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New insights into floral morph variation in *Passiflora incarnata* L. (Passifloraceae) in Tennessee, U.S.A.

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ABSTRACT

Passiflora incarnata is a functionally andromonoecious clonal wildflower, native to the southeastern United States, whose primary pollinator is the carpenter bee, *Xylocopa virginica*. While recent studies looking at reproductive ecology in *P. incarnata* have classified flowers as one of two morphs (male or hermaphroditic) based on stylar deflexion, preliminary field studies conducted in Tennessee indicated there were five distinct morphs present (three male, two hermaphroditic), supported by stylar deflexion, floral size, and pistil development. The present study sought to test the hypothesis that five distinct floral morphs are present in *P. incarnata* by sampling 13 floral characters, and to document variation in nectar constituents, volume, and concentration across the five morphs. Five well-established individual plants were examined at three sites in Cookeville, Tennessee. Two-factor permuted analysis of variance of 13 floral characteristics with floral morph and individual plants as factors suggested that morph:plant interactions explained 6%, individual plant explained 18%, and floral morph explained 36% of variation in floral characteristics. Nectar sampling indicated that all morphs produced nectar comprised exclusively of sucrose. Nectar volume generally increased with floral morph size, while nectar concentration decreased. NMDS analysis indicated that four of the five hypothesized morphs were supported as distinct, with morphs 4a and 4b best classified as submorphs due to substantial overlap. The supported morphs are best distinguished by ovary width, ovary length, style length, and stigma width. These findings support a hypothesis that the morphs result from variation in developmental arrest during floral ontogeny. The ecological implications of the morphs and nectar variation are considered for *X. virginica* with suggestions for additional studies.

1. Introduction

Passiflora incarnata L. is a widespread, native vine that occurs throughout the southeastern United States, reaching north to Pennsylvania and as far west as Texas (USDA NRCS, 2017). This species is most common in open, sunny habitats along roadsides, thickets, along riverbanks, and abandoned agricultural fields. *Passiflora incarnata* is clonal, spreading via underground runners that can extend up to five meters (Foré and Spira, 2002; Ulmer and MacDougal, 2004). This species is an important larval host plant for several species of butterflies, including *Agraulis vanillae* L., *Heliconius charithonia* L., and *Euptoieta claudia* Cramer (Daniels et al., 2011). *Passiflora incarnata* is primarily pollinated by bees (Cunningham, 2000; Frankie and Vinson, 1977) but flowers are also visited by various Lepidoptera, Hymenoptera, Hemiptera, Coleoptera, and Diptera (Frankie and Vinson, 1977; Hardin et al., 1972; McLain, 1983; S. Krosnick, *pers. obs.*). Nectar is produced in the floral nectary, a small ring located at the base of the androgynophore. The nectary is protected by the limen and operculum, which are both outgrowths of the hypanthium (Fig. 1A and B; MacDougal, 1994). This species is characterized by large flowers (ca. 5–7 cm in diameter) and multiple series of coronal filaments. These filaments are outgrowths from the hypanthium that produce scent and serve as both a visual attractant and landing platform for pollinators (Ulmer and MacDougal, 2004). Similar to other species in the genus, the flowers of *P. incarnata* are self-incompatible, short-lived (lasting less than 24 h), and exhibit transient herkogamy, with styles positioned away from the stamens at the start of anthesis (Dai and Galloway, 2011; May and Spears, 1988). This species is especially interesting because it

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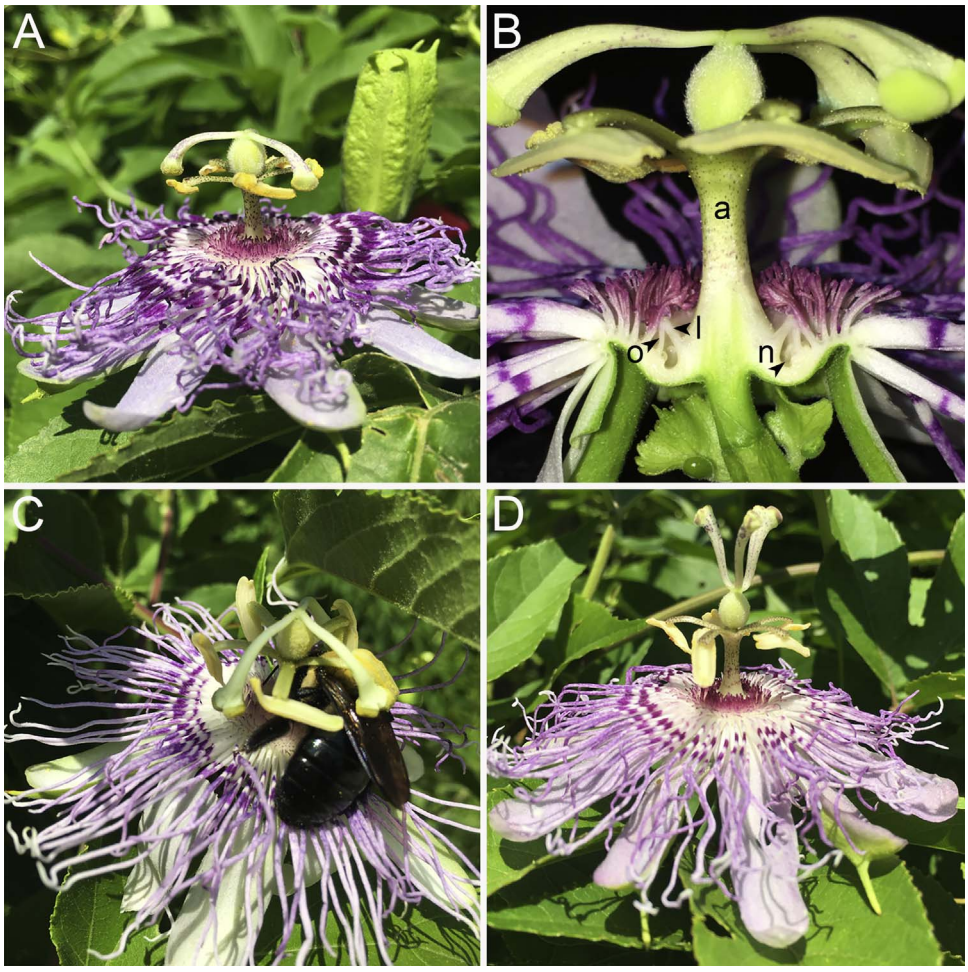

Fig. 1. Floral morphology in *Passiflora incarnata.* A. Typical functionally hermaphroditic flower with styles positioned at same level as anthers within two hours of anthesis. B. Longisection of a flower showing detailed view of androgynophore (a), limen (l), operculum (o), and nectar chamber (n). C. *Xylocopa virginica* visiting a functionally hermaphroditic flower, making contact with both anthers and styles. D. Functionally male flower, where styles remain erect and never make contact with pollinators during legitimate visits.

exhibits functional andromonoecy, where male flowers and hermaphroditic flowers are produced on each individual plant throughout the flowering season, which lasts from mid-June through September throughout its range (McGuire, 1998).

The most common floral visitor to *P. incarnata* is the carpenter bee, *Xylocopa virginica* L. (Fig. 1C; Cunningham, 2000; Foré and Spira, 2002; Hardin et al., 1972; Spears and May 1988), although *Bombus* spp. and halictid mining bees have also been observed (Hardin et al., 1972). *Xylocopa virginica* is a solitary species, often foraging with ranges greater than 1 km (Cunningham, 2000). When carpenter bees arrive at a flower, they land on the coronal filaments and walk in a circular pattern under the anthers, facing the androgynophore as they probe the nectar chamber for a reward (Frankie and Vinson, 1977; Hardin et al., 1972). To reach the base of the nectar chamber, they must grasp the coronal filaments with their legs and pull themselves downward (Frankie and Vinson, 1977). In the process, *X. virginica* incidentally collects large amounts of *P. incarnata* pollen on its body. When floral anthesis is complete in a hermaphroditic flower, both the stigmas and the anthers are positioned to make direct contact with the bee (Fig. 1C), while the stigmas in a functionally male flower will remain untouched by visitors because they remain erect (Fig. 1D). The implications of the interactions between *X. virginica* and the floral gender morphs in *P. incarnata* are not well understood.

Andromonecy is a widely observed condition in angiosperms, with ca. 4000 species across 33 families exhibiting gender-variable floral morphs on the same plant (Miller and Diggle, 2003, 2007; Yadav et al., 2016). The relative ecological advantages associated with andromonoecy include strategic resource allocation, increased rates of outcrossing, and increased male fitness (Bertin, 1982; Solomon, 1986; Spalik, 1991; Spears and May 1988). Andromonoecy is thought to have evolved through developmental reduction in female reproductive organs, and represents one of the first evolutionary steps towards monoecy and dioecy (Bertin, 1982; Primack and Lloyd, 1980). While functional andromonoecy is known to occur in multiple species of *Passiflora* (Krosnick et al., 2015), this condition is best documented in *P. incarnata,* which has been the subject of several ecological studies (Dai and Galloway, 2011, 2012, 2013; Dai et al., 2016; May and Spears, 1988; Spears and May 1988). In *P. incarnata,* flowers have been classified as either hermaphroditic or functionally male based on the final position of the styles once anthesis is complete (Dai and Galloway, 2012; May and Spears, 1988). In a hermaphroditic flower (Fig. 1A–C), anthesis begins with styles erect, but within three hours they have reached their final position level with the anthers. However, in a functionally male flower (Fig. 1D), the styles remain more or less erect (ca. 90°−160°) throughout anthesis, never reaching the same level as the anthers and thus never receiving pollen from a legitimate pollinator (defined as an insect that lands on the coronal filaments and has the appropriate thorax height to make contact with both the anthers and the stigmas). Both male and hermaphroditic flowers open between 11:00 a.m. and noon each day, and regardless of gender, the styles have reached their final positions by early afternoon. Floral gender in *P. incarnata* appears to be related to reproductive success. The greater the fruit set on a vine, the greater the frequency of male flowers relative to hermaphroditic flowers are subsequently produced on the vine (Spears and May 1988). Relative to gametes produced by hermaphroditic flowers, pollen from male flowers sired twice as many seeds as hermaphroditic flowers due to greater pollen production and less self-pollen deposition (Dai and Galloway, 2012). Greater pollen production

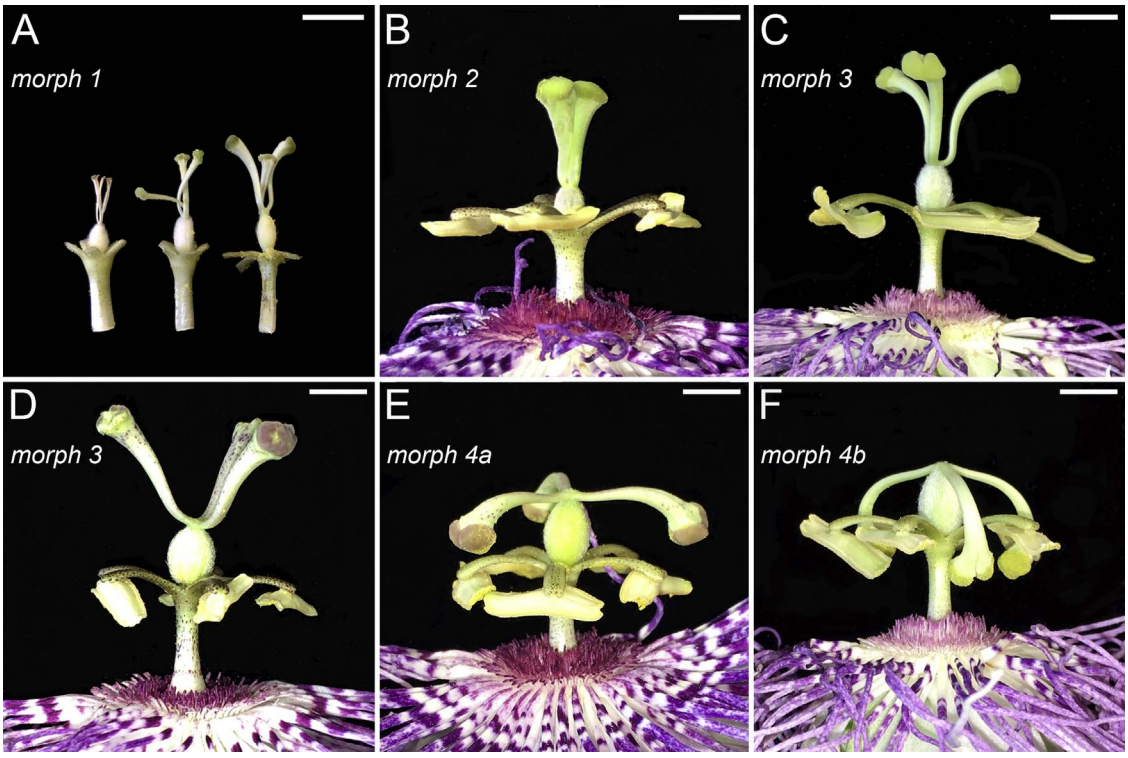

Fig. 2. Floral morph distinctions based on degree of gynoecial development and stylar deflexion during anthesis. A. Examples of morph 1, with aborted gynoecium and under-developed styles that do not deflex. B. Morph 2, with larger ovary and erect styles that remain condensed during anthesis. C. Morph 3, with even larger developed ovary and styles but styles that do not deflex completely. D. Morph 3 variant, with larger ovary and styles of normal size, but styles exhibit partial deflexion. E. Morph 4a, with fully developed ovary and styles that have deflexed to the same level (or slightly above) as the anthers. F. Morph 4b, with fully developed ovary and styles that have deflexed below the level of the anthers. Bars = 5 mm.

in male flowers may be due to decreased resource allocation towards female reproduction (Spalik, 1991; Spalik and Woodell, 1994).

However, the system of andromonoecy in *P. incarnata* is complicated by several factors: (1) hermaphroditic flowers experience a short period of "maleness" at the start of anthesis because it takes ca. 2–3 h for the styles to reach their final position near the anthers; (2) to identify floral morph and gender correctly, all observations must be performed in the late afternoon once anthesis has completed for each newly opened flower; (3) there is a great deal of variation in the distance the styles move downward in both "male" and "hermaphroditic" floral morphs, (4) some flowers exhibit a partially to completely aborted gynoecium (May and Spears, 1988), and (5) hand pollinations performed on functionally "male" flowers have indicated that some of these flowers are, in fact, fertile (Dai and Galloway, 2012; Spears and May 1988). These factors suggest that the andromonoecy observed in *P. incarnata* is not accurately represented by the two categories "functionally male" or "hermaphroditic." Moreover, preliminary observations of this species in 2015 and 2016 in Tennessee suggested the presence of five, rather than two, distinct floral morphs present on individual plants (Fig. 2). These morphs can be distinguished by ovary development and stylar deflexion: morph 1 has an aborted gynoecium with no stylar movement (Fig. 2A); morph 2 has a developed gynoecium but styles remain erect and condensed throughout anthesis (Fig. 2B); morph 3 styles deflex downward, but never reach the level of the anthers (Fig. 2C–D); morph 4a styles deflex to the level of the anthers (Fig. 2E); and morph 4b at least one stigma descends below the level of the anthers (Fig. 2F). For all floral morphs, there appeared to be a gradual increase in size of the perianth and coronal filaments, with the exception of morphs 4a and 4b, which differed only in the final position of the style (either level with, or below the anthers). The gradual increase in floral size and pistil development suggests there may be an ontogenetic basis for the observed floral morph variation; male morphs may represent floral buds that experience developmental arrest prior to complete pistil development. These observations have potential ecological implications. For instance, the identification of additional classes of male morphs (morph 1 being fully male with an aborted gynoecium, vs. morphs 2 and 3 being functionally male with partial gynoecial development), may affect the interpretation of previously published studies with respect to male and female reproductive fitness and plant-pollinator interactions, including nectar rewards.

Similar variation of *P. incarnata* floral morphs has been observed in Alabama, Florida, Georgia, North Carolina, Texas, and Virginia (California Academy of Sciences, 2017) suggesting this is not a locally restricted phenomenon for Tennessee. This raises interesting questions about the connections between floral development, reproductive phenology, and pollinator interactions in *P. incarnata*. In light of these observations, a study was designed to examine these floral morphs in greater detail and explore their potential ecological implications. Specifically, this study sought to: (1) document the variation observed among floral morphs with detailed measurements of each morph across multiple plants (2) evaluate the variation in floral morphs to see if they are consistent with altered floral development, (3) explore the relationship between floral morphs, nectar volume, and nectar constituents, and (4) consider the implications for floral visitors in relation to the observed morph variation.

2. Materials and methods

2.1. Study location

Three locations in Cookeville, Putnam County, Tennessee (TN) were used as study sites (Fig. 3A). Site A (36.132149N, −85.508985W) consisted of an early successional agricultural field, site B (36.176617N, −85.504543W) was a landscaped area along a brick retaining wall on the campus of Tennessee Tech University, and site C (36.165096N,

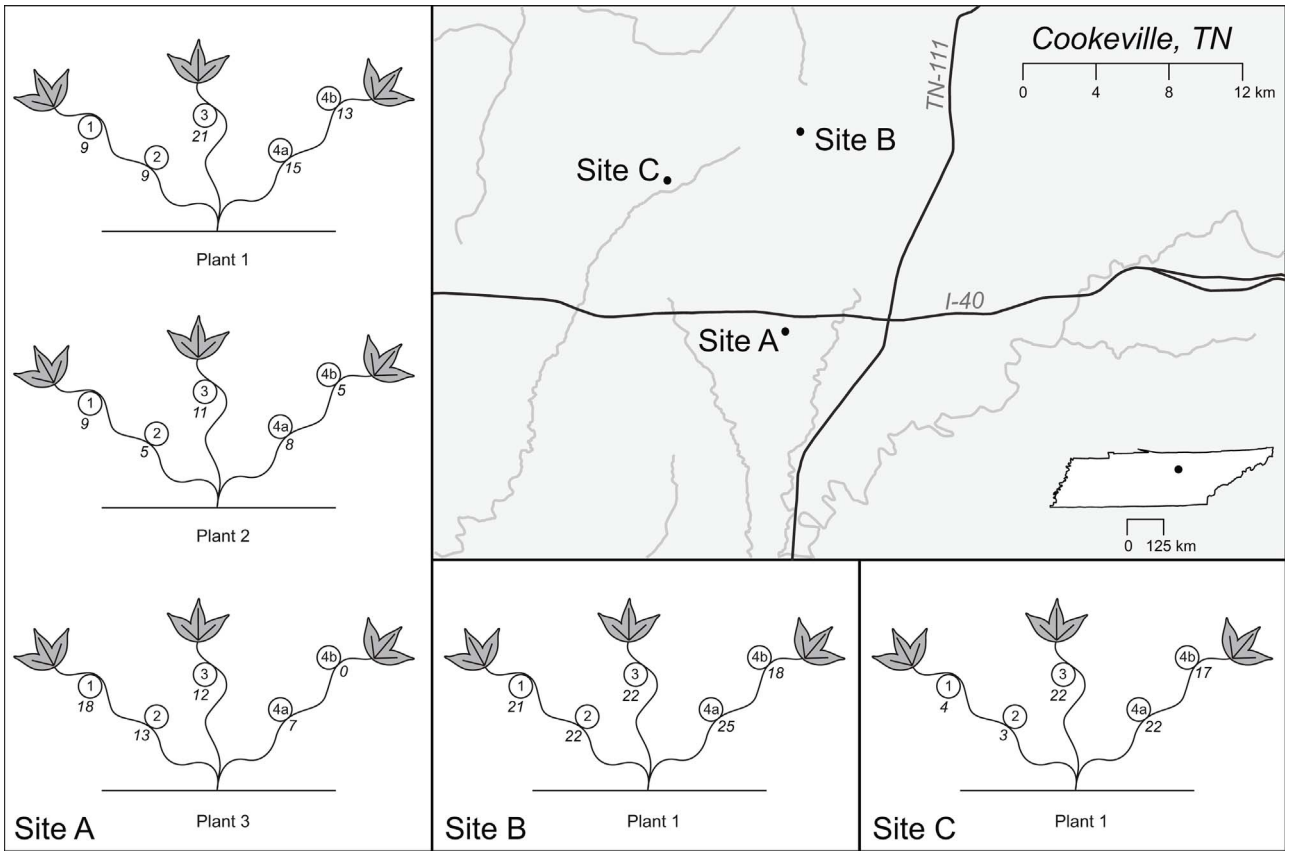

Fig. 3. Sampling of *P. incarnata* in Cookeville, TN. Upper right box shows map of study area and three field sites, left box shows three individual plants studied at site A, bottom center box shows single individual from site B, and bottom right box shows single individual from site C. Numbers within circles indicate floral morph, and numbers beneath each floral morph indicate the number of flowers measured on each individual.

−85.540815W) included the margin of a seasonally mowed field and the unmowed area underneath a recently fallen tree. Three large individual plants were identified from site A (Fig. 3B), each separated by a minimum distance of 10 m, suggesting they are genetically independent plants; sites B and C were each represented by a single sprawling individual plant. All datasets are archived in the Dryad Digital Repository (https://doi.org/10.5061/dryad.8dm63).

2.2. Floral measurements

2.2.1. Sampling

To try to identify differences in floral morphs apart from stylar movement, 13 morphological characteristics were measured for the flowers of *P. incarnata*. The characters chosen for analysis include the size of perianth, length of coronal filaments, and the sizes of individual organs within the androecium and gynoecium. Each floral character is described in Table 1 and illustrated in Fig. 4. Flowers that had opened that same day (ca. 11 a.m.) were collected between 4:00–5:00 p.m. in order to facilitate identification of floral morph, refrigerated to slow senescence, and measured with digital calipers within 72 h of collection.

2.2.2. Statistical analysis

Differences among floral morphs and individual plants were first tested using a two-factor permuted multivariate analysis of variance (PERMANOVA) with individual plant, floral morph, and the interaction between these terms used as factors. Prior to analysis, floral measurements were standardized using a z-score transformation in which the measurement means were rescaled to zero with unit variance using the 'decostand' and 'adonis' functions from the 'vegan' package in Program R (Oksanen et al., 2016). When significant differences among individual plants or floral morphs were detected, pairwise PERMANOVA tests were used to determine which individual plants and floral morphs differed. Multivariate relationships among floral measurements were illustrated using non-metric multidimensional scaling (NMDS) and the Euclidean distances between floral measurements made on individual flowers. We used NMDS over more traditional analyses (e.g., principal components analysis, PCA) because this approach allowed for illustrating best approximate relationships in only two axes, rather than up to 13 axes available with a PCA. The 'metaMDS' function from the 'vegan' package in Program R was employed using two dimensions (i.e., k = 2), and a maximum of 50 iterations. Morph-wise differences in floral measurements were tested using generalized linear mixed models (GLMM) structured with floral measurements as response variables (i.e., 13 individual models), floral morph as a fixed variable, and individual plant and individual flower identities as random variables. Random variables were necessary to account for non-independence among observations made on the same plant. Models were fit using the 'lmer' function from the 'lme4' package, significance of the effect of morph was tested using the 'Anova' function from the 'car' package, and post-hoc pair-wise comparisons based on least squares means were conducted using the 'lsmeans' function from the 'lsmeans' package. Model testing with the 'Anova' function employed a Type III Wald Chi-Square test to assess the significance of floral morph and was necessary because sample sizes were unbalanced among plants and morphs. Because these analyses were exploratory rather than confirmatory, no adjustment for experiment-wise error was used across floral measurements. All statistics were conducted in Program R version 3.1.3 (R Core Team, 2015).

Table 1
Floral measurements, character code, illustration reference, character description, measurement averages for each morph with standard deviation in parentheses, scores along non-metric multidimensional scaling (NMDS) axes 1 and 2, test statistics, and *P*-values for linear mixed models testing differences in floral measurements among morphs. Illustration reference refers to Fig. 4.

Floral Measurement	Code	Illustration Reference	Description	Morph 1 (StDev)	Morph 2 (StDev)	Morph 3 (StDev)	Morph 4a (StDev)	Morph 4b (StDev)	NMDS1	NMDS2	Chi-Square	*P*-Value
Maximum Flower Width	MFW	1	Diameter of flower measured from the tip of one sepal to the tip of the opposite one.	61.80 (4.34)	64.09 (5.22)	66.62 (5.73)	70.61 (6.06)	71.55 (5.53)	−0.008	−0.002	171.88	< 0.001
Maximum Hypanthium Width	MHW	2	Diameter of the floral hypanthium, measured at widest point at base of flower.	9.58 (0.88)	10.24 (0.93)	10.64 (0.91)	10.62 (0.96)	10.84 (0.91)	−0.008	−0.001	23.37	< 0.001
Maximum Coronal Filament Length	MFiL	3	Longest distance from base to tip of coronal filament.	23.94 (2.07)	25.03 (2.77)	26.01 (3.88)	28.68 (3.91)	28.75 (2.56)	−0.006	0.000	82.05	< 0.001
Height of Androgynophore	HA	4	Length from base of androgynophore to point where anther filaments extend outward.	9.25 (0.78)	9.13 (1.51)	9.46 (0.86)	9.75 (1.03)	10.03 (1.17)	−0.012	−0.006	33.69	< 0.001
Ovary Length	OL	5	Height measured from point where anthers extend outward to apex where styles diverge.	3.37 (0.75)	3.77 (0.54)	4.30 (0.56)	5.83 (1.18)	5.80 (0.91)	0.013	0.001	62.38	< 0.001
Ovary Width	OW	6	Width measured at widest portion of ovary.	1.75 (0.36)	2.58 (0.47)	3.05 (0.54)	3.75 (0.46)	4.03 (0.35)	0.026	0.002	713.06	< 0.001
Style Length	SL	7	Length from point of attachment at apex of ovary to stigma.	5.65 (1.43)	10.18 (1.54)	11.56 (1.42)	12.75 (1.46)	13.54 (1.44)	0.026	0.004	785.78	< 0.001
Width of Stigma	WS	8	Maximum diameter of stigma.	1.88 (0.64)	3.23 (0.58)	3.83 (0.58)	4.06 (0.54)	4.21 (0.53)	0.027	0.003	205.46	< 0.001
Anther Length	AL	9	Maximum length of anther.	9.66 (1.06)	9.96 (0.85)	9.97 (1.16)	9.61 (1.65)	9.77 (1.70)	−0.016	0.007	7.23	0.12
Anther Width	AW	10	Maximum width of anther.	3.39 (0.83)	3.45 (0.75)	3.60 (0.71)	3.70 (0.86)	3.57 (1.05)	−0.016	0.021	7.32	0.11
Filament Length	FL	11	Length from androgynophore to attachment point with anther.	7.03 (1.01)	7.25 (0.83)	7.30 (0.97)	8.21 (1.41)	7.59 (1.51)	−0.011	0.006	14.31	0.006
Nectar Chamber Width	NCW	12	Maximum width of nectar chamber.	0.87 (0.12)	0.97 (0.22)	1.10 (0.15)	1.25 (0.26)	1.17 (0.28)	0.003	−0.017	36.72	< 0.001
Nectar Chamber Height	NCH	13	Distance from base of chamber to point of contact between limen and operculum.	2.10 (0.49)	1.96 (0.51)	2.24 (0.40)	2.37 (0.41)	0.50 (2.38)	−0.011	−0.019	22.19	< 0.001

Fig. 4. Schematic longitudinal section of a flower of *Passiflora incarnata*, indicating measurements used for morphometric analysis. Number references are detailed in Table 1.

2.3. Nectar volume

To compare nectar production across floral morphs, individual buds were bagged the evening before anthesis to prevent pollinator access the following day. Preliminary observations of nectar volume indicated production began around anthesis, and continued well into the early hours of the following morning. For this reason, flowers were collected between 7:00–8:00 a.m. the day after anthesis, allowing for ca. 18 h for nectar accumulation within the nectar chamber. Drummond capillary pipettes (Drummond Scientific, Broomall, PA) ranging from 10–50 μL were used to collect and quantify nectar samples. Nectar was then blotted on Whatman grade 3 qualitative filter paper (GE Healthcare, Marlborough, MA), dried, and stored with silica gel. Statistical differences in nectar volume were analyzed using a GLMM as described above for floral measurements.

2.4. Nectar constituent analysis

Dried nectar samples from each individual plant representing all floral morphs were analyzed for constituents using High Performance Liquid Chromatography (HPLC). Due to low individual nectar volumes, samples within each floral morph were pooled across a single individual plant until a desired minimum total volume of 130 μL was obtained. For floral morphs where 130 μL was not available, all blot samples for that morph were pooled for analysis. Blots were dissolved by soaking in 5 mL of Milli-Q Direct purified water (Millipore, Billerica, MA) heated to 95° C. Each sample was then filtered with 0.45 μm IC Millex filter disks (Merck Millipore, LTD, Carrigtwohill, Ireland) to remove particulates, transferred to 1.5 mL glass sample vials (Shimadzu, Columbia, MD), and analyzed using a Shimadzu Prominence twin pump HPLC system with a Shodex Asahipak NH2P-50 4E column (Shimadzu, Columbia, MD) using HPLC grade solvents under isocratic conditions of 75% acetonitrile and 25% water at Southern Arkansas University. Two injections were performed as technical replicates for each sample. Each day of analysis, calibration curves of known sugar amounts were constructed using standard solutions from 5000 ppm (5.0 g/L) to 10,000 ppm (10.0 g/L) of glucose, fructose and sucrose. Data were integrated using SHIMADZU LCSolutions software and peak areas were regressed against sugar weight. For each sample, percentages of glucose, fructose and sucrose of the total sugar weight were calculated as well as total sugar concentration. Differences in mean sugar concentration were tested among morphs using a GLMM as described above; however, because individual flowers were pooled for this analysis, no random effect for individual flowers was included in the model.

3. Results

3.1. Floral measurements

In total, 233 flowers were measured for 13 characters representing all three sites, five genetically unique plants, and each of the five floral morphs (Table 1). Flower measurements differed significantly by floral

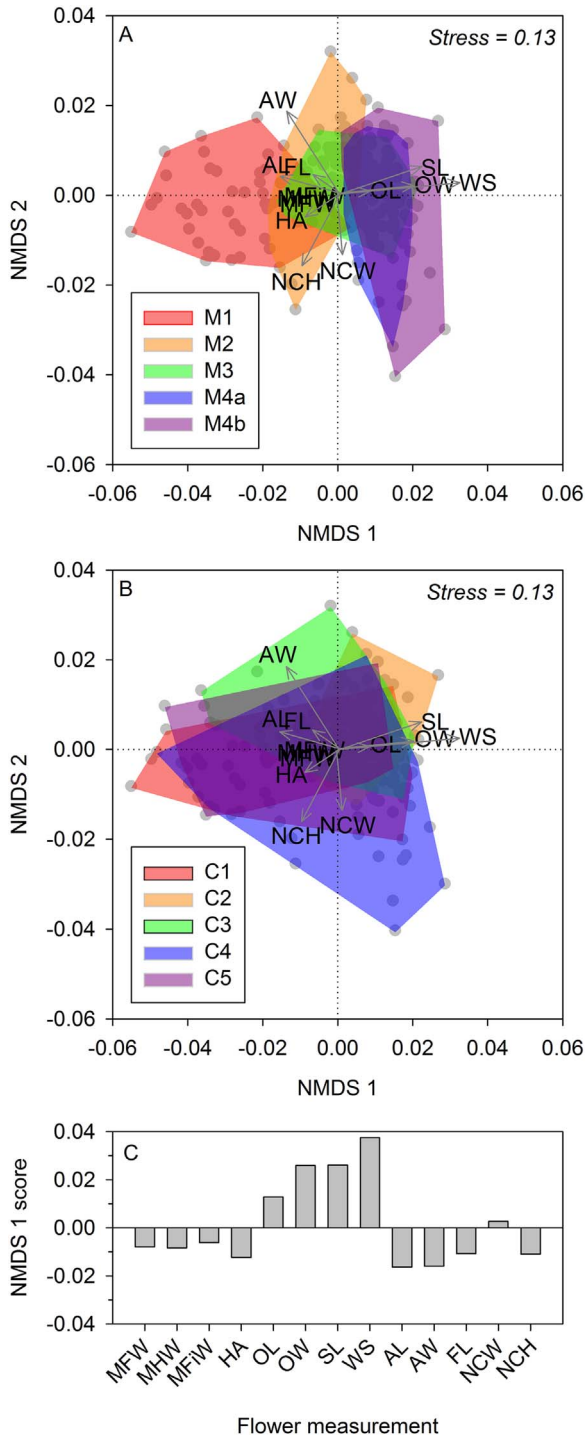

Fig. 5. Non-metric multidimensional scaling (NMDS) plot illustrating two-dimensional Euclidean distances among flower samples (grey dots) based on 13 flower measurements (see Table 1 for abbreviations). A. NMDS plot of five floral morphs. B. NMDS plot of individual plants. C. Flower measurement scores along NMDS 1, the axis representing a gradient of floral morphs.

morph and individual plant, including an interaction between these factors. The floral morph:individual plant interaction (PERMANOVA, *pseudo* $F_{14,210}$ = 2.44, $P < 0.01$) explained 6% of variation in measurements, the main effect of floral morph (*pseudo* $F_{4,210}$ = 47.15, $P < 0.01$) explained 36%, and the main effect of individual plant (*pseudo* $F_{4,210}$ = 23.20, $P < 0.01$) explained 18%. Post-hoc pair-wise comparisons revealed separation among floral morphs (pair-wise PERMANOVA adjusted for multiple tests, $P < 0.01$ for all comparisons) and individual plants ($P < 0.02$ for all comparisons). Multivariate gradients plotted using NMDS expressed low 2-dimensional stress (0.13) and illustrated segregation of floral morphs along NMDS 1 (Fig. 5A), but relatively larger overlap and no apparent pattern among individual plants (Fig. 5B). Flower measurements with the largest scores along NMDS1 included ovary length (OL, 0.013), ovary width (OW, 0.026), style length (SL, 0.026), and width of stigma (WS, 0.027); these characters were also most useful for segregating among floral morphs (Fig. 5C).

Floral measurements differed among morphs for 11 of the 13 characters when analyzed separately (Fig. 6), generally exhibiting an increasing trend from morphs 1 through 4b; anther length and width were the only characters that did not vary significantly across floral morphs (Fig. 6I–J). Significant increases in character size were observed between morphs 1 and 4 (a & b) for eight of the characters (Fig. 6A, C–H, L). Increases were also observed for post-hoc comparisons where morphs 1 and 2 were significantly smaller than morph 4a and 4b (Fig. 6A, C, L), and where morph 1 was smaller than 4a and 4b (Fig. 6D–F). Nectar chamber height (Fig. 6M) showed a significant increase in size from morph 2 to morphs 4a and 4b. Similar to the NMDS analysis (Fig. 5A), OL, OW, SL, and WS (Fig. 6E–H) exhibited the clearest increasing trend across morphs, with a minimum of three significant independent contrasts across the morphs. For all characters, morphs 4a and 4b were not significantly different from one another (Fig. 6A–M), which was consistent with the NMDS plot illustrating a large overlap between these morphs (Fig. 5A).

3.2. Nectar volume, constituents, and concentration

Nectar volumes were quantified for 321 flowers representing field sites A, B, and C, all individual plants, and all floral morphs. Similar to floral measurements, nectar volume exhibited a general increase across morphs (Fig. 7A). The average volume of nectar was 13.11 μL (S.D. = ± 6.1, n = 55) for morph 1, 18.21 μL (S.D. = ± 9.65, n = 52) for morph 2, 27.84 μL (S.D. = ± 9.38, n = 86) for morph 3, 30.07 μL (S.D. = ± 9.87, n = 75) for morph 4a, and 27.34 (S.D. = ± 11.25, n = 53) for morph 4b. Nectar volume differed significantly among the morphs (χ^2 = 83.51, df = 4, $P < 0.001$) and post-hoc contrasts indicated that morph 1 produced significantly less nectar compared to morphs 3 and 4a, and morphs 2 and 4b produced intermediate volumes.

For analysis of nectar constituents, samples were pooled across an average of 5.91 flowers per morph across all individual plants to reach a minimum 130 μL sample volume (min 3, max 13 per floral morph; 142 flowers in total); four pooled samples out of 24 analyzed had less than 130 μL with all available nectar samples combined. HPLC analyses revealed that nectar contained exclusively sucrose across all morphs on each individual plant, with the exception of site B, plant 1, morph 1, which had 91.6% sucrose, 5.4% fructose, and 3.0% glucose. Total sugar percentages calculated for each sample were marginally significant and decreased across morphs (χ^2 = 9.62, df = 4, P = 0.047, Fig. 7B). The average percentage of sugar for each morph across each individual plant was as follows: morph 1, 41.84% (n flowers = 5); morph 2, 39.58% (n = 5), morph 3, 36.79% (n = 5); morph 4a, 36.91% (n = 5); morph 4b, 32.38% (n = 4). Post-hoc contrasts suggested that morphs 1 and 2 had significantly higher sugar concentrations compared to morph 4b, and morphs 3 and 4a were intermediate.

4. Discussion

The present study sought to explore the diversity in floral morphs of *P. incarnata* in Putnam County, TN, and consider the causes and implications for this diversity. Given the environmental diversity among the three field sites, individual plant and site responses were considered in relation to the effect of floral morph to explain the data, and the interaction between these two effects was also considered. Floral morph

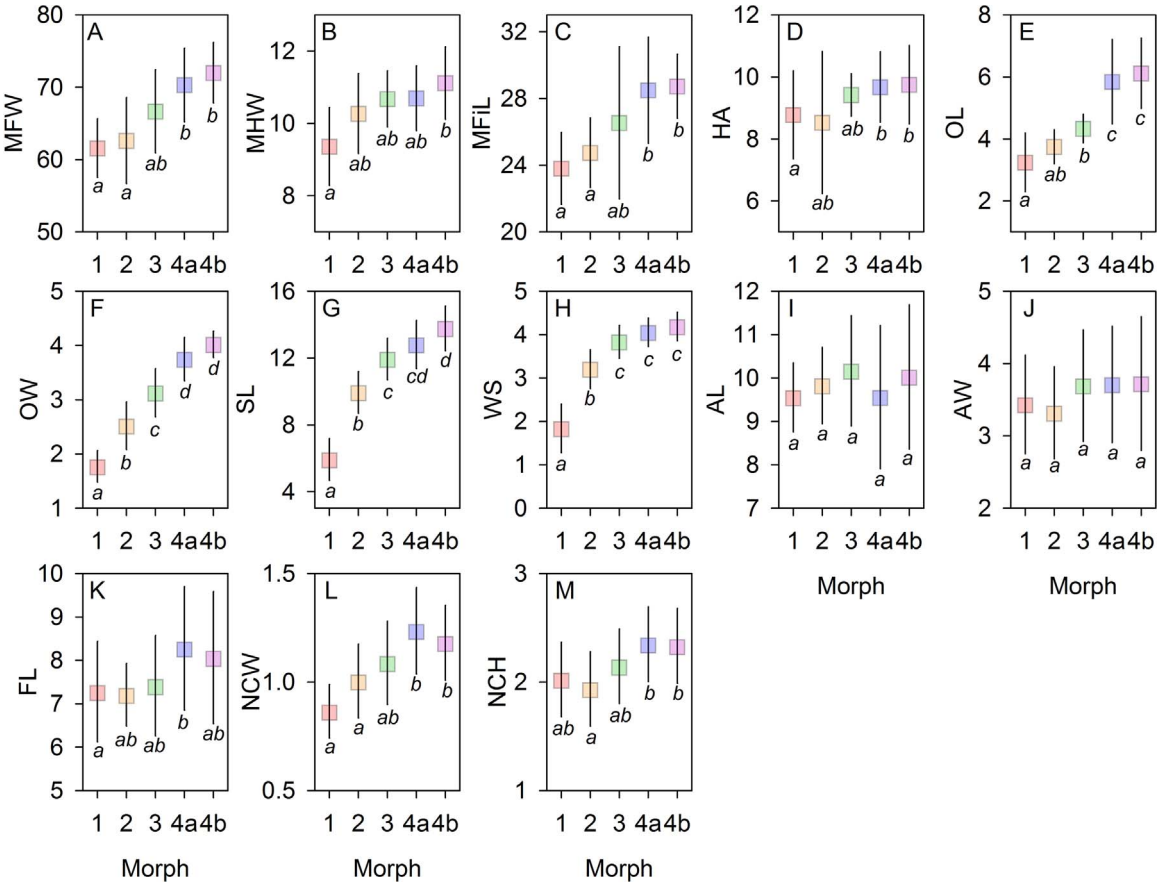

Fig. 6. Mean (± 95% CI) comparison for 13 floral measurements (y-axis) across the five floral morphs (x-axis). Lower-case, italicized letters indicate morph-wise differences in floral measurements tested using generalized linear mixed models and post-hoc pairwise comparisons; symbols with matching letters are statistically similar.

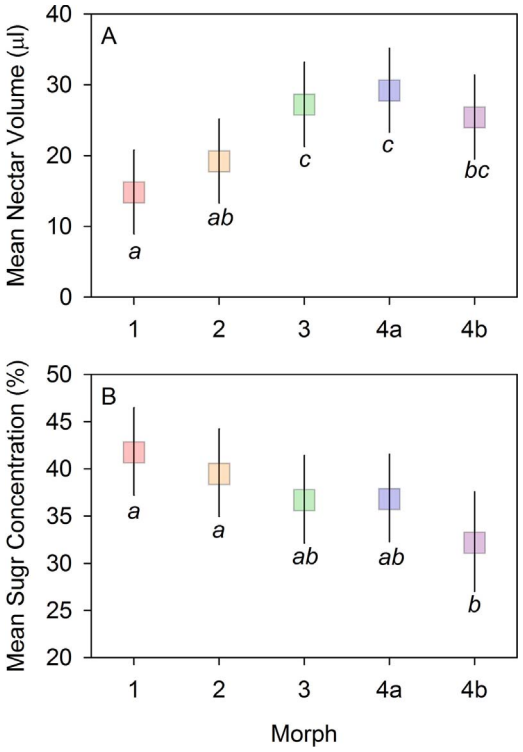

Fig. 7. Mean (± 95% CI) comparison of the five floral morphs regarding nectar volume (A) and nectar concentration (B). A. Nectar volume. B. Nectar concentration. Lower-case, italicized letters represent results of post-hoc pairwise comparisons; symbols with matching letters are statistically similar.

variation explained the greatest proportion of the variance in data (36%), followed by individual plant (18%) and then interaction between the two factors (6%). This supports the original observations that clear floral morph variation is present in *P. incarnata,* even across diverse field sites. Characteristics relating to pistil development provided the clearest criteria distinguishing among each morph. Extensive sampling of flowers that included both floral measurements and analysis of nectar volume and constituents supports the presence of four unique morphs (1 through 4), and two submorphs (4a and 4b). Below, the differences in floral morphs are examined in the context of floral development and compared to other cases of andromonoecy across the angiosperms. In addition, the relationship between floral size, nectar volume, and nectar concentration is also considered, in particular as it relates to plant-animal interactions.

4.1. Floral morph variation

The NMDS plot of the 13 floral characters showed partially overlapping distributions in morphospace for each of the originally hypothesized morphs. Notably, the distribution of morph 4a fell almost entirely within morph 4b, indicating these are better recognized a single morph with two sub-morphs. Given the only obvious distinction between morphs 4a and 4b was in the degree of stylar deflexion, it is not surprising that these two morphs are only weakly supported as unique. Based on the morphometric analysis performed here, the four distinct morphs were best distinguished by ovary length and width, style length, and stigma width. Overall floral size (maximum width of flower, width of floral hypanthium, and length of coronal filaments), and nectar chamber width also increased across morphs 1 through 4. Style deflexion remains a strong characteristic for morph identification: morph 1 had aborted or incompletely developed styles, morph 2 had

erect styles (90°) that remain clustered together, morph 3 had spreading styles oriented between ca. 100°–160°, morph 4a had styles level with the anthers (ca. 170°–180°), and morph 4b had styles positioned below the anthers (ca. 190°–230°). Androecial variation was not informative across morphs (filament length, anther length, anther width), though filament length did increase slightly between morphs 1 and 4a.

Stylar deflexion at anthesis is common in *Passiflora,* but the nature of this movement differs across species: in most species, styles typically descend to the level of the anthers, and then rise back to an erect position at the end of anthesis (MacDougal, 1994); in others, the styles continue downward, curling around the base of the ovary (Krosnick et al., 2015). The variability in the type of stylar movement and their degree of deflection suggests this feature may be an adaptive response to pollinator interactions (Janzen, 1968; May and Spears, 1988). At the start of anthesis, all species of *Passiflora* begin with styles in an erect position and then begin to deflex downward towards the anthers. However, there is a growing body of evidence that suggests styles in individual flowers of *Passiflora* do not always deflex: Amela García and Hoc (2011) cite 14 examples of species where a portion of observed flowers lacked stylar movement, and Krosnick et al. (2015) documented this in *P. herbertiana*. MacDougal (1994), May and Spears (1988), and Amela García and Hoc (2011) have all suggested that andromonoecy is likely more widespread than previously thought in *Passiflora*. Because stylar deflexion occurs over a period of minutes to hours and varies across species, brief observations of flowers (more common than detailed field studies on flowering phenology) will not reveal the stylar variation that may actually be present. It is predicted that careful observations of anthesis in other species of *Passiflora* will yield additional reports of andromonoecy.

The position of the styles relative to the anthers has important implications for reproductive biology in *P. incarnata* and has been extensively studied (Dai and Galloway, 2011, 2013; Dai et al., 2016). Downward deflexion positively correlates with the amount of pollen deposition a flower will experience during insect visitation, but may also increase the amount of self-pollination that occurs (Dai and Galloway, 2012; Dai et al., 2016). Given that *P. incarnata* is self-incompatible, this pollen will not fertilize ovules and may reduce the possibility that outcrossed pollen grains may germinate, depending on where the selfed-pollen is deposited on the stigma (May and Spears, 1988). In morphs 1 and 2, gynoecial development is stunted, making it unlikely that pollen deposited on the stigmas will lead to successful seed development. However, in the case of morph 3, the distinction between male morph and hermaphrodite becomes increasingly ambiguous. This is not only because of the greater deflexion observed in the styles, but the increasing size and development of the gynoecium overall, making it more likely that successful fertilization could occur.

The classification of these unique male morphs is challenging, but is especially relevant when trying to understand the reproductive biology of *P. incarnata*. Previously, male morphs were defined as having less style deflexion than hermaphroditic flowers (Dai and Galloway 2012). Under this definition, morphs 1–3 would be considered as a single "male" morph. Not surprisingly, a small percentage of crosses to male morphs recognized in this single category resulted in the production of fruit: 5% (Dai and Galloway, 2012) and 4.4% (May and Spears, 1988). It is likely the "male" morphs setting fruit in these two studies would have been categorized in the present study as morph 3, having developed enough to allow for at least partial function of the gynoecium. Prior studies focused on male versus hermaphroditic morphs from the standpoint of reproductive ecology and fitness, so the classification of a single male morph was justified and readily identified in the field. In contrast, the work presented here focuses on morphological variation and its developmental causes; the implications of morph 3 as an "intermediate gender" will be interesting to consider in future ecological studies.

4.2. Floral development

With increasing female function, the floral morphs demonstrated a general increase in size, as evidenced by 11 of the 13 characters examined. May and Spears (1988) originally hypothesized that these floral morphs could be explained by developmental arrest of floral buds at various stages near the end of ontogeny. This explanation fits very well with the morph variation observed in the present dataset. During floral ontogeny in *Passiflora,* the androecium is initiated, followed by the gynoecium, and then the androgynophore begins to elevate these structures within the developing bud (Bernhard, 1994, 1999; Claßen-Bockhoff and Meyer, 2015; Krosnick et al., 2006). Underneath, the hypanthium continues to develop. The hypanthium, sometimes referred to as the floral cup, floral tube, receptacle, or calyx tube (MacDougal, 1994) of *Passiflora* is unique in that it is responsible for the production of several novel floral structures. Within the hypanthium, intercalary meristematic activity occurs after all other floral organs have emerged within the existing floral space (Claßen-Bockhoff and Meyer, 2015). This meristematic activity creates room for the coronal filaments, operculum, and limen to initiate and expand. In turn, this growth may affect the size of the developing nectary as the space expands between the operculum and the limen.

Within the bud, each organ initiated will have a unique time to maturity, with certain organs reaching maturity earlier than others. When viewed from this perspective, Fig. 6 could provide some insights into the order and duration of the developmental program for each organ. For example, anther length and width did not vary across morphs. Given the anthers develop prior to the gynoecium, this suggests that any cessation of development across floral morphs occurs at least after the anthers have reached their final size. The hypanthium appears to reach maximum size by morph 4, though this distribution was not statistically significant. The distributions of coronal filament length, nectar chamber width, and nectar chamber height imply they reach full developmental maturity at morph 4, which is consistent with the later meristematic expansion of the hypanthium. As the hypanthium continues to enlarge, so do the coronal filaments and the floral nectary. The androgynophore also reaches its greatest height by morph 4, which provides additional space beneath for the expanding outgrowths of the hypanthium. Style length and stigma width with are maximized by morph 3, while the ovary attains maximum length and width by morph 4. Notably, all morphs are able to produce nectar; even morph 1 has developed enough secretory tissue to produce ca. 13 μL on average. However, only morphs 4a and 4b (and occasionally morph 3) are able to reliably develop seeds and fruit. Depending on how long the gynoecium within each bud is able to develop prior to anthesis, its overall size and potential reproductive function will vary. Yet questions remain as to how and why these floral morphs are induced in *P. incarnata,* and if they exhibit any similarities with other andromonoecious angiosperms.

There is a genetic and ecological component to andromonoecy, in addition to phenotypic plasticity, which may span both aspects (Diggle, 1991; May and Spears, 1988; Meagher 2007; Miller and Diggle, 2003). Diggle (1991) noted two alternative developmental pathways could result in observed andromonoecy: suppression of gynoecial development in the later stages of floral ontogeny, or delayed development of the gynoecium in male flowers relative to hermaphroditic flowers at the onset of ontogeny. In either case, comparative studies of floral development are required to determine when the ontogenetic changes occur that result in the observed floral morphs. In some species, e.g., *Gleditsia triacanthos* L. (Tucker, 1991) and *Apuleia leiocarpa* (Vogel) J.F. Macbr. (Zimmerman et al., 2013), female reproductive organs are completely absent in male flowers. In others, the gynoecium is progressively limited in response to mineral nutrition, water availability, or overall fruit

set on the plant (Gibbs et al., 1999; Zimmerman et al., 2013; Beavon and Chapman, 2011). In the former case, andromonoecy may be genetically pre-determined, while in the latter case ontogenetic changes later in development may limit gynoecial development. While it is likely that functional male morphs in *P. incarnata* result from later ontogenetic changes, a comparative developmental study looking at floral bud development across the morphs identified in this species would confirm this.

Resource allocation directed towards developing fruit has been proposed as one explanation for the presence of male morphs in *P. incarnata* (Dai and Galloway, 2012; May and Spears, 1988). As the number of fruits developing on the plant increases, less energy is directed towards producing hermaphroditic flowers, resulting in increased relative numbers of functionally male flowers. De Jong and Bruinsma (1974) suggested that IAA content in the seeds of developing fruits may inhibit pistil development; it is possible a similar hormonal effect occurs in *P. incarnata.* Only a small number of studies have documented andromonecy induced by fruit development, including *Cleome spinosa* Jacq. (de Jong and Bruinsma, 1974), *Caesalpinia calycina* Benth. (Gibbs et al., 1999), and *Solanum hirtum* Vahl. (Diggle, 1991) among others. However, Spears and May (1988) also found that defoliation of *P. incarnata* plants caused a significant increase in male flowers relative to hermaphrodites. Specifically, removal of the leaf adjacent to the developing floral bud increased the probability of male flowers; larger buds were less affected than smaller buds by leaf removal. Further, Dai and Galloway (2012) observed that while male flowers increased upon fruiting, they were also present at the start and middle of the reproductive season. Thus, there may be multiple factors (genetic, hormonal, resource limitation) that are affecting andromonoecy in *Passiflora.* Additional studies are needed to address these factors in greater detail.

4.3. Nectar variation across morphs

Nectar volume was shown to increase across morphs 1–4a, with morph 4b decreasing slightly; significant differences were only detected between morph 1 and morphs 3 and 4a. Interestingly, this nectar volume pattern is congruent with nectar chamber size, suggesting a connection between the capacity of the chamber and the maximum volume of nectar that the flower produces. Studies across a diverse array of angiosperms have noted that larger flowers are associated with increased nectar volume (e.g., Fenster et al., 2006; Kaczorowski et al., 2005; Tavares et al., 2016), but increased nectar volume is often associated with decreased nectar concentration (Fowler et al., 2016; Pacini and Nepi, 2007). In *P. incarnata,* sucrose was found to be the primary nectar constituent, with concentration ranging from 23 to 35% across all floral morphs. Sugar concentrations were significantly higher in morphs 1 and 2 compared to morph 4b, whereas nectar volumes were much lower. Concentration generally decreased with increasing morph number, consistent with larger volumes and larger floral sizes observed in morphs 3–4b. Pacini and Nepi (2007) noted several examples of species with variation in nectar volume due to floral morph variation, sexual phase (dichogamous flowers), or sexual system (dioecy or monoecy). Almeida et al. (2013) and Kaczorowski et al. (2005) found an inverse relationship between flower size and nectar concentration. In *Passiflora,* the nectar chamber is subtended by secretory tissue and ground parenchyma, interspersed with vascular tissue (Durkee, 1983; Durkee et al., 1981). The subtending tissue and the development of vasculature leading to the secretory cells is responsible for the volume and concentration of nectar secreted. Anatomical studies looking at the subtending tissues across morphs 1–4b might identify differences that can provide new insights into the variation in nectar volume and concentration observed in the present study.

Nectar volume was measured after allowing nectar to accumulate in the chamber over ca. 18 h, resulting in an average volume of ca. 23 μL across morphs 1–4b. Frankie and Vinson (1977) collected nectar hourly from *P. incarnata* (without any distinction of floral gender) between 12:30 p.m. and 6:00 p.m. They found that ca. 3.9 μL per flower was produced upon anthesis, with ca. 0.8 μL per hour accumulating until 5:00 p.m., and an increase to 2.1 μL per hour from 5:00 to 6:00 p.m. The maximum total volume they measured between 12:30 and 6:00 p.m. was 30.5 μL, which is consistent with the present study. In contrast, Dai and Galloway (2012) examined nectar production in male and hermaphroditic flowers, collecting nectar at anthesis and again in the evening immediately before dusk. Nectar volumes were ca. 4.4 μL at anthesis and 1.1 μL at dusk and there was no significant difference in amounts produced in male or hermaphroditic morphs. Differences in methods, locations, and morph designation make comparisons difficult among these datasets. However, it is possible that morph 3 (as defined in the present study) is responsible for an inflated male morph nectar volume in Dai and Galloway's (2012) study, as it was shown in the present study to be one of the highest nectar producing morphs. It is interesting that Frankie and Vinson (1977) noted an increase in nectar production in *P. incarnata* towards dusk, while this was not observed by Dai and Galloway (2012). Ambient humidity, temperature, and water resources can all affect nectar production (Pacini and Nepi, 2007). Additional studies that quantify the volume of nectar produced from anthesis through senescence the following morning are needed for all morphs of *P. incarnata* with appropriate corrections for environmental variables.

4.4. Implications for floral visitors

Field studies conducted on *P. incarnata* in Virginia (Dai and Galloway, 2012), Florida (May and Spears, 1988), New York (McGuire, 1998), Oklahoma (Hardin et al., 1972), South Carolina (Cunningham, 2000) and Texas (Frankie and Vinson, 1977) have indicated that *X. virginica* is the primary effective pollinator, and this appears to be the case in Tennessee, based on preliminary field studies (Krosnick, unpublished data). Sucrose-rich nectar is preferred by long-tongued bees (Baker and Baker, 1982, 1983; Perret et al., 2001) including Apidae, the family to which *X. virginica* belongs. The optimal sugar concentration for bees is 35% (Kim et al., 2011), and certainly the plants studied here produced nectar with approximately this sugar concentration (range: ca. 32–42%), though in practice bees collect nectar across a much wider range (Nicolson, 2007). Nectar concentration can have important implications for bee behavior. For example, Zhao et al. (2016) found that bumblebees visited flowers with higher nectar concentrations within the inflorescences of *Aconitum gymnandrum* Maxim. first, moving to lower concentration flowers secondly. Given that *P. incarnata* varies in both volume and concentration across morphs, total sugar reward (Fenster et al., 2006) should be evaluated (nectar volume × nectar concentration). Optimal foraging theory (Werner and Hall, 1974) predicts that *X. virginica* would spend more time at morphs that provide the greatest total sugar reward for the least foraging effort.

This simplified expectation, however, does not account for many other factors that influence flower choice in bees, including the size of floral display, accumulation of secondary compounds in nectar, and pollen rewards (Nicolson, 2007; Pacini and Nepi, 2007). In addition, *X. virginica,* like many other solitary bees, engage in scent marking of recently visited flowers (Frankie and Vinson, 1977); this marking was found to discourage other bees from visiting the flower for approximately 10 min. Knowing the rate at which the nectar chamber refills after bee visitation could provide new insights into how the bees relate to different concentrations and volumes of nectar offered at each morph. Future studies of pollinator choice for *P. incarnata* are needed to address nectar volume, concentration, flower size, floral display, and the amount of pollen removed and deposited across all morphs. As *P. incarnata* is self-incompatible, insect vectors are essential for natural reproduction. It is likely that important insights will be made regarding how pollinators relate to each morph, thus affecting the degree that each morph is able to contribute gametes to the next generation.

4.5. Future directions

The current study has identified four floral morphs (and two sub-morphs) with distinct trends in size, nectar volume, and nectar concentration. To address the developmental factors that lead to this variation, work is currently underway to examine pistil development and anatomy across morphs. This is being complimented by functional studies using hand-pollinations to assess how well pollen germinates on the stigma and penetrates the style to reach ovules (if present) in each morph. This work will be especially interesting with regard to morph 3, a functionally male morph, which is predicted to have at least some capacity to produce viable seeds. Field studies looking at pollinator interactions and rates of nectar production throughout anthesis are also underway. *Passiflora incarnata* is an accessible model system that provides many opportunities to ask morphological and ecological questions about plant reproductive biology, floral gender, and plant-animal interactions.

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