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Asymmetric reproductive isolation among polymorphic salamanders

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The study of reproductive isolation (RI) as a prerequisite to sympatric speciation has been limited by a focus on species that have already experienced isolation. However, a complete understanding of speciation depends on observing taxa before they complete the speciation process. We estimated RI in field populations of the polyphenic mole salamander, *Ambystoma talpoideum*, by capturing paedomorphic (aquatic) and metamorphic (terrestrial) adults during the breeding season from two natural populations. We found evidence for asymmetric RI between morphs, such that paedomorphic males and metamorphic females had functionally zero RI, whereas metamorphic males and paedomorphic females had substantial RI. Evidence suggests that ecological factors such as the abundance of each morph, timing of rainfall, and water depth of the breeding habitat play a large role in the production of these asymmetries. Spatial aspects of RI had a greater relative impact on overall isolation than temporal differences, in part because metamorphic adults were often captured in shallower water than paedomorphic adults. However, morph separation varied across populations and year, suggesting that environmental heterogeneity likely plays a large role in the potential for RI, particularly between metamorphic males and paedomorphic females. In addition, body-size variation and behavioural differences could also influence the RI estimates presented here. Although facultative paedomorphosis appears to have played a large role in macroevolutionary change via allopatric speciation in some taxa, our results suggest that there is little potential for sympatric speciation in the future within these populations. However, asymmetric RI creates the opportunity for fitness differences between morphs and sexes that would directly affect the maintenance of this polymorphism. Our results suggest that further studies on this and other polyphenisms may provide valuable insight into the evolution of RI and the role of environmental heterogeneity in the production and maintenance of biological diversity. © 2005 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2005, **86**, 265–281.

ADDITIONAL KEYWORDS: body size – breeding phenology – environmental heterogeneity – facultative paedomorphosis – fitness – polyphenism – reproductive behaviour – sympatric speciation.

INTRODUCTION

Speciation continues to be one of the most studied yet least understood features of evolution (Otte & Endler, 1989; Howard & Berlocher, 1998; Barton, 2001; Mallet, 2001). One of the most compelling questions about speciation is the degree to which ecological factors affect species divergence. This ecological hypothesis suggests that reproductive isolation (RI) evolves as a consequence of divergent natural selection on traits between environments (Mayr, 1942; Dobzhansky, 1946; Orr & Smith, 1998). Compelling evidence from studies of a variety of plant, invertebrate, and vertebrate taxa has supported this hypothesis (Schluter, 2001).

Most studies testing this and other speciation models have used extant species and looked for varying degrees of RI based on patterns of ecological or evolutionary separation (Otte & Endler, 1989; Panhuis *et al*., 2001; Schluter, 2001; Via, 2001). Fewer studies have compared examples where speciation has yet to occur, but has the potential to do so in the future (e.g. Grant & Grant, 1989; Johannesson, Rolan-Alvarez & Ekendahl, 1995; Hendry *et al*., 2000). Yet, focusing on these rare systems may provide needed insight into the mechanisms underlying speciation processes,

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*Corresponding author. E-mail: howard.whiteman@murraystate.edu particularly those occurring in sympatry (Barton & Hewitt, 1985; Butlin, 1987, 1989; Grant & Grant, 1989; Via, 2001).

Polymorphisms provide an important model system of RI for sympatric speciation (e.g. models by Maynard Smith, 1966; Felsenstein, 1981; Rice, 1984; Diehl & Bush, 1989; references below). Because polymorphic species may exhibit phenotypic variation that is often dramatically different between morphs, polymorphisms functionally reduce the study of speciation to the study of RI (West-Eberhard, 1986, 1989; Wilson, 1989; see also Kondrasharov, Yampolsky & Shabalina, 1998). Environmentally cued polymorphisms, or polyphenisms, might be especially important for speciation, in that many current models of sympatric speciation rely on a genotype × environment interaction (Via, 2001). Indeed, West-Eberhard (1986, 1989) suggested that environmentally cued morphs can evolve as alternatives along established morphs, allowing a species to develop a new specialization (and perhaps species) without abandoning the established morph.

Although polyphenic phenotypes exist in numerous species across diverse taxa (see Roff, 1996 for a review), facultative paedomorphosis in salamanders provides a unique vertebrate system in which to address these questions. Facultative paedomorphosis occurs when individuals either metamorphose into terrestrial adults or become paedomorphic, branchiate morphs depending on the environmental conditions experienced during larval development (Whiteman, 1994). In facultatively paedomorphic species, paedomorphic and metamorphic adults can coexist in the same population (e.g. Larson, 1968; Anderson, 1971; Patterson, 1978; Sexton & Bizer, 1978; Kalezic & Dzukic, 1985; Semlitsch, 1985a; Whiteman, Wissinger & Brown, 1996; Denoel *et al*., 2001a). The expression of the dimorphism may be a response to the individual's expected success in the aquatic vs. terrestrial environment (Wilbur & Collins, 1973; Whiteman, 1994), and can be viewed as an environmental threshold trait, having genetic variation combined with an environmental trigger (Harris, 1987; Harris *et al*., 1990; Whiteman, 1994; Roff, 1996).

Currently, there is support for the hypothesis that the origin and maintenance of facultative paedomorphosis is a result of selection (Wilbur & Collins, 1973; Whiteman, 1994). For example, it is known that:

1. Populations vary in the frequency of paedomorphs in nature (Sprules, 1974a; Collins, 1981; Kalezic & Dzukic, 1985; Semlitsch, 1985a; Denoel *et al*., 2001a).
2. Individuals from the same population vary in the production of paedomorphic offspring when reared under different environmental conditions (Snyder, 1956; Sprules, 1974b; Harris, 1987; Semlitsch, 1987; Semlitsch & Wilbur, 1989).
3. Individuals from different populations vary in the production of paedomorphic offspring when reared under the same conditions (Semlitsch & Gibbons, 1985; Semlitsch & Wilbur, 1989; Semlitsch, Harris & Wilbur, 1990), suggesting that heritable phenotypic variation is present, at least for some species.
4. Molecular analyses and interspecific crosses are consistent with a genetic basis to paedomorphosis (Shaffer & Voss, 1996; Voss & Shaffer, 1996).
5. Artificial selection for paedomorphosis significantly increased the frequency of the expression of the morph, providing strong evidence for additive genetic variation (Semlitsch & Wilbur, 1989).

However, only recently has differential fitness between the two morphs begun to be explored in detail (e.g. Kalezic *et al*., 1996; Whiteman *et al*., 1996; Whiteman, 1997; Ryan & Semlitsch, 1998; Denoel & Joly, 2000, 2001a; Currens, Niewiarowski & Whiteman, 2002; Denoel, Hervant & Schabetsberger, 2002). Our understanding of fitness differences is especially lacking in terms of mating success (Bovero, Giacoma & Andreone, 1997; Whiteman, Gutrich & Moorman, 1999; Denoel, Poncin & Ruwet, 2001; Denoel *et al*., 2002; Krenz & Verrell, 2002), yet the importance of polymorphism as a mechanism leading to speciation is dependent on its influence on assortative mating and RI (West-Eberhard, 1986, 1989).

Facultative paedomorphosis thus exhibits several characteristics that may facilitate sympatric speciation (Via, 2001), including the potential for discontinuous resource/habitat use by morphs (Patterson, 1978; Whiteman *et al*., 1996; Denoel & Joly, 2001a, b), apparent strong disruptive natural selection on habitat use and other characters associated with resource competition, a genotype × environment interaction in resource use, and apparent genetic variation in habitat preference (via morphology; Semlitsch & Gibbons, 1985; Semlitsch & Wilbur, 1989; Semlitsch *et al*., 1990). However, many of these conditions have yet to be fully supported for this polyphenism. For example,. is habitat use truly discontinuous, given that metamorphic adults return to aquatic habitats for breeding? In addition, many other conditions that would facilitate sympatric speciation have yet to be addressed, e.g. whether habitat choice affects mate choice.

Several aspects of the annual breeding cycle of the facultatively paedomorphic mole salamander, *Ambystoma talpoideum* (Holbrook), make it an excellent candidate for studies of RI between morphs. Mole salamanders exhibit complex courtship behaviour, including male–male competition and female choice for mates (Shoop, 1960). The two morphs can successfully interbreed (Krenz & Verrell, 2002), although the extent to which they do so in natural populations is unclear. The degree to which the morphs interbreed may be influenced by a variety of factors, including temporal, spatial, and behavioural separation among morphs, each of which may help understand how well this polyphenism fits the conditions necessary for incipient sympatric speciation.

For example, Scott (1993) and Krenz & Sever (1995) found that paedomorphic adults begin to breed earlier in the season than metamorphic adults in the same population. In addition, because the immigration of metamorphic adults from terrestrial habitats varies over the season (Semlitsch, 1985b), different numbers of the two morphs will be present in breeding ponds at any point in time. Such a situation should lead to reduced intermorph encounter rate and breeding, and thus partial premating isolation.

Spatial aspects of breeding may also play a role in the degree of premating isolation. Metamorphic adults may be unable to adequately breed in deep water, due to their terrestrial morphology and the physiological rigours of active breeding (Halliday & Sweatman, 1976; Halliday & Worsnop, 1977; but see Currens *et al*., 2002). Such constraints might limit the depth and thus distance from shore that metamorphic adults are able or willing to utilize, providing the potential for spatial isolation between morphs.

Body size is another important variable to consider, because body size distributions of metamorphic and paedomorphic males vary across source populations and time: in some years and ponds, paedomorphic males are larger than metamorphic males, on average; in other years and ponds, the reverse is true (R.D. Semlitsch, unpubl. data). Body size effects also vary within years and populations, because smaller metamorphic adults tend to arrive at breeding ponds before larger individuals (Semlitsch, 1981; Semlitsch *et al*., 1993). Size-specific male mating success has been previously observed in salamanders in which males compete for mates (Janzen & Brodie, 1989; Houck, 1988; Howard, Moorman & Whiteman, 1997), thus body size could strongly influence relative male mating success of each morph, and thus impact the degree of intermorph breeding.

We utilized field observations from two different populations across two years to determine the degree of RI between morphs in *A. talpoideum*. The primary objectives were to estimate: (1) temporal and spatial breeding separation between morphs, both in ecological and RI terms; (2) body-size variation among morphs and over time, and (3) given 1 and 2, the potential for RI and its impact on the polyphenism.

METHODS

FIELD SITES AND SAMPLING PROCEDURES

Field observations were conducted between September and March 1994–95 and 1995–96 at two natural breeding sites located within the United States Department of Energy's Savannah River Site in Aiken County, South Carolina. The two study sites, Ellenton Bay (10 ha) and Flamingo Bay (5 ha), are nearly permanent ponds with large breeding populations of both metamorphic and paedomorphic adults (Semlitsch & Gibbons, 1985; Semlitsch, 1985a). These populations appear to have similar histories of selection for paedomorphosis (Semlitsch *et al*., 1990), but a different genetic basis for the polyphenism (Harris *et al*., 1990). Each had a drift fence with pitfall traps so that incoming terrestrial adults could be monitored. At Ellenton Bay, five pitfall traps were sampled at four compass directions for a total of 20 traps. At Flamingo Bay, a partial drift fence encompassed the north-west side of the bay. Five traps at the two ends of this partial fence were sampled, for a total of ten traps. Each day from October through early March each trap was checked thoroughly for incoming metamorphic adults. Adults were measured for snout–vent length (SVL), sex determined, and released on the opposite side of the fence.

During 1994, we placed two intersecting transects of unbaited plastic minnow traps (Model MT-1; spaced 5 m apart) in the ponds to simultaneously measure the frequencies of the two morphs and sexes during the breeding season. Transects were placed to maximize sampling a variety of depths, including the shoreline. We utilized 34 traps in Ellenton Bay and 24 traps in Flamingo Bay, although the exact number varied because traps near shore were occasionally out of the pond due to low water levels. Traps were checked each morning for 2 days every 1–2 weeks during the breeding season (October–March). Depth was measured at each trap site during each sampling period. Distance from shore was estimated for each trap at the beginning of the project. Although distance from shore varied with changes in water level, each recorded distance provides a relative value for each trap.

We recorded the morph, sex, measured the SVL, and determined reproductive condition by observation of the cloaca for each individual. Sampling during 1994–95 revealed that we could reliably distinguish males in breeding condition by enlarged cloacae, and accurately separate females into those that were not mating (cloaca not swollen), those that were currently mating (swollen cloaca with or without sperm cap present), and those laying eggs (extruding eggs). We used this methodology to record the mating status of males and females during 1995–96. All individuals were measured in the field and immediately released.

STATISTICAL ANALYSES

Throughout our analysis, no direct comparisons were made between bays or years because they were not the main focus of the study. However, apparent differences between bays and years are described and interpreted in the discussion where relevant. Each bay observed during a single year represented a single bay-year, for a total of 4 bay-years during the study.

For pitfall data, individuals captured each day were utilized as independent points, because there was virtually no chance of capturing that individual on subsequent days. Because we used minnow traps over two consecutive days, this assumption could not be made for aquatic sampling. Thus, we utilized the mean value across the two sampling days as our response variable for all subsequent analyses.

All analyses were conducted with *t*-tests, ANOVA, MANOVA and ANCOVA using StatView 5.01 (SAS Institute, 1999). When data did not meet the assumptions of the statistical test they were transformed appropriately. In some cases transformation did not correct heteroscedastic variances, and we subsequently analysed the data with appropriate nonparametric tests.

Because few metamorphic adults were captured in minnow traps, we compared timing of breeding by combining metamorphic migration data from pitfalls and paedomorphic adult captures from minnow traps. Although these metamorphic adults were not captured in the bays, their capture in pitfalls was evidence that they were moving to breed in the bay at this time (Semlitsch, 1985a), allowing body size comparisons. This procedure increased the sample size of metamorphic adults considerably, leading to more powerful and meaningful statistical analyses. Days without metamorphic adult captures were excluded from analysis.

ESTIMATES OF REPRODUCTIVE ISOLATION

We estimated RI among morphs through modification of the methods of Ramsey, Bradshaw & Schemske (2003), who extended the methodology of Coyne & Orr (1989, 1997). These estimates are indicative of the degree of RI between the two morphs, and typically vary between 0 and 1; they were separated by the specific intermorph pairing (paedomorphic males with metamorphic females, metamorphic males with paedomorphic females) in order to assess potential asymmetries in isolation.

We calculated temporal isolation among morphs using the equation:

$$\mathrm{RI}_{temporal} = 1 - \frac{\text{co-occurence time}}{\text{total breeding time}}$$

where co-occurrence time is the total time in days available for intermorph breeding, and total breeding time is the total time a female morph was available for breeding. We calculated these values by estimating the beginning and end of breeding of each morph and sex within each population and year using pitfall and minnow trap data. We also calculated $\mathrm{RI}_{temporal}$ using previous data of the onset of metamorphic adult migrations from Ellenton Bay (table 3 in Krenz & Sever, 1995). We assumed that metamorphic females migrated 3 days after metamorphic males (the average difference for our 1994–95 and 1995–96 data). We utilized data from our 1995–96 data set to estimate the beginning and end of paedomorphic adult breeding, and the end of metamorphic adult breeding. Thus, these data compare the effect of known variation in the onset of metamorphic migrations on $\mathrm{RI}_{temporal}$.

Spatial isolation was calculated using the equation:

$$\mathrm{RI}_{spatial} = 1 - \frac{\text{number of intermorph co-occurences}}{\text{number of intramorph co-occurences}}$$

We utilized our minnow trap data to determine these values, and calculated $\mathrm{RI}_{spatial}$ for each population and year as well as each female morph within each population and year.

Total Reproductive Isolation (T) was calculated as a multiplicative function of the individual RI values (Ramsey *et al*., 2003). We calculated the absolute contribution (AC) of individual RI values at stage *n* as:

$$\mathrm{AC} = \mathrm{RI}_n\left(1 - \sum_{i=1}^{n-1}\mathrm{AC}_i\right).$$

Thus, RI values calculated for earlier stages of an organism's life history will reduce the absolute contribution of RI values calculated for later stages. We then utilized the equation:

$$\mathrm{T} = \sum_{i=1}^{m}\mathrm{AC}_i$$

where *m* is the number of RI measures. $\mathrm{RI}_{temporal}$ was incorporated into the equation first, followed by $\mathrm{RI}_{spatial}$, because the timing of metamorphic migration occurs before any spatial separation among morphs.

We also calculated the relative contribution (RC) of each RI value as:

$$\mathrm{RC}_m = \frac{\mathrm{AC}_m}{\mathrm{T}}.$$

Calculations of AC, T, and RC were made with an Excel spreadsheet developed by Ramsey *et al*. (2003; http://www.plantbiology.msu.edu/schemske.shtml).

RESULTS

TEMPORAL OVERLAP IN BREEDING

Metamorphic adult migrations

In all four bay-years, female metamorphic adults arrived significantly later, in terms of average date of arrival at the breeding site, than males (all $t > 11.7$, all $P < 0.0001$; Fig. 1).

Morph comparisons

In all bay-years, more paedomorphic adults were captured in minnow traps than metamorphic adults (all $F > 108.0$, all $P < 0.0001$; Fig. 2). However, the number of adults of each morph and sex that were captured varied depending on the bay and year. In Ellenton Bay (1994–95) and Flamingo Bay (1995–96) there were significant morph × sex interactions in the number of adults captured (both $F > 28.7$, both $P < 0.0001$), yet the form of the interaction differed. In both bays, metamorphic males outnumbered metamorphic females. However, in Ellenton Bay paedomorphic males far outnumbered paedomorphic females, whereas in Flamingo Bay the reverse was true. In Flamingo Bay (1994–95) there was a significant sex effect, with males outnumbering females in both morphs ($F_{1,1240} = 22.0$, $P < 0.0001$). In Ellenton Bay (1995–96), only the morph effect was significant.

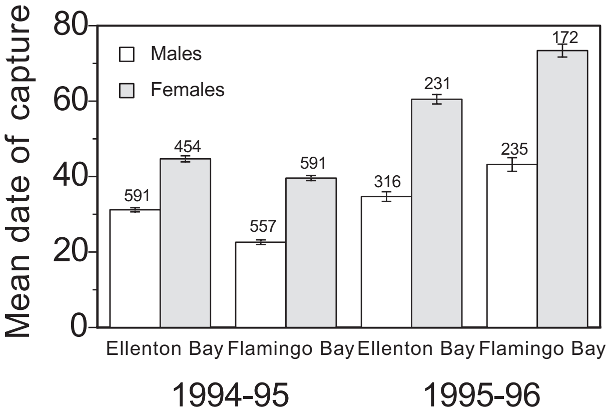

Figure 1. Mean arrival time (± 1 SE) for metamorphic adults captured in pitfall traps during breeding migrations for each bay-year. Date of capture is based on days since the beginning of trapping (1994–95: 25 October; 1995–96: 23 October). Open bars represent males and hatched bars represent females. Sample sizes are shown above each mean; see text for statistics.

Because we captured few metamorphic adults in minnow traps, we compared mean densities of individuals for only those traps that contained metamorphic adults. This provided a comparison of the densities of each morph and sex in areas where metamorphic adults were present. In three of the four bay-years, paedomorphic adults again outnumbered metamorphic adults (all $F > 21.0$, $P < 0.0001$); in Flamingo Bay (1994–95) the reverse was true ($F_{1,488} = 16.9$, $P < 0.0001$; Fig. 3). During 1994–95, both bays showed significantly higher numbers of males captured than females (both $F > 9.7$, $P < 0.003$). However, neither bay in either year showed a significant morph × sex interaction.

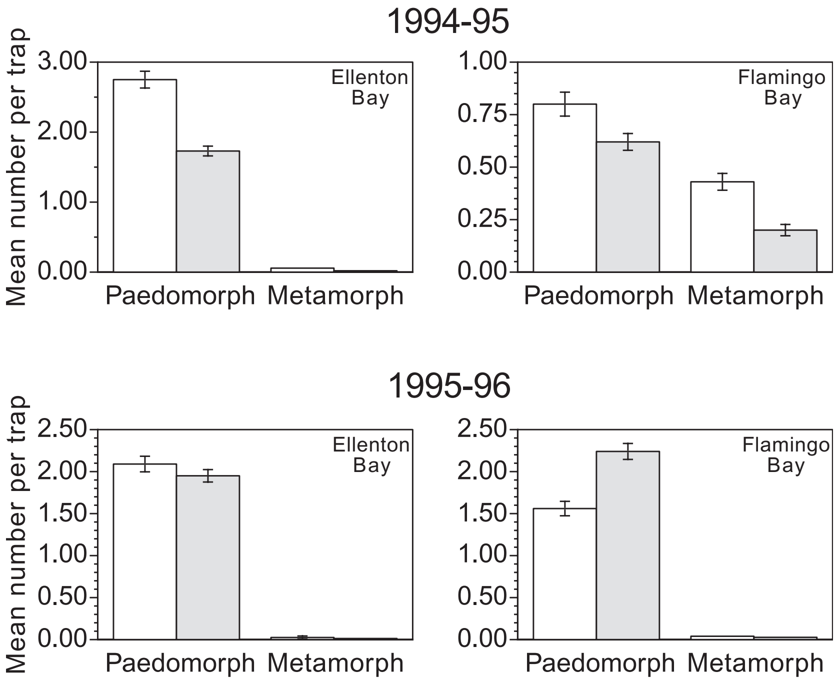

Figure 2. Mean number of captured salamanders per minnow trap (± 1 SE) for each bay-year. Open bars represent males and hatched bars represent females. Sample sizes (number of trap-days) for each morph-sex combination are as follows: Ellenton Bay (1994–95) = 402, Flamingo Bay (1994–95) = 311, Ellenton Bay (1995–96) = 320, Flamingo Bay (1995–96) = 240.

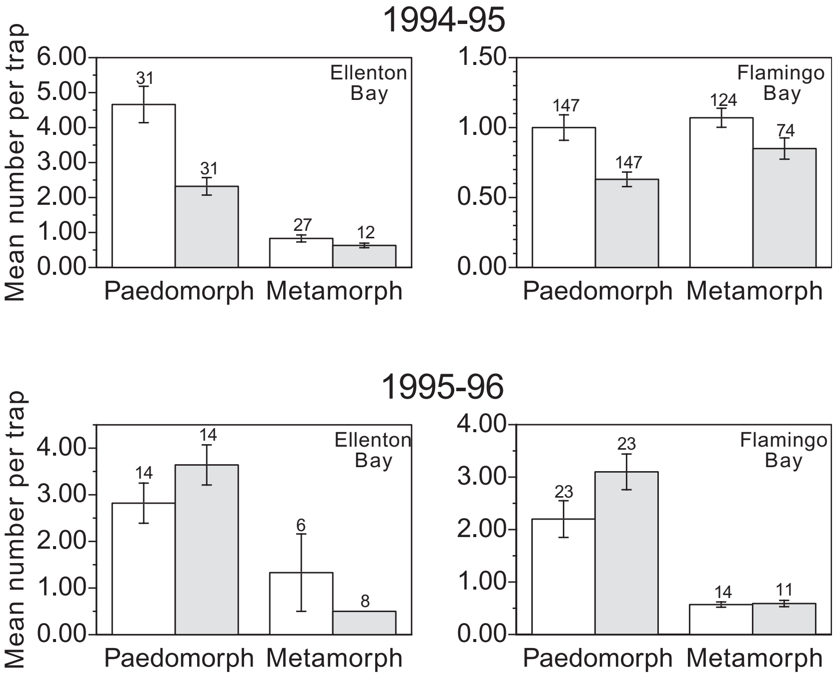

Figure 3. Mean number of captured salamanders per minnow trap (± 1 SE) for each bay-year, when limiting the analysis to those traps with metamorphic adults present. Open bars represent males and hatched bars represent females. Sample sizes are shown above each mean.

The mean number of adults per trap also changed considerably with date. In all bay-years, the mean number per trap increased as the breeding season progressed in both morphs and bays, and then declined as the breeding season waned (Fig. 4). In three of the four bay-years, paedomorphic adults clearly outnumbered metamorphic adults throughout the observation period, and peaked in numbers captured before the peak in metamorphic adults. In contrast, Flamingo Bay (1994–95) exhibited numbers of adults of each morph that were more similar, as mentioned above. This occurred in part because paedomorphic adult numbers were much lower and metamorphic adult numbers were higher in 1994–95 than in 1995–96 in this bay (Fig. 4).

Only 1995–96 data were used to compare the proportion breeding (based on observation of the cloaca) across morph and sex. In both bays, we found a significant morph × sex interaction, with paedomorphic males exhibiting a higher proportion breeding compared to paedomorphic females, whereas metamorphic males and females were similar to each other and paedomorphic males (rank-order ANOVA: both $F > 14.8$, both $P < 0.0003$). However, this comparison averages data across date. Because of few metamorphic adult captures, only paedomorphic adults could be statistically analysed over time. In general, paedomorphic adults tended to increase gradually and then decline (Fig. 5). In both bays, paedomorphic males maintained the highest (> 0.9) proportion breeding for the longest time period, which declined only at the end of the breeding season. In contrast, paedomorphic females had a reduced proportion breeding (both $F > 46.0$, both $P < 0.0001$), with two peaks: one early in the season (day 30 for both bays) and a second, smaller peak later in the season (day 130 for Ellenton, 117 for Flamingo). These data led to a significant interaction between sex and sample date for Ellenton Bay ($F_{9,261} = 2.46$, $P = 0.01$), but not Flamingo Bay ($F_{9,189} = 1.60$, $P = 0.12$), where males and females showed similar patterns in their proportion breeding over time (Fig. 5). Because only a few metamorphic adults were captured during some days, proportion breeding data were sometimes from one trap, and so do not represent mean values. Nonetheless, changes in metamorphic breeding were more abrupt, and when metamorphic adults were captured, they were typically breeding. Paedomorphic male proportion breeding was still high when it overlapped with metamorphic breeding episodes, whereas proportion breeding for paedomorphic females had reached its first peak and was declining by the time metamorphic adults began breeding (Fig. 5).

$RI_{temporal}$ estimates

$RI_{temporal}$ was always zero among paedomorphic males and metamorphic females during this study, but varied from 21.3% to 24.0% isolation (mean = 22.4%)

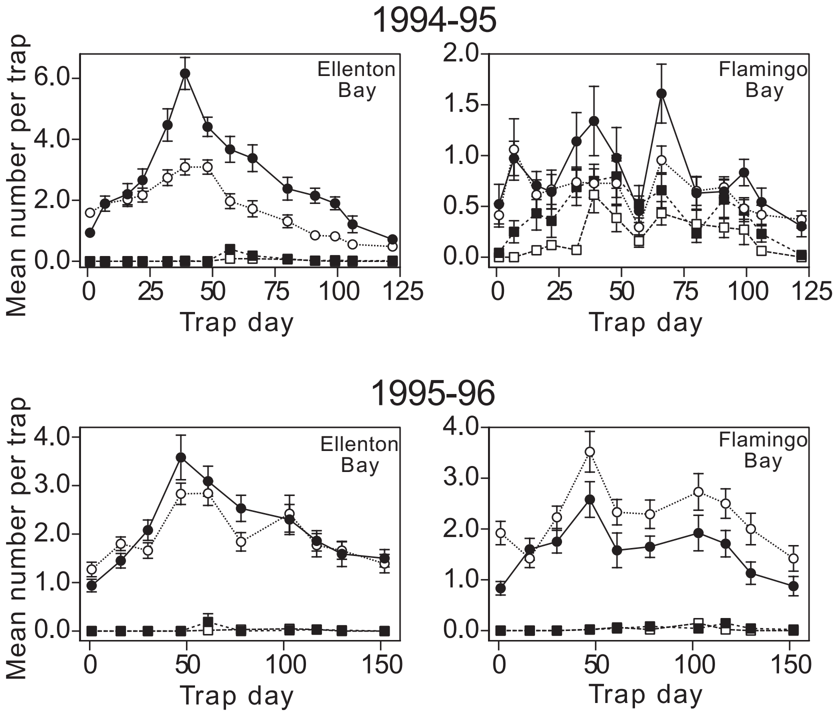

Figure 4. Mean number of salamanders per minnow trap (± 1 SE) over time for each bay-year. Circles indicate paedomorphic adults and squares indicate metamorphic adults; filled symbols represent males and open symbols represent females. Sample sizes (number of traps per trap day): Ellenton Bay (1994–95) = 22–32; Flamingo Bay (1994–95) = 18–24; Ellenton Bay (1995–96) = 32, Flamingo Bay (1995–96) = 24.

among metamorphic males and paedomorphic females, with little variation between the two bays (Table 1). Manipulating the onset of metamorphic migrations with historic data did not alter these estimates dramatically. $\mathrm{RI}_{temporal}$ for paedomorphic males and metamorphic females was zero in six of seven years, and slightly above zero during a year in which metamorphic females began breeding earlier than our 1995–96 data, which was utilized in these calculations (see Methods), suggested for paedomorphic males. Among metamorphic males and paedomorphic females, $\mathrm{RI}_{temporal}$ varied from 0 to 34.0% isolation (mean = 19.4%).

SPATIAL OVERLAP IN BREEDING

Depth and distance to shore

Depth and distance to shore varied among salamanders in some bay-years. The two morphs differed in these spatial parameters during 1994–95 in both bays (MANOVA, both Wilks' $\lambda < 0.950$, $P < 0.0001$). Paedomorphic adults were captured from deeper habitats than metamorphic adults during this sample year (morph effect; both $F > 24.7$, $P < 0.0001$, Fig. 6). In Flamingo Bay, paedomorphic adults were also found significantly farther from shore than metamor-

Table 1. Reproductive isolation estimates based on timing of reproduction of metamorphic and paedomorphic mole salamanders ($\mathrm{RI}_{temporal}$). A, 1994–96 data from this study. B, data previous to 1994–95, based in part on table 3 in Krenz & Sever (1995); see text for details. *Abbreviations*: PM-MF = paedomorphic male and metamorphic female; MM-PF = metamorphic male and paedomorphic female

		$\mathrm{RI}_{temporal}$	
Year	Location	PM-MF	MM-PF
A			
1994–95	Ellenton	0	0.228
	Flamingo	0	0.240
1995–96	Ellenton	0	0.213
	Flamingo	0	0.213
B			
1980–81	Ellenton	0.020	0
1981–82	Ellenton	0	0.040
1986–87	Ellenton	0	0.340
1987–88	Ellenton	0	0.095
1991–92	Ellenton	0	0.313
1992–93	Ellenton	0	0.265
1993–94	Ellenton	0	0.306

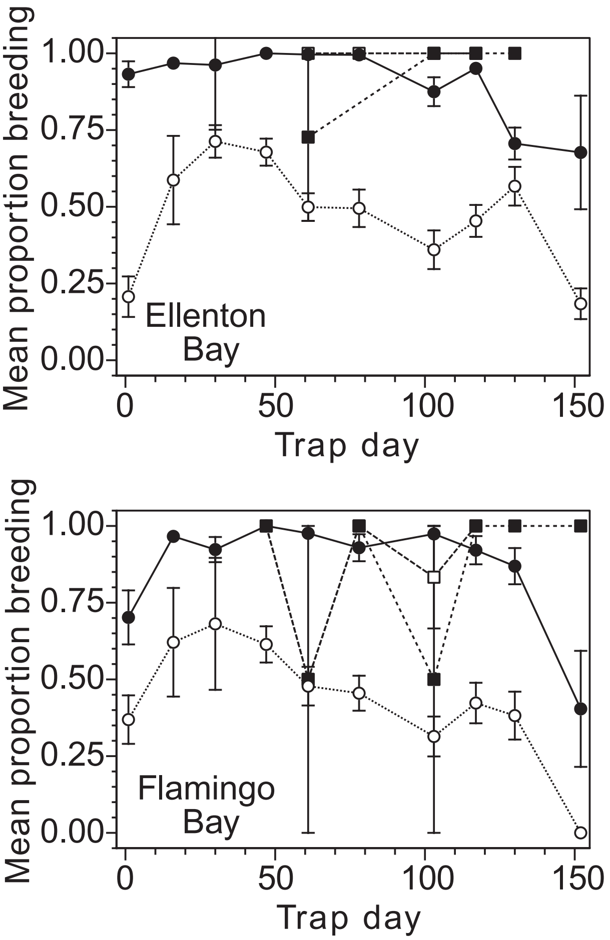

Figure 5. Mean proportion breeding per minnow trap (± 1 SE) over time for Ellenton and Flamingo Bays during the 1995–96 reproductive season. Circles indicate paedomorphic adults and squares indicate metamorphic adults; filled symbols represent males and open symbols represent females. Sample sizes (number of traps per trap day): Ellenton Bay paedomorphs = 26–31, metamorphs = 1–2; Flamingo Bay paedomorphs = 18–24, metamorphs = 1–6.

phic adults during this year (morph effect, $F_{1,587} = 8.3$, $P = 0.004$; Fig. 7). During 1995–96, there was no significant variation in depth or distance to shore detected in Ellenton Bay (all Wilks' $\lambda > 0.994$, $P > 0.22$), while in Flamingo Bay a marginally significant morph × sex interaction was observed (Wilks $\lambda = 0.988$, $P = 0.059$). Metamorphic males were captured closer to shore than metamorphic females in Flamingo Bay, whereas distance to shore did not vary between sexes within paedomorphic adults ($F_{1,470} = 5.5$, $P = 0.02$; Fig. 7). Paedomorphic adults were found intermediate distances from shore when compared to metamorphic males and females during this bay-year.

Table 2. Reproductive isolation estimates based on spatial separation of breeding metamorphic and paedomorphic mole salamanders ($\mathrm{RI}_{spatial}$). Abbreviations per Table 1

		$\mathrm{RI}_{spatial}$	
Year	Location	PM-MF	MM-PF
1994–95	Ellenton	–0.857	0.939
	Flamingo	–0.04	0.528
1995–96	Ellenton	–8	0.985
	Flamingo	–3.50	0.946

$\mathrm{RI}_{spatial}$ estimates

Spatial estimates of RI varied tremendously based on the intermorph combination (Table 2). Paedomorphic males and metamorphic females showed no evidence of RI; each $\mathrm{RI}_{spatial}$ estimate was negative, because paedomorphic males were captured with metamorphic females at a higher frequency than metamorphic males were captured with metamorphic females. In contrast, metamorphic males and paedomorphic females showed evidence for considerable spatial isolation, with $\mathrm{RI}_{spatial}$ varying from 52.8% to 98.5% (mean = 85.0%). Ellenton Bay showed more consistency in this value across the two field seasons (Table 2). Neither spatial variable outlined above (distance from shore or depth) varied consistently with the number of each intermorph pairing.

OVERALL REPRODUCTIVE ISOLATION (T)

Estimates of T varied primarily with intermorph comparison (Table 3). Paedomorphic male and metamorphic female estimates revealed essentially no RI. In contrast, T-values revealed isolation between 64.1% and 98.8% between metamorphic males and paedomorphic females. In both intermorph pairing types, $\mathrm{RI}_{spatial}$ had the largest relative contribution to overall isolation (Table 3).

BODY-SIZE VARIATION

Metamorphic adult migrations

Regression analyses revealed extremely weak but significant positive relationships between date of arrival and body size of metamorphic adults (all $F > 10.9$, all $P < 0.0001$, R^2 range = 0.02–0.22). Only Flamingo Bay females during 1994–95 did not have a significant positive relationship between these two variables ($F_{1,588} = 0.14$, $P = 0.70$). In Ellenton Bay during 1994–95, ANCOVA revealed that the relationship between body size and date of arrival was identical between the sexes. During 1995–96, both bays were significantly

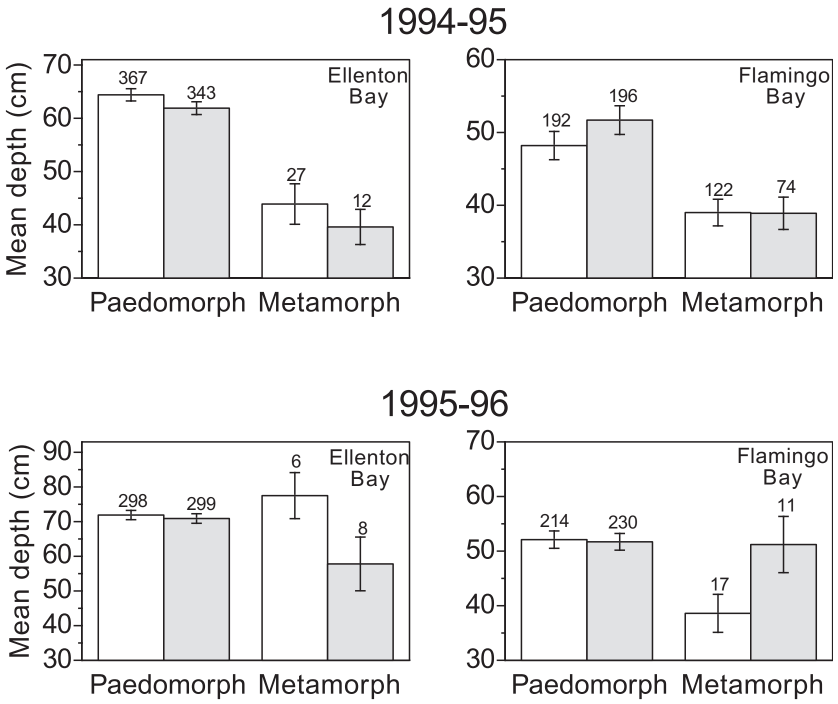

Figure 6. Mean depth of capture (± 1 SE) for paedomorphic and metamorphic adults in each bay-year. Open bars represent males and hatched bars represent females. Sample sizes are shown above each mean.

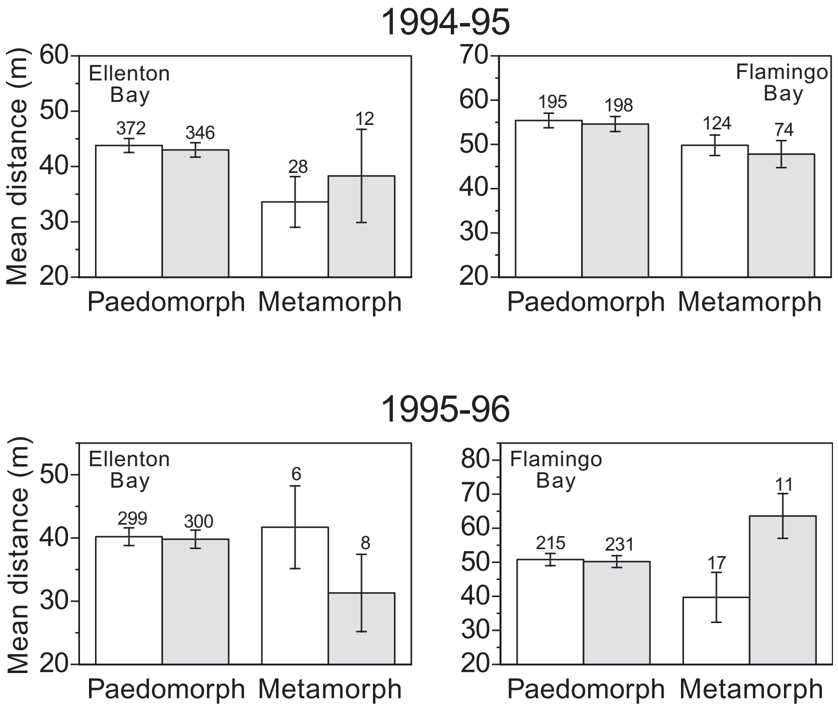

Figure 7. Mean estimated distance from the edge of each bay (± 1 SE) for metamorphic and paedomorphic adults in each bay-year. Open bars represent males and hatched bars represent females. Sample sizes are shown above each mean.

Table 3. Overall reproductive isolation (T) based on temporal and spatial separation of metamorphic and paedomorphic mole salamanders. *Abbreviations*: PM-MF, paedomorphic male and metamorphic female; MM-PF, metamorphic male and paedomorphic female; RI_n, individual reproductive isolation (RI) values at stage *n*; AC, absolute contribution of RI_n to T; RC, relative contribution of RI_n to T

Year	Location	Pairing	Isolation	RI_n	AC	RC	T
1994–95	Ellenton	PM-MF	Temporal	0	0	0	–0.857
			Spatial	–0.857	–0.857	1.0	
		MM-PF	Temporal	0.228	0.228	0.239	0.953
			Spatial	0.939	0.725	0.761	
	Flamingo	PM-MF	Temporal	0	0	0	–0.04
			Spatial	–0.04	–0.04	1.0	
		MM-PF	Temporal	0.240	0.240	0.374	0.641
			Spatial	0.528	0.401	0.626	
1995–96	Ellenton	PM-MF	Temporal	0	0	0	–∞
			Spatial	–∞	–∞	1.0	
		MM-PF	Temporal	0.213	0.213	0.216	0.988
			Spatial	0.985	0.775	0.784	
	Flamingo	PM-MF	Temporal	0	0	0	–3.5
			Spatial	–3.5	–3.5	1.0	
		MM-PF	Temporal	0.213	0.213	0.222	0.958
			Spatial	0.946	0.745	0.778	

different in intercept but not slope, with females having higher intercepts (sex effect; both $F > 4.7$, both $P < 0.032$).

Morph comparisons

Because few metamorphic adults were captured in minnow traps in some bay-years (Fig. 2), we compared body-size differences among morphs using traps that contained metamorphic adults. This provided a comparison of the body-size relationships of each morph and sex that were found in the same area of the bay. In each bay-year, metamorphic adults were significantly larger than paedomorphic adults (all $F > 70.8$, all $P < 0.0001$; Fig. 8). Because of skewed variances that could not be corrected by transformation in three of the four bay-years, this morph effect was also tested using a Mann–Whitney *U*-test, and in each case was significant (all $U > 9.9$, all $P < 0.0001$).

We combined data from pitfall and minnow traps to further assess body-size variation. Across all trap days within a year, morph and sex had significant effects on body size. A significant morph effect was consistent across all bay-years, with metamorphic adults larger than paedomorphic adults (all $F > 928$, all $P < 0.0001$). During 1994–95, males were slightly larger than females in Ellenton, and females slightly larger than males in Flamingo Bay (both $F > 5.9$, both $P < 0.015$), but sex was not a significant factor for either bay during 1995–96. However, significant morph × sex interactions were also present in each bay-year (all $F > 9.5$, all $P < 0.002$). In three of the four bay-years, paedomorphic males were larger than paedomorphic females. Metamorphic females were larger than metamorphic males in another three bay-years. Sex did not affect body size in paedomorphic adults from Flamingo Bay (1994–95) or metamorphic adults from Ellenton Bay (1994–95).

Comparisons of morph and sex when analysed as a function of trap day also showed significant variation in each bay-year. In Ellenton Bay (1994–95), metamorphic males increased in SVL over time, whereas the other morph-sex combinations tended to have similar mean body sizes over time (morph × sex × trap day interaction: $F_{6,2188} = 2.6$, $P = 0.015$; Fig. 9). This relationship was nearly significant ($P = 0.058$) in this bay for 1995–96, although both metamorphic sexes appeared to increase in body size more over time than paedomorphic adults during this bay-year. A similar effect was found in Flamingo Bay during 1995–96 ($F_{4,1816} = 2.81$, $P = 0.02$, Fig. 9), with both metamorphic sexes increasing in body size over time while paedomorphic adults did not change. Flamingo Bay (1994–95) did not show this trend, and all morph-sex combinations remained similar in size over time ($P = 0.16$).

DISCUSSION

From a macroevolutionary perspective, facultative paedomorphosis may act as a transitional phase between two other amphibian life cycles, obligate metamorphosis and obligate paedomorphosis. In the former life cycle, larvae always metamorphose; in the latter, they never do (Whiteman, 1994). Metamorphosis is the primitive life cycle, and obligate paedomor-

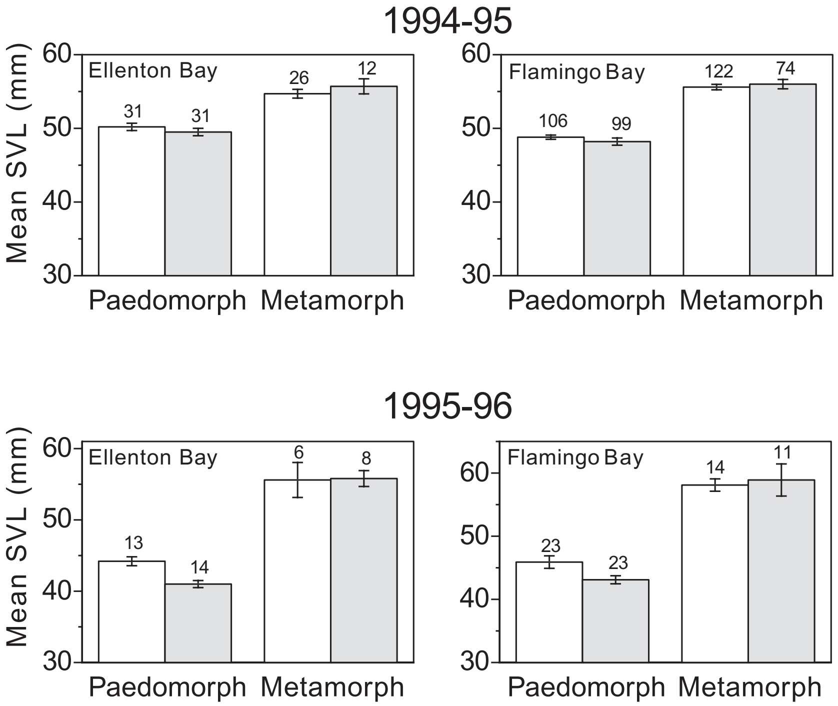

Figure 8. Mean SVL (± 1 SE) of paedomorphic and metamorphic adults for each bay-year, when limiting the analysis to those traps with metamorphic adults present. Open bars represent males and hatched bars represent females. Sample sizes are shown above each mean.

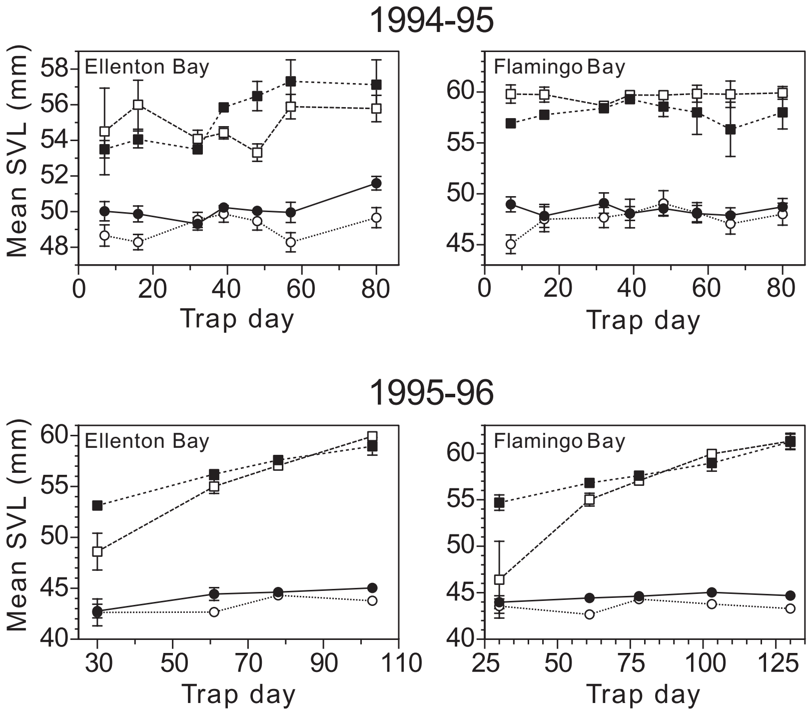

Figure 9. Mean SVL (± 1 SE) of salamanders for each bay-year as a function of date, when combining data from both minnow and pitfall trapping. Circles indicate paedomorphic adults and squares indicate metamorphic adults; filled symbols represent males and open symbols represent females.

phosis is considered most derived (Shaffer & Voss, 1996). Facultative paedomorphosis may thus provide a mechanism for species to fully exploit terrestrial and aquatic habitats via resource partitioning (Denoel & Joly, 2001a) when aquatic habitats remain more permanent. Transitions to a completely obligate paedomorphic state may occur if terrestrial habitats become unsuitable for metamorphic adults. Such a situation appears to have occurred within the Mexican ambystomatid salamanders, where numerous populations have evolved into obligately metamorphic, facultatively paedomorphic, or obligately paedomorphic species, perhaps in response to habitat changes (Shaffer, 1984a; Shaffer & McKnight, 1996; Shaffer & Voss, 1996). Different paedomorphic species are also morphologically and functionally divergent from each other and from transforming sister species (Shaffer, 1984b; Shaffer & Lauder, 1985; Lauder & Shaffer, 1986). These data suggest that facultative paedomorphosis has likely been an important component of macroevolutionary change via allopatric speciation in these lineages. However, the potential for sympatric speciation based on this polyphenism, and the RI mechanisms affecting such change, are currently unknown.

Our results suggest that there is no ecological RI between the morphs *per se*, though there is a clear asymmetry in the overall RI values (T) of the two intermorph pairings (Table 3). Paedomorphic male and metamorphic female pairings revealed *negative* T-values, meaning that this intermorph pairing was more likely than intramorph crosses among metamorphic adults. In contrast, metamorphic male and paedomorphic female pairings showed substantial RI. Although we did not directly observe intermorph matings in this study, our results suggest that the potential for intermorph matings between paedomorphic males and metamorphic females is extremely high.

Our field observations provide some insight into the ecological conditions that have led to these asymmetries in the potential for RI. For example, in all four bay-years, significantly more paedomorphic adults were captured than metamorphic adults (Fig. 2). This relationship held true in three of four bay-years even under a more conservative test in which we included only those traps containing metamorphic adults (Fig. 3). Thus, paedomorphic adults were almost always more abundant than metamorphic adults, even in areas of the bay where metamorphic adults were present. This result suggests that simply based on abundance alone, intramorph matings should be more common than intermorph matings. However, these results also suggest that the numbers of intramorph matings would be asymmetric for each morph: paedomorph × paedomorph crosses should be much more common than metamorph × metamorph crosses, given the abundance of paedomorphic adults with which metamorphic adults could potentially mate. Indeed, this scenario is consistent with the negative T-values among paedomorphic males and metamorphic females.

As in previous studies (Scott, 1993; Krenz & Sever, 1995), we found that paedomorphic adults began breeding earlier in the fall than metamorphic adults (Figs 4, 5), in part because of the dependence on rainfall for metamorphic adult movements. Activity of paedomorphic adults, as measured by number of captures, tended to peak and was beginning to wane in three of the four bay-years by the time metamorphic adults began arriving at the ponds in substantial numbers (Fig. 4). Frequency of breeding data suggested that about 50% of paedomorphic females had bred prior to the arrival of metamorphic males, while paedomorphic male breeding remained high (almost 100%) throughout the sampling period, and waned only during the latter few weeks of the breeding season (Fig. 5). These results produce a situation in which paedomorphic males had access to metamorphic females throughout their breeding period, whereas metamorphic males were more limited in terms of their access to paedomorphic females, producing $\mathrm{RI}_{temporal}$ values in which no isolation occurred among paedomorphic males and metamorphic females, whereas moderate isolation occurred among the reciprocal intermorph pairing. $\mathrm{RI}_{temporal}$ values did not vary considerably when the onset of metamorphic breeding was altered using data from seven earlier breeding seasons (Table 1). Although limited in scope, such isolation could be reinforced by the two-fold advantage of paedomorphs that breed earlier in the season: males could obtain more females at reduced levels of male–male competition (i.e. few metamorphic males present, advantage via sexual selection) and offspring produced early could accrue a competitive growth advantage (advantage via natural selection).

$\mathrm{RI}_{spatial}$ had a greater relative contribution to overall isolation than $\mathrm{RI}_{temporal}$ (Table 3), which suggests that spatial ecology might also be of greater relative importance than temporal differences between morphs. During 1994–95, there were significant differences in depth of capture between morphs, with metamorphic adults residing in shallower water than paedomorphic adults, on average (Fig. 6). These data suggest that morphs might separate spatially with depth, with metamorphic adults utilizing shallower habitats, possibly due to physiological constraints (Halliday & Sweatman, 1976; Halliday & Worsnop, 1977; but see Currens *et al*., 2002) or foraging behaviour (Denoel & Joly, 2001a). However, this same pattern was not observed during 1995–96. During this field season, there were substantially lower sample sizes for metamorphic adults in both bays (Fig. 6). In addition, average depth was greater across all samples in 1995–96 compared to 1994–95 in both bays (both $t > 2.4$, $P < 0.014$), which might produce less separation among morphs, because metamorphic adults along shore would be in deeper water due to variation in bay morphology. Spatial separation based on distance from shore was also inconclusive. Only one bay-year (Flamingo Bay 1994–95, Fig. 7) was consistent with the hypothesis that metamorphic adults would be captured closer to shore, yet these data are *not* consistent with the observed asymmetries in $\mathrm{RI}_{spatial}$. In Ellenton Bay, both morphs were captured at similar distances from shore in both years. In Flamingo Bay (1995–96), limited samples showed that metamorphic females were captured further from shore, and metamorphic males closer to shore, than paedomorphs, which is the only distance comparison that is consistent with our $\mathrm{RI}_{spatial}$ results.

Body-size variation among morphs and sexes could not be directly evaluated in terms of its impact on overall RI. Nonetheless, previous studies on size-specific mating success in salamanders (Houck, 1988; Janzen & Brodie, 1989; Howard *et al.*, 1997) suggest that body-size differences among morphs might influence RI values. Although body-size variation between morphs depended in part on bay-year, in general metamorphic adults were larger than paedomorphic adults (Fig. 8). The larger body size of metamorphic adults has been documented previously (Semlitsch, 1985a), and may be related in part to the age structure differences between the two morphs. It has been suggested that in some ponds, paedomorphs of this species may successfully metamorphose after one or two years of breeding (Scott, 1993), although the extent to which this occurs is unknown. However, such metamorphosis patterns would likely produce differences in age structure, and thus size structure, between the two morphs.

Perhaps in part because smaller metamorphic adults arrived at breeding ponds earlier than larger individuals (Semlitsch, 1981; Semlitsch *et al.*, 1993; this study), the body size separation between morphs tended to increase with time in three of four bay-years (Fig. 9). Because body-size differences were greatest at the end of the breeding season, when paedomorphic breeding activity was lowest, paedomorphic males would be predicted to be at an extreme disadvantage in male–male interactions with metamorphic males during this time period. Furthermore, female fecundity varies with body size (Semlitsch, 1985a); thus differential migration of small vs. large metamorphic females would influence the reproductive benefit accrued by males of each morph, and perhaps provide a mechanism of reinforcement for morph-specific mating. These results suggest that the RI values calculated in this study might be tempered based on the advantages of large body size accrued by metamorphic males, particularly during the later parts of the breeding season.

Our results make no assumptions about the behavioural differences that might occur during intermorph courtship, and their effect on RI. Krenz & Verrell (2002) provided evidence that the two morphs of this species could successfully interbreed in the laboratory, and found little evidence for sexual incompatibility during their experiments. H.H. Whiteman, J.D. Krenz & R.D. Semlitsch (unpubl. data) observed successful interbreeding between morphs in both laboratory and field experiments, but found that the success of intermorph encounters was reduced in the latter parts of the breeding season when metamorphic adults were most abundant. These data suggest that breeding experiments that manipulate variables such as morph densities, time of year, and water depth might provide important insight into the degree of RI between morphs.

Overall, our RI estimates suggest there is no isolation between the morphs *per se*. Thus, at least in this facultatively paedomorphic species, there is little potential for a future sympatric speciation event, and behavioural studies suggest that this conclusion may be generalized across other species as well (Whiteman *et al.*, 1999; Denoel *et al.*, 2001b, 2002). However, the degree to which the morphs were temporally and spatially separated, differed in temporal and spatial RI values, differed in overall RI (T) values, and differed in body size depended in part on the source population and year, suggesting that spatial and temporal environmental heterogeneity play a significant role in any RI between these two morphs.

This conclusion, when combined with the asymmetric nature of the RI among morphs, suggests that *Ambystoma talpoideum* may be useful as a unique vertebrate model for studies of the evolution of RI, and particularly how environmental variation might impact isolation. For example, initiation of breeding migrations in metamorphic adults is dependent in part on the timing of fall rains (e.g. Semlitsch, 1985a; Scott, 1993; this study). Thus, when rains occur earlier in the fall, more temporal overlap in breeding will occur with paedomorphic adults. When rainfall is delayed until later in the winter, less temporal overlap occurs between morphs. Because the body size of migrating metamorphic adults increases over time, delayed breeding due to lack of rainfall will interact with temporal differences and lead to different degrees of intermorph breeding. Our results also suggest that in years when ponds have low variation in depth because of the interaction of rainfall patterns and pond morphology, there will be more overlap between morphs than in years when there is substantial variation in depth.

Finally, our results have important implications for the maintenance of this polyphenism and the fitness consequences of males and females of the two morphs. Paedomorphic males have a potentially large advantage over metamorphic males because they were significantly more likely to be found with metamorphic females than metamorphic males were, leading to negative RI values, and because there was substantial isolation between metamorphic males and paedomorphic females. Their numerical advantage over metamorphic males (Fig. 1) also suggests substantial reproductive advantages for paedomorphic males. Paedomorphic adults of both sexes may gain fitness benefits via a competitive growth advantage for their offspring over those produced by metamorphic adults or intermorph pairings later in the season. These results suggest that, all else being equal (e.g. body size effects, courtship differences between morphs), paedomorphic males should accrue higher reproductive success than metamorphic males in these populations.

Although the advantages to females of each morph are less clear, it is possible that metamorphic females gain benefits by breeding with paedomorphic males via the production of variable offspring, i.e. given a genetic basis for paedomorphosis, intermorph offspring may be more flexible in terms of life history plasticity. Paedomorphic females might gain from earlier breeding through the competitive growth advantage described above.

These results suggest fitness benefits to becoming paedomorphic, particularly among males, in these populations. In addition, paedomorphic *A. talpoideum* mature earlier than metamorphic adults (Semlitsch, 1985a; Ryan & Semlitsch, 1998), which should lead to increased fitness, barring trade-offs with other fitness components (Whiteman, 1994; Ryan & Semlitsch, 1998, 2003). Such trade-offs could include the larger body size in metamorphic adults, which may lead to increased mating success among males (Houck, 1988; Janzen & Brodie, 1989; Howard *et al*., 1997) at least later in the season, and provide increased fecundity in females (Semlitsch, 1985a), which could moderate or even outweigh any fitness cost due to delayed breeding. These trade-offs, combined with the dispersal abilities of metamorphic adults and the flexibility of metamorphosis during drought years, should maintain the metamorphic phenotype even under considerable fitness gains by paedomorphic adults (Whiteman, 1994). Furthermore, intermorph breeding between paedomorphic males and metamorphic females should also maintain the genetic basis of the polymorphism. Because our understanding of the fitness payoffs to each morph and sex is incomplete, our ability to test hypotheses about the evolutionary maintenance of facultative paedomorphosis will require further studies aimed at estimating lifetime reproductive success (Whiteman, 1994, 1997; Denoel *et al*., 2002).

This study supports the importance of polyphenism for studies of RI and the potential for macroevolutionary change (West-Eberhard, 1986, 1989; Via, 2001). Because most studies testing current speciation models have used extant species groups (Panhuis *et al*., 2001; Schluter, 2001; Via, 2001) rather than model taxa where speciation has yet to occur (Grant & Grant, 1989; Johannesson *et al*., 1995; Hendry *et al*., 2000), future studies on polyphenism may provide unique opportunities to clarify the mechanisms underlying speciation processes. Polyphenisms besides facultative paedomorphosis that have appropriate systems and sufficient background for such research include trophic polyphenisms in fish (e.g. Ehlinger & Wilson, 1988; Skulason, Noakes & Snorrason, 1989; Ehlinger, 1990; Skulason *et al*., 1993; Robinson & Wilson, 1996; Robinson, Wilson & Shea, 1996) and wing polymorphism in insects (Harrison, 1980; Roff, 1986; Roff & Fairbairn, 1991; Zera & Denno, 1997). Research on these and other polyphenisms may help clarify our understanding of speciation as well as the role of environmental heterogeneity in the production and maintenance of biological diversity.

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