


RESEARCH ARTICLE

Acid waters in tank bromeliads: Causes and potential consequences

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Abstract

Premise: The consequences of acidity for plant performance are profound, yet the prevalence and causes of low pH in bromeliad tank water are unknown despite its functional relevance to key members of many neotropical plant communities.

Methods: We investigated tank water pH for eight bromeliad species in the field and for the widely occurring *Guzmania monostachia* in varying light. We compared pH changes over time between plant and artificial tanks containing a solution combined from several plants. Aquaporin transcripts were measured for field plants at two levels of pH. We investigated relationships between pH, leaf hydraulic conductance, and CO₂ concentration in greenhouse plants and tested proton pump activity using a stimulator and inhibitor.

Results: Mean tank water pH for the eight species was 4.7 ± 0.06 and was lower for *G. monostachia* in higher light. The pH of the solution in artificial tanks, unlike in plants, did not decrease over time. Aquaporin transcription was higher for plants with lower pH, but leaf hydraulic conductance did not differ, suggesting that the pH did not influence water uptake. Tank pH and CO₂ concentration were inversely related. Fusicocin enhanced a decrease in tank pH, whereas orthovanadate did not.

Conclusions: *Guzmania monostachia* acidified its tank water via leaf proton pumps, which appeared responsive to light. Low pH increased aquaporin transcripts but did not influence leaf hydraulic conductance, hence may be more relevant to nutrient uptake.

KEYWORDS

aquaporins, Bromeliaceae, foliar uptake, leaf hydraulic conductance, low pH, proton pumps, tropical epiphytes

Substrate pH can have profound impacts on a plant's ability to acquire resources. The influence of pH is so pervasive that many species have adaptations that modify their local environment to make the pH more favorable for resource uptake, as exemplified by root exudation of organic acids to mobilize soil nutrients, primarily phosphorus (Egle et al., 2003). Leaves can also modify the acidity of their surfaces (Gilbert and Renner, 2021) and the aqueous contents enclosed in the leaf traps of carnivorous plants (Moran et al., 2010), again to assist in mineral uptake. The impacts of substrate pH on water uptake are less well known,

although low pH can reduce root water uptake by inhibiting transport through aquaporins (membrane-embedded proteinaceous pores; Kamaluddin and Zwiazek, 2004; Zhang and Zwiazek, 2016). Epiphytic tank bromeliads (Bromeliaceae) acquire most of their water and nutrients not through their roots but through their tanks—external reservoirs of water trapped by the overlapping bases of their leaves (Benzing and Burt, 1970; Benzing, 2008; Zotz, 2016; but see Leroy et al., 2019), implying that the pH of their tank contents may be functionally related to their acquisition of resources. The amount of acidity has been shown to

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influence or even regulate the community composition of living organisms that occupy the tanks, including bacteria (Goffredi et al., 2011, 2015; Louca et al., 2017), invertebrates (Laessle, 1961; Lopez et al., 2001, 2009), and vertebrates (primarily frogs; Domingos et al., 2014; Mageski et al., 2016). Moreover, the pH of bromeliad tank contents is relevant to community structure not only within the tank but also outside due to the large biomass of tank bromeliads in neotropical forests and the correspondingly large mass of water they contain (Males, 2016; Zotz, 2016; Pereira et al., 2022). Thus, the relevance of pH to tank bromeliad function is ecologically important and worthy of closer examination.

Field studies of several species of tank bromeliad in a wide range of habitats have shown that the tank water tends to be acidic, with a pH usually between 4.0 and 6.5 (Laessle, 1961; Maguire, 1971; Benzing, 2008; Jocque and Kolby, 2012). These values are not surprising, given that the pH of rainfall reported in several tropical rainforests has a similar range (Forti and Moreira-Nordemann, 1991; Eklund et al., 1997; Gioda et al., 2013). However, tank water pH is more variable than that of precipitation, changing on a diel and seasonal basis in response to a variety of environmental factors. For example, living tank occupants and debris from host trees and surrounding vegetation can alter the pH; in one study, the presence of a snail shell was associated with disproportionately high pH readings in a few leaf axils (Laessle, 1961). The chemical composition of host tree leaves is known to affect canopy soils and epiphytes rooted in them (Cardelús et al., 2009), but the effect on tank pH in bromeliads has not been reported. The release of acids during decomposition is thought to be a likely cause of low pH in tank water (Jocque and Kolby, 2012), as is the respiratory release of CO₂ by inhabitants of the tank and the bromeliad itself. In addition, tank pH could be lowered by proton extrusion accompanying various metabolic processes, such as the assimilation of ammonium (NH₄⁺), the predominant form of mineral N available in bromeliad tanks (Inselsbacher et al., 2007). Conversely, photosynthetic uptake of CO₂ and production of O₂ by the plant, resident algae, and photosynthetic microbes have been implicated in raising the pH (Benzing et al., 1972).

In our preliminary investigations of bromeliad tank water in a lowland tropical forest in Costa Rica, pH was frequently low (below 5), and often lower in high light than in shade. Several studies have shown that the pH of tank water changes on a diel basis and in sun vs. shade, although not always in the same direction (Laessle, 1961; Benzing et al., 1972; Guimarães-Souza et al., 2006). A somewhat paradoxical corollary between pH and light exposure is that, presumably, transpirational demands would be greatest when water uptake from the tank could be limited by possible inhibition of aquaporins due to low pH (Kamaluddin and Zwiazek, 2004; Zhang and Zwiazek, 2016). In the case of the species central to this study, *Guzmania monostachia* (L.) Rusby ex Mez (Bromeliaceae), stomatal opening is quite sensitive to light, and stomatal conductance decreases markedly with decreasing light

(Maxwell et al., 1994; North et al., 2013, 2016, 2019); thus, low tank pH would seem to be disadvantageous when transpiration is high. On the other hand, one possible benefit of aquaporin inhibition by low pH could be to prolong the availability of water and nutrients by limiting water uptake from the tank at times of peak demand.

Tank bromeliads exhibit several adaptations to resource limitation in addition to the tank habit. Perhaps the best-studied adaptation to a limited and variable water supply is the wide range of water-conserving CAM photosynthetic pathways shown in the group, from full CAM to weak CAM, including the C₃-CAM intermediacy of the focal species in this study, *G. monostachia* (Smith et al., 1986; Maxwell et al., 1994; Crayn et al., 2015; Pikart et al., 2020). Adaptations to limited mineral nutrients include high-affinity membrane transporters for nitrogen (Inselsbacher et al., 2007), phosphorus (Winkler and Zotz, 2009), and potassium (Winkler and Zotz, 2010), and the capacity to acquire organic sources of N such as urea (Matiz et al., 2019; Gonçalves and Mercier, 2021). The most fundamental adaptation to limitation of both water and nutrients may be the slow growth rate of most epiphytes, including tank bromeliads (Schmidt and Zotz, 2002).

The primary goal of our study was to investigate acidity in the water of tank bromeliads in the context of resource limitation. We chose to focus on water as a resource primarily because many studies have indicated that water availability is the principal, though certainly not the only, determining factor in epiphyte distribution in the neotropical forests (Laube and Zotz, 2003; Graham and Andrade, 2004; Males, 2016). The specific questions we sought to answer were:

- Q1. What is the mean pH of tank contents in a representative group of species in a lowland tropical rainforest?
- Q2. How does light level affect tank water pH?
- Q3. Are acidity levels in the tanks the result of activity by the tank constituents or by the plants themselves?
- Q4. How does pH affect gene transcription of aquaporins?
- Q5. How does pH affect leaf hydraulic conductance?
- Q6. Is tank water pH determined by CO₂ concentrations or by the activity of proton pumps in the leaves?

To address Q1, we surveyed tank pH for several species of bromeliad growing at La Selva Biological Station, a lowland tropical rainforest in Costa Rica. Subsequently, we carried out experiments to investigate pH variation more intensively for the widely distributed, broadly adapted, and well-documented species *Guzmania monostachia*. We addressed Q2 in a 7-week shading experiment on plants of *G. monostachia* in a naturally occurring forest gap at La Selva. To address Q3, we compared the pH of a solution of tank contents obtained from field plants placed in artificial tanks versus that in intact plant tanks. To address Q4, we collected tissue samples from plants of *G. monostachia* with

a range of tank water pH in the field and processed them for aquaporin gene expression. In this regard, our lab group previously identified a mercury-sensitive aquaporin gene in *G. monostachia* (*GmPIP*; archived at GenBank; MK880047; North et al., 2019). We addressed Q5 by measuring leaf hydraulic conductance for greenhouse-grown plants of *G. monostachia* under two levels of pH. To address Q6, we measured diel patterns of tank water pH simultaneously with CO₂ concentrations for greenhouse-grown plants. In addition, because several plant species utilize proton pumps to adjust pH in the rhizosphere (Neumann and Römheld, 2012) and in carnivorous leaf traps (An et al., 2001), we tested the effects of a proton pump inhibitor and a stimulator on the pH of the tank water.

We recognize that our focus on pH and water uptake sidesteps the rich and extensive literature on mineral nutrition in tank bromeliads (Pittendrigh, 1948; Benzing and Renfrow, 1974; Givnish et al., 2014) and that adaptations to water and nutrient limitation tend to occur together (Gonçalves and Mercier, 2021). In view of our results and in the more general context of resource limitation, we briefly discuss possible implications—for epiphytes as well as other species with foliar uptake of water and nutrients (Schreel and Stepe, 2020)—of low pH for resource acquisition by tank bromeliads.

MATERIALS AND METHODS

Field site and pH survey

The tank pH was measured for eight commonly encountered species of tank bromeliad growing in a wet, lowland tropical forest managed by the Organization for Tropical Studies, La Selva Biological Station (84°00'12"W, 10°25'52" N) in northeastern Costa Rica. Plants were accessed at trail sides, from observation towers, from bridges, and from the canopy using single rope climbing techniques. With the exception of one species, *Werauhia ringens*, which was encountered at only one site, plants were measured at several sites per species. No effort was made to standardize light exposure or time after rainfall, but pH was measured mid-morning in early June in almost all cases. A narrow-diameter glass pH probe attached to a handheld pH meter (model UP 5, Denver Instrument Company, Denver, Colorado, USA) was calibrated daily and inserted into either the central tank or into an axial tank close to the center. An attached thermocouple was used to correct for temperature. At least eight plants were measured for each species, except for only four plants of *Androlepis skinneri*.

Shading experiment

A tree-fall at La Selva created a large light gap that persisted long enough for bromeliads previously in the tree canopy to reorient and establish in upright positions; according to

observers at the station, the gap was several months old when we set up our experiment. Plants were growing in full sunlight, and their leaves and tanks were intact. Ten plants of *Guzmania monostachia* approximately 0.3 m in diameter were selected, and five were chosen at random to receive shade treatments, which consisted of a PVC frame placed over each plant to be shaded and covered with two layers of 60% shade cloth. The sides were left open to allow air movement. Light (photosynthetically active radiation [PAR]) in the shade treatment was reduced to about 25% of that measured at midday using a handheld quantum meter (Apogee Instruments, Logan, UT, USA). Temperature-corrected measurements of pH were made every 2 days at mid-morning for 7 weeks. The shade cloth was removed for the last 2 weeks of the experiment.

Artificial tank experiment

To distinguish between pH-altering activity by the bromeliads versus the tank water constituents (primarily microbes and microscopic eukaryotes such as protozoa, algae, and fungi; Goffredi et al., 2015), we monitored pH in plants and in artificial tanks. We collected several newly fallen plants of *G. monostachia* after a storm and placed them upright outside an ambient lab at La Selva where temperature and relative humidity were similar to those outside due to screened openings. The plants received rainfall for 5–7 days before the contents of their tanks were collected and combined in a clean plastic container. Tank contents were completely removed from four plants, which were then rinsed with rainwater. Four tapered plastic tubes that held approximately the same volume of water as the central tanks of *G. monostachia* were used as artificial tanks, and a ring of construction-paper leaves was placed on the rim of each tube to mimic plant light interception above the tank. The sides of the tubes were covered with aluminum foil to exclude light. The collected solution of tank water was diluted with an equal volume of rainwater, mixed thoroughly, and distributed between the plants and plastic tubes, and a pH probe attached to a datalogger (Model 850060, Sper Scientific Ltd., Scottsdale, AZ, USA) was inserted in each. Temperature probes were placed in each plant and tube, and all pH values were temperature-corrected. The plants and tubes were placed near a window where they received several hours of indirect sun each day. Tank pH was recorded for 92 h; the solution was then removed from the artificial tanks and placed in four newly rinsed plants of *G. monostachia*, and pH was recorded for 70 more hours.

Aquaporin transcription for plants in the field

The pH of tank water was repeatedly measured for plants of *G. monostachia* growing 0.5–3.0 m above the forest floor in the understory at La Selva using handheld pH meters. Plants

were measured during mid-morning hours over a period of 4 d, yielding a pH range of 3.2–6.3. For all plants, the first fully expanded leaf (~leaf 4) was removed, and 10-mm sections of leaf tissue were excised at the leaf base, placed in 500 μL DNA/RNA Shield (Zymo Research, Irvine, CA, USA), chopped into 1- to 2-mm sections, and stored at 4°C until use.

Total RNA was extracted from all tissue segments using the Quick-RNA MiniPrep kit (Zymo Research), and RNA concentration was determined using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Single-stranded cDNA was synthesized from 0.5 μg RNA using oligo dT (Integrated DNA Technologies, Coralville, IA, USA) as primer and Maxima H Minus reverse transcriptase (Thermo Fisher Scientific). qRT-RT-PCR analyses of total RNA were performed using gene-specific primers (North et al., 2019) and following the Apex 2X GREEN Mix, Low Rox manufacturer's protocol (Genesee Scientific, San Diego, California, USA). qRT-RT-PCR was run on a 7500 Real Time PCR System (Thermo Fisher Scientific) using the following program: polymerase activation at 95°C for 15 min and 40 cycles of denaturation at 95°C for 15 s, annealing at primer-specific temperature for 30 s, and extension at 72°C for 31 s, followed by a plate read. Melt-curve analyses were performed for each plate using the following program: denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and 70 cycles of 0.5°C increments every 10 s starting at 60°C, followed by a plate read. Data for aquaporin-specific primers were analyzed relative to the *matK* reference gene and normalized to the geographic mean (Remans et al., 2014). Gene-specific primer efficiencies ranged from 90–110% (Bustin et al., 2009). Significant outliers for the data were removed based on a 0.05 significance level using a Grubbs test.

Effect of pH on leaf hydraulic conductance

For this and subsequent experiments, plants of *G. monostachia* were purchased from a commercial nursery (Michael's Bromeliads, Venice, FL, USA) and grown in a shaded greenhouse at Occidental College (Los Angeles, CA, USA; 34°7'39"N, 118°12'37"W) for at least 60 d before experiments were begun. Light levels in the greenhouse were ca. 20% of ambient solar radiation (with a maximum PAR of ca. 360 $\mu\text{mol m}^{-2} \text{s}^{-1}$); daily average maximum/minimum temperatures were ca. 30.5°/21.5°C. To investigate the short-term effect of pH on leaf hydraulic conductance, K_{leaf} ($\text{m}^3 \text{m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$ or $\text{mmol m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$) was measured using the evaporative flux method (Sack et al., 2002) for leaves treated with buffer solutions at pH 4.0 and pH 6.0 following methods described in North et al. (2019). Briefly, a mature leaf (4th from the center) was removed from the plant, and the leaf blade (lamina) was recut under water just above the tank region (because the tank region was not included, K_{leaf} was measured for the exposed region of the leaf blade only, with water entry through the xylem exposed by cutting the leaf base). The leaf

base was immersed in a vial of dilute phosphate buffer prepared in previously filtered, de-gassed water adjusted to either pH 4.0 or 6.0, and the leaf was allowed to transpire for 30 min under red/blue LED lights at 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR. The vial was then placed on a 0.1-mg analytical balance, under the same lighting, and its mass was recorded every 10 s. A fan was used to reduce boundary layers during measurement, and leaf temperature averaged 23°C, air temperature 20–22°C. When mass-loss readings stabilized, usually within 10 min, the mass was recorded and graphed for 30 min, and the slope of the line was used to calculate volumetric flow, which is equivalent to leaf transpiration E expressed on a leaf area basis ($\text{mmol m}^{-2} \text{s}^{-1}$). After the leaf was removed from the balance, it was bagged for 5 min, and its water potential Ψ_{leaf} (MPa) was measured with a pressure chamber (PMS Instruments, Portland, OR, USA). Leaf area was determined from digital photos analyzed with ImageJ (Schneider et al., 2012). Leaf hydraulic conductance, K_{leaf} , was calculated from the average slope of the volumetric flow divided by Ψ_{leaf} :

$$K_{\text{leaf}} = \frac{E}{-\Psi_{\text{leaf}}}.$$

CO₂ concentration and pH of tank water

Diel measurements of CO₂ concentration and pH of tank water were made for two plants, each for 3 d in the laboratory with a 12-h dark period and 12 h under red/blue LED lights at 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR. To measure CO₂, a microelectrode (MI-720 Micro-Carbon Dioxide Electrode, Microelectrodes Inc., New Bedford, NH, USA) was first calibrated with standard gases (three concentrations of CO₂) and suspended in the center of the water in an axial (side) tank, formed by leaves that typically are most photosynthetically active in *G. monostachia*. The electrode was attached to a datalogger set to record in millivolts every 10 min for 3 d. The CO₂ concentration of the tank fluid was calculated from a standard curve corrected for temperature obtained from the millivolt reading, based on Henry's law (McGuire and Teskey, 2002). Tank water pH was then calculated based on carbonate chemistry for freshwater systems with native pH < 5.0 (McGuire and Teskey, 2002), using an online calculator (Robbins et al., 2010). A calibrated pH microelectrode (MI-406 flat-membrane pH, with reference electrode MI-402 [Microelectrodes Inc.]) was suspended in an adjacent tank of the same plant along with a temperature probe, set to record simultaneously with the CO₂ microelectrode.

Effects of proton pump stimulation and inhibition on pH of tank water

In separate experiments, we used the fungal toxin fusaric acid (10 μM ; Sigma-Aldrich, St. Louis, MO, USA) to stimulate the activity of plasma membrane proton pumps (H⁺-ATPases; DUBY and BOUTRY, 2009; Neumann and

Römheld, 2012) and sodium orthovanadate (500 μM ; Sigma-Aldrich; Bogoslavsky and Neumann, 1998) to inhibit proton pumps. In both experiments, the solutions were added to the tanks of four plants, and the initial pH of the tank water was adjusted to 7.0 with HCl or KOH in both treated and control plants. The plants were kept in the greenhouse under natural lighting, and Sper datalogging pH meters recorded the pH of the tank water every 10 min for 3 d (fusicoocin) or 4 d (sodium orthovanadate).

Statistical analysis

Statistical analyses were performed using SigmaPlot 13.0 (Systat Software, San Jose, California, USA) as described in the Results, with data transformations when necessary along with the Bonferroni correction for multiple comparisons. Data are usually reported as means \pm 1 SE except where error bars interfered with graph legibility; in these cases, the range of the SE is reported. In cases where data were not normally distributed despite transformation, nonparametric tests were used.

RESULTS

Survey of pH in field plants

A multiyear survey of tank water pH for 156 plants from the eight common species of bromeliads yielded individual values ranging from 2.6 to 6.5, with a mean of 4.7 ± 0.06 and a median of 4.6. The mean was significantly lower than the volume averaged mean of 5.2 ± 0.35 (SD) for rainfall collected at La Selva during June and July 1983 (t -test, $t = 3.756$, $df = 1.99$, $P < 0.001$; Parker, 1994; rainfall pH indicated by dashed line, Figure 1). Only two of the eight species had a mean pH greater than the mean pH of rainfall. Within each species, the ranges in pH were usually large, and the variances were unequal among the groups (Figure 1). The species with the lowest median pH, *Werauhia ringens*, was significantly different in this respect from the four species with the highest median pH, *Aechmea mariae-reginae*, *A. nudicaulis*, *Androlepis skinneri*, and *Werauhia gladioliflora* (Kruskal–Wallis test, followed by Dunn's pairwise test; $H = 39.518$, $df = 7$, $P < 0.001$). For *Guzmania monostachia*, the subject of further investigation in this study, the median pH was 4.35, and the mean was 4.63 ± 0.14 , $N = 29$.

Shading experiment

The mean pH of tank water for plants of *G. monostachia* that were artificially shaded in the 7-week field experiment was 4.59 ± 0.03 , compared with the mean of 4.45 ± 0.03 for unshaded plants at the same site (Figure 2; Friedman repeated measures ANOVA on ranks [used in place of repeated measures ANOVA due to non-normal distribution of data], followed by Tukey test, $q = 5.692$, $df = 1$, $P < 0.001$). Soon after

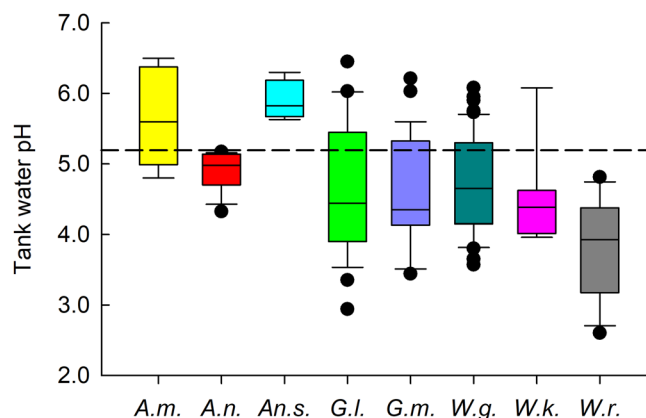


FIGURE 1 Tank water pH measured at La Selva Biological Station, with outliers, quartiles, and median values indicated for eight bromeliad species: *Aechmea mariae-reginae* (A.m.), *A. nudicaulis* (A.n.), *Androlepis skinneri* (An.s.), *Guzmania lingulata* (G.l.), *G. monostachia* (G.m.), *Werauhia gladioliflora* (W.g.), *W. kupperiana* (W.k.), and *W. ringens* (W.r.). The horizontal dashed line indicates the mean pH of rainfall collected in June–July 1983 (Parker, 1994).

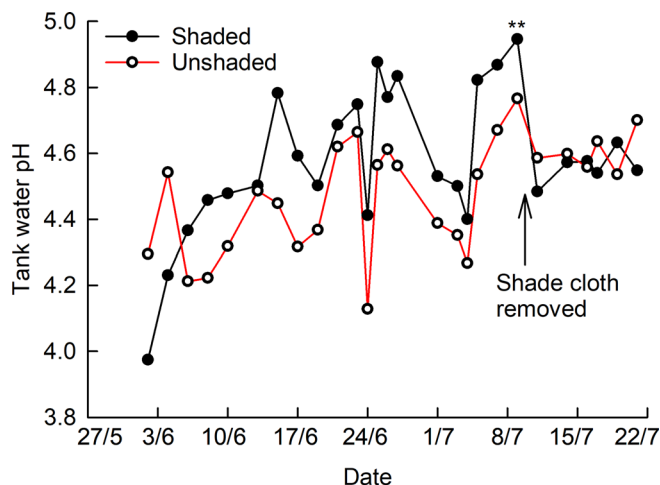


FIGURE 2 Changes in pH of tank water for 10 plants of *Guzmania monostachia* in a forest gap at La Selva Biological Station, Costa Rica, five shaded with shade cloth (black line) and five unshaded (red line); the significant difference in pH for the two sets (indicated by asterisks; $P < 0.001$) disappeared when the shade cloth was removed at week 5. Error bars are not shown for the sake of clarity; SE averaged less than 5% of the mean.

the shade cloth was removed from previously shaded plants on 9 July (Figure 2), the difference in pH between the two groups disappeared (t -test; $t = 1.172$, $df = 5$, $P = 0.293$).

Artificial tank experiment

To investigate the influence of the plants themselves on tank water pH, the tank contents of several plants of *G. monostachia* were removed, combined, and used to refill the tanks of four rinsed plants (Figure 3, black line) and four artificial tanks (Figure 3, red line) in an ambient lab at La

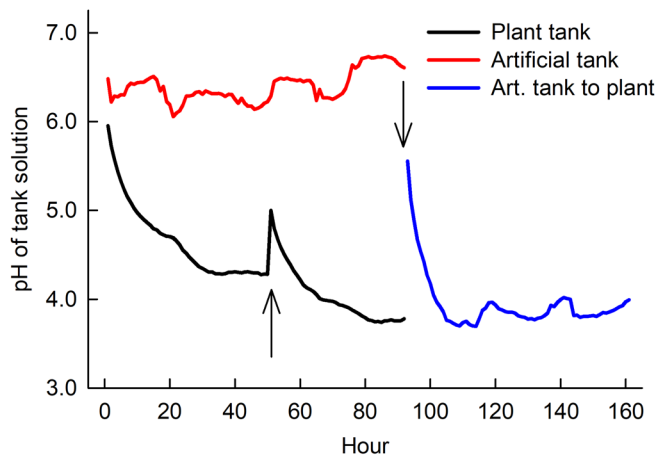


FIGURE 3 Changes in pH of tank water solution placed in four plants of *Guzmania monostachia* (black line) and four plastic artificial tanks (red line) in an ambient lab at La Selva Biological Station for 92 h, when the solution from artificial tanks was placed in four new plants (blue line). Upward arrow indicates when fresh solution was added to tanks to compensate for evaporation; downward arrow indicates shift of solution from artificial tanks to new plants. Lines represent mean values of pH; SE was less than 10% of the mean in all cases. Values of pH for solutions in plant tanks did not differ from each other ($P = 0.849$) and both were significantly different from pH of the solution in the artificial tanks ($P < 0.001$).

Selva. After 92 h, the contents of the artificial tanks were put into four additional rinsed plants of *G. monostachia* (Figure 3, blue line). Within a few hours of adding the tank solution to both the first and the second group of plants, the pH decreased, whereas the pH of the solution in the artificial tanks remained relatively unchanged (Figure 3). At the endpoint of each treatment, hour 92 for the first group of plants and the artificial tanks and hour 160 for the solution removed from the artificial tanks and put in the second group of plants, the pH was significantly lower for plants than for the artificial tanks ($N = 4$; one-way repeated measures ANOVA followed by Holm Sidak test, $F_{3,2} = 35.544$, $P < 0.001$).

Aquaporin transcription

Transcription of the previously identified aquaporin gene *GmPIP*, normalized relative to the reference gene *matK*, was higher for plants with tank water pH lower than 4.3 (Figure 4, light gray bars) than for those with pH higher than 5.4 (dark gray bars; $N = 3$; two-way ANOVA, $F_{1,1} = 8.877$, $P = 0.014$). Transcription of *GmPIP* was also higher for the leaf blade region just above the tank than for the tank leaf tissue ($N = 3$; two-way ANOVA, $F_{1,1} = 15.634$, $P = 0.003$). There was no significant interaction between pH and leaf region.

Leaf hydraulic conductance

Leaf hydraulic conductance (K_{leaf}) was measured for six plants of *G. monostachia* from an established greenhouse

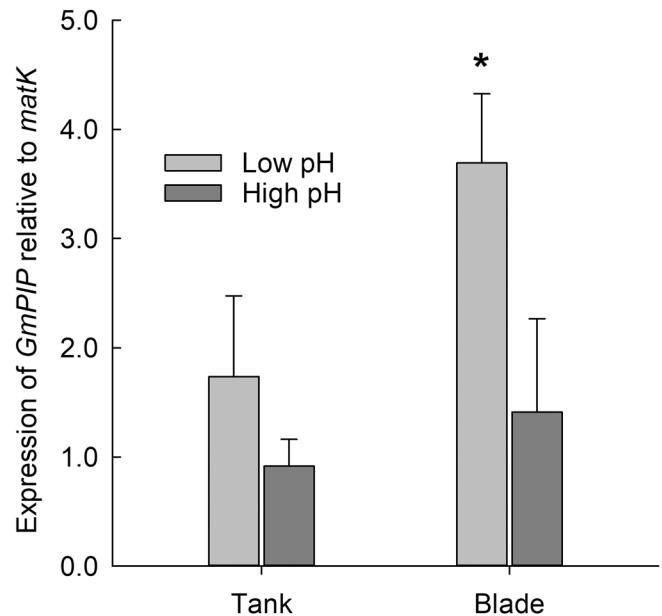


FIGURE 4 Expression of aquaporin gene *GmPIP* relative to reference gene *matK* for plants of *Guzmania monostachia* growing in the field at La Selva Biological Station. Low pH (light gray bars) indicates plants with tank water pH lower than 4.3 and high pH (dark gray bars) indicates pH higher than 5.4; data are means \pm SE for three biological replicates for the leaf blade region (the green region just above the tank) and the leaf tank region (the submerged leaf bases). Differences due to pH and leaf region were significant ($N = 3$; two-way ANOVA, $F_{1,1} = 8.877$, 15.634 ; $P = 0.014$ and 0.003 , respectively, with no interaction).

population at Occidental College (Figure 5). Plants were similar in size to those used for pH measurements at La Selva, and the pH of their tank water in the greenhouse was 4.6 ± 0.14 . Two adjacent mature leaves from each plant were used to measure K_{leaf} , one with its cut end immersed in solution of pH 4.0, one in pH 6.0. After 30 min under lights in the lab, K_{leaf} was measured using the transpirational flux method, which took about 40 min per leaf. There was no significant difference in K_{leaf} for leaves in solutions of different pH (Figure 5; $N = 6$; t -test; $t = -0.0521$, $df = 10$, $P = 0.96$).

Changes in CO₂ concentration

Diel changes in CO₂ concentration (Figure 6, blue line) and pH (black line) of the tank water were measured simultaneously using microelectrodes for two plants brought into the lab and put on a 12-h lights on/lights off schedule; because of background lighting in the lab, the lights-off period was not completely dark. Due to equipment limitations, plants were measured sequentially; data for only one set of measurements is shown (Figure 6), but the curves for both sets of measurements were similar. The three curves showed pronounced diel differences, with maxima and minima occurring in similar patterns for recorded pH and CO₂ concentration of the tank water. In contrast, the curve representing the pH calculated based on Henry's law and

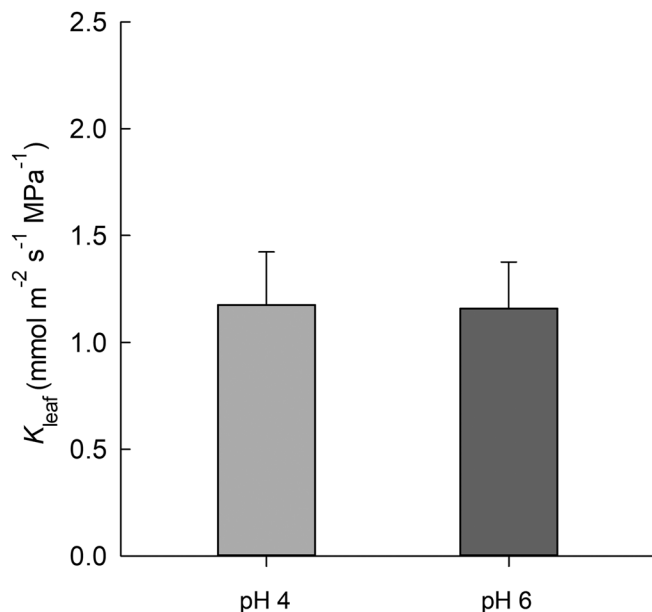


FIGURE 5 Leaf hydraulic conductance (K_{leaf}) for two leaves from each of six plants of greenhouse-grown *G. monostachia*, one placed in buffer solution at pH 4.0 (light gray bar) and one in pH 6.0 (dark gray bar). Data are means \pm SE; means did not differ significantly among treatment groups ($N = 6$; $P = 0.96$).

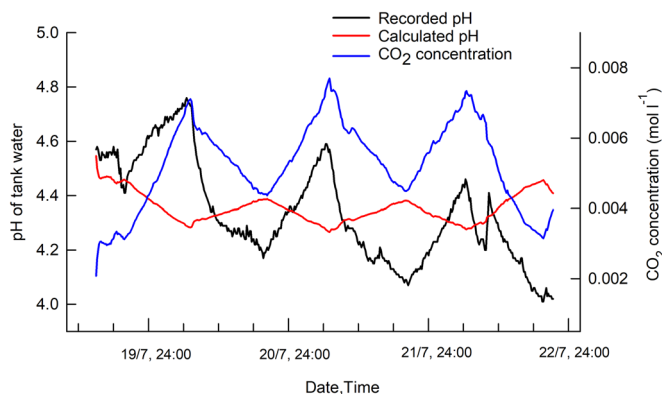


FIGURE 6 Changes in CO_2 concentration (blue line) and pH (black line) measured by microelectrodes in the lab, and pH calculated from CO_2 concentration for two plants of greenhouse-grown *G. monostachia* (curves shown are for one plant).

carbonate chemistry (Figure 6, red line) showed diel maxima when the measured pH and CO_2 concentration were lowest and minima when the other two measurements were highest (Figure 6), suggesting that the contribution of CO_2 concentration to measured pH of the tank water was negligible.

Proton pumps

The activity of proton pumps, specifically H^+ -ATPases in plasma membranes, in the alteration or maintenance of tank

water pH was investigated using a proton pump stimulator (Figure 7) and an inhibitor (Figure 8). The tank water of plants treated with the stimulator fusicoccin (FC) had an overall mean pH of 3.84 ± 0.05 ($N = 2$), while for control plants the mean pH was 4.82 ± 0.08 ($N = 2$). Changes in tank pH over 3 d indicated that the difference between FC-treated plants and control plants due to treatment was highly significant (Figure 7; Friedman repeated measures ANOVA on ranks, $q = 29.431$, $df = 1$, $P < 0.001$). Treatment with the proton pump inhibitor sodium orthovanadate had the opposite effect on tank water pH, with treated plants maintaining a higher pH (6.86 ± 0.24 , $N = 3$) than control plants (6.38 ± 0.13 , $N = 2$; Figure 8). Changes in tank pH for these two groups differed significantly over 4 d (Friedman repeated measures ANOVA on ranks, $q = 33.317$, $df = 1$, $P < 0.001$). Strong diel changes (seen in Figure 8) occurred during four cloudless days in the greenhouse. In contrast, the 3-day period when plants were treated with fusicoccin was cloudy, perhaps accounting for the lack of a pronounced diel pattern (Figure 7).

DISCUSSION

The range of pH measured in the tank water for 156 plants from eight common species of tank bromeliad at La Selva was 2.6–6.5, and the mean was 4.7 ± 0.06 , a value significantly lower than the volume averaged mean of 5.2 ± 0.35 for rainfall collected at La Selva during June and July, 1983 (Parker, 1994); importantly, although rainfall pH can vary seasonally at La Selva (Parker, 1994), the plants in the current study were also sampled in June. Mean tank water pH was higher than rainfall pH for only two of the eight species, *Aechmea mariae-reginae* and *Androlepis skinneri*. There were significant differences among the species, though only between the species with the lowest pH and those with the highest. Many explanations have been offered for the acidity of bromeliad tank water, most often citing acids released during microbial decomposition (Jocque and Kolby, 2012), the pH of leaf litter and other debris accumulated in the tank (Laessle, 1961), and carbonic acid buildup due to CO_2 produced in respiration by microbes and other occupants of the tank (Benzing et al., 1972). While these processes and others have been shown to lower pH (Benzing, 2008), our results with one species, *Guzmania monostachia*, suggest that the plants themselves are responsible for much of the tank water acidity. Moreover, our results also point to the activity of proton pumps in the leaves as a principal mechanism of tank water acidification.

The clearest indication that plants of *G. monostachia* regulated the pH of their tank water was the comparison between plant tanks and artificial tanks (Figure 3). When combined tank water was divided between rinsed plants and artificial containers, the pH of the solution in the plant tanks decreased to 4.6, about 75% of its initial value, within 24 h, whereas the pH of the solution in the artificial tanks remained unchanged. After 92 h, pH in the plant tanks was 3.8, or 63% of

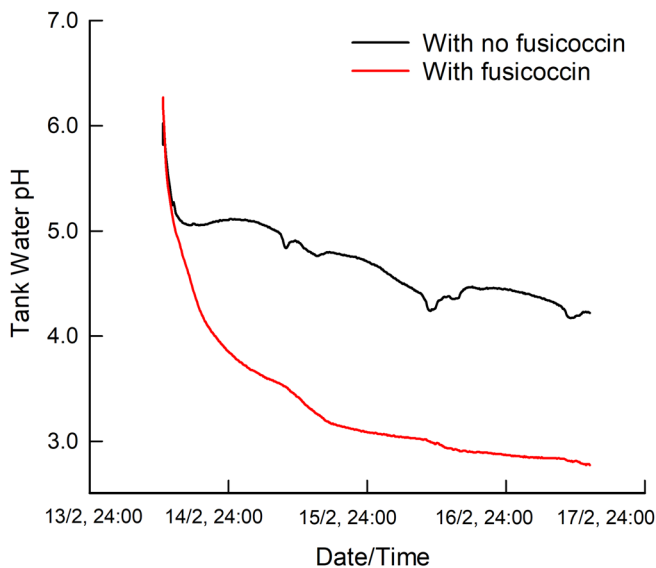


FIGURE 7 Tank water pH for two greenhouse-grown plants of *G. monostachia* with the proton pump stimulator fusicoccin added to the tank (red line) and for two plants with no fusicoccin added (black line). Values for the treatment groups were significantly different ($N = 2$, $P < 0.001$).

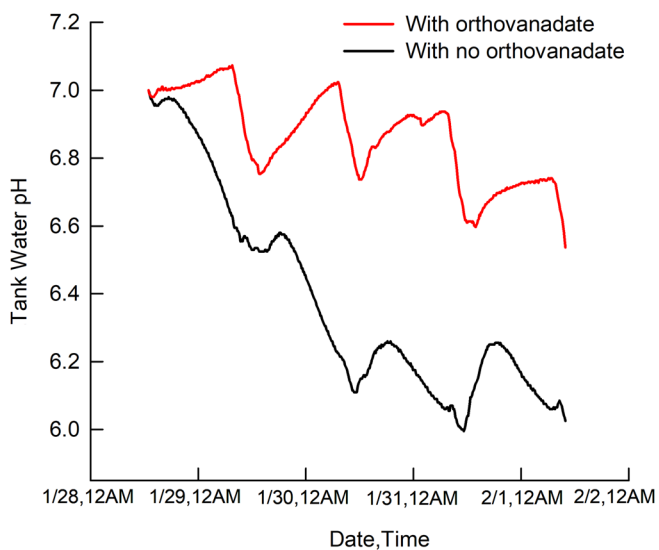


FIGURE 8 Tank water pH for three greenhouse-grown plants of *G. monostachia* with the proton pump inhibitor sodium vanadate added (red line) and for three plants with no orthovanadate added. Values for the treatment groups were significantly different ($N = 3$, $P < 0.001$).

its initial value, while the pH of the solution in the artificial tanks showed no decrease. These results are similar to those obtained in an experiment conducted in Brazil with plants of *Aechmea nudicaulis* and artificial containers (Lopez, Rios, 2001). In addition, our experiment showed an abrupt change in pH when the solution was removed from the artificial tanks and returned to plant tanks, decreasing from 5.6 to 3.9 within 24 h (Figure 3). The implication is that plant activity, not that of microbes or other tank constituents, was responsible for tank water acidification.

One suggested cause of low tank water pH in bromeliads has been carbonic acid buildup during plant respiration, previously investigated by several researchers, with mixed results. Early work with bromeliads in Jamaica found that the CO_2 concentration in tank water did increase at night, but found no consistent relationship between CO_2 levels and pH (Laessle, 1961). Work by Benzing et al. (1972) showed a similar pattern, with higher levels of CO_2 at night yet no inverse correlation between CO_2 concentrations and tank water pH. Our results with greenhouse-grown plants also demonstrate that high CO_2 was not the cause of low pH. On the contrary, microelectrode measurements over a 3-d period showed a positive, not negative, relationship between CO_2 concentrations and pH (Figure 6). As seen in previous studies, CO_2 was higher at night, when recorded pH values were also at their highest. According to our results, diel curves for the measured values of pH were roughly mirror images of those for pH values calculated based on CO_2 . Thus, there is no indication that plant production (or uptake) of CO_2 caused the changes in tank water pH that we observed.

Plants have been shown to acidify their surroundings in a number of ways, the best known of which are organic acid exudation and proton (H^+) extrusion, most often by roots. Proton extrusion can occur during root mineral uptake processes that maintain cation–anion balance at the root–soil interface (e.g., Hinsinger et al., 2003; Neumann and Römheld, 2012) and can also lower the pH of the soil solution to improve uptake of minerals in short supply, particularly phosphorus (Dakora and Phillips, 2002; Barrow, 2017). Aboveground, the best-known cases of proton extrusion by plants are by the trap leaves or pitchers of carnivorous plants (An et al., 2001; Moran et al., 2010), which utilize proton pumps, specifically, plasmalemma H^+ -ATPases, in the epidermal cells to produce a pH in the pitcher fluid that is favorable for the action of externally released digestive enzymes (Saganová et al., 2018). Fluid in the pitchers of certain *Nepenthes* species in Borneo has the same range of pH as that encountered in bromeliad tanks (Moran et al., 2010), one of the reasons we chose to investigate plasmalemma proton pumps in *G. monostachia*. Indeed, the activity of proton pumps was implicated by the rapid decline in pH of the tank water for plants treated with the proton pump stimulator fusicoccin (Figure 7) and the lack of pH decline in the plants treated with the H^+ -ATPase inhibitor sodium orthovanadate (Figure 8). We did not test the tank water for the presence of organic acids, so we cannot rule out their influence on changes in pH in this study. In this regard, H^+ -ATPases have been shown to function in organic acid exudation, including in soil acidification by the cluster roots of white lupin (Tomasi et al., 2009); by analogy, both H^+ and organic acids may be involved in lowering the pH of bromeliad tanks.

With respect to the diel pattern of pH (clearest in Figures 6 and 8), greater acid production occurred during daylight than at night, with the lowest pH tending to occur at the end of the light period and the highest pH at the end

of the dark period, early in the morning. These results complement the results of the shading experiment, in which the tank water pH of shaded plants in the field was higher than that of unshaded plants (Figure 2). A study on tank bromeliads in Brazil also found negative correlations between the pH of tank water and the amount of light received (Guimarães-Souza et al., 2006). Tank bromeliads in Ecuador had lower pH during the day than at night, but the authors attributed this difference to temperature (Jocque and Kolby, 2012). The pH values reported in our study were all temperature-corrected; thus, we suggest that light was the responsible environmental variable. It is tempting to suggest that high light helps to provide the energy needed to drive the activity of proton pumps in the bromeliad leaf bases. This suggestion is supported by research showing that proton pumps in leaf discs from *Vicia faba* are stimulated by light (Petzold and Dahse, 1988; Shabala and Newman, 1999). In the experiments on *V. faba* discs, the epidermis was removed; when present, it prevented proton extrusion, as did the cuticle in *Olea europaea* (Rinaldelli and Bandinelli, 1999). The proton extrusion shown here for *G. monostachia* was for intact leaves in natural conditions, similar to the studies on *Nepenthes* and other carnivorous plants.

With respect to possible consequences of low pH in the tanks, we investigated its effect on leaf water uptake in two ways, by comparing aquaporin transcription in plants with contrasting values of pH and by measuring leaf hydraulic conductance for leaves fed with solutions of pH 4.0 versus 6.0. Typically, low pH such as that associated with anoxic bathing solutions reduces aquaporin activity (reviewed by Kapilan et al., 2018), most frequently shown in roots but also in leaves (Martre et al., 2002; Parent et al., 2009). In a previous study on *G. monostachia*, aquaporins were shown to facilitate leaf hydraulic conductance, as indicated by a reduction in K_{leaf} when aquaporin activity was reduced by the addition of mercuric chloride (North et al., 2019). In the current study, leaf hydraulic conductance did not differ according to the pH of the bathing solution, suggesting that aquaporin-mediated uptake was not affected by external pH, or that at least some aquaporins present were pH-insensitive. Interestingly, in a study of pH effects on root hydraulic conductance, a mutated aquaporin isoform was shown to be both less sensitive to pH and less efficient in water transport (Tournaire-Roux et al., 2003). Plants of *G. monostachia* in the field with tank water pH lower than 4.3 had significantly higher expression of the aquaporin gene *GmPIP* than did plants with pH higher than 5.4. One possible explanation for this difference is that lower pH stimulated *G. monostachia* to produce more aquaporin transcripts as compensation for reduced efficiency, as found for plants exposed to other abiotic stresses such as low temperature (Lee et al., 2012). However, because we did not measure *GmPIP* protein accumulation or activity, a more conservative interpretation is that higher transcription of *GmPIP* in plants with lower tank water pH may represent a response to other, unmeasured factors. In addition, PIP

aquaporins are a large and varied group (Mercier et al., 2019), and we measured transcription for only a subset of them.

Water may be the chief limiting factor for tropical epiphytes such as bromeliads (Zotz and Bader, 2009; Males, 2016), particularly as episodes of drought increase in frequency and duration due to global warming (Colwell et al., 2008; Corlett, 2016), but the relative scarcity of mineral nutrients in the canopy also requires special adaptations (Zotz, 2016). For example, aquaporins in the leaves of the bromeliads *Vriesea gigantea* and *G. monostachia* can take up urea and other organic forms of nitrogen from the tank (Matiz et al., 2019; Gonçalves and Mercier, 2021), and several species have been shown to obtain mineral N through their association with decomposing microbes and N-fixing cyanobacteria (Bermudes and Benzing, 1991; Inselsbacher et al., 2007). Phosphorus may be even more limiting than N for tank bromeliads, which have numerous biochemical adaptations to improve P-uptake and storage (Winkler and Zotz, 2009). Another adaptation to low nutrients may be the ability of tank bromeliads to acidify their tank contents to create an environment favorable to the activity of nutrient-releasing enzymes in resident bacteria and fungi, as occurs in the rhizosphere in P-deficient soils (Richardson et al., 2011). The energy cost of acidifying the tank water via the activity of proton pumps could help to explain a functional association between higher light and lower pH if low pH contributes to greater nutrient uptake by the tank bromeliads when resource demands are high.

CONCLUSIONS

This survey of eight species of tank bromeliads in a neotropical forest showed that their tank water pH was lower on average than that of the local rainwater. Based on results with the widespread species *Guzmania monostachia*, low pH was due to plant activity, specifically, to plasma-lemma proton pumps in the leaves. Shading experiments and diel patterns of acidity showed that pH became lower under higher light conditions, and decreases in CO₂ concentration in the tank water were not responsible for the measured decrease in pH. Applications of a proton pump stimulator and inhibitor indicated that H⁺-ATPases were involved in tank water acidification. Low pH was associated with higher aquaporin expression by the leaves yet had no effect on leaf hydraulic conductance, suggesting that acidification had little to do with water uptake. Future studies should explore the interplay between pH and nutrient absorption, not only for tank bromeliads but for epiphytes in general and other plants that perform foliar nutrient uptake.

AUTHOR CONTRIBUTIONS

G.N. was involved in conceptualization, data curation, formal analysis, funding acquisition, methodology, project administration, and writing; E.B. was involved in

conceptualization, formal analysis, investigation, methodology, and writing; K.F., T.K., F.M., A.F., M.R., and J.S. were involved in formal analysis, investigation, and methodology; and all authors were involved in writing.

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DATA AVAILABILITY STATEMENT

Raw data for this study are deposited in the public repository FigShare under the collection “Acid waters in tank bromeliads: causes and potential consequences” (<https://doi.org/10.6084/m9.figshare.c.6275019.v1>).

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