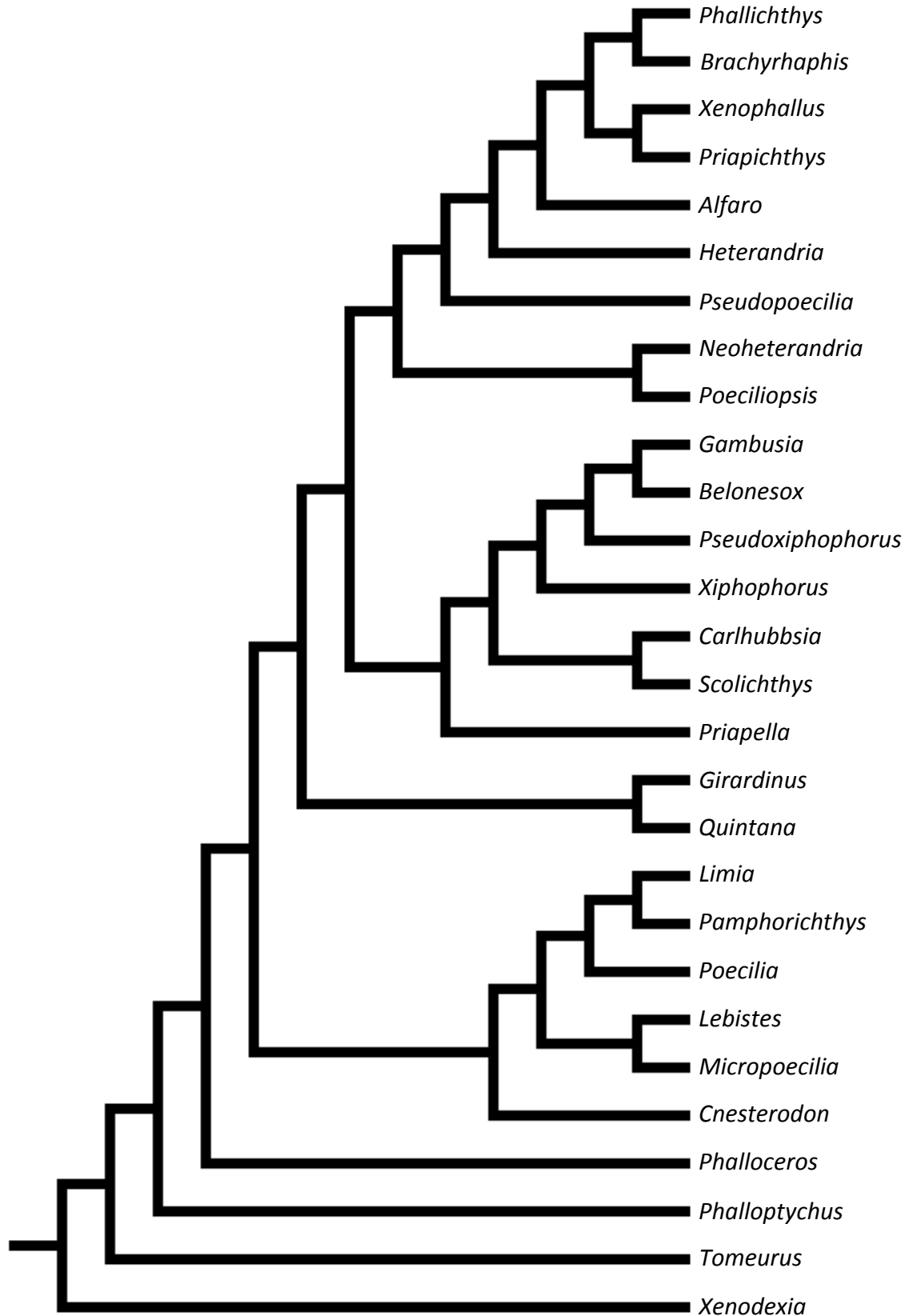


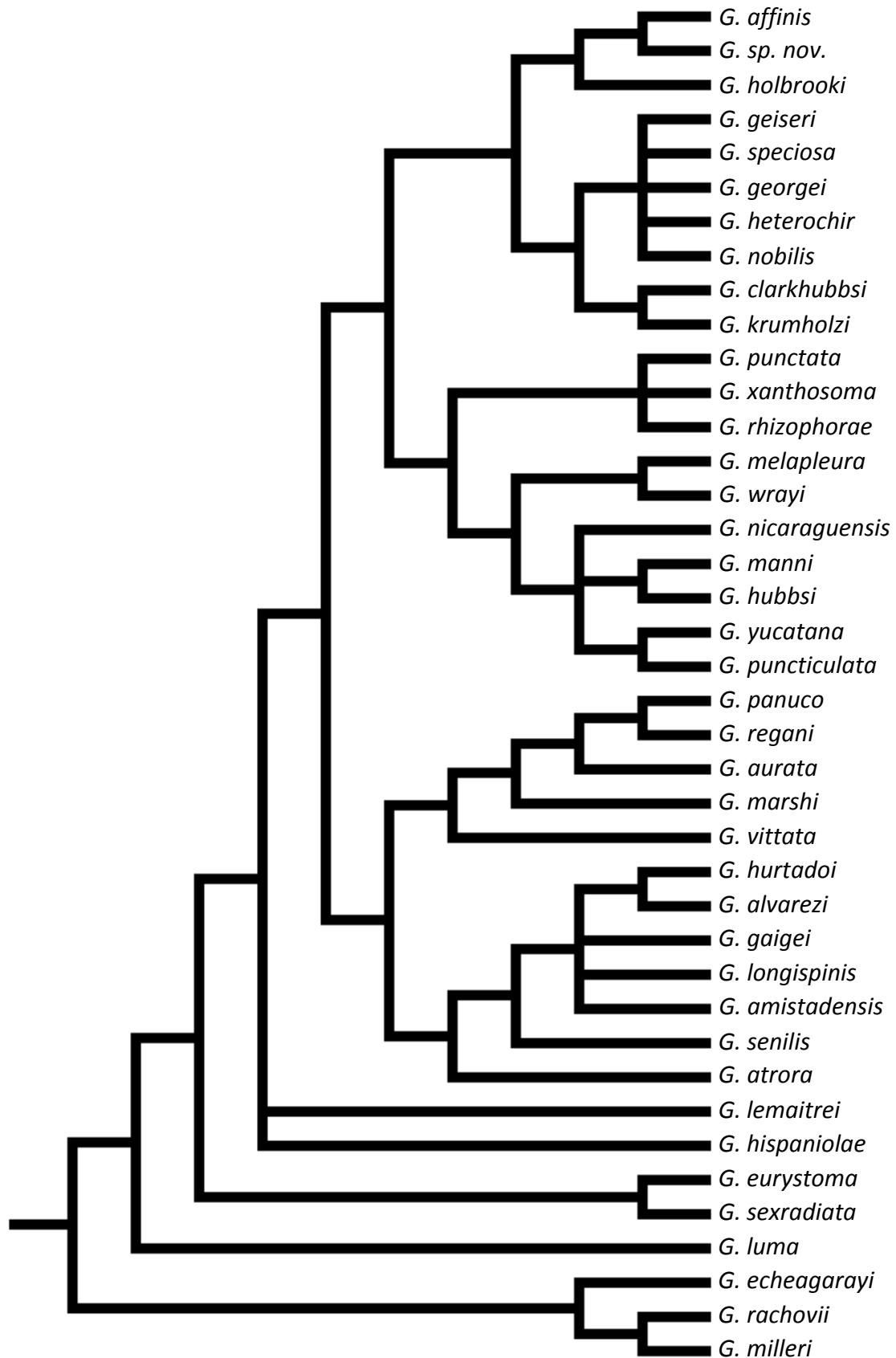
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Appendix 21.1 Phylogenetic hypothesis of Poeciliidae used in genera-scale analyses (from Hrbek et al. 2007). *Lebistes* represents the *Poecilia reticulata* lineage, which is genetically distinct from the rest of *Poecilia*. *Pseudoxiphophorus* represents the *Heterandria* lineages from Mexico and Central America, which are genetically distinct from *H. formosa*, found in the United States.



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Appendix 21.2 Phylogenetic hypothesis of *Gambusia* used in analyses (from Lydeard et al. 1995; Rauchenberger 1989; R.B. Langerhans, M.E. Gifford, O. Domínguez-Domínguez, I. Doadrio unpubl. data).



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Appendix 21.3 Character data examined in the genera-scale analyses. See text for descriptions of character values (appendices 21.5 – 21.8).

Genus	Courtship Display	Gonopodium Color	Gonopodium Length	Gonopodium Symmetry	Holdfasts	Accessory Structures
<i>Alfaro</i>	0	0	0	1	1.5	2
<i>Belonesox</i>	1	2	0	1	4	1.5
<i>Brachyrhaphis</i>	1	3	0	1	1.5	0.5
<i>Carlhubbsia</i>	0	1	1	0	2	1
<i>Cnesterodon</i>	0	0	1	1	3	1
<i>Gambusia</i>	1	2	0	1	4.5	2
<i>Girardinus</i>	0	1	1	1	4	1.5
<i>Heterandria</i>	0	0	1	1	2	0
<i>Lebistes</i>	1	0	0	1	4	3
<i>Limia</i>	1	1	0	1	2.5	2.5
<i>Micropoecilia</i>	1	1	0	1	2	3
<i>Neoheterandria</i>	0	0	1	1	2.5	0.5
<i>Pamphorichthys</i>	1	-	0	1	2	3
<i>Phallichthys</i>	0	0	1	0	2	0
<i>Phalloceros</i>	0	1	1	1	2	1
<i>Phalloptychus</i>	0	1	1	0	2	0
<i>Poecilia</i>	1	0	0	1	2	3
<i>Poeciliopsis</i>	0	1	1	0	1.5	0
<i>Priapella</i>	0	0	0	1	3	1
<i>Priapickthys</i>	0	0	1	1	3	0.5
<i>Pseudopoecilia</i>	0	0	1	1	2	1
<i>Pseudoxiphophorus</i>	0	-	1	1	2	0
<i>Quintana</i>	0	0	1	0	3	0
<i>Scolichthys</i>	0	0	0	1	3	1
<i>Tomeurus</i>	0	0	1	1	3	2
<i>Xenodexia</i>	0	-	1	0	2	2
<i>Xenophallus</i>	0	3	1	0	0	0.5
<i>Xiphophorus</i>	1	0	0	1	5.5	2

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Appendix 21.4 Character data examined in *Gambusia* analyses. See text for descriptions of character values (appendices 21.9 – 21.10).

Species	Gonopodial Tip Shape	Female Genital Morphology	Female Anal Spot Location	Female Anal Spot Size
<i>G. affinis</i>	2	4	3	1
<i>G. alvarezi</i>	1	2	1	3
<i>G. amistadensis</i>	1	2	1	3
<i>G. atrora</i>	1	2	3	1
<i>G. aurata</i>	1	4	1	1
<i>G. clarkhubbsi</i>	0	2	1	1
<i>G. echeagarayi</i>	3	4	0	0
<i>G. eurystoma</i>	1	4	1	2
<i>G. gaigei</i>	1	2	1	1
<i>G. geiseri</i>	2	4	0	0
<i>G. georgei</i>	1	2	2	1
<i>G. heterochir</i>	0	1	2	3
<i>G. hispaniolae</i>	2	4	0	0
<i>G. holbrooki</i>	2	4	3	1
<i>G. hubbsi</i>	1	4	0	0
<i>G. hurtadoi</i>	1	2	1	3
<i>G. krumholzi</i>	0	2	1	1
<i>G. lemaitrei</i>	2	3	0	0
<i>G. longispinis</i>	1	2	1	2
<i>G. luma</i>	0	2	0	0
<i>G. manni</i>	1	4	0	0
<i>G. marshi</i>	0	1	1	1
<i>G. melapleura</i>	1	4	0	0
<i>G. milleri</i>	3	4	-	-
<i>G. nicaraguensis</i>	2	4	3	1
<i>G. nobilis</i>	0	2	3	3
<i>G. panuco</i>	0	1	1	1
<i>G. punctata</i>	0	2	0	0
<i>G. puncticulata</i>	1	4	1	1
<i>G. rachovii</i>	3	4	0	0
<i>G. regani</i>	1	1	-	-
<i>G. rhizophorae</i>	0	2	0	0
<i>G. senilis</i>	1	2	-	-
<i>G. sexradiata</i>	1	4	0	0
<i>G. sp. nov.</i>	2	4	3	2
<i>G. speciosa</i>	1	4	0	0
<i>G. vittata</i>	0	0	0	0
<i>G. wrayi</i>	1	4	0	0
<i>G. xanthosoma</i>	0	2	0	0
<i>G. yucatanana</i>	1	4	-	-

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Appendix 21.5 A comparative test for the role of female mate choice in the evolution of gonopodium color.

To test the hypothesis that species with mating displays have evolved more colorful gonopodia due to female mating preference, I collected relevant data at the level of genera. Genera were assigned one of two mating strategies following prior work (Rosen & Tucker 1961; Bisazza 1993; Martin et al. In press), in which the genus either did not comprise any species that exhibit courtship displays (0) or did comprise at least some species that exhibit courtship displays (1). I collected genus-level data on gonopodium color using photographs, personal observations, and prior descriptions, scored on a scale of 0-3: 0 = no color, 1 = dark pigmentation only, 2 = bright coloration, 3 = strong, contrasting coloration, such as black and orange. Some genera contain species varying in the presence of gonopodial coloration, and these genera were scored according to the species that do exhibit coloration. Results suggest that gonopodial coloration has evolved in association with male display, as genera with bright or contrasting coloration also tend to include species exhibiting mating displays (25 genera, PIC: $r = 0.41$, one-tailed $P = 0.021$). This admittedly crude test provides cautious support for the hypothesis that female mate choice plays an important role in the evolution of gonopodium color. However, an alternative explanation is that males are generally more colorful in species with courtship displays, as female preference might have driven a general increase in male color, not in gonopodia *per se*. This would be consistent with the previous finding that sexual dichromatism may be associated with display behavior in poeciliids (Farr 1989; Bisazza 1993). While a trend between genera with sexual dichromatism and those exhibiting more brightly colored gonopodia does not seem apparent, the possible confounding role of correlated traits (i.e. brightly colored fins and bodies) deserves further examination.

Appendix 21.6 A comparative test for the role pre-mating sexual conflict in the evolution of gonopodium size.

Gathering generic-level data, I tested the hypothesis that longer gonopodia have evolved to effectively prevent/overcome female choice. Specifically, I examined the association between gonopodium length and mating display, where species with relatively long gonopodia are not expected to exhibit courtship. Genera were assigned to a mating strategy as described earlier (see appendix 21.5). Genera were classified as either having short (< ~35% of SL) or long (> ~35% of SL) gonopodia based on previous work (Rosen & Tucker 1961; Martin et al. In press). I found a significant evolutionary relationship between the two characters, as genera with longer gonopodia tend to also exhibit a lack of mating displays (28 genera, Pagel's 1994 test: one-tailed $P = 0.0003$).

Appendix 21.7 Comparative analyses for the evolution of gonopodial symmetry/asymmetry.

Rosen and Tucker (1961) suggested that gonopodial asymmetry should be disfavored in species with short gonopodia because it would interfere with the function of bilaterally symmetric accessory structures, such as pelvic and pectoral fins, effectively reducing insemination and fertilization success. Presumably, this selection for enhanced insemination/fertilization success results from sperm competition (but could additionally result from sexually antagonistic selection to thwart female control of mating), and predicts that gonopodial asymmetry should primarily evolve in species with long gonopodia. While prior work has suggested this association indeed exists (Rosen & Tucker 1961; Rosen & Bailey 1963; Greven 2005), no explicit test performed within a phylogenetic context has yet been performed. Here I provide such a test. Genera were assigned as either exhibiting asymmetric (0) or symmetric (1) gonopodia, and either exhibiting short or long gonopodia as

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described earlier (see appendix 21.6). The two characters exhibited a significant evolutionary association (28 genera, Pagel's 1994 test: one-tailed $P = 0.020$). While this association points to a possible factor responsible for the evolution of gonopodial asymmetry (functionally enhancing insemination/fertilization success for long gonopodia), it does not explain the existence of symmetric gonopodia.

One hypothesis for the evolution of gonopodial symmetry is that it is a byproduct of selection via sperm competition favoring holdfast devices at the gonopodium tip. That is, symmetric holdfasts might be more effective at enhancing insemination or fertilization than asymmetric ones, incidentally leading to the evolution of bilaterally symmetric gonopodia to ensure increased functionality. If accurate, gonopodial symmetry should evolve in concert with holdfasts. To test this prediction, I estimated holdfasts as the total number of potential types of holdfast structures (hooks, spines, and serrae) present on each fin ray (data from Rosen & Gordon 1953; Rosen 1967). If a holdfast type was invariably absent or present within a genus, it was scored as 0 or 1, respectively; if a type was variable in its presence within a genus, it was scored as 0.5. The sum of these values was used as the overall holdfast score for each genus. I found significant correlated evolution between gonopodial symmetry and holdfasts (28 genera, PIC: $r = 0.45$, one-tailed $P = 0.008$). Because gonopodial symmetry and gonopodium length also exhibit correlated evolution, it is possible that the relationship between symmetry and holdfasts is a spurious result of both characters evolving in concert with gonopodium length. However, this does not appear to be the case, as holdfasts and gonopodium length are not strongly associated (see appendix 21.8) and the relationship remains significant when examined within a multiple regression context (one-tailed $P = 0.023$).

Appendix 21.8 Comparative tests for correlated evolution of gonopodium length, holdfasts, and accessory structures.

To test the oft-suggested correlated evolution of gonopodium length, holdfasts, and accessory structures (Rosen & Tucker 1961; Rosen & Bailey 1963; Greven 2005), I examined these relationships across poeciliid genera within a phylogenetic context. Gonopodium length and holdfasts were estimated as described earlier (see appendices 21.6, 21.7 respectively). Accessory structures were estimated for each genera as the sum of the number of structures present that are thought to aid the gonopodium during copulation through tactile or kinesthetic means: hood/palp, bony extension, modified paired fins, and ventral spines. First, I did not find a particularly strong relationship between gonopodium length and holdfasts (28 genera, PIC: $r = -0.27$, $P = 0.17$). Second, a moderate, positive relationship between holdfasts and accessories was suggested by the data (28 genera, PIC: $r = 0.35$, $P = 0.069$). Finally, a strong association between gonopodium length and accessory structures was uncovered (28 genera, PIC: $r = -0.59$, $P = 0.0009$).

Appendix 21.9 A comparative test of coevolution of male and female genitalia in *Gambusia*.

Extending Peden's (1972a) findings, I recorded a crude score for male and female genital morphology for most species of *Gambusia*. For males, I recorded gonopodial tip shape using a range of 0-3: 0 = blunt/rounded, 1 = broadly acute, 2 = acute, 3 = strongly and narrowly acute (data for 18 species from Peden [1972b], data for 22 additional species gathered from previously published figures or personal examination of specimens). External female genital morphology was recorded using a range of 0-4: 0 = no papilla in aperture, aperture completely covered by tissue protuberance, 1 = no papilla in aperture, aperture partially enclosed by external tissue, 2 = small or absent papilla in small- to moderately-sized aperture, 3 = small to

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moderate papilla in large aperture, 4 = large papilla in large aperture (data for 18 species from Peden [1972b], data for 22 additional species gathered from personal examination of specimens). The phylogeny contained eight polytomies (Appendix 21.2), which I resolved by inserting branches of zero length. The degrees of freedom in the analysis was then adjusted for the number of polytomies (Purvis & Garland 1993; Garland & Diaz-Uriarte 1999). Because genital morphology was measured using ranks, the values were non-normally distributed, and their association was thus tested with the nonparametric Spearman rank correlation test. Strong evidence for coevolution among the sexes in genitalia was found (40 species, PIC: $\rho = 0.79$, one tailed $P < 0.0001$; Fig. 21.4).

Appendix 21.10 A comparative test of reproductive character displacement in *Gambusia*.

The genus *Gambusia* represents an excellent system to test the lock-and-key hypothesis, as gonopodial morphology is highly variable (see Fig. 21.3), we have a high degree of knowledge of female genital morphology compared to other poeciliid groups, *Gambusia* species exhibit a wide range of sympatry/allopatry status, and hybridization is known to occur in the wild (Hubbs 1955; Hubbs 1959; Scribner & Avise 1993). The pattern of reproductive character displacement might be observed at two scales, both of which could be examined in *Gambusia*: 1) populations of two species exhibit greater genitalic differences in sympatry than when in allopatry, and 2) species pairs found in sympatry exhibit greater genitalic differences than species pairs that are exclusively allopatric. Here I test for the latter pattern.

I estimated four external genitalic characters in *Gambusia*: male gonopodial-tip shape, female urogenital aperture morphology, female anal-spot location, and female anal-spot size. The first two traits were measured for 40 species as described earlier (see appendix 21.9), while anal-spot morphology was measured for 36 species (15 species from Peden [1973], 21 species from personal examination). Anal-spot location was scored on a scale of 0-3: 0 = absent, 1 = between anus and urogenital aperture, 2 = on or beside urogenital aperture, 3 = posterior to urogenital aperture. Anal-spot size was also scored on a scale of 0-3: 0 = absent, 1 = small, 2 = medium, 3 = large. I tested the prediction of greater differences in genital morphology between sympatric species than allopatric species, controlling for phylogenetic effects, by calculating matrices of pair-wise distances among species pairs (distances in genital morphology, phylogenetic distance), and testing for matrix association using one-tailed partial Mantel tests (using 9999 randomizations). Significant support for the prediction of reproductive character displacement was found for gonopodial tip shape (one-tailed $P = 0.021$) and urogenital aperture morphology (one-tailed $P = 0.036$), and suggestive support was found for anal-spot location (one-tailed $P = 0.113$); while the hypothesis was clearly rejected for anal-spot size (one-tailed $P = 0.794$) (Fig. 21.5). As a means of circumventing possible errors in the phylogeny, I also conducted analyses restricting the dataset to a well-supported clade of 12 *Gambusia* species in northern Mexico / southwest Texas that exhibit considerable variation in sympatry/allopatry status. Results are similar to that from the full dataset, regardless of whether phylogeny is included in the analyses (gonopodium tip: both one-tailed $P < 0.010$, urogenital aperture: both one-tailed $P < 0.028$, anal spot location: both one-tailed $P < 0.101$, anal spot size: both one-tailed $P > 0.79$). Thus, these results appear fairly robust.

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