

ORIGINAL ARTICLE

Salicaceae detoxification abilities in Florida tiger swallowtail butterflies (*Papilio glaucus maynardi* Gauthier): Novel ability or Pleistocene holdover?

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Abstract Florida populations of the eastern tiger swallowtail butterfly, *Papilio glaucus* L., have unique morphological features and ecological adaptations that have contributed to their subspecies status (*P. g. maynardi* Gauthier). We describe geographically unique abilities for detoxification of Carolina willow, *Salix caroliniana* Michx. (Salicaceae), for several Florida populations of *P. g. maynardi*. Of all the approximately 570 worldwide species of the Papilionidae, such Salicaceae detoxification abilities exist only in the allopatric North American western and northernmost species (*P. rutulus* Lucas, *P. eurymedon* Lucas and *P. canadensis* Rothschild & Jordan). Females of *P. glaucus* collected from populations in southeastern USA were examined for oviposition preference in 5-choice assays, and displayed a low preference for Salicaceae (<5%), but larvae from Florida populations exhibited a high survival (>60%) on these plants. Detoxification abilities have previously shown to be autosomally inherited, and can be transferred via natural or hand-paired interspecific hybrid introgression. However, these Florida populations are at least 700–1 500 km from the nearest hybrids or the hybrid species, *P. appalachiensis* Pavulaan & Wright, which possess these detoxification abilities. In any case, the Z (= X)-linked oviposition preferences for Salicaceae are lacking in these Florida populations, illustrating genetic independence of oviposition preference determination and larval survival/performance abilities. The origins of detoxification abilities are unlikely to be due to recent climate-driven introgression, and may represent ancestral trait carry-overs from interglacial refugium populations of the Pleistocene epoch.

Key words eastern tiger swallowtail, genetic introgression, interglacial island refugia, *Papilio glaucus maynardi*, Papilionidae host plants, Salicaceae detoxification

Introduction

Nutritional and allelochemical differences among host plants of herbivorous insects have been the subject of

extensive research and theory during the past 6 decades, especially in the swallowtail butterflies (Papilionidae) (Ehrlich & Raven, 1964; Feeny, 1976; Scriber & Slansky, 1981; Herms & Mattson, 1992; Scriber, 2002a, 2010; Berenbaum & Feeny, 2008). One of the most unique families of plants, used by only two or three species of the approximately 570 species of swallowtail butterflies, is the Salicaceae (willows, poplars and aspens). Only *Papilio rutulus* Lucas, *P. canadensis* Rothschild & Jordan, and to a lesser degree, *P. eurymedon* Lucas, can use

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Salicaceae as a host (Scriber, 1984, 1991; Dowell *et al.*, 1990; Ayres & Scriber, 1994); the phenolic glycosides are toxic to other swallowtail species in the *P. glaucus* group (Lindroth *et al.*, 1986, 1988a), and larval survival in the critical neonate stage (Zalucki *et al.*, 2002) has been essentially zero for all *P. glaucus* L. populations tested (Scriber, 1988, 2002a, 2004). The detoxification abilities of Salicaceae by *P. rutulus* and *P. canadensis* are genetic (autosomal inheritance; Scriber, 1986b) and transferable interspecifically to *P. glaucus* via natural or artificial (hand-paired) hybridization and backcrosses (Scriber *et al.*, 1989, 1995, 1999).

Host use of Salicaceae plant species in the field requires adult oviposition on such plants. Using interspecific hybrids with *P. glaucus*, the genetic basis of quaking aspen, *Populus tremuloides* Michx., acceptance and use has been shown to be sex-linked in *P. canadensis*, controlled by a factor on the Z (= X) chromosome (Scriber *et al.*, 1991b; Mercader & Scriber, 2007; Mercader *et al.*, 2009). The eastern tiger swallowtail, *P. glaucus* usually avoids *Populus* sp. for oviposition (including *P. tremuloides*, Scriber, 1993; Mercader & Scriber, 2008; and *P. deltoides* Bartram ex. Marsh., Scriber, 2004). Preliminary oviposition assays of the morphologically distinct Florida tiger swallowtail populations (Lehnert *et al.*, 2011) (putative *P. glaucus maynardi* Gauthier; Maynard, 1891) subspecies showed nearly total avoidance of Salicaceae (<5%) in 3- and 5-choice preference arrays that included Rosaceae, Magnoliaceae and Oleaceae plants (Bossart & Scriber, 1995). However, these ecologically monophagous Florida populations have not been assayed for larval survival capabilities on Salicaceae (Scriber, 1986a; Scriber *et al.*, 1995). We report the results of oviposition assays for *P. glaucus* sampled from southeastern USA populations, and larval survival assays for Florida populations on one of the most common and widely distributed Salicaceae species, Carolina willow, *Salix caroliniana* Michx. (Nelson, 1994). Other host plants commonly used by *P. glaucus*, including the local favorite, sweetbay, *Magnolia virginiana* L. (Magnoliaceae), were included for comparison.

Materials and methods

In order to evaluate the willingness to accept Salicaceae for oviposition and larval survival abilities, wild females of *P. glaucus* were allowed to oviposit in the laboratory by placing individual females and different host plant leaves (aqua-picked), inside clear plastic arenas (Fig. 1 shows a 3-choice array); the arenas were placed on rotating platforms in front of a bank of 100 W incandescent lights (see Scriber, 1993). The collecting locations are listed in Table 1 (see also Fig. 2). Four-choice assays consisted



Fig. 1 Photograph of oviposition arena setup used to determine oviposition preference of females of *Papilio glaucus*. In this image, a female was placed inside the arena to demonstrate a setup for a 3-choice assay (only 4- and 5-choice assays were used in this study).

of sweetbay, *M. virginiana* (SB), black cherry, *Prunus serotina* Ehrh (Rosaceae) (BC), green ash, *Fraxinus pennsylvanica* Marshall (Oleaceae) (GA) and Carolina willow, *S. caroliniana* (W); 5-choice assays added tulip tree, *Liriodendron tulipifera* L. (Magnoliaceae) (TT). Carolina willow was selected because it is common and widely distributed throughout most of Florida, perhaps more so than any other Salicaceae species (Nelson, 1994).

Females were fed 20%–25% honey water solution daily. The eggs were counted according to which plant they were oviposited upon and collected daily. Eggs not oviposited directly onto leaves (including plastic arena or filter paper lining), but oviposited within 2 cm of a leaf were counted; eggs laid further than 2 cm were not counted. Eggs were placed into a Petri dish and left in a laboratory room for larval emergence (18 : 6-h L : D photoperiod: 22–25°C). Upon eclosion, neonate larvae were immediately transferred (using camel hair brushes) into plastic rearing dishes (Rubbermaid TakeAlongs®, Atlanta, GA, USA) that contained an aqua-picked host plant. Distribution of these split broods was randomly made across the five host plant treatments. Survival through completion of the first larval instar and to pupation was recorded.

Additional data from similar assays conducted during the previous 30 years were presented to illustrate the geographic pattern of Salicaceae and Magnoliaceae host plant use differences between the *P. glaucus* and *P. canadensis* butterfly populations and their hybrids. These studies include larvae from populations of *P. glaucus* (168 families, 2 282 initial larvae set up; 23 counties sampled, 15 states), the hybrid zone (154, 2 180; 21 counties, 8 states), and *P. canadensis* (232, 2 772; 41 counties, 4 states and

Table 1 Oviposition preferences of individual females of *Papilio glaucus* from seven populations in southeastern USA (percentage of total eggs in 5-choice bioassays; 2008).

Location	GPS Coordinates	<i>n</i>	SB	TT	BC	GA	W	Total eggs
Lake Placid, FL	27°19'58"N 81°18'55"W	4	36 a	40.4 b	5.1	16	2.5 ab	191
Sebring, FL	27°28'0"N 81°32'52"W	17	15.8 ab	57.7 ab	6.7	15.9	3.8 a	1622
Cedar Key, FL	29°12'49"N 83° 2'12"W	6	21.1 ab	48.9 b	4.4	20.4	5.15 a	848
Waycross, GA	31° 5'51"N 82°13'38"W	5	5.5 b	67.0 a	7.0	17.9	2.7 ab	420
Fairmount, GA	34°56'5"N 85°16'43"W	4	19.5 ab	53.3 ab	11.2	13.4	2.5 ab	477
Elkton, TN	35° 2'32"N 86°58'51"W	4	6.0 b	55.7 ab	7.7	28.8	1.8 ab	514
Fayette, AL	33°41'16"N 87°48'26"W	3	11.1 ab	48.1 ab	8.9	31.9	0 b	103
Mean ± SE			16.3 ± 2.5	54.7 ± 2.4	6.9 ± 1.1	18.8 ± 2.3	3.2 ± 0.5	
Totals		43						4 175

SB, sweetbay; TT, tulip tree; BC, black cherry; GA, green ash; W, Carolina willow.

Mean percentages in columns followed by the same letter are not significantly different ($P = 0.05$; each pair Student's *t*-tests).

Data represents average percentage of eggs oviposited for each population, the transformed data used to determine significant differences is not presented.

Manitoba) given quaking aspen, *P. tremuloides* (Salicaceae), in a split-brood regime to determine percent survival. All populations tested also were fed tulip tree, *L. tulipifera* (Magnoliaceae) (*P. glaucus* 176, 2372; hybrid zone 162, 1486; *P. canadensis* 232, 2765). The Florida populations of *P. glaucus* (15, 94; 5 counties, 1 state; Fig. 2) represent the putative *P. g. maynardi* subspecies, and were given *S. caroliniana* (Salicaceae) and *L. tulipifera* to compare larval survival. The data were arcsine transformed for normality and uploaded in JMP® 9.0 (SAS Institute Inc., Cary, NC, USA) for analysis. An each pairs Student's *t*-test was applied to determine significant differences ($P = 0.05$) in oviposition preferences between populations sampled per host plant. The Tukey-Kramer Honestly Significant Difference (HSD) test was used to determine significant differences ($P = 0.05$) in larval survival abilities between the tiger swallowtail butterfly genotypes (*P. glaucus*, *P. canadensis*, hybrids, and *P. g. maynardi*) tested.

Results

The 43 females of *P. glaucus* from the southeastern USA assayed for oviposition preferences in the 5-choice arrays clearly avoided *S. caroliniana* (Table 1). A total of 4 175

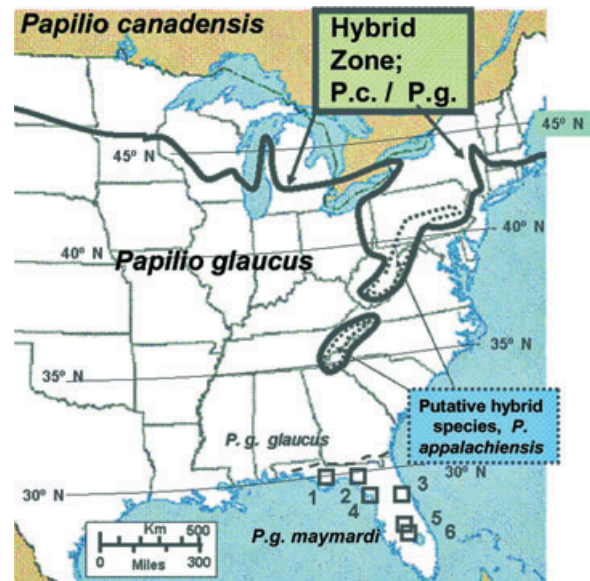


Fig. 2 The geographic distribution of tiger swallowtail butterflies in eastern North America. *Papilio glaucus*, *P. canadensis*, the center of the historical hybrid zone, (see Scriber *et al.*, 2008a; Scriber, 2011 for details), and the putative *P. g. maynardi* (Scriber, 1986a) are shown with Florida populations used for neonate survival assays indicated (1 = Wakulla, 2 = Perry, 3 = Barberville, 4 = Cedar Key, 5 = Sebring, 6 = Lake Placid).

Table 2 Larval survival on five Florida plant species (2008). The first, second and third columns under each host plant heading are the total numbers of larvae set up on that host plant (A), the number that survived to second instar (B), and the number that pupated (C), respectively.

Location	GPS coordinates	Family ID	SB			TT			BC			W			GA		
			A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Cedar Key, FL	29°12'49"N	8020	0	0	0	–	–	–	3	3	0	6	0	0	5	2	0
	83°2'12"W	8308	25	24	17	29	27	23	9	9	9	1	1	0	–	–	–
		8309	2	2	0	10	7	6	1	1	1	10	1	0	–	–	–
		8313	34	23	9	4	3	1	4	4	4	6	3	1	11	8	1
		8318	53	37	13	17	14	7	21	20	19	11	7	0	10	8	1
		8324	9	8	2	20	17	12	7	7	4	3	3	0	4	4	0
Sebring, FL	27°28'0"N	8173	8	3	1	–	–	–	2	0	0	6	3	0	–	–	–
	81°32'52"W	8341	13	12	6	36	34	21	10	10	10	5	3	0	–	–	–
		8353	19	15	8	7	5	4	1	1	0	2	2	0	–	–	–
		8355	4	4	2	23	17	13	11	5	4	2	2	0	–	–	–
		8485	27	20	8	17	13	9	19	12	5	5	2	0	2	1	0
Barberville, FL	29°11'36"N	8063	81	74	28	–	–	–	72	52	10	18	10	7	33	6	0
	81°24'8"W																
Perry, FL	29°58'24"N	8114	9	4	3	–	–	–	11	1	1	6	3	0	5	0	0
	83°28'58"W																
Lake Placid, FL	27°19'58"N	8239	30	29	18	–	–	–	12	8	8	7	7	2	3	2	2
	81°18'55"W																
Wakulla, FL	30°12'51"N	8242	10	10	5	–	–	–	15	13	11	5	4	0	–	–	–
Totals	84°11'46"W		324	265	120	163	137	96	196	146	86	94	51	10	73	31	4
% Survival				83.3	36.5		80.2	54.5		75.9	51.6		63.9	5.6	53.4	10.7	

SB, sweetbay; TT, tulip tree; BC, black cherry; GA, green ash; W, Carolina willow.

eggs were oviposited, but the mean percentage of eggs placed on willow was less than 5%. In 4-choice assays, the total number of eggs placed on *S. caroliniana* was less than 10% of the 953 eggs oviposited by 15 females from Florida (data not shown).

Neonate larval survival (total larvae from all families) on these host plant species for Florida populations was greatest for *M. virginiana* (SB = 83%), followed closely by *L. tulipifera* (TT = 80%), and *P. serotina* (BC = 76%) (Table 2). The overall neonate larval survival was lowest on *F. pennsylvanica* (GA = 53%) and survival on *S. caroliniana* was intermediate (CW = 64%); however, the only significant difference in larval survival was between *M. virginiana* (high survival) and *F. pennsylvanica* (low survival) (each pair Student's *t*-test, $P = 0.05$). The survival to the pupal stage was lower: 37% on SB, 55% on TT, 52% on BC, 11% on GA and 6% on CW.

The various tested populations of *P. canadensis* and *P. glaucus* genotypes from Canada to Florida show a major reciprocal difference in their host plant use abilities with very poor survival of *P. glaucus* on quaking aspen, *P. tremuloides* (Salicaceae), and of *P. canadensis* on tulip tree

(Magnoliaceae) (Fig. 3). The populations within 100 km of the historical hybrid zone showed considerable introgression of host use abilities for both of these species. Percent larval survival of *Papilio canadensis* on Magnoliaceae was significantly different (lower) than the other genotypes tested (Tukey-Kramer HSD, $P \leq 0.0001$); all other populations of *P. glaucus* and hybrids were similar in ability to survive on Magnoliaceae (Fig. 3). Significant differences were found between the *P. glaucus*, *P. canadensis* and hybrid zone populations in ability to survive on Salicaceae (Tukey-Kramer HSD, $P \leq 0.0001$). The Florida 2008 populations were only significantly different from *P. glaucus* populations; they were similar in Salicaceae detoxification abilities to *P. canadensis* and hybrid zone populations (Fig. 3).

Discussion

Similar to previous studies, *P. glaucus* showed a low oviposition preference for Salicaceae (Bossart & Scriber, 1995). Results from this study suggest that wild Florida

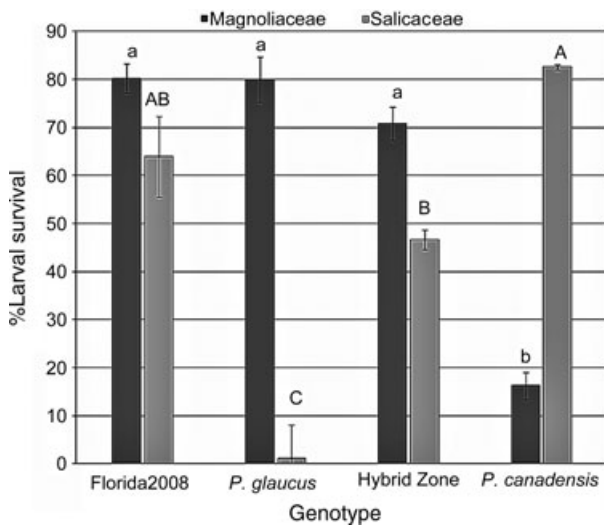


Fig. 3 Neonate survival summaries for populations of *Papilio glaucus*, *P. canadensis*, hybrid zone, and Florida 2008 genotypes fed on Salicaceae and Magnoliaceae. The Florida 2008 genotype represents the putative *P. g. maynardi* subspecies. Survival is expressed as the mean \pm SE of all populations per genotype. Significant differences between percent means were tested for each plant family between each genotype (Tukey-Kramer HSD, $P \leq 0.001$), as indicated by different letters (capital letters, Salicaceae; lowercase letters, Magnoliaceae). Data for *P. glaucus*, *P. canadensis*, and hybrid zone genotypes combined from previous studies (Scriber, 1983; Scriber & Ordning, 2005; Mercader *et al.*, 2009; Scriber, unpublished data).

populations of *P. glaucus* likely do not use, or rarely use, *S. caroliniana* as a host plant when other host plants such as sweetbay, tulip tree, black cherry and green ash are available (Table 1). Sweetbay is the only host plant available to southern populations of *P. g. maynardi* (Scriber, 1986a; Bossart, 2003); although *S. caroliniana* is also found in these southern regions, the lack of oviposition on this plant likely maintains the ecological monophagous status of these populations.

Although lower than observed on the favorite Magnoliaceae and Rosaceae host plants (see Scriber *et al.*, 1991a; Bossart, 2003), Florida populations of *P. glaucus* neonate larval survival on *S. caroliniana* (Salicaceae) was unexpectedly high (64%, 15 families from 6 different Florida populations), with some larvae surviving to pupation (Table 2). Previous host use survival studies of *P. glaucus* on various Salicaceae species ($n = 7$ species of *Populus* and 4 species of *Salix*) resulted in essentially total mortality of neonate larvae; only on weeping willow, *S. babylonica* L., did any neonates survive ($> 9\%$), while *P. canadensis* had high survival on all 12 Salicaceae species (Scriber, 1988; Ayres & Scriber, 1994).

Carolina willow was not assayed in these earlier studies, but the phytochemical toxins of this plant include similar concentrations (Lindroth *et al.*, 1988b; Soetens *et al.*, 1998) of the phenolic glycosides salicin, salicortin and tremulacin (Prudic *et al.*, 2007) found in other *Populus* and *Salix* species (Palo, 1984; Julkunen-Tiito, 1989; Nyman & Julkunen-Tiito, 2005). These glycosides have previously been shown to be either toxic (Lindroth *et al.*, 1986, 1988a), or serve as a repellent (larval refusal to eat leaves; Scott, 1986; Scriber, 1988) to *P. glaucus* larvae.

Phenolic (salicylic) glycosides and other phytochemicals may differ significantly among *Salix* species (Ruuhola *et al.*, 2001). However, local adaptations of Salicaceae-specialized insect herbivores remain somewhat plastic and host shifts can occur to and from species that exhibit high or low salicylic glycoside levels (Weingartner *et al.*, 2006; Zvereva *et al.*, 2010). Generalist species (such as *P. glaucus*) have been negatively impacted by such chemical variation in glucoside concentrations (Tahvanainen *et al.*, 1985; Lindroth *et al.*, 1988a; Ruuhola *et al.*, 2001).

The eastern tiger swallowtail butterfly is the most polyphagous of all Papilionidae worldwide (Scriber, 1973, 1984) and has been shown to feed successfully on dozens of species from more than 12 families (Scriber, 1986a, 1988). Historical records of Salicaceae use as a host plant by *P. glaucus* exist (Gosse, 1840; Edwards, 1884, 1885a, b, 1886; Scudder, 1889; Clark & Clark, 1951; McGugan, 1958; Tietz, 1972; Scott, 1986); however, most of these references relate to weeping willow and may have been the source of some subsequent literature citation duplication. In any case, most of these reports are likely in reference to *P. canadensis* (then believed to be a subspecies of *P. glaucus*; see Scriber, 1988; Hagen *et al.*, 1991) or hybrids in what today has been recognized as a hybrid zone from the Great Lakes to New England (Scriber, 2010, 2011; Scriber *et al.*, 2003, 2008a).

Salicaceae detoxification in Florida populations: An ancestral ability or a novel trait?

Recent gene flow from the mountain refuges (possibly supporting *P. appalachiensis* genotypes; Scriber & Ordning, 2005; Scriber *et al.*, 2008a; Ordning *et al.*, 2010; Scriber, 2011; Kunte *et al.*, 2011) may explain some of the recent abilities to use quaking aspen, *P. tremuloides*, by females of *P. glaucus* near these regions. In the 1980s, *P. glaucus* from this region had low (but hybrid-like) survival on *P. tremuloides* (38.7%, 12 families, 31 neonates), but subsequent to the 1998 regional climate warming, most females assayed from the southern Appalachian

Mountains (Habersham & Rabun Counties, GA, near southernmost *P. appalachiensis* types; see Fig. 2) showed significant survival on this plant species (95.5%, 12 families, 44 neonates). In contrast, the Clark and Oglethorpe Counties populations that are further south by only 100–200 km showed essentially no introgression of abilities to survive on Salicaceae (only 1.7% of 162 neonate larvae from 19 families in 2002–2003 survived on *P. tremuloides*).

However, the general movements of Salicaceae and Magnoliaceae detoxification abilities in the historical *P. canadensis* and *P. glaucus* hybrid zone have been disproportionate since the recent warming, despite being autosomally controlled (Scriber, 1986b, 2011). Recent introgression of Magnoliaceae detoxification abilities northward into *P. canadensis* populations has not been reciprocated by movement of Salicaceae detoxification abilities southward (Scriber, 2002b, Scriber *et al.*, 2008a). Other local adaptations are doubtlessly involved (Aardema *et al.*, 2011; Scriber, 2011), but oviposition preferences and detoxification abilities are critically important for any host shifts.

Other than in these northernmost historical hybrid zone populations (and *P. canadensis*), the only tiger swallowtail populations in eastern North America with Salicaceae detoxification abilities in neonate larvae greater than 10% are Pendleton and Clay Counties in West Virginia, and Habersham and Rabun Counties in Georgia (both of these mountainous populations are near the recombinant hybrid species, *P. appalachiensis*; see Fig. 2 and Kunte *et al.*, 2011). It would seem that recent gene flow from the southern Appalachian Mountains is an unlikely explanation of Salicaceae detoxification abilities in these Florida *P. glaucus* populations. The alternative suggests that the Salicaceae detoxification abilities are ancestral and were retained (possibly due to a lack of antagonistic pleiotropy, or trade-offs) by these Florida populations.

Florida populations of *P. glaucus* (*P. g. maynardi* subspecies) may represent remnant refuge populations that inhabited Florida during the Pliocene or interglacial (warm) periods of the Pleistocene when much of the northern Florida and southern Georgia region was below sea level, leaving a chain of islands in central Florida (Ellsworth *et al.*, 1994; Lane, 1994; Soltis *et al.*, 2006). The populations of organisms on these islands were likely separated from mainland populations, and their subsequent secondary contact upon the retreat of the seawater likely contributed to the formation of the numerous hybrid zones within the Northern-Florida Suture Zone (Remington, 1968). The isolated populations of *P. glaucus* that inhabited the islands of Florida may have retained the Salicaceae

detoxification abilities while it was lost in northern mainland populations.

Florida populations of *P. glaucus* have somehow retained abilities to detoxify and process some Salicaceae leaves for survival and growth, despite their strong avoidance of these leaves for oviposition. Such independence of oviposition preference and larval performance in insects is not uncommon (but see Berenbaum & Feeny, 2008). However, in the *Papilio glaucus* group pleiotropic traits conveying the ability to detoxify ancient plant families appear to persist (Scriber *et al.*, 2008b; Scriber, 2011).

The eastern tiger swallowtail has retained the ability to feed on the phylogenetically basal Rutaceae hosts as well as some Australian host species that it has never encountered (at least during the past millions of years; Scriber *et al.*, 2008b, 2008c). These abilities to feed on new hosts without losing abilities to feed on ancestral hosts may have something to do with the detoxification abilities it possesses (cytochrome P-450 enzymes; Li *et al.*, 2002, 2003, 2004; Scriber, 2010). The likely phytochemical mechanisms of why the Salicaceae are nearly uniformly toxic to this species has been elucidated (Lindroth *et al.*, 1988a; Scriber *et al.*, 1989, 1999; Fig. 3), but the Florida populations assayed here seem to be an exception; the survival through the neonate stage and also to pupation was relatively high. We suggest that introgression enhanced by the recent climate-driven forces (Scriber 2011; Aardema *et al.* unpublished data) is less likely as an explanation than is historical detoxification trait retention (Putnam *et al.*, 2007; Scriber, 2008b), potentially in reproductively isolated Pleistocene interglacial *Papilio* populations of Florida refugial islands (Lane, 1994), as in southern Appalachian Mountain populations (Scriber & Ording, 2005; Kunte *et al.*, 2011).

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