1 **Replicated radiations in the South American Marsh Pitcher Plants (*Heliamphora*)**
2 **lead to convergent carnivorous trap morphologies**

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6

7 **Abstract**

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

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

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

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

26 **摘要 (Simplified Chinese)**

27 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，
28 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
29 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
30 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
31 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
32 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
33 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、
34 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
35 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
36 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
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Introduction

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, Peroutka, and Lendl 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, Robins, and Joel 1989; Givnish et al. 2018).

Among the independently evolved pitcher plant lineages, *Nepenthes* (Caryophyllales), Sarraceniaceae (Ericales), and *Cephalotus follicularis* (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* and *Paepalanthus bromelioides*), which have structurally simpler pitchers morphologically similar to their tank forming relatives (Magallón et al. 2015; Cross et al. 2018). With the exception of the monotypic Australian pitcher plant *C. follicularis*, *Nepenthes* (the 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 et al. 2011; McPherson and Schnell 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 (Pavlovic 2012; Clarke et al. 2018; Thorogood, Bauer, and Hiscock 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 (Pavlovic 2012; Clarke et al. 2018; Thorogood, Bauer, and Hiscock 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*) or novel nutrient acquisition strategies (e.g. detritus-feeding in *N. ampullaria*, fecal-feeding in *N. lowii*, and subterranean-feeding in *N. pudica*) (Thornham et al. 2012; Moran et al. 2012; Gilbert et al. 2022; Dančák 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* and *Sarracenia psittacina*), trapping mechanisms (e.g. superhydrophilic hairy surface of *Heliamphora nutans*), symbiotic relationship (e.g. *S. purpurea* and commensal *Wyeomyia smithii*), 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

79 neighboring plants) and abiotic factors (e.g. extreme microclimate) (Brewer 1999; McPherson et
80 al. 2011; McPherson and Schnell 2011; Clarke et al. 2018).

81 Despite the wide variety of morphologies associated with pitcher traps, few comparative studies
82 have focused on the morphological evolution of carnivorous traps, and none with quantitative
83 methods. Morphometric analyses allow us to define the spaces occupied by phenotypic
84 combinations as well as those that are empty (Stayton 2019; Segall et al. 2020; Jardine et al.
85 2022) and, in the context of well supported phylogenies (Givnish et al. 1997), identify instances
86 of morphological convergence (Papadopulos et al. 2013; Smith and Kriebel 2018). This
87 quantitative shape information also sets the stage for rigorous testing of ecological and
88 evolutionary hypotheses (Smith and Kriebel 2018; Dellinger et al. 2019; Kriebel et al. 2022).

89 Here we focus on the evolution of pitcher morphologies in the South American marsh pitcher
90 plants *Heliamphora* (Sarraceniaceae). This genus is endemic to the Guiana Highlands, and is the
91 most species-rich genus in the family, with 23 extant species and several yet to be described
92 (McPherson et al. 2011). The major clades are thought to have emerged through both vicariance
93 and dispersal during the Miocene with more recent diversification in each clade driven by
94 vertical displacement during the Pleistocene glacial-interglacial cycles (Liu and Smith 2021).
95 The adult pitchers vary dramatically in pitcher shape, size, nectar spoon structure and many other
96 taxonomically important characters across species (Figure 1) (McPherson et al. 2011). It has
97 been suggested these diverse morphologies may relate to differences in microclimate (Nerz
98 2004), prey attraction strategies (Wistuba, Harbarth, and Carow 2001; Wistuba, Carow, and
99 Harbarth 2002), and/or shifts in prey composition (Jaffe et al. 1992). Furthermore, structural
100 stability of the pitcher could impose constraints on the morphological evolution of *Heliamphora*
101 pitchers, limiting available pitcher morphospace.

102 In the present study, we used comparative morphometric approaches to quantify the shape space
103 occupied by *Heliamphora* pitchers and to trace evolutionary trajectories through this space.
104 Building on previous phylogenetic work in *Heliamphora* (Liu and Smith 2021), we also tested
105 for phylogenetic signal in pitcher shape and investigated how pitcher shape evolution may be
106 related to pitcher size. In particular, we hypothesize that aspects of shape evolution may be
107 tightly correlated with height given the structural constraints imposed by supporting high volume
108 of pitcher fluid. In light of the dense packing in many natural populations (McPherson et al.
109 2011), we also considered how pitcher development responded to crowding and tested the
110 hypothesis that pitcher angle (the angle between two most recently developed pitchers) shrinks in
111 crowded conditions to allow for tighter packing. In interpreting these results, we discuss the
112 potential ecological significance of *Heliamphora*'s diverse pitcher morphologies and ability to
113 modify pitcher development in response to varying conditions.

114

MATERIALS AND METHODS

Taxon sampling and phylogenetic framework

117 We sampled 23 described and 2 additional yet to be described taxa of *Heliamphora* for this study
118 (Appendix S1, Table S1; see the Supplementary Data with this article). All specimens were

119 grown under controlled environmental conditions [photoperiod 15hr per day (full sun);
120 temperature range 12–25 °C; relative humidity > 80%], resembling the climate of their native
121 habitats (Jaffe et al. 1992; Adlassnig, Pranjic, et al. 2010). Species determinations of the living
122 collections were confirmed by comparing their morphological traits to the original descriptions
123 (Bentham 1840; Nerz and Wistuba 2000; Carow, Wistuba, and Harbarth 2005; Fleischmann,
124 Wistuba, and Nerz 2009; McPherson et al. 2011).

125 We used the recently published phylogeny of 24 of the 25 *Heliamphora* taxa (Liu and Smith
126 2021) to reconstruct the evolution of adult pitcher shape, maximum adult pitcher size and pitcher
127 angle. This phylogeny lacks *H. macdonaldae*, which is thought to be sister to *H. tatei* based on
128 similarities in pitcher morphology and distributional range (Maguire 1978; McPherson et al.
129 2011; Liu and Smith 2021). We manually included this species in the tree (Figure 1) by placing it
130 sister to *H. tatei* and setting their split to 0.75 mya, which is the average node depth of other
131 sister pairs in the W lineage, i.e., (*H. ceracea, H. hispida)* and *(H. neblinae, H. parva*).

Morphometric analysis of pitcher shapes

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

145 A set of six landmarks were place on each outline to delineate pitcher functional zones (Figure 2)
146 according to McPherson et. al. (2011). In *Heliamphora* pitchers, Zone 1 (landmarks 2-4)
147 corresponds to the nectar spoon, which is responsible for attracting prey. Zone 2 (landmarks 1, 2,
148 4, 5) represents the upper section of the pitcher above the narrow band and plays a role in prey
149 capture and retention. Zone 3 (landmarks 1, 5) consists of a narrow restriction of the pitcher
150 interior, located close to the waterline of the pitcher fluid. The position of the drainage hole
151 (indicated by landmark 1; the drainage hole is further adapted into drainage slit in E1 clade)
152 determines the maximal level of pitcher fluid in the pitcher (landmarks 1, 5), which is crucial in
153 maintaining carnivorous and other ecological functions in *Heliamphora* (see discussion). Zone 4
154 (landmarks 1, 5, 6) comprises the bottom part of the pitchers and is involved in prey digestion as
155 well as nutrient absorption. Despite morphological diversity, these functional zones are
156 homologous and easily identifiable across *Heliamphora* species, making them reliable reference
157 points for the landmarks and preventing any distortion of the outlines.

158 Using the landmarks, shape variation of adult pitchers was quantified using elliptical Fourier
159 analysis (EFA) that decomposed the outlines into 21 harmonics, each with four coefficients.
160 After aligning the outlines using Full Generalized Procustes alignment in Momocs, a principal

161   component analysis (PCA) was used to summarize the 84 coefficients resulting from the EFA.
162   Taxa were plotted along the PC axes to illustrate their distribution in morphospace. Blomberg's
163   K (Blomberg, Jr, and Ives 2003) for the shape PCs (PC1, PC2, and PC3) and adult pitcher size
164   were estimated to infer phylogenetic signals in pitcher shapes and size by using 1000
165   randomizations of the data across the phylogeny to test if the value was significantly different
166   from K=0 (no phylogenetic signal).

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

172   Effects of crowding on pitcher angle

173   We used living collections of *Heliamphora* species to characterize the angle of pitcher
174   emergence and examine the effect of crowding on this angle. Depending on the availability of
175   material, we sampled 3 to 63 individuals per taxon for all 25 taxa. These individuals were
176   sampled from our collections and those of G. Pipis; in possessing adult pitchers, these plants
177   were three or more years of age. For each sampled individual, images were taken directly above
178   each growing point, i.e., where new pitchers are constantly produced (Appendix S2, Figure S1).
179   For each growing point, a line was drawn from the growing point (rosette center) to the tip of the
180   nectar spoon of the youngest (the most recently fully developed) pitcher. Similarity, a second
181   line was drawn for the second youngest pitcher. Pitcher angle (PA) was measured as the angle
182   between the two straight lines using the software ImageJ (Schneider, Rasband, and Eliceiri
183   2012). We replicated these measurements for both juvenile and adult pitchers, since pitchers are
184   dimorphic in *Heliamphora* (McPherson et al. 2011) and may respond differently to crowding. To
185   test whether PA is affected by the presence of neighboring individuals (i.e., if crowding reduces
186   PA), phylogenetic paired t-tests were performed for both adult and juvenile pitchers using the
187   'phyl.pairedttest 'function in R package phytools (Revell 2012). Similarly, the effect of
188   developmental stage on PA was also tested with phylogenetic paired t-tests. In addition to
189   phylogenetic t-tests, regular t-tests were also conducted for each comparison. An additional chi-
190   squared test was performed to test whether the drastic bending observed in juvenile pitchers was
191   associated with the presence of neighbors. Similar to the PCs, we computed Bloomberg's K for
192   PA in adult and juvenile individuals growing with and without neighbors, and then tested if the
193   value was significantly different from zero.

194   Model testing for correlations among morphological traits

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

5

203 and ones growing with neighbors present. Assuming BM process, the PCs were regressed against
204 pitcher angle with PGLM. Taxa with incomplete pitcher angle data due to sampling limitations
205 were dropped from the analysis.

206 **RESULTS**

207 **Morphospace of *Heliamphora* Pitchers**

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

218 All of the measured pitcher traits, except for curvature, lacked a significant phylogenetic signal.
219 Specifically, we found Blomberg's K was not significantly different from zero for nectar spoon
220 elaboration (PC2, K = 0.06, p = 0.886), pitcher stoutness (PC3, K = 0.09, p = 0.391), or
221 maximum pitcher size (K = 0.12, p = 0.198), consistent with the frequent patterns of
222 convergence across the phylogeny (Figure 4). By contrast, significant phylogenetic signal was
223 detected in pitcher curvature (PC1, K = 0.18, p = 0.018) (Figure 4). Looking at the phylogeny,
224 pitcher curvature appears to be conserved in the W+E1 and E2a clades but not in other lineages.
225 In the W+E1 clade, all species were associated with convex pitcher bodies to varying degrees
226 (PC1 > 0.02), with the exception of *H. chimantensis*, which was associated with a straighter
227 pitcher body (PC1 = -0.00321). In E2a, all adult pitchers were associated with very concave
228 pitcher body shapes (PC1 < -0.04).
229
230 **Relationships between Pitcher Shape and Size**

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

238 **Relationship of pitcher angle to growth conditions and pitcher shape**

239 Our results show that *Heliamphora* growing in crowded conditions tend to put out new pitchers
240 at a tighter angle than those in isolated conditions. A significant difference in pitcher angle (PA)
241 was detected between adult pitchers growing in isolated vs. crowded conditions with both
242 phylogenetic and non-phylogenetic t-tests (phylogenetic mean difference [PMD] = 13.64°, p =

243   0.002; p < 0.0001for regular t-test, Figure 6). In juveniles, a significant difference was detected
244   in regular t-test (p < 0.0001) but not in the phylogenetic t-test (PMD = 6.36°, p = 0.199).
245   Moreover, we found juvenile pitchers tend to bend unnaturally when their growth was obstructed
246   by neighboring pitchers (chi-squared test, p < 0.001), possibly due to the more slender and
247   parallel-to-ground body shape. No significant difference in PA was found between juvenile and
248   adult pitchers within each condition (crowded or not) using either t-test (Figure 6). Raw
249   measurements for all taxa, stages and conditions are shown in Appendix S2, Figure S2. No
250   phylogenetic signal was detected in PA across developmental stages and crowding conditions (K
251   = 0.12, p = 0.481 [juvenile, not crowded]; K = 0.11, p = 0.353 [juvenile, crowded]; K = 0.10, p =
252   0.494 [adult, not crowded], K = 0.09, p = 0.597 [adult, crowded]).

253   To investigate the association between pitcher shape and PA, similar PGLM analyses were
254   performed for each crowding condition using data for adult pitchers. The analyses indicated a
255   strong quadratic relationship between pitcher curvature and pitcher angle only in crowded
256   conditions (Appendix S1, Table S2). When crowded, species with straighter pitcher bodies
257   generally put out new pitchers at a wider angle than those with curvier bodies (Appendix S2,
258   Figure S3). Similarly, a strong linear relationship between pitcher stoutness and pitcher angle
259   was only found in crowded condition. No relationship was found between nectar spoon
260   elaboration (PC2) and pitcher angle in either condition, indicating that, in crowded conditions,
261   the degree of pitcher angle reduction depends on the morphology of the pitcher body rather than
262   the morphology of nectar spoon.

263

DISCUSSION

Divergent and convergent evolution of pitcher morphologies

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

275   Accompanying these patterns of divergence in shape and size among close relatives, we also
276   documented frequent convergence across the phylogeny. For example, small pitchers (< 20 cm)
277   evolved convergently in three lineages (Figure 4). Interestingly, these small pitcher species can
278   be found across all major areas of distribution but none of them occur sympatrically (e.g. *H.*
279   *hispida*, Neblina Massif; *H. puchella*, Chimantá Massif; *H. minor*, Auyán Tepui; *H. nutans* and
280   *H. arenicola*, different parts of Eastern Tepui Chain). Such widespread convergence is often
281   associated with adaptation to a common ecological niche (Donoghue et al. 2022), and previous
282   authors suggest that small pitchers may be favored in habitats with suboptimal growth
283   conditions, e.g., shallow or infertile substrates, heavy shade, and periodic drought or heat stress

284   (Wistuba, Carow, and Harbarth 2002; Givnish et al. 2018). Small pitchers impose lower
285   construction costs (Karagatzides and Ellison 2009; McPherson et al. 2011) and are more drought
286   and heat tolerant due to the reduced transpiration from the pitcher and evaporation from the
287   pitcher fluid (Adlassnig, Pranjic, et al. 2010).

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

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

307   Gaps in pitcher morphospace due to evolutionary constraints

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

321   This general trade-off between pitcher size and shape may allow some conservation of pitcher
322   volume, as pitchers can achieve similar volume by expanding laterally or vertically. Pitcher
323   volume is expected to be under strong selection as maintaining a high level of fluid is crucial to
324   carnivory in *Heliamphora* (Jaffe et al. 1992; Nerz 2004; Bauer et al. 2013). At a high level, the
325   pitcher fluid is able to effectively rise along the pubescent inner pitcher wall up to the rim by

326 capillary action, forming superhydrophilic (slippery) trapping surface that facilitates prey capture
327 (Jaffe et al. 1992; Bauer et al. 2013). The regulation and maintenance of the pitcher fluid level
328 might be important in other ecological functions, such as thermal regulation, water reservoir
329 during hot and dry periods, and microhabitat for commensal microbiomes to facilitate digestion
330 (Jaffe et al. 1992; Nerz 2004; Adlassnig, Pranji, et al. 2010). We expect that there are also lower
331 limits on pitcher fluid level, bounded by the minimum volume needed to allow for carnivorous
332 functions.

333 **Plasticity in pitcher development**
334
335 Like all leaves, the pitcher development is plastic and can be affected by various environmental
336 factors (Brewer 1999; Ellison and Gotelli 2002; Fukushima et al. 2021). This is the first study to
337 examine plasticity in *Heliamphora* pitcher rosette development, specifically focusing on a
338 common natural condition — crowding due to the presence of neighboring plants. In nature,
339 *Heliamphora* populations are often crowded and restricted to habitats with suitable growth
340 conditions, such as small depressions on tepui summits and open clearings in the montane forests
341 (McPherson et al. 2011). Our study found that rosettes in such crowded conditions put out new
342 pitchers at tighter angle while those in growing without neighbors grow out at wider angle. This
343 effect is apparent in both juvenile and adult pitchers (Figure 6). In adult pitchers, the effect is
344 dependent on pitcher curvature and pitcher stoutness (Appendix S1, Table S2; Appendix S2,
345 Figure S3), suggesting the degree of angle reductions is further constrained by pitcher body
346 shape.

347 To visualize how the plastic differences in pitcher angle would translate to spacing of pitchers
348 and rosettes, we created two sets of diagrams based on two angles of emergence (140° and 125°,
349 Appendix S2, Figure S5). The wider angle, roughly the mean estimated in uncrowded conditions
350 (Figure 6), leads to evenly spaced pitchers in the mature rosette (Figure 7 A & B). Mature
351 rosettes typically comprise five active pitchers (e.g, Butschi, Huber, and Ammann 1989; Wistuba
352 et al. 2005; Wrazidlo 2019; Golos 2020), and we added a sixth pitcher to demonstrate how a new
353 pitcher would overlap with an older inactive pitcher (Figure 7 B). Furthermore, we envisioned
354 how a clonal cluster of rosettes emerging from the rhizomes of a single plant could fill a
355 confined space, such as a depression in the rock substrate (Figure 7 C). When we simulated the
356 same developmental process with the narrower pitcher angle observed under crowded conditions
357 (Figure 7), we found that individual pitchers on each rosette exhibited greater overlap (Figure 7
358 D & E), but more new rosettes could be packed into the confined space (Figure 7 F), resulting in
359 increased pitcher density. While these visual models are only hypothetical, they illustrate how
360 pitcher angle will have direct consequences for the packing of pitchers into confined spaces and
361 likely affect fitness through access to light, rainwater, and prey resources.

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

370 While we chose to focus on the effects of crowding, other biotic and abiotic factors may
371 influence pitcher development and represent adaptive plasticity. For example, *Heliamphora*
372 grown under drought stress produce smaller pitchers, a response which may serve to minimize
373 transpiration (McPherson et al. 2011). Pitchers growing in heavy shade typically lose
374 carnivorous activity and primarily function as photosynthetic structures, as indicated by the
375 elongated pitcher bodies, and the absence of pitcher coloration, nectar spoon structure, or
376 attractants (McPherson et al. 2011). Similar plastic responses are found in *Darlingtonia*,
377 *Sarracenia* and all other pitcher plants, suggesting pitcher plasticity commonly associated with
378 trade-offs between carnivory and photosynthesis (Givnish et al. 1984; Ellison and Gotelli 2002;
379 Ellison and Farnsworth 2005; Pavlovič and Saganová 2015; Givnish et al. 2018; Fukushima et al.
380 2021). Further ecological studies should be conducted to investigate and better understand the
381 dynamic interplay of pitcher development, carnivory, and photosynthesis. Furthermore, it would
382 be interesting to investigate whether crowding has a similar effect on pitcher angle in the closely
383 related *S. purpurea* and *S. psittacina*, which also have a similar rosette growth form to
384 *Heliamphora*.
385
386
387 **CONCLUSION**
388
389 Both divergent and convergent evolution of *Heliamphora* pitcher morphologies was likely a
390 result of adaptive radiation across the dynamic Pantepui landscape over the last 20 million years
391 (Liu and Smith 2021). We observed repeated diversification of pitcher shape and size within
392 geographically structured clades as well as frequent convergence in form across the entire genus.
393 This convergence upon similar combinations of shapes and sizes suggest that these suites of
394 traits may be adaptive, as has been proposed for convergently evolved pitcher trait combinations
395 in other carnivorous plant lineages (Clarke et al. 2018; Thorogood, Bauer, and Hiscock 2018).
396 Many traits beyond shape and size contribute to pitcher function (e.g., volatiles, pigmentation,
397 external nectaries, pubescence), and we predict that these traits may show similar patterns of
398 convergence and correlated evolution. While many studies have demonstrated phenotypic and
399 even genomic convergence associated with carnivory across angiosperms (e.g. Givnish et al.
400 1997; Fukushima et al. 2017; Bittleston et al. 2018; Clarke et al. 2018), our study further
401 contributes to this understanding by demonstrating that the diversification and convergent
402 evolution of carnivory-related traits can occur on finer evolutionary timescales. The radiation of
403 pitcher forms in Sarraceniaceae, including *Heliamphora*, reinforces the notion that extreme
404 ecological specialization, such as the carnivorous lifestyle, may not necessarily be an
405 evolutionary dead end (Tripp and Manos 2008; Wim A. Ozinga 2012; Day, Hua, and Bromham
406 2016; Thorogood, Bauer, and Hiscock 2018).
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Author Contributions

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Data Availability
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).

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Supporting Information
Additional Supporting Information may be found online in the supporting information section at
the end of the article (Appendix S1, Table S1: Sampling information; Appendix S2, Table S2:
Model fit results; Appendix S2, Figure S1: Pitcher Angle Measurements; Appendix S2, Figure
S2: Pitcher Angle by Taxa; Appendix S2, Figure S3: Associations between Adult Pitcher Shapes
Variations and Pitcher Angle in Different Crowding Conditions; Appendix S2, Figure S4: Adult
Pitcher Morphosapce of PC1 and PC3; Appendix S2, 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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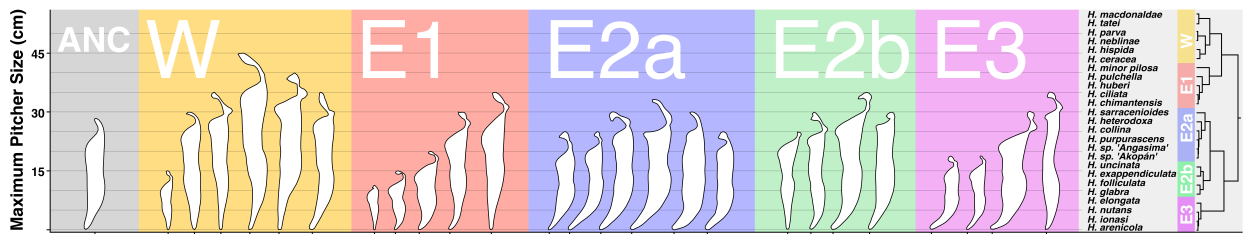657
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Figures

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, H. ciliata, H. huberi,* and *H. chimantensis*. **E2a:** *H. heterodoxa, H. collina, H. sp.* "Angasima"*, H. sp.* "Akopán"*, H. sarracenioides,* and *H. purpurascens*. **E2b:** *H. exappendiculata, H. glabra, H. uncinata,* and *H. folliculata*. **E3:** *H. nutans, H. arenicola, H. ionasi,* and *H. elongata*. 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.
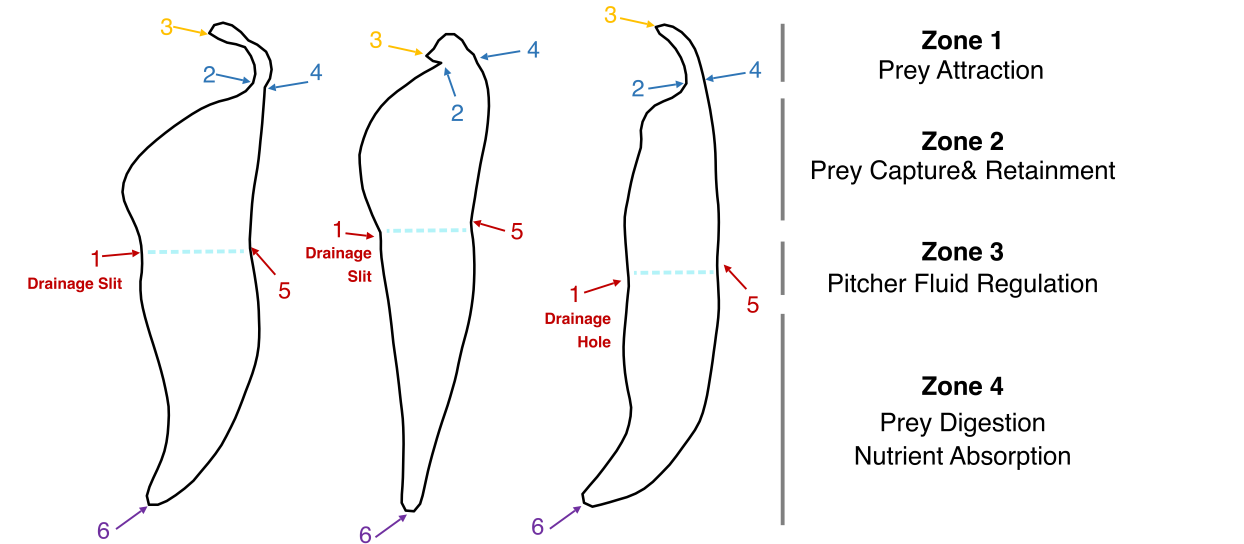
Figure 2. Landmarking examples of three *Heliamphora* species: *H. ciliata* (left), *H. pulchella* (middle) and *H. sarracenioides* (right). The adult pitcher functional zones (McPherson et al. 2011) were delineated by six landmarks indicated above. Morphologically, *Heliamphora* pitchers are similar 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

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688 and 5) while letting the excess to drain out and prevent the pitchers from becoming overfilled
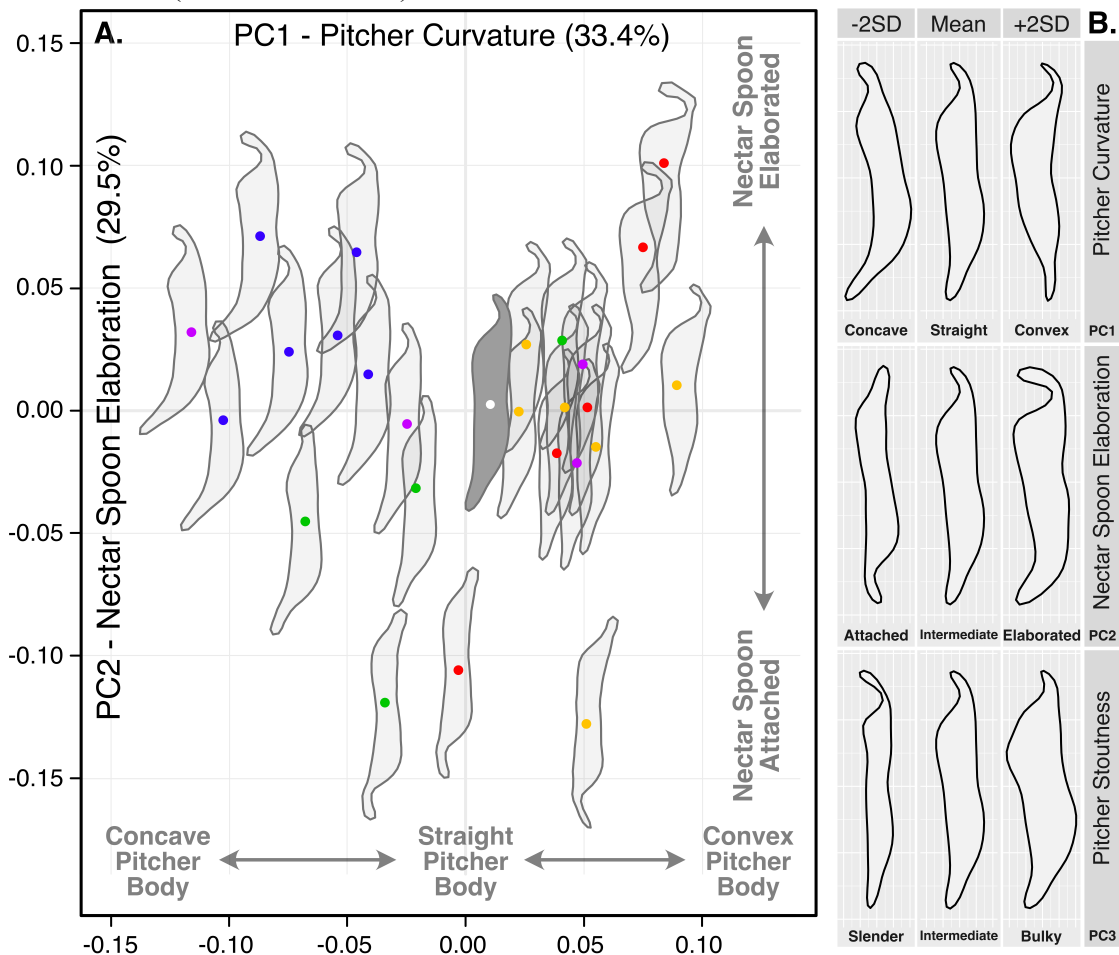689 and unstable (Bauer et al. 2013).

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Figure 3. A. Adult pitcher morphospace of pitcher curvature (PC1) and nectar spoon elaboration (PC2) with outlines of each taxa and the reconstructed ancestral *Heliamphora* adult pitcher (darker gray). Taxa are colored by clades: W (orange), E1 (red), E2a (blue), E2b (green), 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.
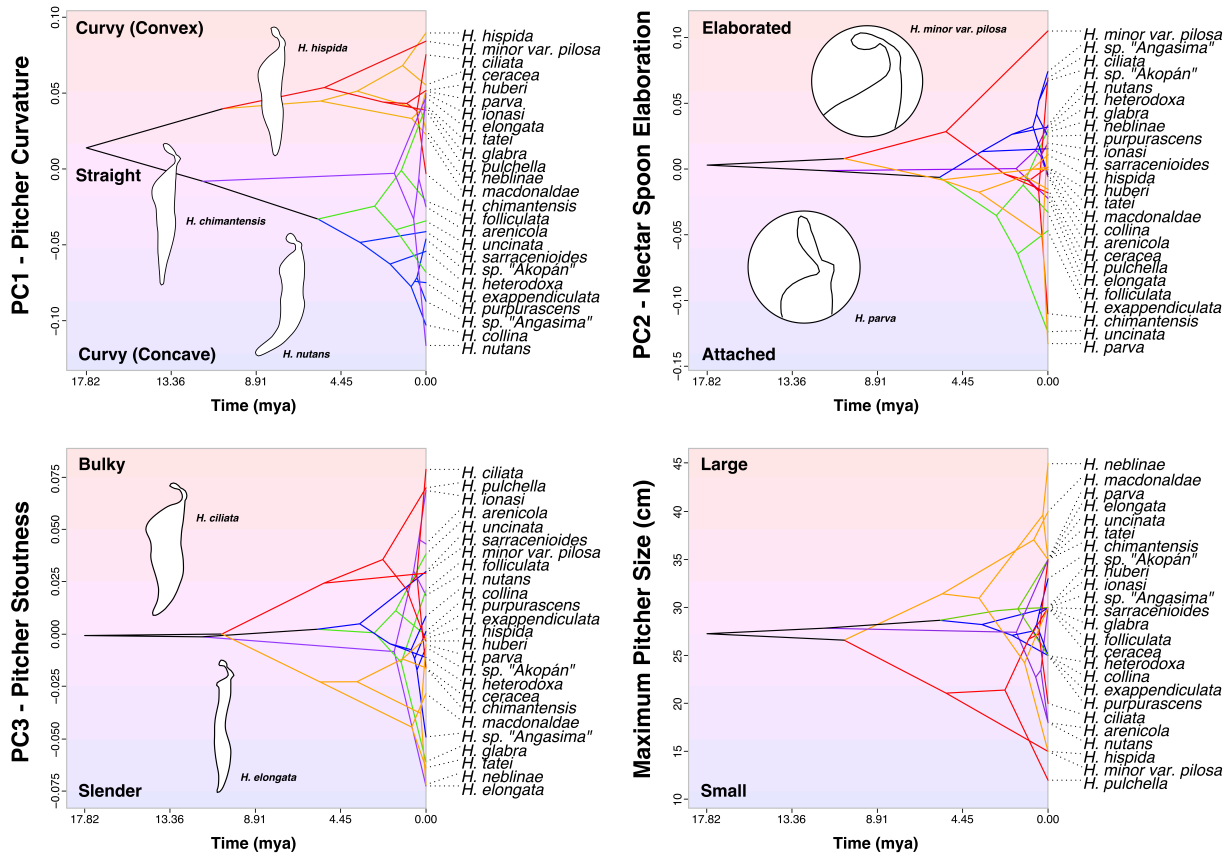
Figure 4. Traitgrams of pitcher curvature (PC1), nectar spoon elaboration (PC2), pitcher stoutness (PC3), and maximum pitcher size. Branches are colored by clades. Examples of pitcher and nectar spoon morphologies are shown in the traitgrams.
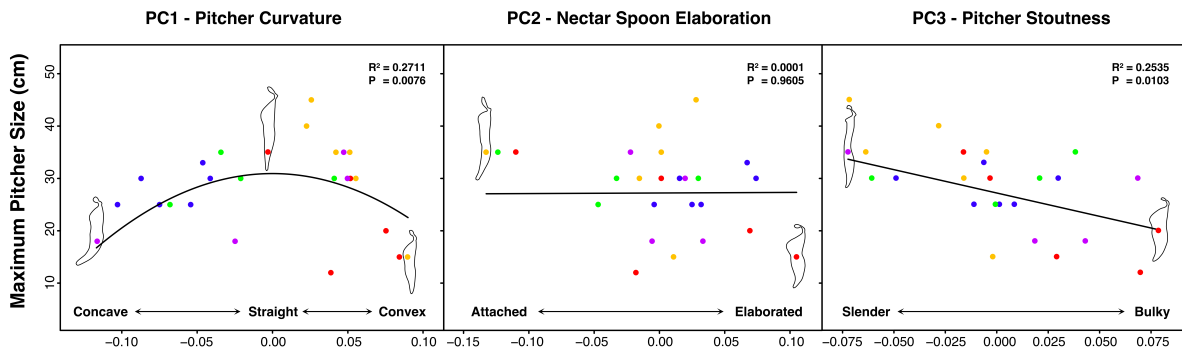

Figure 5. Associations between adult pitcher shape variations and maximum pitcher size in pitcher morphospace. Taxa are represented by solid circles and colored by clades. Regression lines were drawn based on the best fit phylogenetic generalized linear models. Example pitcher shapes (corresponding to the taxa encircled) are shown in each morphospace.
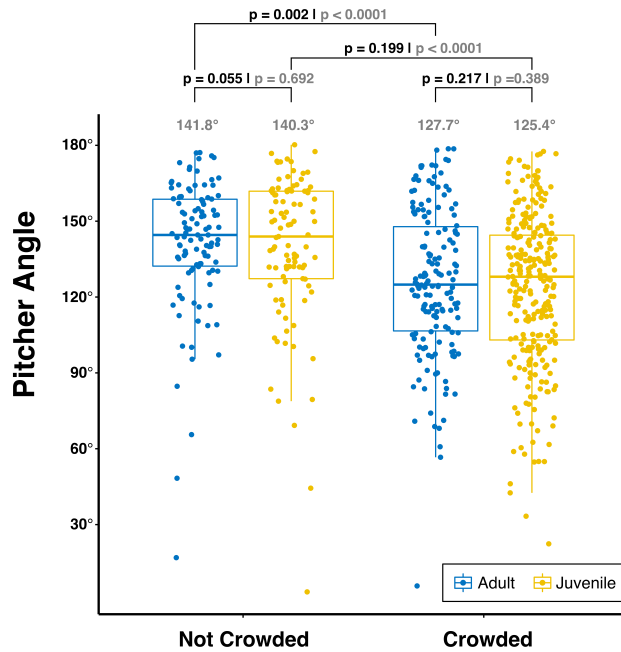
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Figure 6. Effect of crowdedness on pitcher angle across developmental stages. Both
phylogenetic and simple unpaired t-tests were conducted for each comparison with test statistics
shown in black and gray, respectively. Note the angles shown above each condition were
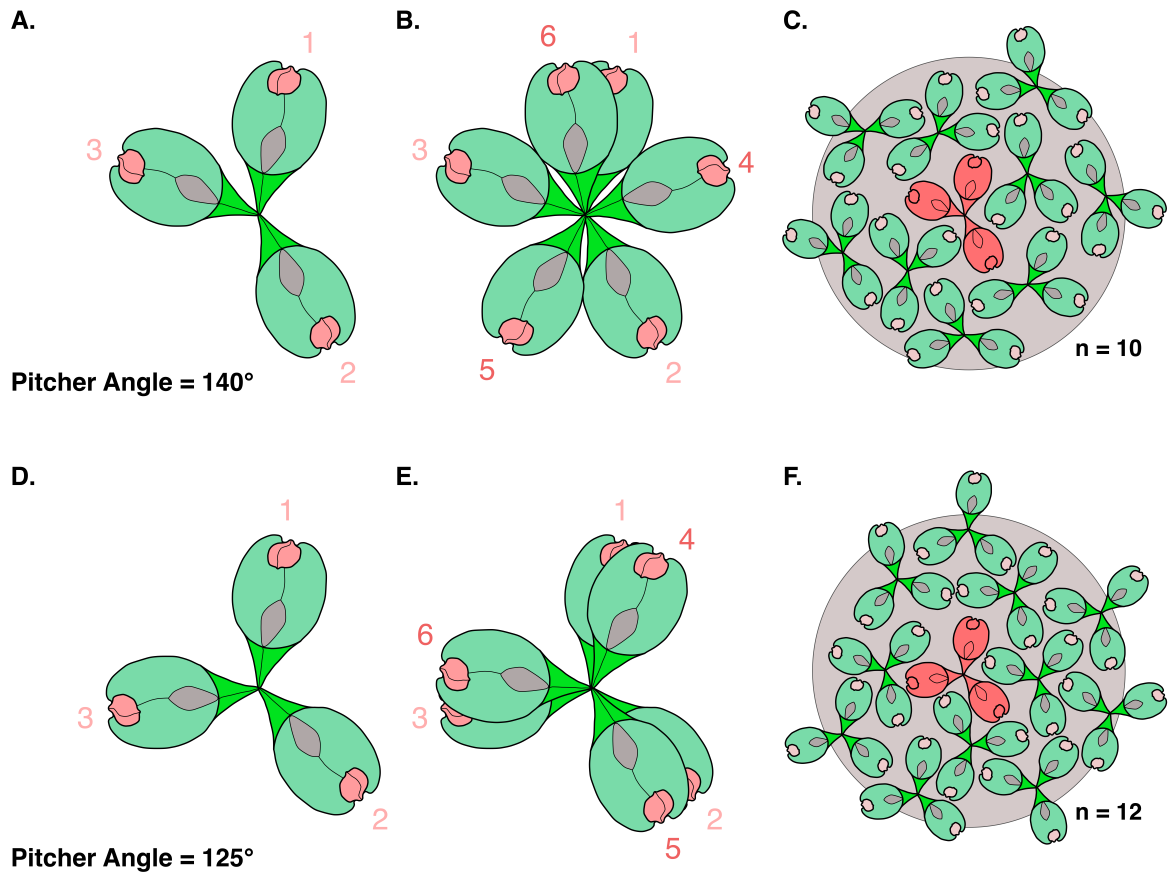averages pooled from all samples, with potential bias toward species that were over sampled.

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.
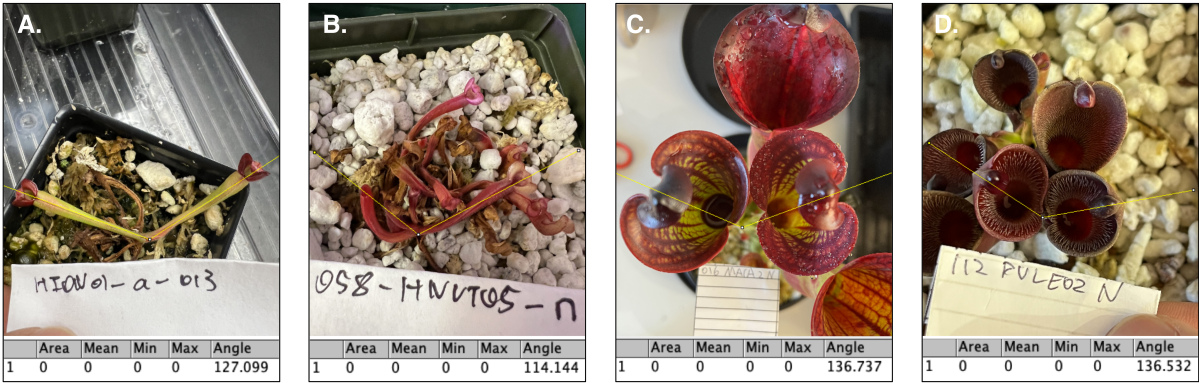
	Area	Mean	Min	Max	Angle
1	0	0	0	0	127.099

	Area	Mean	Min	Max	Angle
1	0	0	0	0	114.144

	Area	Mean	Min	Max	Angle
1	0	0	0	0	136.737

	Area	Mean	Min	Max	Angle
1	0	0	0	0	136.532

Figure S1. Pitcher Angle Measurements. A. *H. ionasi* juvenile growing in isolated condition (growing singly). **B.** *H. nutans* juvenile in crowded condition (neighbors present). Note the juvenile pitcher being measured here does not have a linear pitcher body and curves significantly away from the straight line drawn for pitcher angle measurement. **C.** *H. macdonaldae* adult growing singly. **D.** *H. pulchella* adult in crowded condition.
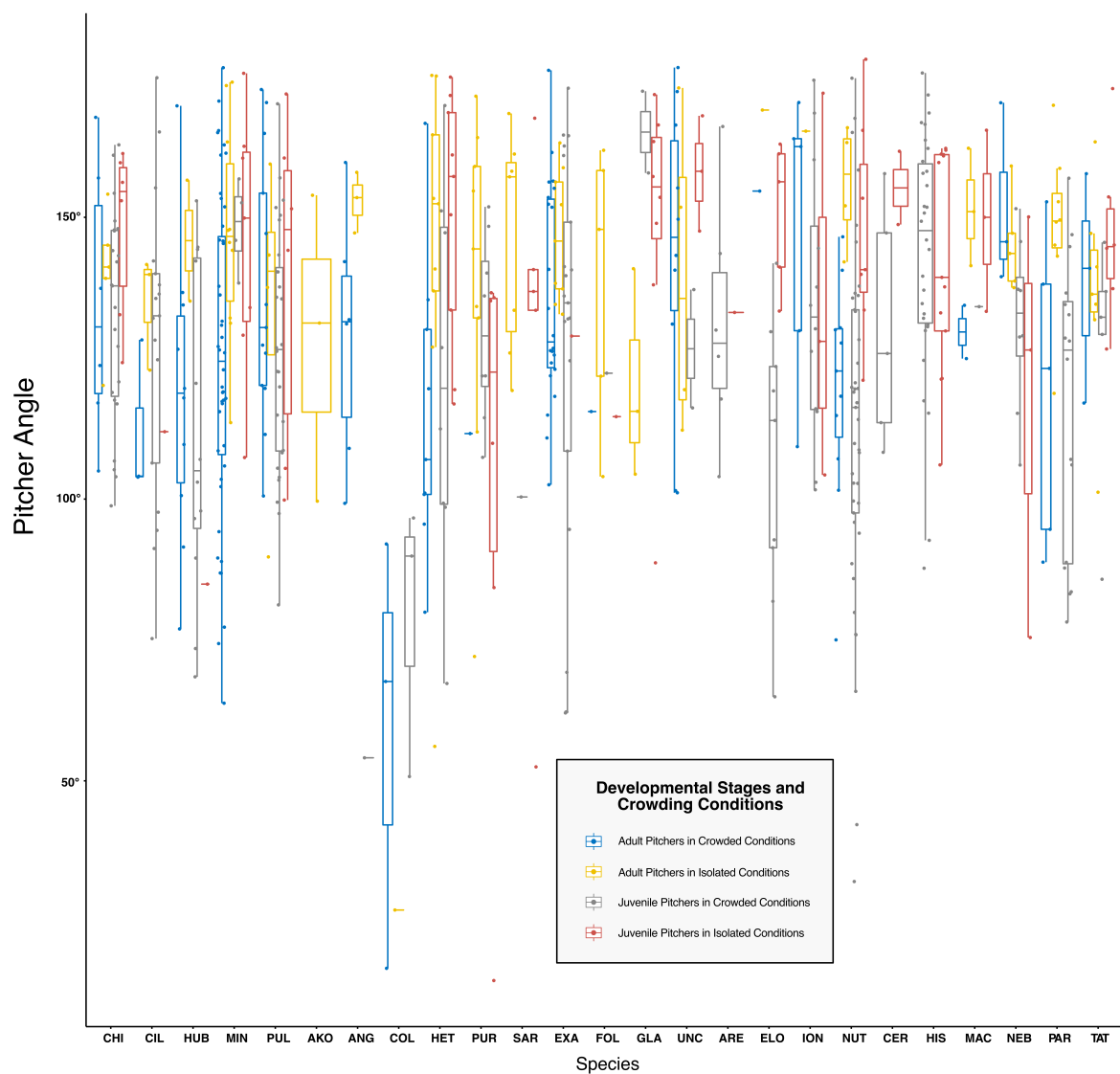
722

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Figure S2. Pitcher angle by taxa across developmental stages and crowding conditions (box plot). Each measurement is indicated by the solid circle. Taxa names are abbreviated to the first three letters (e.g. CHI = *H. chimantensis* & AKO = *H. sp.* "Akopán").
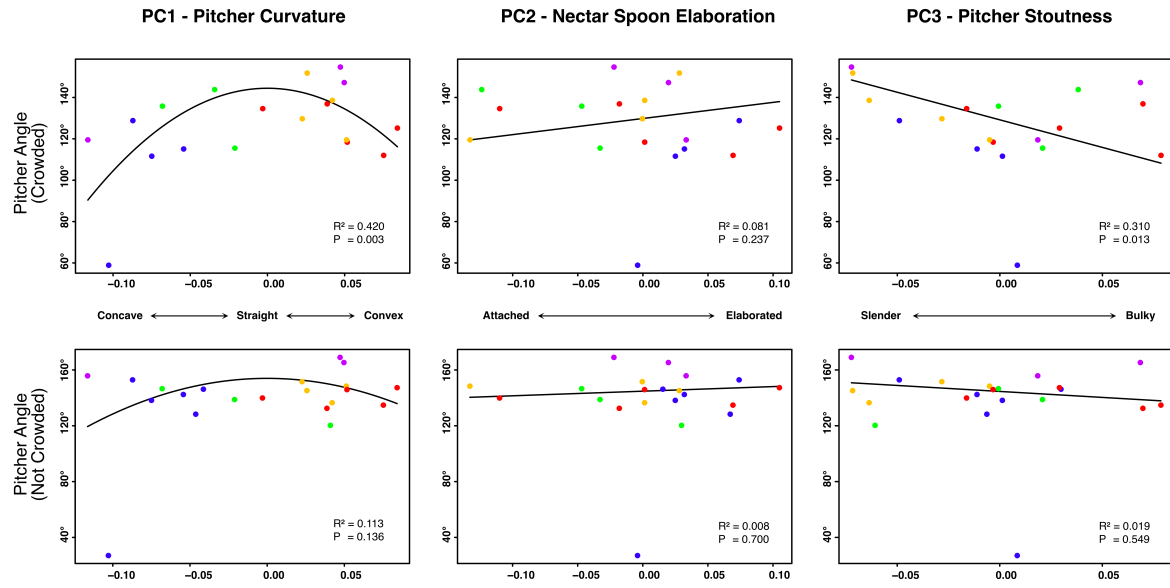
723

Figure S3. Associations between adult pitcher shape variations and pitcher angle in different crowding conditions. Taxa are represented by solid circles and colored by clades. Regression lines were drawn based on the best fit phylogenetic generalized linear models.
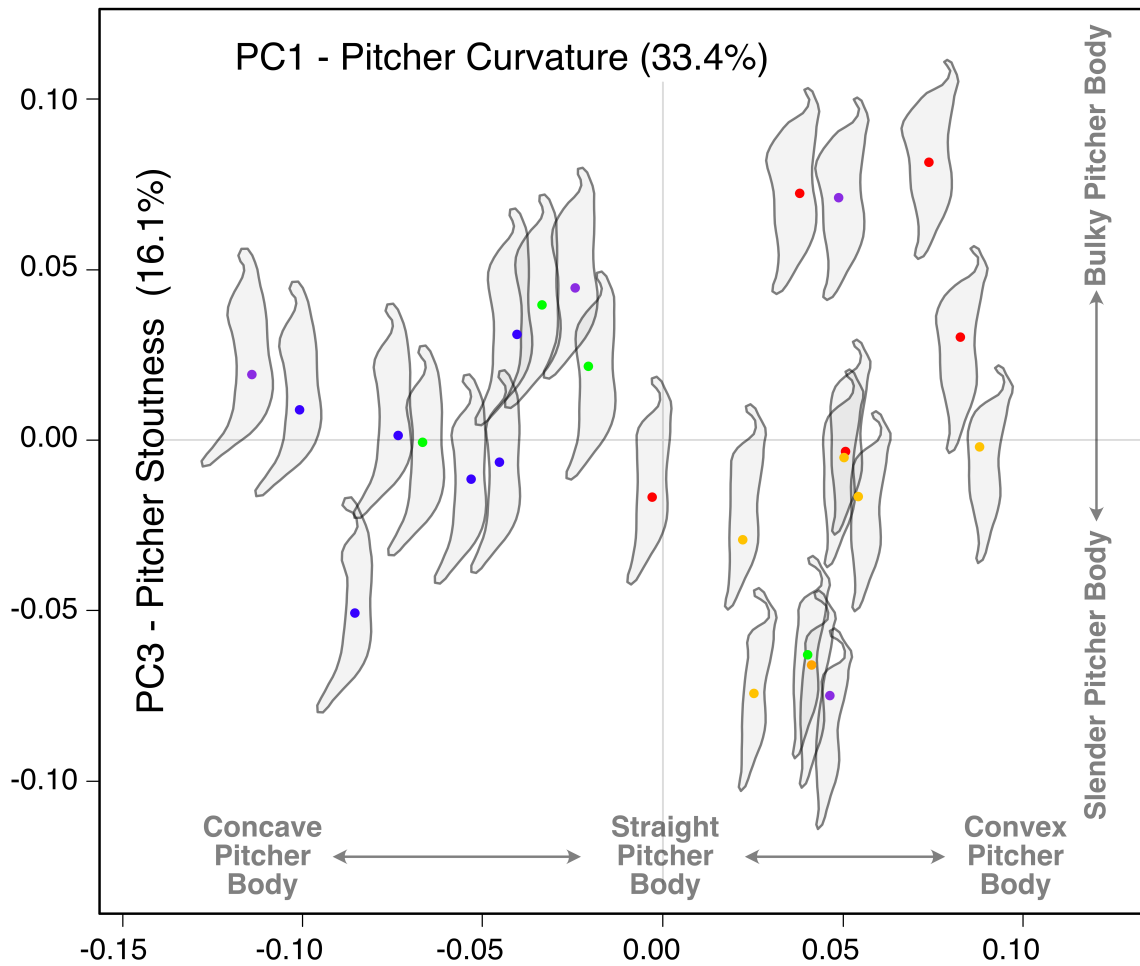
724

Figure S4. Adult pitcher morphospace of pitcher curvature (PC1) and stoutness (PC3) with outlines of each taxa colored by clades: W (orange), E1 (red), E2a (blue), E2b (green), E3 (purple).

Pitcher Angle = 140°

* Isolated conditions

Pitcher Angle = 125°
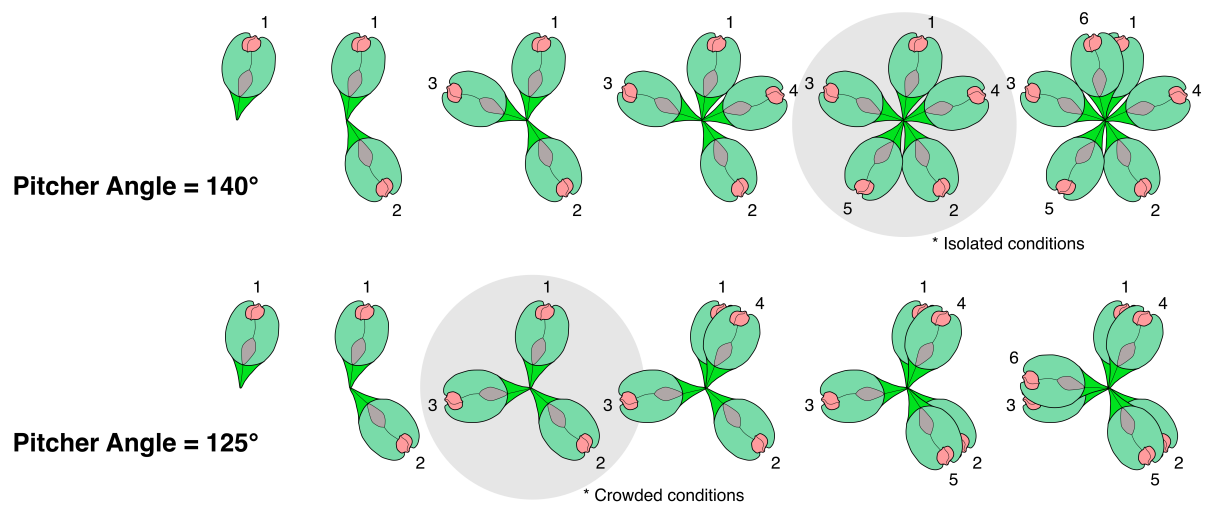
* Crowded conditions

Figure S5. Developmental timeline of *Heliamphora* adult pitcher rosettes associated with wider and sharper pitcher angles. Rosette morphologies frequently found in isolated and crowded conditions are indicated with grey circles.

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