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RESEARCH ARTICLE

Replicated radiations in the South American marsh pitcher plants (*Heliamphora*) lead to convergent carnivorous trap morphologies

Sukuan Liu | **Stacey D. Smith**

Department of Ecology and Evolutionary Biology, University of Colorado Boulder, 1900 Pleasant Street, Boulder, Colorado 80309, USA

Correspondence

Sukuan Liu, Department of Ecology and Evolutionary Biology, University of Colorado Boulder, 1900 Pleasant Street, Boulder, Colorado 80309, USA.

Email: sukuan.liu@colorado.edu

Abstract

Premise: The evolution of carnivorous pitcher traps across multiple angiosperm lineages represents a classic example of morphological convergence. Nevertheless, no comparative study to-date has examined pitcher evolution from a quantitative morphometric perspective.

Methods: In the present study, we used comparative morphometric approaches to quantify the shape space occupied by *Heliamphora* pitchers and to trace evolutionary trajectories through this space to examine patterns of divergence and convergence within the genus. We also investigated pitcher development, and, how the packing of pitchers is affected by crowding, a common condition in their natural environments.

Results: Our results showed that *Heliamphora* pitchers have diverged along three main axes in morphospace: (1) pitcher curvature; (2) nectar spoon elaboration; and (3) pitcher stoutness. Both curvature and stoutness are correlated with pitcher size, suggesting structural constraints in pitcher morphological evolution. Among the four traits (curvature, spoon elaboration, stoutness, and size), all but curvature lacked phylogenetic signal and showed marked convergence across the phylogeny. We also observed tighter packing of pitchers in crowded conditions, and this effect was most pronounced in curved, slender pitchers.

Conclusions: Overall, our study demonstrates that diversification and convergent evolution of carnivory-related traits extends to finer evolutionary timescales, reinforcing the notion that ecological specialization may not necessarily be an evolutionary dead end.

KEYWORDS

carnivorous plants, morphological evolution, phylogenetic comparative methods, pitcher plants, Sarraceniaceae

摘要

食虫植物的瓶状捕虫器在不同被子植物谱系中的演化是形态趋同进化的经典案例。然而，迄今为止还没有研究使用系统发育比较分析法与计量方法来研究瓶状捕虫器的演化。本研究使用了上述手段来研究南美沼泽瓶子草（*Heliamphora*）的瓶状捕虫器是如何占据形态空间，并在捕虫器的形态空间内追踪它们的演化轨迹，以研究沼泽瓶子草属捕虫器的趋异与趋同的演化模式。同时，本研究还探讨了沼泽瓶子草属植株 (捕虫瓶) 的发育模式，特别是在沼泽瓶子草在原生地的发育是如何受到种群密度过高 (拥挤) 的影响。本研究的结果表明，沼泽瓶子草属捕虫瓶在形态空间里沿着三个主要形态轴分化:捕虫瓶的弯曲度、蜜匙的精细程度和捕虫瓶的粗壮程度。捕虫瓶的弯曲度和粗壮度均与瓶子的大小相关，表明捕虫瓶在形态进化中存在结构约束。在这四个形态特征中 (捕虫瓶曲率、蜜匙精细程度、捕虫瓶粗壮度与大小)，除了捕虫瓶曲率，其他的形态特征都没有显示系统发育信号。在南美沼泽瓶子草属的进化中，蜜匙精细程度、捕虫瓶粗壮度与大小都显示了趋同演化。本研究还阐明了瓶子草植株在拥挤条件下，捕虫瓶会更加紧密地排列，而且这种效应在有着弯曲、细长的捕虫瓶的物种中最为显著。总体来说，本研究显示与食肉有关的特征的趋异和趋同演化发生在更微观的时间尺度上，也表明了生态专化或许并不是生物进化的死胡同。

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Pitcher plants are one of the most dramatic examples of convergent evolution in carnivorous plants, with at least 6 independent origins in angiosperms (Fleischmann et al., 2018). The pitchers are specialized leaves that form cavities filled with an often-viscous liquid containing a suite of digestive enzymes (Adlassnig et al., 2011). Pitcher plants utilize these pitchers to attract, drown, and consume small animal prey in order to obtain nutrients not available from the abiotic environment (Juniper et al., 1989; Givnish et al., 2018).

Among the independently evolved pitcher plant lineages, *Nepenthes* (Nepenthaceae, Caryophyllales), Sarraceniaceae (Ericales), and *Cephalotus follicularis* Labill. (Cephalotaceae, Oxalidales) are commonly considered as the true pitcher plants due to their highly specialized carnivorous pitchers. Moreover, the true pitcher plants are considerably older (all emerged during the Cretaceous) as compared to the more recently evolved (<3 mya) pitcher lineages in Poales (e.g., *Brocchinia reducta* Baker [Bromeliaceae] and *Paepalanthus bromelioides* Silv. [Eriocaulaceae]), which have structurally simpler pitchers morphologically similar to their tank-forming relatives (Magallón et al., 2015; Cross et al., 2018). Except for the monotypic Australian pitcher plant *Cephalotus follicularis*, *Nepenthes* (the Old-World tropical pitcher plants) and Sarraceniaceae (the American pitcher plants) both represent true pitcher lineages with high species diversity, together comprising more than 98% of all extent pitcher plant taxa (McPherson and Schnell, 2011; McPherson et al., 2011; Fleischmann et al., 2018; Murphy et al., 2020).

In addition to dispersal and vicariance (Ellison et al., 2012; Naczi, 2018; Murphy et al., 2020), adaptive radiation played an important role in the diversification of *Nepenthes* and Sarraceniaceae (Pavlovič, 2012; Clarke et al., 2018; Thorogood et al., 2018). The emergence of pitcher traps has likely facilitated the diversification in *Nepenthes* and Sarraceniaceae by allowing them to adapt to new ecological niches, resulting in dramatic morphological variations in pitcher traps (Pavlovič, 2012; Clarke et al., 2018; Thorogood et al., 2018). Many *Nepenthes* species have diversified and further modified their pitcher traps to specialize in novel symbiotic relationships (e.g., ant-mutualism in *N. bicalcarata* Hook.f.) or novel nutrient acquisition strategies (e.g., detritus-feeding in *N. ampullaria* Jack, fecal-feeding in *N. lowii* Hook.f., and subterranean-feeding in *N. pudica* Dančák & Majeský) (Moran et al., 2012; Thornham et al., 2012; Dančák et al., 2022; Gilbert et al., 2022). A similar radiation has occurred across Sarraceniaceae lineages, giving rise to many species with novel trap types (e.g., lobster traps of *Darlingtonia californica* Torr. and *Sarracenia psittacina* Michx.), trapping mechanisms (e.g., superhydrophilic hairy surface of *Heliamphora nutans* Benth.), symbiotic relationship (e.g., *S. purpurea* L. and the commensal mosquito *Wyeomyia smithii* Coquillett), and nutrient specializations (Jaffe et al., 1992; Peterson et al., 2008; McPherson et al., 2011; Bauer et al., 2013; Naczi, 2018). Pitcher morphology in Sarraceniaceae and *Nepenthes* is also plastic to varying degree, affected by both biotic (e.g., crowding or competition from neighboring plants) and abiotic factors (e.g., extreme microclimate) (Brewer, 1999; McPherson and Schnell, 2011; McPherson et al., 2011; Clarke et al., 2018).

Despite the wide variety of morphologies associated with pitcher traps, few comparative studies have focused on the morphological evolution of carnivorous traps, and none with quantitative methods. Morphometric analyses allow us to define the spaces occupied by phenotypic combinations as well as those that are empty (Stayton, 2019; Segall et al., 2020; Jardine et al., 2022) and, in the context of well-supported phylogenies (Givnish et al., 1997), identify instances of morphological convergence (Papadopulos et al., 2013; Smith and Kriebel, 2018). This quantitative shape information also sets the stage for rigorous testing of ecological and evolutionary hypotheses (Smith and Kriebel, 2018; Dellinger et al., 2019; Kriebel et al., 2022).

Here we focus on the evolution of pitcher morphologies in the South American marsh pitcher plants *Heliamphora* (Sarraceniaceae). This genus is endemic to the Guiana Highlands and is the most species-rich genus in the family, with 23 extant species and several yet to be described (McPherson et al., 2011). The major clades are thought to have emerged through both vicariance and dispersal during the Miocene with more recent diversification in each clade driven by vertical displacement during the Pleistocene glacial-interglacial cycles (Liu and Smith, 2021). The adult pitchers vary dramatically in pitcher shape, size, nectar spoon structure at the apex of the pitcher, and many other taxonomically important characters across species (Figure 1) (McPherson et al., 2011). It has been suggested these diverse morphologies may relate to differences in microclimate (Nerz, 2004), prey attraction strategies (Wistuba et al., 2001, 2002), and/or shifts in prey composition (Jaffe et al., 1992). Furthermore, structural stability of the pitcher could impose constraints on the morphological evolution of *Heliamphora* pitchers, limiting available pitcher morphospace.

In the present study, we used comparative morphometric approaches to quantify the shape space occupied by *Heliamphora* pitchers and to trace evolutionary trajectories through this space. Building on previous phylogenetic work in *Heliamphora* (Liu and Smith, 2021), we also tested for phylogenetic signal in pitcher shape and investigated how pitcher-shape evolution may be related to pitcher size. In particular, we hypothesize that aspects of shape evolution may be tightly correlated with height given the structural constraints imposed by supporting the high volume of pitcher fluid. Considering the dense packing in many natural populations (McPherson et al., 2011), we also considered how pitcher development responded to crowding and tested the hypothesis that pitcher angle (the angle between two most recently developed pitchers) shrinks in crowded conditions to allow for tighter packing. In interpreting these results, we discuss the potential ecological significance of diverse pitcher morphologies in *Heliamphora* and its ability to modify pitcher development in response to varying conditions.

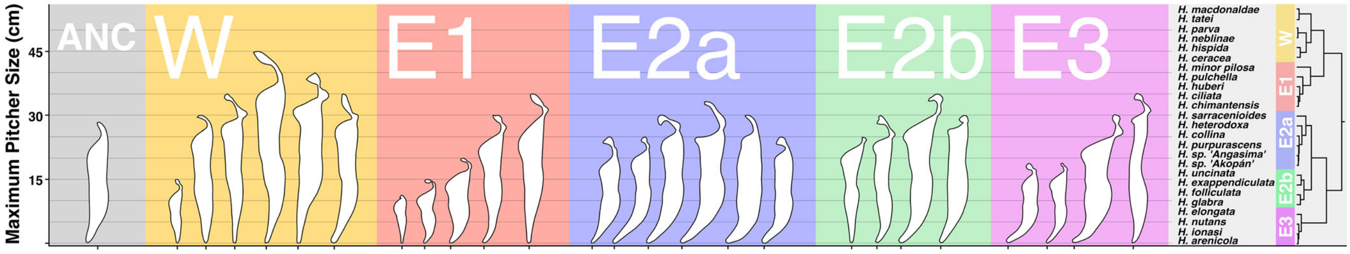

FIGURE 1 Left: Adult pitcher morphologies of the reconstructed ancestral *Heliamphora* (ANC) and extant species grouped by clades. Right: Phylogeny of *Heliamphora* adapted and modified from Liu and Smith, 2021. Taxa names in each clade are ordered aesthetically from left to right. **W:** *H. hispida*, *H. ceracea*, *H. tatei*, *H. neblinae*, *H. macdonaldae*, and *H. parva*. **E1:** *H. pulchella*, *H. minor var. pilosa* A. Fleischm., A. & J. R. Grande, *H. ciliata* Wistuba, Nerz & A. Fleischm., *H. huberi* A. Fleischm., Wistuba & Nerz, and *H. chimantensis*. **E2a:** *H. heterodoxa* Steyerm., *H. collina* Wistuba, Nerz, S. McPherson & A. Fleischm., *H. sp.* "Angasima", *H. sp.* "Akopán", *H. sarracenioides* Carow, Wistuba & Harbarth, and *H. purpurascens* Wistuba, A. Fleischm., Nerz & S. McPherson. **E2b:** *H. exappendiculata* (Maguire & Steyerm.) Nerz & Wistuba, *H. glabra*, *H. uncinata* Nerz, Wistuba & A. Fleischm., and *H. folliculata*. **E3:** *H. nutans*, *H. arenicola*, *H. ionasi*, and *H. elongata* Nerz. The ancestral *Heliamphora* pitcher morphology was generated based on estimated ancestral values for each of the three shape PCs and the estimated ancestral size and maximum pitcher size. Note that the ancestral pitcher is based on the expected values at the root, which carry a degree of uncertainty.

MATERIALS AND METHODS

Taxon sampling and phylogenetic framework

We sampled 23 described and 2 additional yet-to-be described taxa of *Heliamphora* for this study (Appendix S1, Table S1). All specimens were grown under controlled environmental conditions [photoperiod 15 hr per day (full sun); temperature range 12–25°C; relative humidity >80%], resembling the climates of their native habitats (Jaffe et al., 1992; Adlassnig et al., 2010). Species determinations of the living collections were confirmed by comparing their morphological traits to the original descriptions (Bentham, 1840; Nerz and Wistuba, 2000; Carow et al., 2005; Fleischmann et al., 2009; McPherson et al., 2011).

We used the recently published phylogeny of 24 of the 25 known *Heliamphora* taxa (Liu and Smith, 2021) to reconstruct the evolution of adult pitcher shape, maximum adult pitcher size, and pitcher angle. This phylogeny lacks *H. macdonaldae* Gleason, which is thought to be sister to *H. tatei* Gleason based on similarities in pitcher morphology and distributional range (Maguire, 1978; McPherson et al., 2011; Liu and Smith, 2021). We manually included this species in the tree (Figure 1) by placing it sister to *H. tatei* and setting their split to 0.75 mya, which is the average node depth of other sister pairs in the W clade lineage (Figure 1), i.e., (*H. ceracea* Nerz, Wistuba, Grantsau, Rivadavia, A. Fleischm. & S. McPherson – *H. hispida* Nerz & Wistuba) and (*H. neblinae* Maguire – *H. parva* (Maguire) S. McPherson, A. Fleischm., Wistuba & Nerz).

Morphometric analysis of pitcher shapes

Data for maximum pitcher size (i.e., the maximum length of adult pitchers observed growing under optimal conditions, in situ) were obtained from the monograph of *Heliamphora* by McPherson et al. (2011). Adult pitcher shape was characterized using images of fully opened and matured pitchers taken from living individuals. For each taxon, a single adult pitcher from the individual sampled for the phylogeny was photographed in a lateral view, providing a two-dimensional representation of the adult pitcher shape along the proximal-distal axis. The lateral view captured shape variation among major structural components involved in carnivorous functions, such as the shapes of nectar spoon and pitcher body associated with prey attraction and prey capture/retention, respectively (Figure 2). These images were converted into silhouettes in Affinity Designer version 1.10.5 (https://affinity.serif.com/designer), which were then transformed into outlines using the R package Momocs (Bonhomme et al., 2014). Also using Momocs, the outlines were converted into lists of two-dimensional coordinates describing the polygons and then centered.

A set of six landmarks were place on each outline to delineate four pitcher functional zones (Figure 2) according to McPherson et al. (2011). In *Heliamphora* pitchers, Zone 1 (landmarks 2-4) corresponds to the nectar spoon, which is responsible for attracting prey. Zone 2 (landmarks 1, 2, 4, 5) represents the upper section of the pitcher above the narrow band and plays a role in prey capture and retention. Zone 3 (landmarks 1, 5) consists of a narrow restriction of the pitcher interior, located close to the waterline of the pitcher fluid. The position of the drainage hole (indicated by landmark 1; the drainage hole is further adapted into a drainage slit in the E1 clade) determines the maximal level of pitcher fluid in the pitcher (landmarks 1, 5), which is crucial in maintaining carnivorous and other ecological functions in *Heliamphora* (see Discussion). Zone 4 (landmarks 1, 5, 6) comprises the bottom part of the pitchers and is involved in prey digestion as well as nutrient absorption. Despite morphological diversity, these functional zones are homologous and easily identifiable across *Heliamphora* species, making them reliable reference points for the landmarks and preventing any distortion of the outlines.

Using the landmarks, shape variation of adult pitchers was quantified using elliptical Fourier analysis (EFA) that decomposed the outlines into 21 harmonics, each with four coefficients. After aligning the outlines using Full

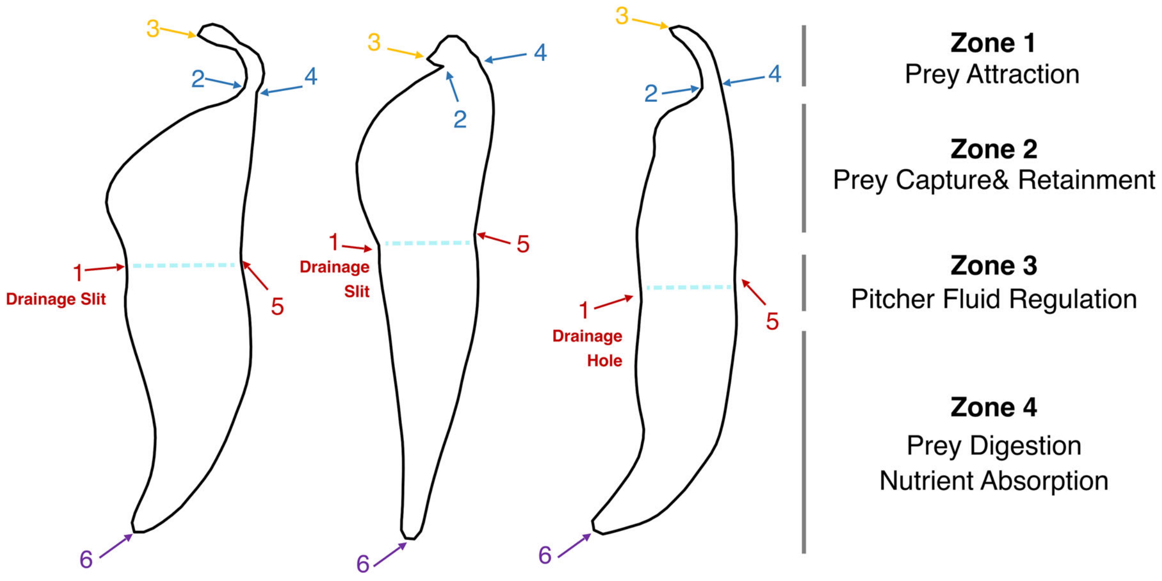

FIGURE 2 Landmarking examples of three *Heliamphora* species: *H. ciliata* (left), *H. pulchella* (middle) and *H. sarracenioides* (right). The adult pitcher functional zones (McPherson et al., 2011) were delineated by six landmarks as indicated in the text. Morphologically, *Heliamphora* pitchers are similar to those of *Sarracenia* and *Darlingtonia*, except that they lack a covering at the pitcher openings to prevent rainwater from overfilling the pitchers. Instead, *Heliamphora* evolved the drainage hole (further modified into drainage slit in E1 clade, indicated by landmark 1) to allow rainwater to accumulate in the pitcher (thus maintaining a high level of pitcher fluid, with maximum fluid level shown with the dotted blue line and roughly align with landmark 1 and 5) while letting the excess to drain out and prevent the pitchers from becoming overfilled and unstable (Bauer et al., 2013).

Generalized Procustes alignment in Momocs (Bonhomme et al., 2014), a principal component analysis (PCA) was used to summarize the 84 coefficients resulting from the EFA. Taxa were plotted along the PC axes to illustrate their distribution in morphospace. Blomberg's K (Blomberg et al., 2003) for the shape PCs (PC1, PC2, and PC3) and adult pitcher size were estimated to infer phylogenetic signals in pitcher shapes and size by using 1000 randomizations of the data across the phylogeny to test if the value was significantly different from $K = 0$ (no phylogenetic signal).

Finally, we used ancestral state estimation to visualize the expected ancestral pitcher shape in *Heliamphora*. We estimated the ancestral values for each of the three shape PCs and for maximum pitcher size using the 'fastAnc' function in R package phytools (Revell, 2012). We visualized the reconstructed shape given the estimated ancestral PC values using Momocs and scaled the size of the pitcher based on the estimated ancestral size.

Effects of crowding on pitcher angle

We used living collections of *Heliamphora* species to characterize the angle of pitcher emergence and examine the effect of crowding on this angle. Depending on the availability of material, we sampled 3 to 63 individuals per taxon for all 25 taxa. These individuals were sampled from our collections and those of G. Pipis (Aurora, Colorado, USA); in possessing adult pitchers, these plants were three or more years of age. For each sampled individual, images were taken directly above each growing point, i.e., where new pitchers are constantly produced (Appendix S2, Figure S1). For each growing point, a line was drawn from the growing point (rosette center) to the tip of the nectar spoon of the youngest (the most recently fully developed) pitcher. Similarity, a second line was drawn for the second youngest pitcher. Pitcher angle (PA) was measured as the angle between the two straight lines using the software ImageJ (Schneider et al., 2012). We replicated these measurements for both juvenile and adult pitchers, since pitchers are dimorphic in *Heliamphora* (McPherson et al., 2011) and may respond differently to crowding. To test whether PA is affected by the presence of neighboring individuals (i.e., if crowding reduces PA), phylogenetic paired t-tests were performed for both adult and juvenile pitchers using the 'phyl.pairedttest' function in R package phytools (Revell, 2012). Similarly, the effect of developmental stage on PA was also tested with phylogenetic paired t-tests. In addition to phylogenetic t-tests, regular t-tests were also conducted for each comparison. An additional chi-squared test was performed to test whether the drastic bending observed in juvenile pitchers was associated with the presence of neighbors. Like the PCs, we computed Blomberg's K for PA in adult and juvenile individuals growing with and without neighbors, and then tested if the value was significantly different from zero.

Model testing for correlations among morphological traits

We used phylogenetic generalized linear models (PGLM) (Paradis and Claude, 2002) to test the relationship between shape variation and maximum pitcher size in adult pitchers. For some combinations of shape PCs and size, we predicted a non-linear relationship. Thus, we compared linear and quadratic models for each pair and present results for the best-fitting model (that with the lowest AIC score). We fit these models using the R package 'phylolm' with 1000 bootstraps (Ho and Ane, 2014). We assumed a correlation structure based on the Brownian motion (BM) process, which models stochastic evolution in those traits. Similarly, the relationships between shape variation and pitcher angle were also tested using PGLM in adult pitchers growing singly and ones growing with neighbors present. Assuming BM process, the PCs were regressed against pitcher angle with PGLM. Taxa with incomplete pitcher angle data due to sampling limitations were dropped from the analysis.

RESULTS

Morphospace of *Heliamphora* pitchers

The PCA of harmonic coefficients from the elliptic Fourier analysis captured 79% of shape variation (SV) of adult pitchers in the first three PCs (Figure 3). PC1, accounting for 33.4% of SV, describes pitcher body curvature or concavity. While the negative and positive PC1 values described concave and convex pitcher shapes, respectively, PC1 values closer to zero represented pitchers with straighter bodies. PC2, comprising 29.5% of SV, describes the elaboration (shape, relative size, and position) of nectar spoon with negative and positive values representing erect (attached) and elaborated structures, respectively. PC3, explaining 16.1% of SV, is related to pitcher stoutness, or the general pitcher body length-to-width ratio. On the PC3 axis, negative values described more slender pitcher body shapes whereas positive values corresponded to bulkier body shapes.

All of the measured pitcher traits, except for curvature, lacked a significant phylogenetic signal. Specifically, we found Blomberg's K was not significantly different from zero for nectar spoon elaboration (PC2, K = 0.06, p = 0.886), pitcher stoutness (PC3, K = 0.09, p = 0.391), or maximum pitcher size (K = 0.12, p = 0.198), consistent with the frequent patterns of convergence across the phylogeny (Figure 4). By contrast, significant phylogenetic signal was detected in pitcher curvature (PC1, K = 0.18, p = 0.018) (Figure 4). Looking at the phylogeny, pitcher curvature appears to be conserved in the W + E1 and E2a clades but not in other lineages. In the W + E1 clade, all species were associated with convex pitcher bodies to varying degrees (PC1 > 0.02), except for *H. chimantensis* Wistuba, Carow & Harbarth, which was associated with a straighter pitcher body (PC1 = −0.00321). In E2a, all adult pitchers were associated with very concave pitcher body shapes (PC1 < −0.04).

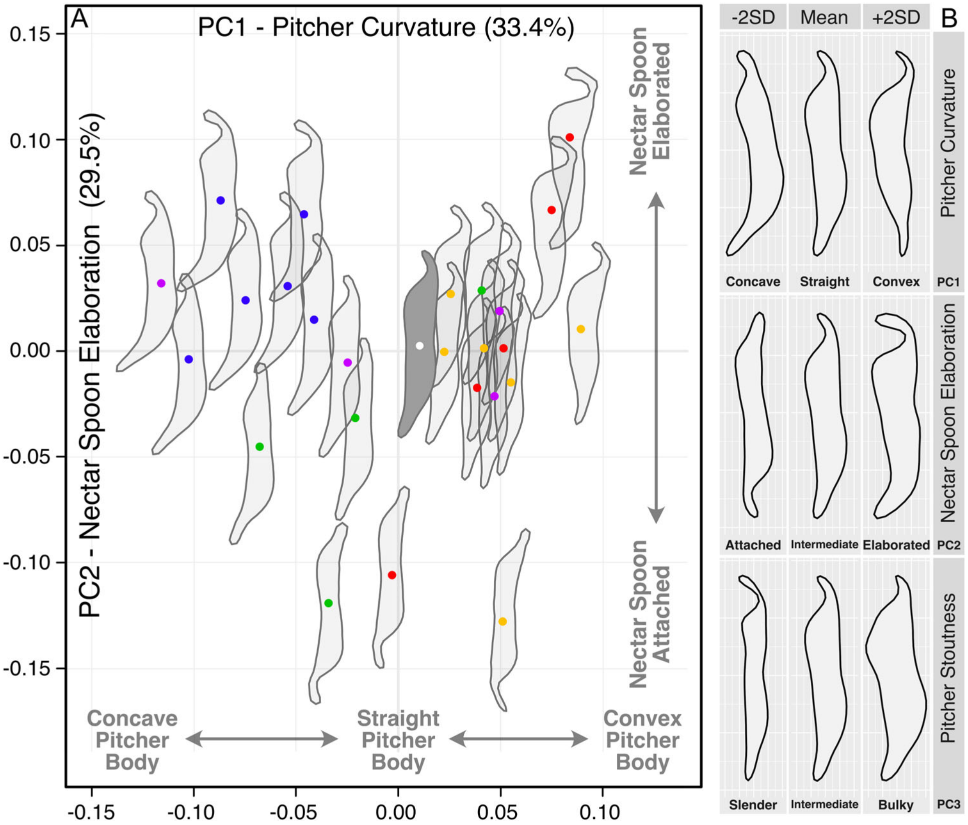

FIGURE 3 (A) Adult pitcher morphospace of pitcher curvature (PC1) and nectar spoon elaboration (PC2) with outlines of each taxon and the reconstructed ancestral *Heliamphora* adult pitcher (darker gray). Taxa are noted by clades by colored circles: W (orange); E1 (red); E2a (blue); E2b (green); and E3 (purple). (B) Share variation along PC axes. For each of the first three PCs, the mean is shown along with shapes corresponding to ±2 standard deviation (SD). +2 SD was chosen to better visualize the morphological variation along shape PC axes.

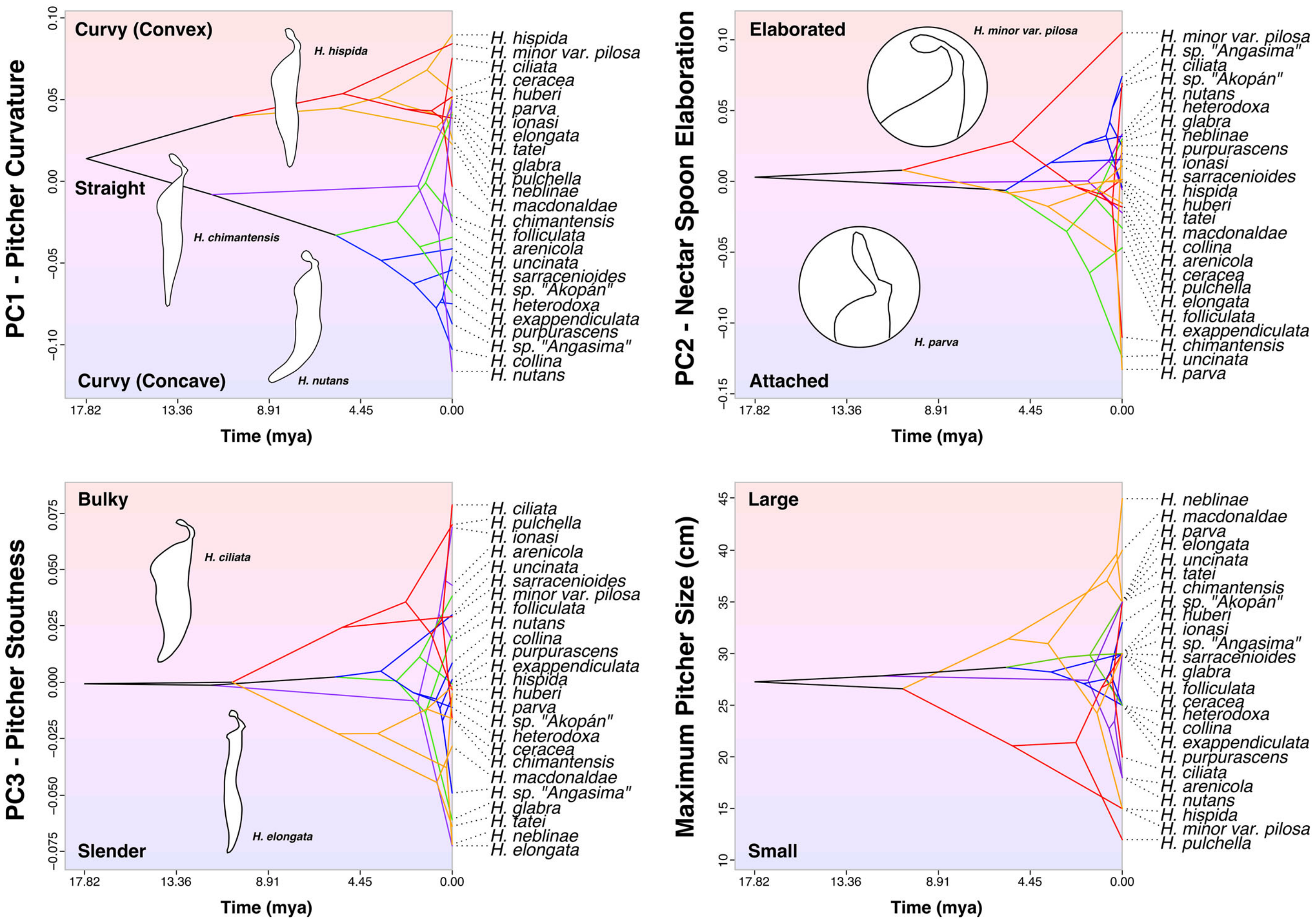

FIGURE 4 Traitgrams of pitcher curvature (PC1), nectar spoon elaboration (PC2), pitcher stoutness (PC3), and maximum pitcher size. Branches are colored by clades. Examples of pitcher and nectar spoon morphologies are shown in the traitgrams. Colors as in Figure 3.

Relationships between pitcher shape and size

Model comparisons indicated a strong quadratic relationship between pitcher curvature (PC1) and maximum pitcher size (Appendix S1, Table S2). As pitchers becomes straighter (i.e., less concave or convex), the maximum pitcher size generally increases (Figure 5), consistent with the expectation that more erect pitchers can accommodate more pitcher fluid (see discussion). A strong linear relationship was found between pitcher stoutness (PC3) and maximum pitcher size, with larger pitchers being stouter (Figure 5). No relationship was found between nectar spoon elaboration (PC2) and maximum pitcher size (Figure 5).

Relationship of pitcher angle to growth conditions and pitcher shape

Our results show that *Heliamphora* growing in crowded conditions tend to put out new pitchers at a tighter angle than those in isolated conditions. A significant difference in pitcher angle (PA) was detected between adult pitchers growing in isolated vs. crowded conditions with both phylogenetic and non-phylogenetic t-tests (phylogenetic mean difference [PMD] = 13.64°, $p = 0.002$; $p < 0.0001$ for regular t-test; Figure 6). In juveniles, a significant difference was detected in regular t-test ($p < 0.0001$) but not in the phylogenetic t-test (PMD = 6.36°, $p = 0.199$). Moreover, we found juvenile pitchers tend to bend unnaturally when their growth was obstructed by neighboring pitchers (chi-squared test, $p < 0.001$), possibly due to the more slender and parallel-to-ground body shape. No significant difference in PA was found between juvenile and adult pitchers within each condition (crowded or not) using either t-test (Figure 6). Raw measurements for all taxa, stages and conditions are shown in Appendix S2, Figure S2. No phylogenetic signal was detected in PA across developmental stages and crowding conditions ($K = 0.12$, $p = 0.481$ [juvenile, not crowded]; $K = 0.11$, $p = 0.353$ [juvenile, crowded]; $K = 0.10$, $p = 0.494$ [adult, not crowded], $K = 0.09$, $p = 0.597$ [adult, crowded]).

To investigate the association between pitcher shape and PA, similar PGLM analyses were performed for each crowding condition using data for adult pitchers. The analyses indicated a strong quadratic relationship between pitcher curvature and pitcher angle only in crowded

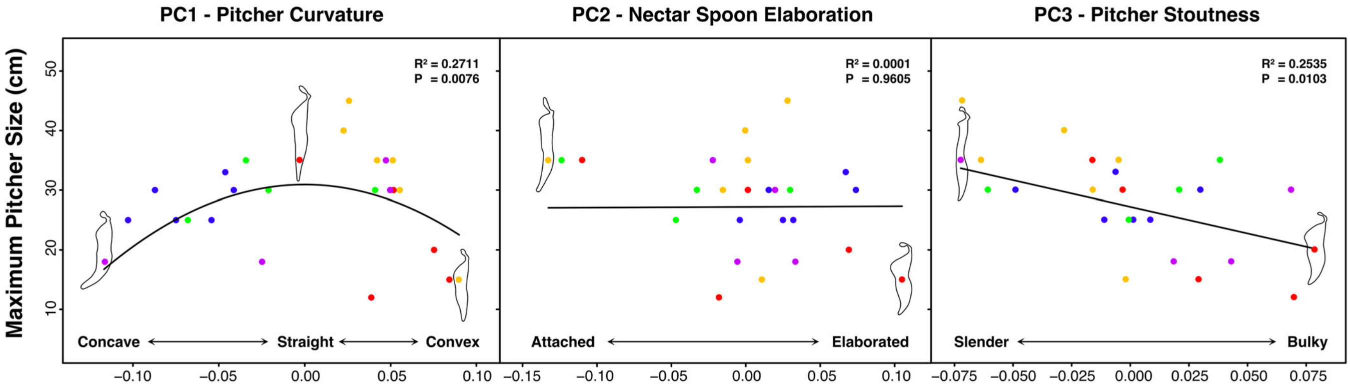

FIGURE 5 Associations between adult pitcher shape variations and maximum pitcher size in pitcher morphospace. Taxa are represented by solid circles and colored by clades as in Figure 3. Regression lines were drawn based on the best-fit phylogenetic generalized linear models. Example pitcher shapes (corresponding to the taxa encircled) are shown in each morphospace.

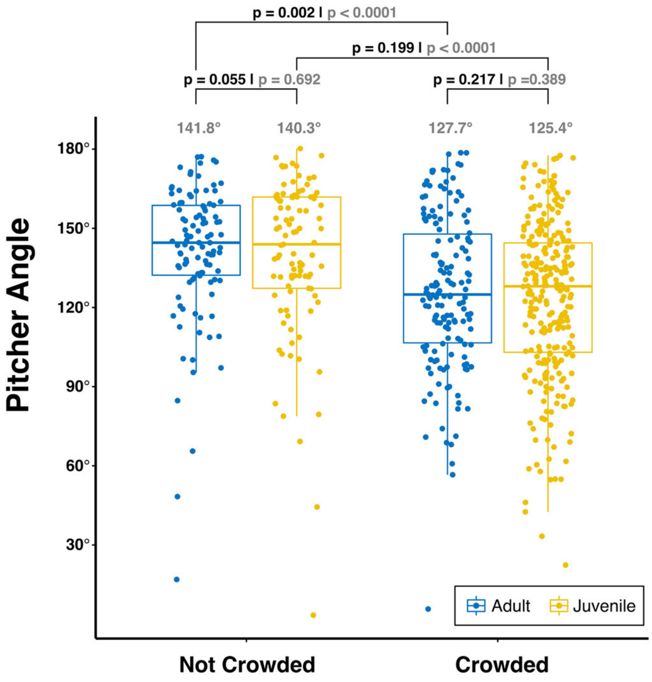

FIGURE 6 Effect of crowdedness on pitcher angle across developmental stages. Both phylogenetic and simple unpaired t-tests were conducted for each comparison with test statistics shown in black and gray, respectively. Note the angles shown above each condition were averages pooled from all samples, with potential bias toward species that were over-sampled.

conditions (Appendix S1, Table S2). When crowded, species with straighter pitcher bodies generally put out new pitchers at a wider angle than those with curvier bodies (Appendix S2, Figure S3). Similarly, a strong linear relationship between pitcher stoutness and pitcher angle was only found in crowded condition. No relationship was found between nectar spoon elaboration (PC2) and pitcher angle in either condition, indicating that, in crowded conditions, the degree of pitcher angle reduction depends on the morphology of the pitcher body rather than the morphology of nectar spoon.

DISCUSSION

Divergent and convergent evolution of pitcher morphologies

Our morphospace analysis revealed three main shape axes across which *Heliamphora* pitchers have diversified, namely in curvature (PC1), nectar spoon elaboration (PC2), and pitcher stoutness (PC3). In accordance with the generally low phylogenetic signal in these traits, we observed that most clades contain a diversity of shapes, e.g., curved and straight, with and without elaborate spoons (Figure 1). *Heliamphora* pitchers also vary widely in size (almost five-fold difference across the genus), even between closely related species (e.g., the sympatric *H. chimantensis* at 35 cm and *H. pulchella* Wistuba, Carow, Harbarth & Nerz at 10 cm). Across these axes, *Heliamphora* species are clustered in distinct regions of morphospace, e.g., curved with elaborate spoons or straight and slender (Figure 3; Appendix S2, Figure S4; Appendices S3 and S4).

Accompanying these patterns of divergence in shape and size among close relatives, we also documented frequent convergence across the phylogeny. For example, small pitchers (<20 cm) evolved convergently in three lineages (Figure 4). Interestingly, these small pitcher species can be found across all major areas of distribution but none of them occur sympatrically (e.g., *H. hispida*, Neblina Massif; *H. pulchella*, Chimantá Massif; *H. minor* Gleason, Auyán Tepui; *H. nutans*; and *H. arenicola* Wistuba, A. Fleischm., Nerz & S. McPherson, different parts of Eastern Tepui Chain). Such widespread convergence is often associated with adaptation to a common ecological niche (Donoghue et al., 2022), and previous authors suggest that small pitchers may be favored in habitats with suboptimal growth conditions, e.g., shallow or infertile substrates, heavy shade, and periodic drought or heat stress (Wistuba et al., 2002; Givnish et al., 2018). Small pitchers impose lower construction costs (Karagatzides and Ellison, 2009; McPherson et al., 2011) and are more drought and heat tolerant due to the reduced transpiration from the pitcher and evaporation from the pitcher fluid (Adlassnig et al., 2010).

Instances of small pitcher species occurring sympatrically with species of larger pitchers (e.g., *H. hispida* and *H. ceracea*; *H. pulchella* and *H. chimantensis*; *H. nutans* and *H. glabra* (Maguire) Nerz, Wistuba & Hoogenstrijd) can be found across all major distributional areas (McPherson et al., 2011), suggesting a potential case of adaptive radiation into different ecological niches (Givnish et al., 1997). Sympatric pairs of small and tall pitcher plant species may be associated with different capture strategies and, consequently, prey composition (e.g., crawling insects like ants for short pitchers and flying insects for tall pitchers), as has been shown in *Sarracenia* (Gibson, 1983; Givnish, 1989) and *Brocchinia* (Givnish et al., 1997). Overall, the diversity of habitats and prey types provided by the topologically complex Pantepui province of the Guiana region combined with the geological isolation between tepuis may account for the repeated diversification of pitcher size in each clade and the resulting convergence across the genus.

Similarly marked convergence is also observed in aspects of pitcher shape, although the underlying drivers are less clear. The degree of elaboration in the nectar spoon may relate the available prey and the reward needed to attract them (Jaffé et al., 1995; Plachno et al., 2007). Differences in shape have been related to the surrounding vegetation, for example, with *Heliamphora* growing through dense vegetation tending to have straight pitchers to protrude above the rest to capture prey (Brewer-Carias, 1972; Jaffe et al., 1992; McPherson et al., 2011). The stoutness of the pitcher may be more directly related to the overall size of the pitcher and may represent a developmental constraint (see below).

Gaps in pitcher morphospace due to evolutionary constraints

Unoccupied regions in the space of possible phenotypes are often attributed to constraints on evolution, be they structural, ecological, or developmental (e.g., Vernescu and Ryser, 2009; Stayton, 2019; Chitwood and Mullins, 2022]. We observed several gaps in pitcher space represented the absence of certain trait combinations, such as large and curvy pitchers or small and slender pitchers. The strong correlations between size and shape, in particular curvature and stoutness (Figure 5), are consistent with structural constraints, i.e., that stout and/or curved pitchers are limited in height while straight, slender pitchers can remain erect at greater heights. We postulate that, compared to species with straight body shapes, taxa with curvy shapes are less structurally stable due to the center of their pitcher mass being further away from the growing point on the rosette which provides structural support. Nevertheless, the potential structural instability resulting from the curvy shape or large size can be mitigated (e.g., *H. ionasi* Maguire [McPherson et al., 2011]).

This general trade-off between pitcher size and shape may allow some conservation of pitcher volume, as pitchers can achieve similar volume by expanding laterally or vertically. Pitcher volume is expected to be under strong selection as maintaining a high level of fluid is crucial to carnivory in *Heliamphora* (Jaffe et al., 1992; Nerz, 2004; Bauer et al., 2013). At a high level, the pitcher fluid can effectively rise along the pubescent inner pitcher wall up to the rim by capillary action, forming superhydrophilic (slippery) trapping surface that facilitates prey capture (Jaffe et al., 1992; Bauer et al., 2013). The regulation and maintenance of the pitcher fluid level might be important in other ecological functions, such as thermal regulation, water reservoir during hot and dry periods, and microhabitat for commensal microbiomes to facilitate digestion (Jaffe et al., 1992; Nerz, 2004; Adlassnig et al., 2010). We expect that there are also lower limits on pitcher fluid level, bounded by the minimum volume needed to allow for carnivorous functions.

Plasticity in pitcher development

Like all leaves, the pitcher development is plastic and can be affected by various environmental factors (Brewer, 1999; Ellison and Gotelli, 2002; Fukushima et al., 2021). This is the first study to examine plasticity in *Heliamphora* pitcher rosette development, specifically focusing on a common natural condition—crowding due to the presence of neighboring plants. In nature, *Heliamphora* populations are often crowded and restricted to habitats with suitable growth conditions, such as small depressions on tepui summits and open clearings in the montane forests (McPherson et al., 2011). Our study found that rosettes in such crowded conditions put out new pitchers at tighter angle while those in growing without neighbors grow out at wider angle. This effect is apparent in both juvenile and adult pitchers (Figure 6). In adult pitchers, the effect is dependent on pitcher curvature and pitcher stoutness (Appendix S1, Table S2; Appendix S2, Figure S3), suggesting the degree of angle reductions is further constrained by pitcher body shape.

To visualize how the plastic differences in pitcher angle would translate to spacing of pitchers and rosettes, we created two sets of diagrams based on two angles of emergence (140° and 125°; Appendix S2, Figure S5). The wider angle, roughly the mean estimated in uncrowded conditions (Figure 6), leads to evenly spaced pitchers in the mature rosette (Figure 7A, B). Mature rosettes typically comprise five active pitchers (e.g., Butschi et al., 1989; Wistuba et al., 2005; Wrazidlo, 2019; Golos, 2020), and we added a sixth pitcher to demonstrate how a new pitcher would overlap with an older inactive pitcher (Figure 7B).

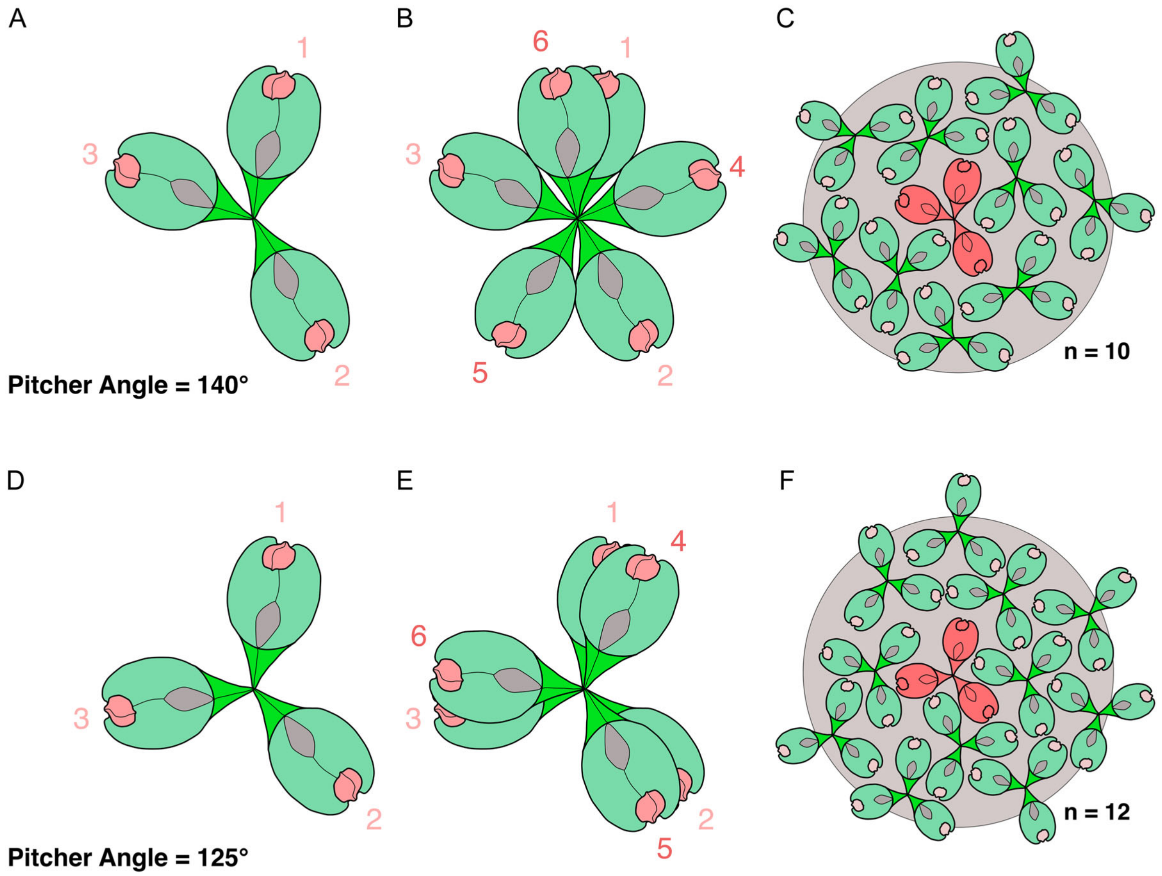

FIGURE 7 Morphologies of *Heliamphora* rosettes associated with wider (A, B) and sharper pitcher angles (D, E). For each pitcher angle, rosettes are illustrated with three (A, D) to six pitchers (B, E) to demonstrate the effect of shading from overlapping pitchers. In each rosette, pitchers are numbered increasingly from the oldest to the youngest pitcher (most active pitcher). Hypothetical scenario illustrating the effect of wider (C) and sharper pitcher angles (F) on optimal packing in crowded conditions. In this hypothetical scenario, the wider and sharper pitcher angles, respectively, allowed the populations to fit in up to 10 and 12 rosettes when space is limited. The sharper pitcher angle allowed *Heliamphora* population to occupy available space more efficiently, with an increase of 20% more active pitchers.

Furthermore, we envisioned how a clonal cluster of rosettes emerging from the rhizomes of a single plant could fill a confined space, such as a depression in the rock substrate (Figure 7C). When we simulated the same developmental process with the narrower pitcher angle observed under crowded conditions (Figure 7), we found that individual pitchers on each rosette exhibited greater overlap (Figure 7D, E), but more new rosettes could be packed into the confined space (Figure 7F), resulting in increased pitcher density. While these visual models are only hypothetical, they illustrate how pitcher angle will have direct consequences for the packing of pitchers into confined spaces and likely affect fitness through access to light, rainwater, and prey resources.

It is possible that the increased density of pitchers could lead to a decreased prey capture rate per pitcher due to competition from surrounding pitchers, as evidenced in *Sarracenia* (Gibson, 1983; Givnish, 1989). *Heliamphora* often form dense clonal populations, as they tend to produce new growing points (rosettes) along the rhizome of existing rosettes, whether in situ (Wistuba et al., 2002; Fleischmann and Grande Allende, 2011) or in cultivation (Ziemer, 1979; McPherson et al., 2011). Even if the individual pitchers receive less prey during crowding, the entire clonal population may benefit from the tight packing of rosettes with higher total prey capture and higher growth by maximizing area for photosynthesis.

While we chose to focus on the effects of crowding, other biotic and abiotic factors may influence pitcher development and represent adaptive plasticity. For example, *Heliamphora* grown under drought stress produce smaller pitchers, a response which may serve to minimize transpiration (McPherson et al., 2011). Pitchers growing in heavy shade typically lose carnivorous activity and primarily function as photosynthetic structures, as indicated by the elongated pitcher bodies, and the absence of pitcher coloration, nectar spoon structure, or attractants (McPherson et al., 2011). Similar plastic responses are found in *Darlingtonia*, *Sarracenia*, and all other pitcher plants, suggesting pitcher plasticity commonly associated with trade-offs between carnivory and photosynthesis (Givnish et al., 1984; Ellison and Gotelli, 2002; Ellison and Farnsworth, 2005; Pavlovič and Saganová, 2015; Givnish et al., 2018; Fukushima et al., 2021). Further ecological studies should be conducted to investigate and better understand the dynamic interplay of pitcher development, carnivory, and photosynthesis. Furthermore, it would be interesting to investigate whether crowding has a similar effect on pitcher angle in the closely related *S. purpurea* and *S. psittacina*, which also have a similar rosette growth form to *Heliamphora*.

CONCLUSIONS

Both divergent and convergent evolution of *Heliamphora* pitcher morphologies were likely a result of adaptive radiation across the dynamic Pantepui landscape over the last 20 million years (Liu and Smith, 2021). We observed repeated diversification of pitcher shape and size within geographically structured clades as well as frequent convergence in form across the entire genus. This convergence upon similar combinations of shapes and sizes suggests that these suites of traits may be adaptive, as has been proposed for convergently evolved pitcher trait combinations in other carnivorous plant lineages (Clarke et al., 2018; Thorogood et al., 2018). Many traits beyond shape and size contribute to pitcher function (e.g., volatiles, pigmentation, external nectaries, pubescence), and we predict that these traits may show similar patterns of convergence and correlated evolution. While many studies have demonstrated phenotypic and even genomic convergence associated with carnivory across angiosperms (e.g., Givnish et al., 1997; Fukushima et al., 2017; Bittleston et al., 2018; Clarke et al., 2018), our study further contributes to this understanding by demonstrating that the diversification and convergent evolution of carnivory-related traits can occur on finer evolutionary timescales. The radiation of pitcher forms in Sarraceniaceae, including *Heliamphora*, reinforces the notion that extreme ecological specialization, such as the carnivorous lifestyle, may not necessarily be an evolutionary dead end (Tripp and Manos, 2008; Ozinga, 2013; Day et al., 2016; Thorogood et al., 2018).

AUTHOR CONTRIBUTIONS

S.L.: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, validation, visualization, writing—original draft, writing—review & editing. **S.D.S.:** Conceptualization, formal analysis, investigation, methodology, project administration, resources, supervision, writing—review & editing.

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DATA AVAILABILITY STATEMENT

Raw pitcher angle measurements, pitcher shape outlines, modified *Heliamphora* phylogeny, and R Markdown file containing scripts used in this study can be accessed via Figshare (https://doi.org/10.6084/m9.figshare.23646414.v3).

ORCID

Sukuan Liu http://orcid.org/0000-0003-3561-9623
Stacey D. Smith http://orcid.org/0000-0003-2010-4459

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

APPENDIX S1.

Table S1. Sampling information.

Table S2. Model fit results.

APPENDIX S2.

Figure S1. Pitcher angle measurements.

Figure S2. Pitcher angle by taxa.

Figure S3. Associations between adult pitcher shapes variations and pitcher angle in different crowding conditions.

Figure S4. Adult pitcher morphospace of PC1 and PC3.

Figure S5. Developmental timeline of *Heliamphora* adult pitcher rosettes associated with wider and sharper angles.

APPENDIX S3. Morphospace of PC1, PC2, and PC3.

APPENDIX S4. Morphospace of PC1, PC3, and maximum pitcher size.

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