forage easily for light, water, and mineral nutrients.

Vriesea is a taxonomically difficult genus and major efforts have been made to understand its evolutionary history and define monophyletic clades. Gomes-da-Silva & Souza-Chies (2018) argued that Barfuss et al., (2016) poorly sampled *Vriesea* and had other flaws in their experimental design that make the taxonomic changes they proposed premature. With the use of genome skimming, Machado et al., (2020) reassessed the monophyly of the genera in subtribe Vrieseeae and investigated phylogenetic relationships at the intergeneric level. They found that subtribe Vrieseinae in fact was monophyletic with strong support containing the genera and groups *Alcantarea*, *Vriesea s.s.* (sensu stricto) and *Stigmatodon*. *Vriesea s.s.* is largely distributed and most abundant in the Brazilian Atlantic Forest (BAF), but there are a few species that occur in Central America and other South American countries. This disjunction in distribution appears to have been facilitated by the brejos in northeastern Brazil which provided a humid steppingstone in the Caatinga during the Pleistocene (Melo Santos et al., 2007, Kessous et al., 2019). Within *Vriesea s.s.*, 12 main clades from genome skimming were retrieved that are also supported by morphological characters or geography. The evolution of similar reproductive characters are distributed throughout the different the clades, in contrast to the prior hypothesis of the sections being based on two "clear" morphologies. *Vriesea* taxa are often epiphytes in the BAF, where the humid montane surroundings favor the leaf tank habit. Amongst all vascular epiphytes in the BAF, *Vriesea* is one of the most species rich genera (Martinelli et al., 2008). Certain species of *Vriesea* have flexibility in where they grow. These taxa can be found in a variety of terrestrial spaces but can commonly be found outside of humid habitats in open montane grasslands as lithophytes on rock outcrops as well as being found on inselbergs (Machado et al., 2020, Neves et al., 2020).

The genus *Billbergia* Thunb. is the third largest in the subfamily Bromelioideae and is split into two subgenera based on petal form. Subgenus *Helicodea* petals coil back into a spring shape while subg. *Billbergia* slightly bend at the tip to give a flared appearance. Recent molecular work has found that the two subgenera which are still used today are not supported by the molecular data (Evans et al., 2015). *Billbergia* is not monophyletic as there are taxa that form a clade with *Aechmea* subgenus *Ortgiesia* (Sass & Specht, 2010). The genus is

predominantly epiphytic, distributed from Mexico to Argentina with centers of diversity being found in the Amazon and BAF (Barros & Costa, 2008). Vegetatively, *Billbergia* species rosettes form tall, narrow tank rosettes (Baensch & Baensch, 1994). Most aspects of floral biology and pollination in this genus have not been heavily researched (Marques et al., 2015), though *Billbergia* is thought to predominantly be pollinated by hummingbirds (Benzing, 2000). There is however one case of a bat pollination syndrome in *Billbergia horrida* Regel. (Marques et al., 2015, Aguilar-Rodriguez et al., 2019). The long tubular flowers of *Billbergia* are often odorless, with petal color varying from green to dark violet. Fruits of Bromelioideae and more specifically *Billbergia* are fleshy and change pigments to further attract vertebrates to disperse the seeds. The reflective surfaces of the fruits is noted in many species of *Billbergia*, and in *B. robert-readii* E.Gross & Rauh the presence of scales in the pericarp have led researchers to hypothesize bats as the main mammal to disperse fruits of the genus (Benzing, 2012, Silva et al., 2020) as the reflective surfaces help increase the brightness of the fruits at night through reflecting dim light.

Biogeography background and role of Brazilian Atlantic Forest (BAF) in Bromeliaceae diversification

Bromeliaceae first arose 97.5 Mya in the infertile tepuis and adjacent sand plains of the Guiana Shield located at the northern tip of South America. The divergence of extant lineages we see today did not begin until around 22.7 Mya. Many Neotropical plant groups also began to diversify around the Middle Miocene (15.97-13.82 Mya) due to the uplift of the northern Andes, Serro do Mar and ranges in southeastern Brazil as well as their coevolution with hummingbird pollinators in fertile montane, moist regions (Givnish et al., 2014). Tillandsioideae and Bromelioideae along with most of the current diversity of Bromeliaceae began to expand their range towards both Central America and the northern regions of South America beginning 15 Mya (Givnish et al., 2007). The Brazilian Shield began to be inhabited by Bromelioideae 5.7 Mya and was the region where many Core Bromelioid lineages originated (Aguirre-Santoro et al., 2020). Tillandsioideae lineages diversified via vicariance through the uplift of the Andes and

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the formation of the Paranaean Sea during the Mid-Upper Miocene (12.0–7.9 Mya). These events possibly influenced the isolation of the Cipuropsidinae and Tillandsieae tribes in the Andes from the subtribe Vrieseinae located in the Atlantic Forest and Chacoan dominion (Kessous et al., 2019). During drying of the Paranaean sea, the Caatinga, Cerrado and Chaco were formed between the Atlantic and Amazon Forest. The BAF and Caatinga (Giulietti et al., 2003) are areas of endemism and diversification of bromeliads while the grassland dominant Chaco and Cerrado as well as Amazon Forest were nearly devoid of terrestrial bromeliads and very few epiphytic species in the Amazon. The southeast-to-northwest connection between the Atlantic Forest and Amazon Forest system has been noted to be the most important connecting path between these regions. The ancient route was most likely established during the start of the Andean uplift which was occurring at the same time as the formation of the South American monsoon system. The route helped the distribution of cold- and warm-adapted plant communities at various times in the past (Ledo & Colli, 2017). In the present, the northern BAF broadly shares floristic similarities with the eastern section of the Amazon Forest while the southern part of the BAF shares similarity in floristics with the south-western parts of the Amazon. The Amazon Forest does contain bromeliads but since there is less topographic diversity across the forest, it is not a hotspot of diversity of the family. Ultimately, all parts of the BAF are closer in floristic aspects than with portions of the Amazon (Oliveira-Filho & Fontes, 2000).

The BAF is a tropical rainforest found on the eastern coast of Brazil. The seasonally xeric shrubland regions of Caatinga, Cerrado (tropical savanna), and the semiarid lowland Chaco region to the south isolate the BAF from the Amazonian and Andean forests (Fig. 2). As this region is spatially and biogeographically distinct presently from the Amazon and Andes due to the "dry diagonal" (Fig. 2) (*sensu* Ledo & Colli, 2017). The BAF has an intricate topography over short geographical distances, resulting from tectonic activity in the Tertiary. Changes to the climate during the last glacial maximum and last interglacial drastically affected the sea-level. At its peak, ocean levels dropped to a maximum of around 150 m which allowed for the BAF to expand onto the newly exposed continental shelf. This widespread expansion of forest occurred considerably over hundreds of kilometers on the Brazilian continental shelf even during the

cooler climate of the Quaternary glacial period that additionally had slightly lower amounts of precipitation (Cardoso da Silva et al., 2004, Leite et al., 2016, Costa et al., 2018).

While the BAF is now tropical rainforest, grassland biomes were the predominant type 2,000 years ago, before being progressively replaced as precipitation increased (Leite et al., 2016). When grasslands were dominant, regions in the BAF were thought to have evolved differently with the change of climate, and the BAF was thought not to have been homogenous throughout. Climate oscillations affected the southern part of the BAF the most as the glacial/interglacial cycles led to more fragmentation in that section than in the northern BAF (Carnaval & Moritz, 2008, Aguiar-Melo et al., 2019, Neri et al., 2021). More specifically, this fragmentation in the southeast BAF yielded a mosaic of humid rain forest and dry grassland. The exact distribution pattern of both types of fragments during this time is still up for debate, but when looking at forest recovery times, it has been suggested that forest habitat was not as severely fragmented as has been suggested (Thomé et al., 2010). Small forest refugia most likely helped repopulate, but widespread coastal forest where biodiversity remained intact is thought to have primarily facilitated this recovery (Leite et al., 2016, Costa et al., 2018).
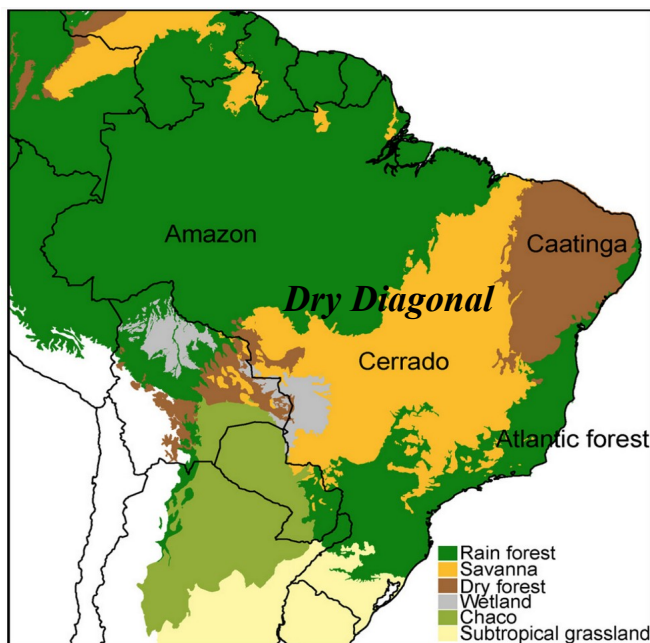

Figure 2. Reprinted from (Simon et al., 2009). Map of the major biomes in the central countries of South America

Tank-forming Bromelioideae found shelter as epiphytes in this mesic coastal forest cover where they had lower extinction rates than non-tank forming Bromelioideae that could have possibly been affected by the shift to a drier biome. The increased risk of fire found in grassland biomes also would have put those taxa at a greater risk of extinction (Silvestro et al., 2014). Tank forming Bromelioideae were also uncommon in lowlands where it is arid, and rainfall is tied to seasonality. Young tanks are quite

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susceptible to desiccation with their high evaporative surface to water volume ratio. Epiphytic bromeliads with smaller body plans deal with a similar issue of desiccation in these arid regions and are thus equally less common (Krömer et al., 2006, Givnish et al., 2014).

The expansion of the BAF into the exposed Atlantic Ocean plain during the last glacial maximum during the Pliocene coincides specifically with the crown age of the genera in the subtribe Vrieseinae (Kessous et al., 2019). *Vriesea* notably is the most biodiverse in the BAF (Martinelli et al., 2008). This diversification also occurred in other tillandsioids, where the expansion and retraction of humid and arid environments due to glacial cycles, helped facilitate the movement of species between these fragments during the late Pleistocene (Palma-Silva et al., 2009, Kessous et al., 2019). Stable refugia areas that appear to be drivers of biodiversity in the northern BAF have been identified (Costa et al., 2018). Populations of bromeliads here are older than southern population species lineages (Carnaval et al., 2009, Uribbe et al., 2020). Overall, the various climatic conditions that occurred historically in this region have been the driver of biogeographical shifts of biota and biodiversity of Neotropical species both in the BAF and across South America (Costa et al., 2018, Neri et al., 2021).

The Brazilian Atlantic Forest current distribution and ecological role of bromeliads

In the present day, the BAF is a distinct biogeographical region in South America and a global hotspot of biodiversity (Myers et al., 2000). The main region of the BAF today occurs along the eastern coast and is comprised of several forest types that form an almost continuous zone from the states of Rio Grande do Norte to Rio Grande do Sul (Cardoso da Silva et al., 2004). High levels of diversity and endemism are associated with the portions of the BAF that have been historically stable. In the present day these are the portions of the forest that were historically a part of the continental shelf forest that extended into the Atlantic Ocean (Leite et al., 2016).

There are a few regions disjunct from the coastal forests that are considered to also be a part of the BAF. The first disjunct regions are called brejos, which are evergreen to semideciduous montane forest fragments in the Caatinga. The semisolated forests along the middle section of the São Francisco River and in southern Piauí are also in the BAF, and there

are isolated deciduous and semideciduous forests inside the Serra da Bodoquena National Park. These areas when grouped together are about 1,400,000 km^2 (Cardoso da Silva et al., 2004). The BAF is one of the main geographical regions globally where large remnants are being fought to be conserved for their endemism (Ribeiro et al., 2009). In the present day, four areas of endemism have been identified for passerine birds and woody plants. While not specifically studied, these areas have shown similar congruence of endemism for other organisms in the BAF (Prance, 1982, Cardoso da Silva et al., 2004). The 4 areas are Pernambuco, Central Bahia, Coastal Bahia and Serra do Mar. Serra do Mar is the only of the four areas that shows recognized nested subareas of endemism within the larger region (Cardoso da Silva et al., 2004).

Bromeliaceae is one of the most crucial families in Neotropical forests due to the ecosystem services they provide as well as habitat for both vertebrates and invertebrates (Greeney, 2001, Killick et al., 2014, Ladino et al., 2019). The distribution of pollinators in the Neotropics are shaped by climatic and geographic conditions and relate to physiological preferences of these pollinator groups (Kessler et al., 2020). Insects, especially Hymenoptera, can store nectar or pollen to utilize as food during arid periods and or hibernate if in pupal stages in dry climatic regions (Krömer et al., 2006). Bromeliads in more dry regions have diversified with insects and present entomophily pollinator syndromes as insects require warm and dry conditions for optimal foraging. Bats thrive in the nocturnal airspace of humid lowland forests, and bromeliads with bat pollinators are the most diverse in the BAF. The long-tongued glossophagine bats are major pollinators in the BAF, pollinating in both the lowland and highland forest (Sazima et al., 1999). Like bats, hummingbirds can still pollinate in wet conditions and thermoregulate themselves to withstand cooler temperatures. Bromeliads pollinated by hummingbirds are found the most in mid- to high elevation areas. While these distribution patterns describe most bromeliad-pollinator interactions, there are plenty of interactions of hummingbirds across all the climatic and geographic regions (Abrahamczyk & Kessler, 2015, Aguilar-Rodriguez et al., 2019, Kessler et al., 2020).

In the BAF, Bromeliaceae are one of the families with the greatest richness and diversity with many species being endemic to the region (Martinelli et al., 2008). Unfortunately, the BAF

has been severely destroyed through human modification and is now approximately 13% of its original area. This human occupation is the main threat to BAF flora and fauna in the forest (Ribeiro et al., 2009). In the Southern and Southeastern regions of the BAF, only a few large fragments of forest remain which support the profuse number of smaller, disturbed fragments (Palma-Silva et al., 2009). This fragmentation has serious consequences for endemic species. The BAF contains at least 653 endemic bromeliads, with 40% of them endangered (Zanella et al., 2012). As with greater fragmentation, endemic taxa abundances are declining and have been listed as endangered or vulnerable due to being outcompeted and succeeded by generalist species with wider geographical ranges and climate change. Generalist species are increasing in abundance as they can succeed over a wider variety of environmental conditions and changed ecology that occurs with degraded fragments (de Lima et al., 2020).

To summarize, both *Vriesea* and *Billbergia* are predominantly epiphytic genera that recruit vertebrates as pollinators. Research on pollination syndromes is not comprehensive in *Billbergia*, however the putative pollinators are hummingbirds due to the long tubular flowers that are often odorless, with petal color varying from green to dark violet. *Billbergia* fruits are fleshy and change pigments to attract vertebrates to disperse the fruits. *Vriesea* species pollinated by bats have floral bracts that are not showy, and have a green, brown, or purple pigment. Flower shape is campanulate and anthesis occurs at dusk/night with pungent odors (Neves et al., 2020). *Vriesea* fruits are a capsule that once dehisced allow the seeds to be dispersed by the wind via a plumose appendage.

Section II: Inflorescence development & mating systems in Bromeliaceae and two focal species

Reproductive effort components in Bromeliaceae

Reproductive effort can be defined as the proportion of biomass invested by a plant towards structures for sexual reproduction (such as flowers, fruits, seeds, floral stems, and bracts) compared to vegetative structures (Mantovani & Iglesias, 2009). Reproduction competes with defense and growth for energetic resources (Bazzaz et al., 1987) and the interaction between these three expenditures affect how a plant lineage evolves. In most Bromeliaceae taxa, the development of an inflorescence starts with the transition from a shoot apical meristem into a floral meristem. Plants process and utilize stored photosynthate from the vegetative body to invest into the reproductive structures, which are of high cost to the plant as they often lack chlorophyll or do not return as much energy to the system as they expend (Orozco-Ibarrola et al., 2015). Conversion of the apical meristem to the inflorescence is a major phase in life history, marking the end of vegetative body growth as that individual cannot produce additional leaves on the parental rosette. There are some bromeliad species that produce inflorescences though the conversion of axillary meristems that allow a parental rosette the ability to produce multiple inflorescences during its life. Few bromeliads that have this single-ramet iteroparous life history are found in the family with most taxa being multi-ramet iteroparous (Jabaily et al., 2021), only able to continue on after flowering by producing axillary ramets, each capable of producing a terminal inflorescence.

Inflorescence structure

Inflorescence size and structure is quite diverse across Bromeliaceae (Fig. 1). A comprehensive understanding of reproductive effort in bromeliads begins with the overall inflorescence structure, which shapes how the flowers are presented spatially and temporally (Kirchoff & Claßen-Bockhoff, 2013). While the closely related grass family Poaceae parallels Bromeliaceae in the diversity of inflorescence forms found across species (Benzing, 2000), Bromeliaceae taxa devote more energy towards reproductive effort because the family is almost entirely reliant upon animals for pollination, and for fruit dispersal in Bromelioideae. While there are lineages that have relatively small reproductive structures such as *Neoregelia* (Fig. 1h) (Santos-Silva et al., 2017), many bromeliads often have relatively large inflorescences

relative to their vegetative body (Benzing, 2000, Males, 2016). Bromeliad taxa that have a branching rachis can have more flowers and a larger display while a taller inflorescence may recruit pollinators from a greater distance on top of aiding in enhanced fruit and seed dispersal. Many species employ conspicuous colorful bracts that together with other structures visually attract pollinators. These bracts vary in shape, size, and number across the family and even among species in the same genera. In hummingbird-pollinated plants, bracts play a crucial role in the overall attractiveness of inflorescences. While they appear costly, they are important in the floral display because of their ability to attract pollinators at long distance by signaling the reward of nectar. Furthermore, bracts can help increase the attractiveness of the floral display for pollinators without having to increase the number of flowers open (Ishii et al., 2008, Pélabon et al., 2012, Bergamo et al., 2019).

Floral structure and bromeliad mating systems

How the flower develops and is presented is interconnected with the inflorescence and both must be considered as a cohesive unit when seeking to understand the reproductive system and reproductive effort (Harder et al., 2004, Remizowa et al., 2013). Floral organs are expensive but are important for pollinator-mediated outcrossing. Bromeliaceae flowers have both a colorful calyx and corolla, augmenting the attractiveness to pollinators. Fruits with calyx's help provide a visual indication once ready for dispersal for frugivores (Bergamo et al., 2019). Septal nectaries, specialized tissues embedded within an ovary where nectar is release at a pore, are developmentally complex and have evolved due to the costly reliance most bromeliad taxa have on vertebrate pollinators (hummingbirds and bats in particular) (Benzing, 2000, Sajo, et al., 2004b). Floral costs of carbon and water have not yet been quantified across bromeliads, but is suspected to be considerable, especially for epiphytes or taxa that do not have easy access to water (Males, 2016). Petal appendages deep in the corolla throat are a unique flower trait in the family known to be found in around 35% of bromeliads (Brown & Terry, 1992). The appendages help in nectar retention and delivery as well as possibly limiting access to nectar for smaller floral visitors who are often inefficient pollinators (Sajo et al., 2004, Santa-Rosa et al., 2020).

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Pollination modes in Bromeliaceae are diverse, with ornithophily, chiropterophily, entomophily, mixed/unspecific (both selfing and outcrossing), and autogamy (selfing) modes fixed within various taxa. Estimations of how abundant a certain syndrome have been hypothesized and directly measured. For example, in mountainous ranges such as the Andes and BAF, 40–85% of bromeliad species are thought to be principally attracting hummingbirds through their ornithophilous pollination syndrome (Kessler et al., 2020). These estimates have been speculative as there are no published family-wide accounts of the number of bromeliads with hummingbird pollination. Recent work has been completed to discern the ancestral pollination syndromes of the 42 extant bromeliad species known to be pollinated by bats. Most of the species belong to the Tillandsioideae subfamily, with the genera *Werauhia* and *Stigmatodon* appearing to be exclusively bat-pollinated (Aguilar-Rodriguez et al., 2019). As shown in *Pitcairnia flammea* when multiple flowers are open per day, geitonogamy (fertilization by another flower on the same plant) is common especially with hummingbird pollinators (Wendt et al., 2002). Some species possess floral traits such as herkogamy (spatial separation of anthers and stigma) and dichogamy (temporal separation of anther dehiscence and stigma receptivity), which help prevent selfing and promote outcrossing (Zanella et al., 2012). Self-compatibility has been observed to be the most common mating system in Tillandsioideae (Bush & Guilbeau, 2009, Matallana et al., 2010) as most genera rely on selfing and mixed systems (Zanella et al., 2012). This tendency to favor autogamy has been noted in many species such as *Werauhia gladioliflora* (H.Wendl.) J.R.Grant , *Pitcairnia brittoniana* (Mez) Mez and *Tillandsia juncea* (Ruiz & Pav.) Poir. (Cascante⬚Marín et al., 2005, Bush & Guilbeau, 2009, Ramírez-Rosas et al., 2020). Self-compatibility is less frequent in Bromelioideae, with about 48% of the species studied across 7 genera in the subfamily being able to to set fruits spontaneously in the absence of pollinator vectors (Matallana et al., 2010).

Selfing is thought to have evolved in Bromeliaceae because of the reproductive assurance it provides species which is quite necessary in an epiphytic habitat where water and pollinators can be limiting. Selfing in the family is also thought to have been favored in related species that occur in the same habitat with the same pollinator or flower phenology to avoid

hybridization, as observed in the genus *Pitcarnia* (Matallana et al., 2010, Palma-Silva et al., 2011).

Figure 3. Floral variation in the *Vriesea rafaelii* study population. *(a)* A herkogamous flower with a separated style and stamen with filaments that are below the stigma. *(b)* Mismatch in male and female reproductive structures. *(c)* An example of a large petal scale that obstructs access to pollen and the stigma. *(d)* A slender terminal flower smaller than other flowers lower on the inflorescence. *(e)* Visual of what a flower looked like after emasculation and cross pollination. *(f)* Reproductive structure mismatch where the stamen are below the stigma. *(g)* Petal scale that is sitting on top of the stigma obstructing access. *(h)* A flower that has stamen above a separated style.

Reproductive overview of two focal species *Vriesea rafaelii* and *Billbergia brasiliensis*

This study seeks to understand the difference in reproductive effort between *Vriesea rafaelii* Leme *and Billbergia brasiliensis* L.B.Sm. These two species are good representatives of their subfamilies Tillandsioideae and Bromelioideae, because they both are mixed mating tank forming epiphytic species. The species also have different pollinator syndromes (bat, hummingbird respectively) and are both self-compatible, thus are ideal representatives to study resource allocation towards reproductive structures. We specifically seek to determine if there are any differences in reproductive allocation between flowers that are outcrossed vs selfed in both species. We particularly want to test this relationship for both species because in Bromeliaceae, the number of resources invested in reproductive structures has been found to correlate with the size of the plant, albeit not always proportionally (Mantovani & Iglesias, 2009).

Now we will focus on the two focal species, situating their reproductive morphologies and mating systems within Bromeliaceae. Prior to my work on *Vriesea rafaelii*, only a species description, some localities and placement into a phylogeny was known (Gomes-da-Silva & Souza-Chies, 2018). The first focal species for my project, *Vriesea rafaelii* forms a tank from its suberect funnelform basal rosette leaves that are whitish green with visible but not prominent parallel nerves. *Vriesea rafaelii* accumulates leaves during development as its mode of mass acquisition (Jabaily et al., 2021). The scape is glabrous, dark green, and erect with the inflorescence form a simple raceme. Floral bracts are not showy but are a dark green color that is similar to the color of the scape and leaves (Fig. 5). The floral bracts exude a mucilaginous substance throughout their development and is thought to be a deterrent for ant nectar thieves (Benzing, 2000) and has been noted to protect against florivory from wasps in *Werauhia gladioliflora* (Cascante-Marín et al., 2009).

Vriesea rafaelii has a steady state flowering pattern, which is described as floral resources being available over many days where 1-2 flowers open per night (Kamke et al., 2011, Aguilar-Rodriguez et al., 2019). *Vriesea rafaelii* inflorescences (n = 17 individual plants studied) contained an average of 16.5 ± 3.3 flowers, with a range of 7 to 20. Anthesis was observed to

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occur at dusk depending on the season, with flowers closing the following midday. Nectar was produced during twilight and production began to taper off close to midnight. The calyx is inconspicuous as it is the same color as the bracts and scape, while the corolla is primarily yellowish white but has purple apical margins (Fig. 3). *Vriesea rafaelii* floral structures do not need to be attractive as bat pollinators are attracted to the flowers by a garlic odor which I observed to not be present until the anthers were dehisced. The scent was strongest approaching midnight. The stigma is convolute-bladed, yellow, and densely papillose. Anthers arrange themselves basally in the flower, although exceptions were observed in herkogamous flowers. *Vriesea rafaelii* can self-fertilize their flowers but due to anthesis timing, I hypothesize this species has a mixed mating system that includes bats. *Vriesea rafaelii* has an erect spike where flowers are spread out along the inflorescence, which is necessary to provide space for the wing movements of bats to navigate and successfully pollinate flowers without damaging the inflorescence (Krömer et al., 2006). This is an example of how pollinator foraging preference affects the spatial arrangement of flowers (Claßen-Bockhoff, 2009). Seeds are dispersed by the wind via a plumose appendage once capsules dehisce (Fig. 4a).
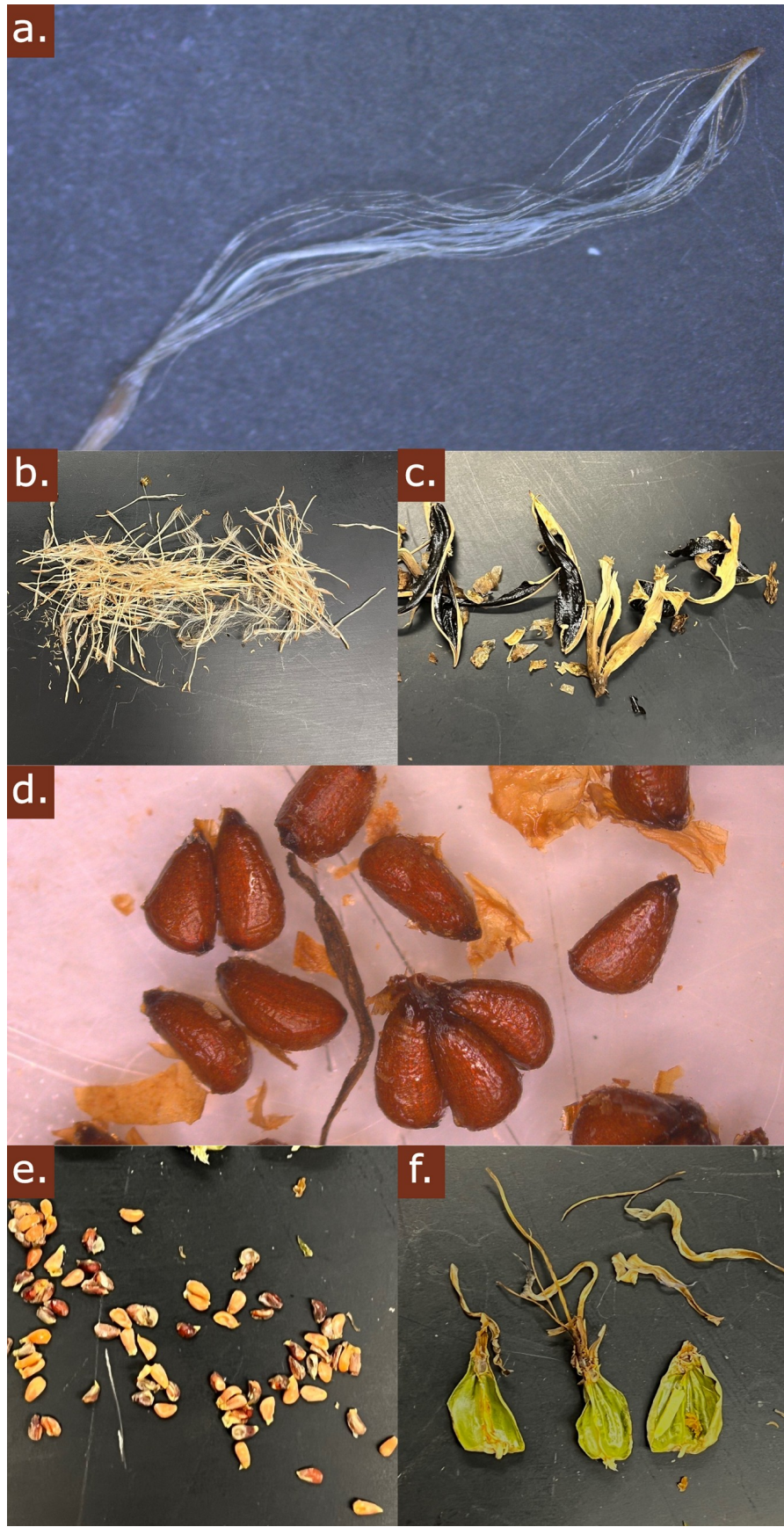
Figure 4. (a) Immature Vriesea rafaelii seed in the bottom left corner with plumose appendage coming off the seed. (b) Pooled seeds from a single immature fruit of V. rafaelii. (c) V. rafaelii Fruit structure after dissection to remove seeds. (d) Billbergia brasiliensis seeds where the mucilaginous coat has dried (Photo by R. Jabaily). (e) Pooled seeds from a single immature fruit of B. brasiliensis. (f) Photo of B. brasiliensis fruit structure and affiliate floral material taken after dissection to remove seeds

Figure 5. inflorescence development in *Vriesea rafaelii*.

Variation was noted in relative reproductive organ length and position in some individual flowers of *V. rafaelii*. Most individuals had anthers at the same length as the stigma, aiding in selfing. Mismatch in anther and pistil length was noted particularly however in individuals who began their development later than others in the population. The most common mismatch observed was the stamen being shorter than the pistil length, so much so that selfing did not appear to have been able to occur (Fig. 3f). Petal scale development also

varied in flowers and in certain cases appeared to cover the stigma, potentially hindering both selfing and pollen export (Fig. 3c,g). Bromeliaceae inflorescence development is presumed to occur acropetally, but for some study individuals, anthesis started from the distal tip, as would be expected of a determinate inflorescence. Another pattern that was observed were two individuals developing and opening flowers in the middle of the inflorescence first (Fig. 5b). This pattern of opening flowers in the middle has also been observed in *Vriesea gigantea* Gaudich.,

Figure 5. Inflorescence variation in *Vriesea rafaelii. (a)* Typical acropetal inflorescence development. *(b)* An inflorescence that primarily developed flowers in the middle of the scape while aborting development on flowers above and below. *(c)* An inflorescence that had every flower open. Various amounts of development occurred in each flower with some carpels that did not develop fruits.

in where anthesis occurs in the center of the inflorescence first and proceeds upwards and downwards (Martinelli, 1995).
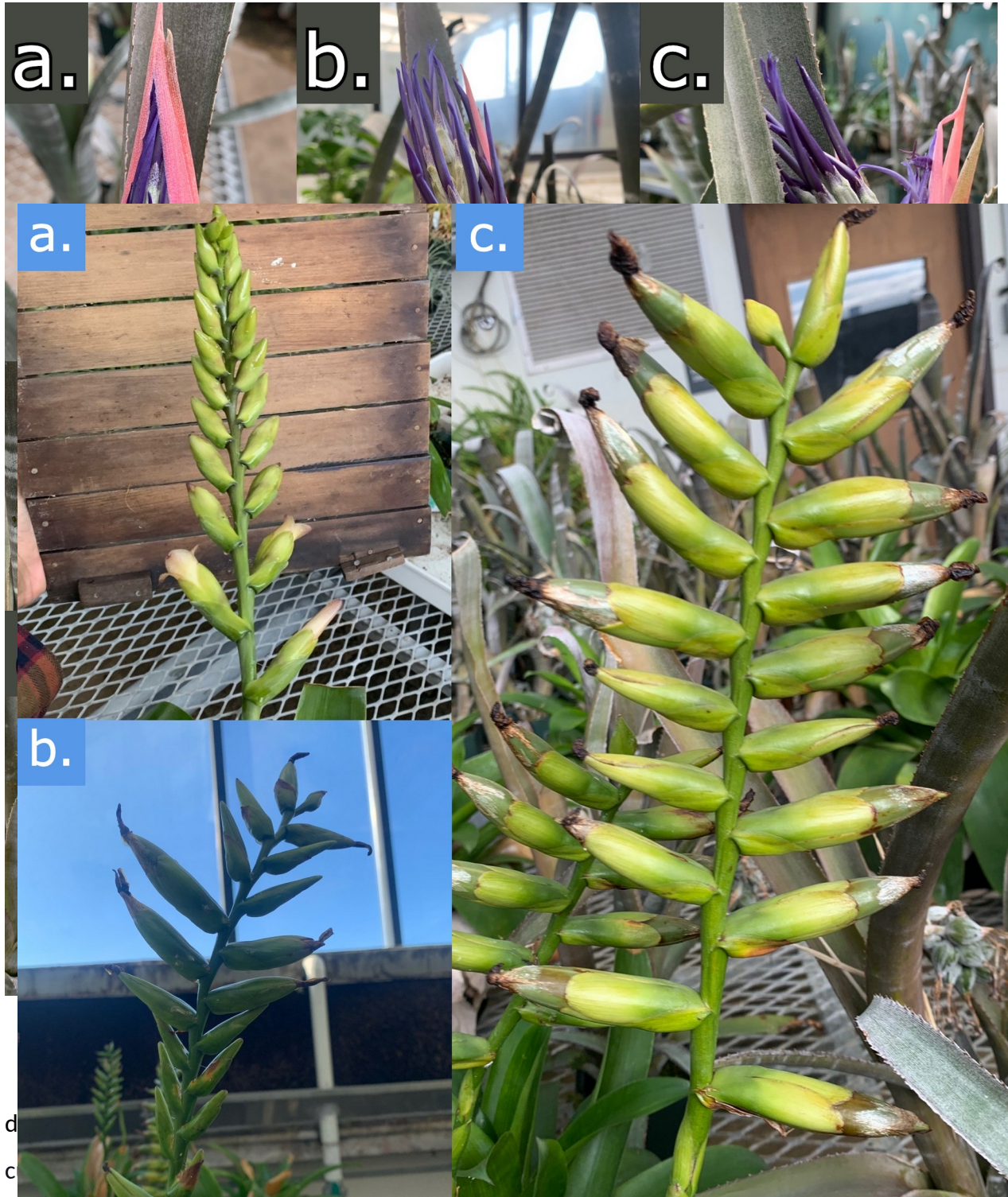
Southeast of the state of Rio de Janeiro in the BAF. Additional species including *Billbergia*

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velascana Cárdenasm and *Billbergia kuhlmannii* L.B.Sm are treated as *B. brasiliensis* by the Global Biodiversity Information Facility (GBIF) and are synonymized into *B. brasiliensis*, extending the range into the Bolivian Amazon (Grant S & Niezgoda C, 2020, Solomon J & Stimmel H 2021). More taxonomic work is needed as this disjunct distribution seems unlikely. *Billbergia brasiliensis* forms a tight, relatively tall tubular tank from leaves that are grayish green from a plethora of trichomes. Silver cross-banding is present and varies in intensity among leaves and individuals. The leaves are succulent and curl back at the tip. The leaf margin in spinose-serrate, a feature shared across Bromelioideae, and the species primarily lengthens their leaves during development as their mode of mass increase (Jabaily et al., 2021). Flowers are arranged in a spiral on the inflorescence, which is pendent out of the tank, a common trait shared by most species in the genus (Faria et al., 2004). A pendant inflorescence has a lower cost as there is less need for supporting structural elements. In mistletoes and the genus *Heliconia*, a pendant inflorescence helps lower the plants center of gravity (Tadey & Aizen, 2001, Iles et al., 2017). Bracts are large, thin and are present lower on the scape spatially isolated from the flowers on the inflorescence, a feature noted across all *Billbergia* (Smith & Downs, 1979). The flowers open in a "cornucopia" flowering pattern (*sensu* Gentry, 1974), which is where a plant opens many flowers over a short time period (Kamke et al., 2011). *Billbergia brasiliensis* inflorescences (n = 14 individual plants) contained an average of 17.5 ± 5.7 flowers, with a range of flower number from 9 to 33. Ovaries are inferior and are spherical with triangular edges. The outside of the ovary is white from a fuzzy coat, and the flower has purple petals that recoil into a spiral at anthesis. Fruits change pigment from green to yellow once ripe (at least 6 months post anthesis) and are subrotund with ribs.

Anthesis was observed to occur around midday with flowers staying open for multiple days. The calyx is inconspicuous as it is the same color as the bracts and scape, while the corolla is a dark hue of purple made up of delicate petals. *Billbergia brasiliensis* bracts were often a bright pink color providing visual contrast to the purple petals. I observed no scent from the flowers. *Billbergia brasiliensis* can self-fertilize their flowers but due to the colorful corolla and bracts, I hypothesize this species has a mixed mating system that includes hummingbirds.

The typical showy pink bracts varied in pigment between individuals in *B. brasiliensis*. There were multiple individuals with bracts that were brown and looked dead at anthesis. Other individuals that were not used in this study also had brown bracts and many of these individuals appear to have halted their inflorescence development with the structure looking slightly developmentally younger than what is seen in figure 5a. Scape length also varied in *B. brasiliensis* with multiple scapes not elongating enough to become pendant and instead were erect, primarily within the tubular vegetative rosette. Flowers on these scapes were difficult to access, particularly the flowers that were most basal (Fig. 7b,e,h).

To summarize, reproductive effort is quantified by how much biomass is being invested towards sexual reproduction compared to vegetative structures. A comprehensive understanding of reproductive effort in bromeliads includes inflorescence and flower form as these structures are quite diverse across Bromeliaceae. Pollination modes in Bromeliaceae are diverse throughout the family with self-pollination being a part of the mating system of more species than thought previously (Benzing, 2000, Matallana et al., 2010). This study seeks to investigated reproductive effort in *Vriesea rafaelii* Leme *and Billbergia brasiliensis* L.B.Sm. Before this study, only a species description, some localities and placement into a phylogeny for *Vriesea rafaelii* was known (Gomes-da-Silva & Souza-Chies, 2018). These two species have different pollinator syndromes (*Vriesea:* bat *Billbergia:* hummingbird) and are good representatives of the subfamilies Tillandsioideae and Bromelioideae where epiphytism and other linked key adaptations have led to diversification. Both species also have a mixed mating breeding system. We specifically seek to determine if there are any differences in reproductive allocation between flowers that are outcrossed vs selfed in both species.
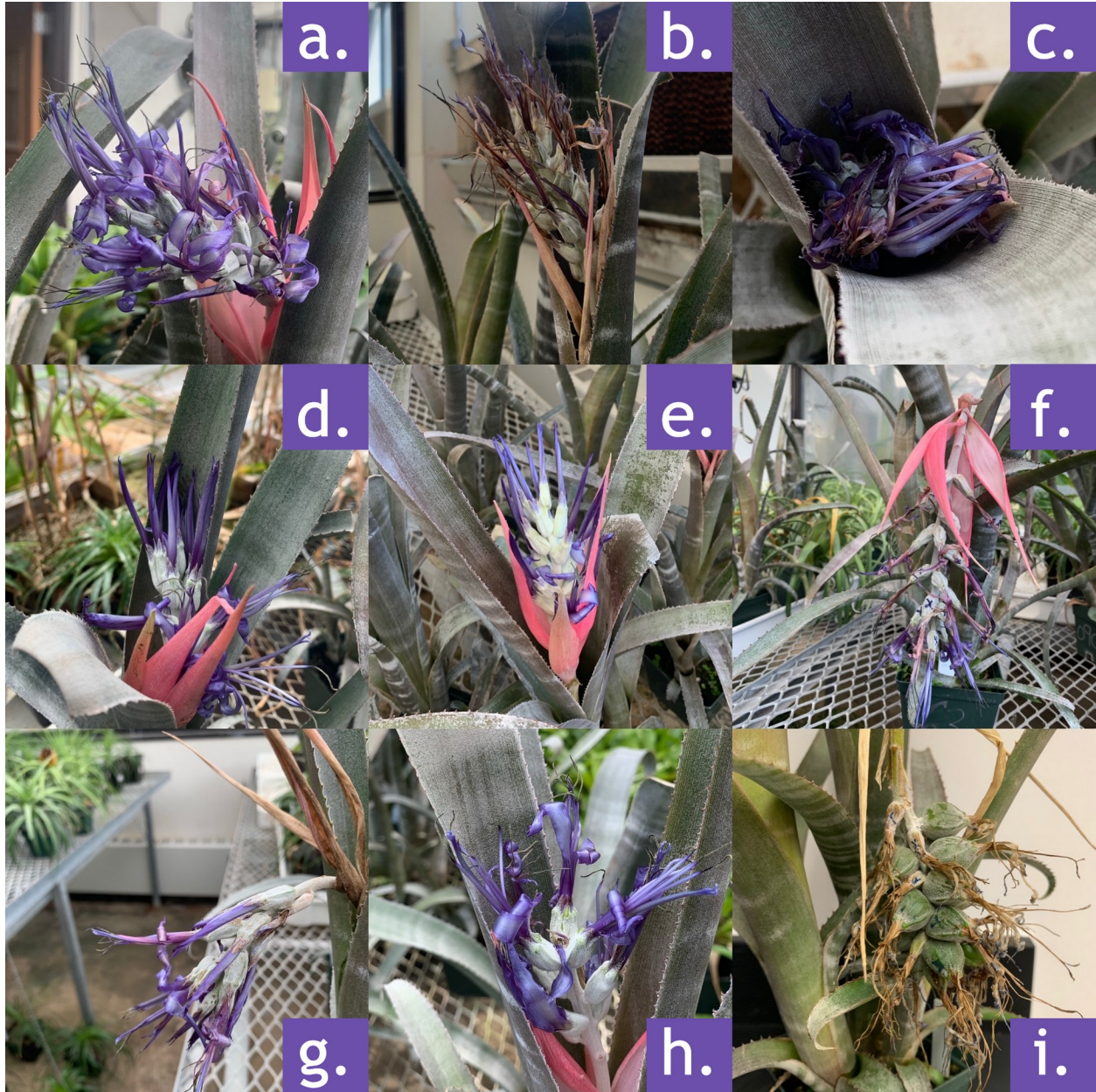
Figure 7. Floral and inflorescence variation in *Billbergia brasiliensis* individuals. (a) A semi pendant scape. (b) An erect scape with flowers partially obstructed by the rosette. (c) Top-down view on a scape that did not elongate past the top of the tank showing flowers obstructed by the rosette. (d) Another short scape with flowers that opened into leaves. (e) A short scape. (f) A pendant scape that developed as expected. (g) A scape with a few underdeveloped brown ovaries and bracts with no pigment and brown during flower anthesis. (h) Close up of an erect scape where flowers were pressed up against the leaves. (i) Maturing infructescence.

Section III: Crossing effects on reproductive effort and fitness

Bromeliaceae reproductive structures are showy and large compared to their vegetative structure. This most likely benefits the family in sexual reproduction as the family is almost entirely reliant upon animals for pollination and some are also dependent on an organism to disperse their fruit. Dependance on organisms for reproduction can be difficult in stressful habitats such as in the tree canopy where nutrients can be limiting (Lasso & Ackerman, 2013). Fragmentation of habitats can also hurt pollinator populations (Paggi et al., 2007), thus limiting pollen flow. Selfing is thought to have evolved in Bromeliaceae because of the reproductive assurance it provides species. However, I am curious about the efficacy of selfing in two representatives of the largest subfamilies in Bromeliaceae. I seek to understand if there are differences in reproductive allocation between flowers that are outcrossed vs selfed in *Vriesea rafaelii* and *Billbergia brasiliensis*.

Methods

Study population

Over 100 individuals of each species were initially purchased from Tropiflora (https://tropiflora.com; Sarasota, FL) and were seed grown from one parental plant. Individuals were kept at Tropiflora during germination and several months of their early growth before being purchased in summer 2017 (*Vriesea rafaelii*) and winter 2018 (*Billbergia brasiliensis*). Many individual plants were sacrificed for other measurements (Jabaily et al. 2021) prior to this work. The individuals included in these experiments were minimally impacted by prior measurements. *Vriesea rafaelii* individuals were ~3.5 years old and *Billbergia brasiliensis* individuals were closer to three years old when data collection began. Seventeen individuals of *V. rafaelii* were selected from the greenhouse population to be included in this study in January

of 2021 once inflorescence induction was visualized but before inflorescences were fully developed. Fourteen *B. brasiliensis* individuals with inflorescences induced during June of 2021.

Vriesea crossings

The *Vriesea rafaelii* experimental population was observed frequently for two weeks to discern the developmental timing and cues necessary to emasculate the flowers before anthers dehisce and pollen grains become visible. Before opening, the corolla looks similar to a balloon, expanding in size from the dark colored calyx until anthesis. Petals first start to open around 5pm, some flowers noted to open sometime in the hour before as well as flowers opening in the hours past 5pm. Petals fully open half an hour after initial opening. Anthers at this time are not dehisced, with dehiscence occurring about an hour to two hours after anthesis. Typically, only one flower per individual opened per day, with the petals closing by midday the following afternoon.

Flowers that were not manipulated were inferred to have selfed as the earliest blooming *Vriesea rafaelii* in our study population had been previously observed to have highly self-compatible flowers readily producing seeds without manipulation. In some cases where petal scales obstructed the anthers ability to touch the stigma, flowers had to be manually selfed. Crossings occurred from late February to late April. *Vriesea rafaelii* individual plants were split into either a selfing-only group (n = 8) or a group designated for the flowers to be outcrossed (n = 9). In the outcrossing group, the majority but not all of the flowers on the individuals were outcrossed as there were times when I missed them blooming, and so they were selfed. Individual flowers were numbered and tracked on the group designated for the flowers to be outcrossed. The flower closest to the proximal end was where numbering began and was tracked until the distal tip.

Before cross-pollinating flowers, individuals were observed during the late afternoon to determine if there would be flower crosses needing to be done that night as well as if there an available donor flower that was ideally in the selfing group had opened. When there were no flowers blooming in the selfing-only group available to be a pollen donor, flowers on outcrossed individuals were used as pollen donors. The pollen donor flower was first selfed with any

remaining pollen used for the subsequent outcross. At dusk or around 5 pm, flowers were observed again to check for petals that had opened but had not yet dehisced their anthers. Flowers were then emasculated by removing their stamens (Fig. 3e) which then were frozen at -20°C to be used as donor pollen in the case a single flower bloomed (n = 9). Frozen pollen was determined to be OK for crossing experiments later through discussion with Andy Siekkenin (pers. comm.). If anther sacs had dehisced on a flower that belonged to the outcrossing group, it was often used as a donor for another flower and was recorded as a selfed flower. Between 9 pm and 12 am when the stigmas were receptive, pollen transfer occurred. Stigma receptivity was checked by visually observing if the stigma appeared glossy with a hand lens or through a phone camera. Pollen was transferred with a dissecting needle with ample clumps of pollen grains being deposited on the stigma and then spread around with the flat backside of the needle. Hand pollination was first conducted on the recipient flower and then the donor flower was selfed with its own pollen. Sucrose concentration (BRIX) of nectar was taken on the donor and recipient flowers for as many flowers that were open when crosses were performed.

Billbergia crossings

Billbergia brasiliensis was also observed for a week to discern when would be the time of day to emasculate the flowers before the anther sacs dehisced. I found that when *Billbergia* petals curled back, anthers sacs were always dehisced. Thus, stamen prior to dehiscence were removed after manually opening the closed petals carefully with dissecting needles. Group size of both outcrossed and selfed individuals was slightly smaller than *V. rafaelii* (each group n = 7). Crossings occurred from mid-July to late August 2021.

The days before crosses were conducted, individuals were observed for developmental stage. Photos were taken and bract color was noted at the time of flowering to infer if any issues occurred during development, deducing if the inflorescence was fully healthy. When petals had not curled back, flowers were manually opened using a dissecting needle to separate the petals. Flowers were emasculated and then hand crossed. An anther sac attached to the filament was held with tweezers and rubbed against the stigma to perform the crosses. Stigmas were checked for receptivity via observing with the zoom function of a phone camera. Flowers

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that had anthers that had already dehisced were self and then used as donors to perform crosses. Once the stamen was taken from the flower, a mesh bag was placed over the flower to ensure that it would self only (Fig. 6g). Mesh bags were only placed on selfed only flowers in *Billbergia brasiliensis* and not *Vriesea rafaelii* as the proximity of *B. brasiliensis* flowers to each other, the pendent inflorescence and the cornucopia flowering pattern led us to implement bags to ensure no crossing would occur. Individuals in the selfing-only group did not receive bags placed over their flowers as many of these individuals bloomed before protocol was established.

## Reproductive and vegetative measurements

Inflorescence mass in this study is defined as the scape itself, any bracts on it but does not include fruits. Fruit development takes at least 7 months in *Billbergia brasiliensis* and at least 18 months in *Vriesea rafaelii*. As time was limited to complete the study, the entire inflorescence structure and all developing fruits were harvested after ~2-4 months of development. The longest leaf length was measured when the entire scape was removed from as near as possible to the base of the rosette without removing the leaves. The entire scape length was measured, and fruits were counted. Inflorescence, scape bracts, and floral bracts were separately bagged for drying. Fruits including residual sepal, petals, and stamen were separately bagged in brown lunch sacks for a minimum of 4 days, with most drying for ~5 days, at ~60°C in a benchtop drying oven. Using a razor blade, forceps, and a variety of metal tools in a pimple removing kit, fruits were then dissected to remove seeds and affiliate material (plumose appendages in *Vriesea*, fleshy exterior for *Billbergia*). Fruit mass, including total carpels and remaining floral parts, were massed for individual flower. Seeds and affiliate material were massed collectively from each fruit. Massing was completed on a Sartorius analytical balance.

Vegetative dry mass was collected for all individuals ~5-9 months after flowering. To collect masses that were similar to what the individual's mass would have been during anthesis, axillary ramets that developed subsequently were removed. The largest ramet was removed from *Vriesea rafaelii* before massing and the smallest ramet was removed from *Billbergia*

brasiliensis. If there was only one pup that was similar size to the parental ramet for *B. brasiliensis*, it was not removed. Removal of axillary ramets was done to control for any new clonal growth since flowering. *Vriesea rafaelii* began axillary ramet production in the summer of 2021 after flowering in the spring of that year. *Billbergia brasiliensis* began ramet production prior to flowering (Jabaily et al., 2021). Axillary and parental ramets were both dried ~ 80°C for a day and then ~60°C for two days before massing.

Statistical analyses

To determine if outcrossing or selfing had an effect on seed and fruit mass for both study species, we ran a nested analysis of variance (ANOVA) between species type, plant number nested in each species as a random effect, cross-type (selfed vs outcrossed) and the interaction between species and cross-type. We performed a correlation analysis to understand whether degree of difference (DoD) in selfed vs. outcrossed traits was correlated with reproductive or vegetative traits. The traits we tested were fruit mass degree of difference (DoD = absolute value of selfed minus outcrossed), seed mass DoD, inflorescence mass, scape length, longest leaf length (LLL) and vegetative mass for each study species. We also examined how biomass was being allocated to reproductive structures depending on the size of plants regardless if they were fully selfed or partially outcrossed. Correlation matrices across selfed and outcrossed plants within each species were created with the following traits: scape length, inflorescence mass, fruit mass, seed mass. Regression analyses were then performed with all of the plants in the study population as a directed approach of considering the precise relationships of vegetative mass and the reproductive traits. Once all trait relationships were evaluated, one-way ANOVAS of ratios of fruit mass to individual mass (inflorescence mass, seed mass, vegetative mass) and inflorescence mass to individual mass (fruit, seed and vegetative mass) were calculated to closely discern if one species allocates more energy towards attracting pollinator and dispersal attraction/ability. All statistical analyses were performed with version 4.0.4 of JMP (SAS Institute, 2001).

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Average fruit mass

Legend: B. brasiliensis, V. rafaelii
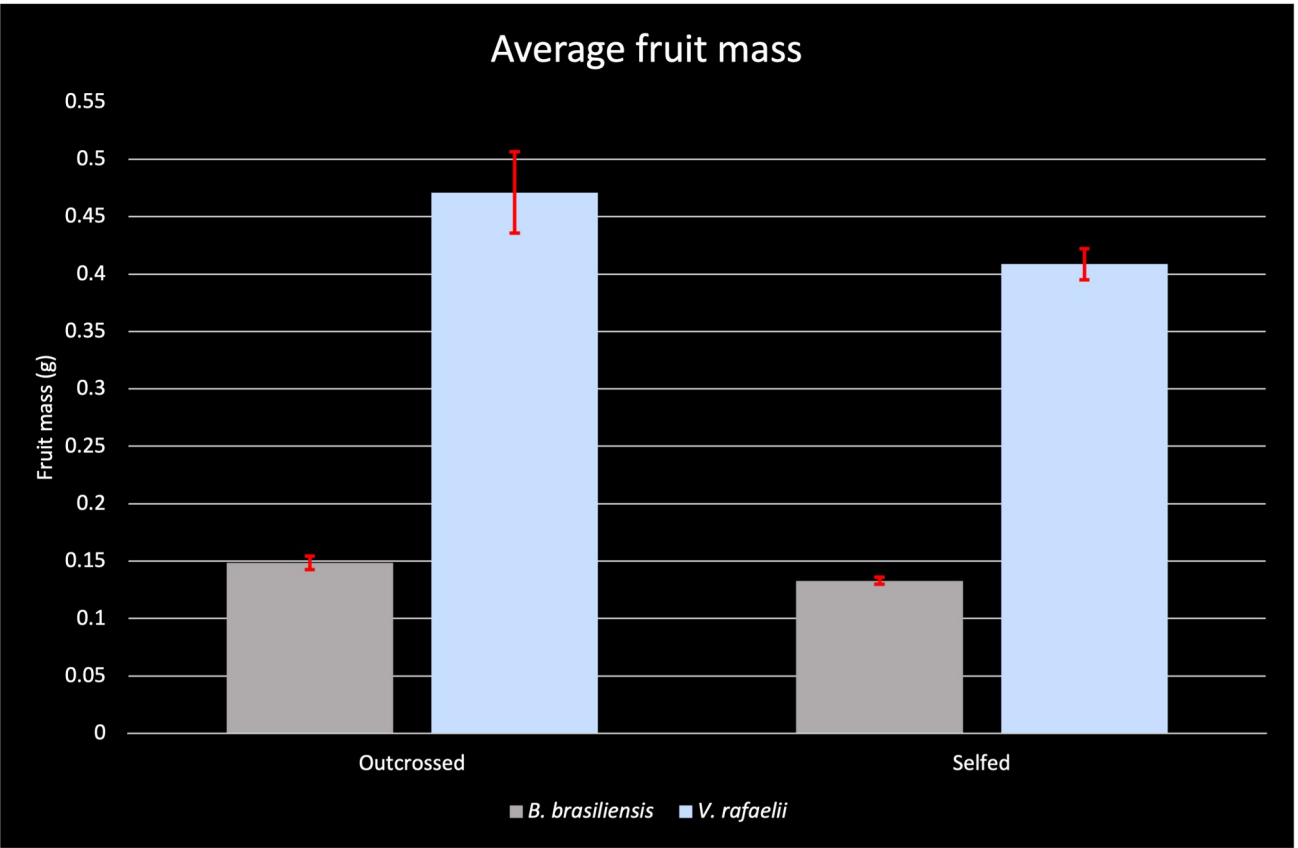
Results

Figure 8. Average fruit mass in outcrossed and selfed flowers for each study species. Sd error is labeled on each bar.
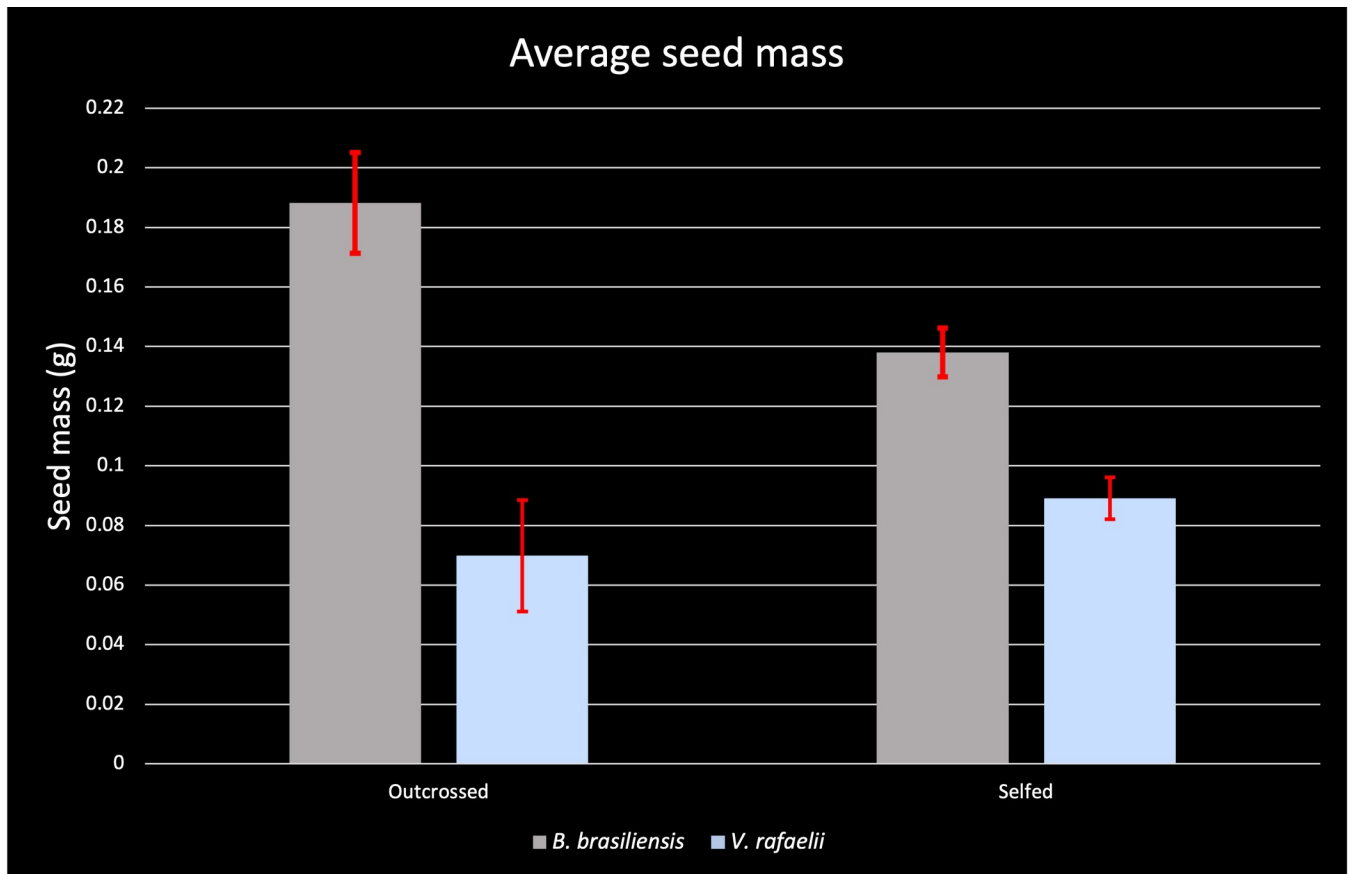
Figure 9. Average seed mass in outcrossed and selfed flowers for each study species. Sd error is labeled on each bar.

Fruit mass was marginally significant between selfed and outcrossed flowers ($F = 3.5499$, df = 1, P = .0601) (Fig. 8). Between the study species, there was a significant difference in selfed vs outcrossed seed mass

SOURCE	SS	DF	F	P
SPECIES	5.82004	1	24.0678	<.0001
PLANT # [SPECIES]&RANDOM	13.7834	30	19.194	<.0001
SELF OR OUTCROSSED	0.08497	1	3.5499	0.0601
SPECIES*SELF OR OUTCROSSED	0.03089	1	1.2903	0.2565

Table 1. ANOVA for the effect of species, plant ID nested within each species (random effect) and cross type on carpel mass.				
SPECIES	0.45549	1	9.0274	0.0047
PLANT # [SPECIES]&RANDOM	2.68866	30	7.9698	<.0001
SELF OR OUTCROSSED	0.01339	1	1.1912	0.2756
SPECIES*SELF OR OUTCROSSED	0.06771	1	6.0209	0.0145

| Table 2. ANOVA for the effect of species, plant ID nested within each species (random effect) and cross type on seed mass. |
|---|

Flowers that were outcrossed in *Billbergia brasiliensis* had higher fruit mass than selfed flowers (F = 4.0761, df = 1, P = .0447). *Vriesea rafaelii* outcrossed flowers had marginally higher fruit mass than flowers that were selfed (F = 2.4817, df = 1, P = .1163). *Billbergia brasiliensis* seed mass was higher in outcrossed fruits than fruits that were selfed (F = 7.2327, df = 1, P = .0077). *V. rafaelii* seed mass did not differ significantly between fruits that were selfed or outcrossed (F = .8548, df = 1, P = .3560).

Seed mass DoD and fruit mass DoD were correlated in *Billbergia brasiliensis* (r = .8459, P = .0165). Scape length and inflorescence mass were correlated (r = .9495, P = .0011). LLL and seed mass DoD were marginally correlated (r = .7254, P = .065), whilst all other correlations were not significant for *B. brasiliensis*. Scape length and inflorescence mass were correlated in *Vriesea rafaelii* (r = .8450, P = .0041). The correlation of seed mass DoD and fruit mass DoD was marginally significant in *V. rafaelii* (r = .6157, P = .0775). LLL was correlated with both inflorescence mass (r = .9262, P = .0003) and scape length (r = .6806, P = .0436). All other traits

for *V. rafaelii* were not correlated with each other. Vegetative mass did not significantly correlate with any traits for both species. Inflorescence and fruit mass are slightly correlated for *Billbergia brasiliensis* (r = .1318, P = .0392). Scape length and fruit mass are also slightly correlated in *B. brasiliensis* (r = .1669, P = .0089). Scape length and inflorescence mass were strongly correlated (r = .9027, P = <.0001). Seed mass and fruit mass were strongly correlated across individuals of *Vriesea rafaelli*. Scape length and inflorescence mass were correlated (r = .4087, P = <.0001)

Vegetative and inflorescence mass for *Vriesea rafaelii* was fit by a positive line that was significant (Slope = 7.60, t = 3.75, P = .0002). Scape length was also significantly related to vegetative mass for *V. rafaelii* (Slope = .714, t = 7.86, P = <.0001). None of the traits from the correlation matrix analysis (scape length, inflorescence mass, fruit mass, seed mass) were fit by a line for *Billbergia brasiliensis*.

Vriesea rafaelii individuals allocated significantly more biomass towards inflorescence mass (F = 71.3668, df = 1, P = <.0001) (Fig. 10) and towards fruits during fruit development (F = 12.9397, df = 1, P = .0012) (Fig. 11). This difference could be due to *V. rafaelii* having an erect
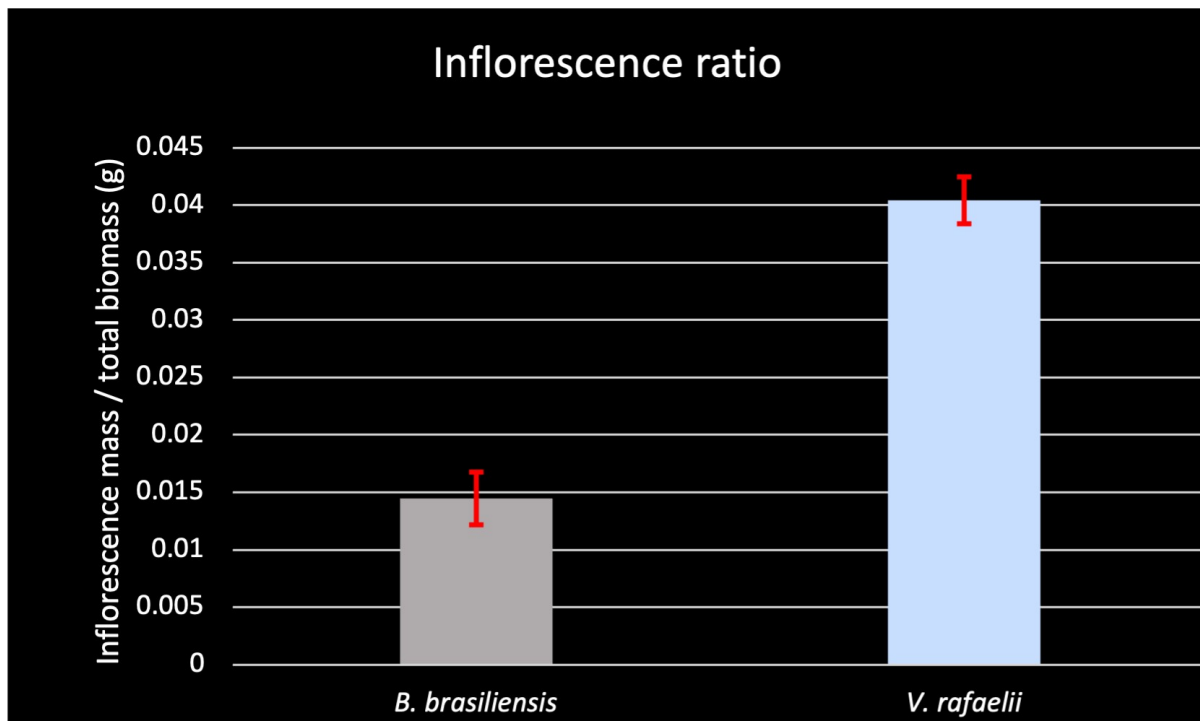
inflorescence which requires more structural material to support the fruits, which also had to fit

Figure 10. Average ratio of mass allocated towards the inflorescence compared to total biomass for each study species. Sd error is labeled on each bar.

in all of the plumose appendage from the seeds into the fruit. *Vriesea rafaelii* nectar was on average 14.856 BRIX across 79 sampled flowers.
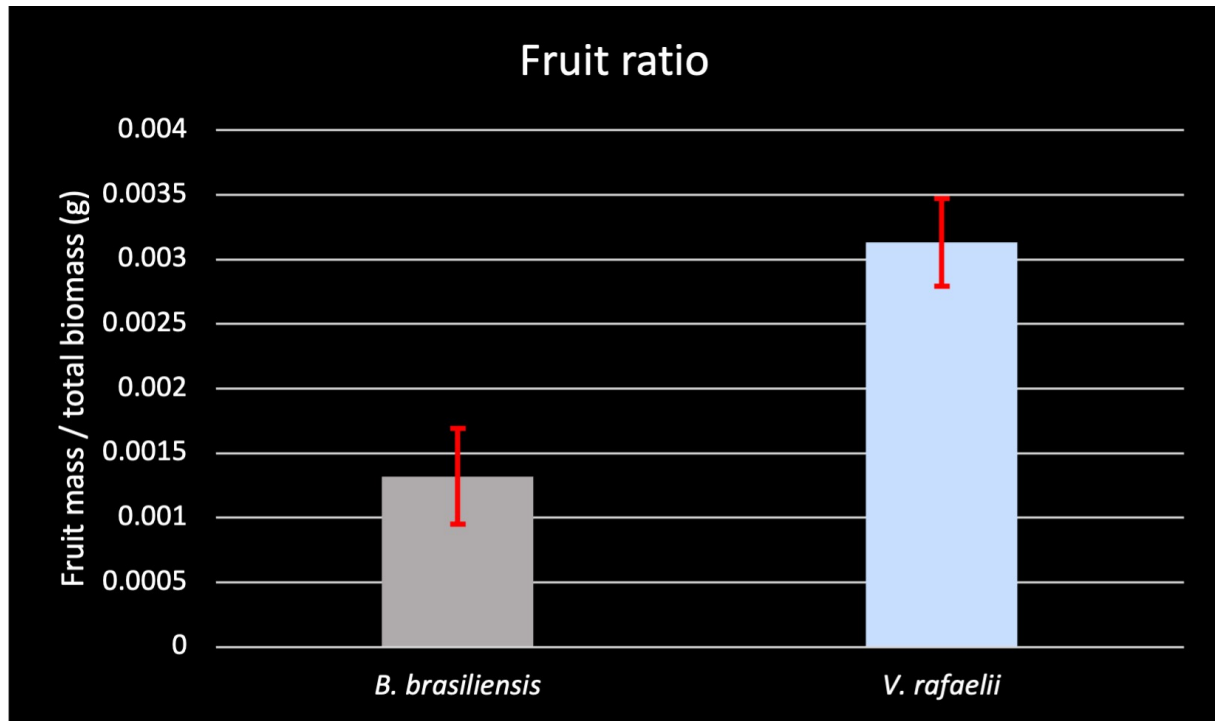

Figure 11. Average ratio of mass allocated towards the fruit compared to total biomass for each study species. Sd error is labeled on each bar.

Discussion

Our aim was to evaluate how resource allocation and fitness differs between flowers that were outcrossed or selfed in our two self-compatible study species. Resource allocation is evaluated in this study as the biomass put towards fruit and inflorescence mass compared to vegetative mass while fitness is approximated as total seed mass of a fruit. We detected significantly more inbreeding depression for *Billbergia brasiliensis*; Outcrossed fruits and seeds in *Billbergia brasiliensis* had higher mass than selfed fruits and seeds. Outcrossed fruits only were marginally higher in mass in *Vriesea rafaelii* and there was no difference in total seed

mass between outcrossed or selfed. *Vriesea rafaelii* individuals allocated significantly more biomass towards inflorescence mass and fruits during fruit development.

        Vriesea rafaelii had flowers that were open for a maximum of 24 hours while *Billbergia brasiliensis* flowers were open for multiple days. In *B. distachia,* the flowering period (comparable in duration to *B. brasiliensis*) is thought to be related to the breeding system which is self-incompatible and exclusively visited by a single hummingbird species (Zambon et al., 2020). The difference in time of a flower open between these two species likely affects the individual rates of outcrossing, expecting *Billbergia brasiliensis* to have more chance to have flowers outcrossed. On the other hand, as *Vriesea rafaelii* flowers are open for only a day, they may rely on selfing for reproductive assurance. Bromeliaceae puts a lot of energy towards ensuring their reproductive structures promote outcrossing and are historically considered as predominant outcrossers (Benzing, 2000). The investment into outcrossing appears to be important for *Billbergia brasiliensis* where selfed fruits were overall smaller but not so much for *Vriesea rafaelii*.

        For *Vriesea rafaelii* scape length and inflorescence mass being correlated is logical as an increase of length would thus produce larger structures that would have more biomass. *Vriesea rafaelli* scapes were all similar in circumference in the population thus making length the predictor of biomass for this species inflorescence mass. Height of release of seeds is important in wind dispersed seeds, as they would have a better chance of dispersal away from the parent rosette if released higher. A larger fruit in *V. rafaelii* does not impart a dispersal advantage due to the anemochorous seeds (Benzing, 2000). The difference observed in fruit mass is most likely due to those fruits having more seeds, thus requiring a larger fruit.

    Only *Billbergia brasiliensis* shows inbreeding depression in both fruit and seed mass (Fig. 9 & 10). The lower masses of selfed flowers emphasizes the reduction of fitness if a flower is not outcrossed for *B. brasiliensis. B. brasiliensis* seed and fruit mass degree of differences were correlated which would coincide with the finding of inbreeding depression in fruits for this species. As inbreeding depression is observed in fruits, seeds were also affected by inbreeding depression for this species. Scape length and inflorescence mass were correlated. Scape length

by fruit mass has a positive relationship in *B. brasiliensis* as well as inflorescence mass by fruit mass.

Seeing noticeable effects of inbreeding in *Billbergia brasiliensis* while not *Vriesea rafaelii* could be due to each species history of population bottlenecks or pollinator limitation. Taxa that have long histories of dealing with these issues are likely to support self-fertilization while species with historically large populations that primarily outcross would be expected instead to have substantial inbreeding depression when selfing occurs (Paggi et al., 2007). Both taxa are endemic to the BAF. *Vriesea rafaelii* is only known from two locations and IUCN listed as critically endangered (Versieux & Wendt, 2007). *Billbergia brasiliensis* has not been IUCN listed but is designated as "in danger" by Centro Nacional de Conservação da Flora. While both species have most likely dealt with bottlenecks and limitations in pollen availability, *V. rafaelii* populations may have maintained higher heterozygosity in the wild compared to *Billbergia brasiliensis* leading to deleterious recessive alleles being maintained and expressed when selfing occurs. Genetically based fitness reductions from the result of smaller population sizes has been found in bromeliads and other plants (Heschel & Paige, 1995). Because environmental stressors exacerbate the negative effects of inbreeding, changing climate and greater fragmentation of the BAF may further hurt *Billbergia brasiliensis* populations.

Future research will discern if *Vriesea rafaelii* is possibly putting more energy towards sexual reproduction because it delays axillary ramet production until well after flowering in contrast to *Billbergia brasiliensis* which produces numerous axillary ramets prior to flowering(Jabaily et al., 2021) *Billbergia brasiliensis* fruits were ripe ~8 months after flowering with seeds being dispersed through a vertebrate while *Vriesea rafaelii* fruits that flowered in October 2020 took ~17 months to fully develop. Long fruit maturation time is potentially adaptive and was also found in *Tillandsia fasciculata*, in the subfamily Tillandsioideae where individuals required one more year after the first dry season to reach seed dispersal phase (Cascante‐Marín et al., 2006). Developing the plumose appendage may be one reason for the stark difference in fruit maturation time. If conservation efforts are put into place, life history of clonal and sexual reproduction, population genetic structure and mating systems should be considered as important factors when working to develop successful conservation methods for Bromeliaceae

(Zanella et al., 2012). Future studies should confirm inbreeding depression in *B. brasiliensis* through examination of cross- and self-pollen tube growth and molecular marker-based assessment of inbreeding coefficients. Investigating if selfed individuals have smaller and lighter inflorescences through generational crossing experiments can aid our understanding of how inbreeding affects reproductive structures in these species.

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