

Howard H. Whiteman · John D. Krenz ·  
Raymond D. Semlitsch

## Intermorph breeding and the potential for reproductive isolation in polymorphic mole salamanders (*Ambystoma talpoideum*)

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**Abstract** The study of reproductive isolation as a prerequisite to sympatric speciation has been limited by the focus on species that have already experienced such isolation. However, a complete understanding of how such processes evolve depends on observing taxa before they complete the speciation process. We studied the potential for sexual isolation in the polyphenic mole salamander, *Ambystoma talpoideum*, using a series of laboratory and field experiments. This species consists of aquatic paedomorphic adults and terrestrial metamorphic adults which are exhibited by both sexes and which mate in the same aquatic habitat. Previous field studies on this species suggested that intermorph breeding would be less common during the winter months, because paedomorphic adults begin breeding in early autumn and thus may have less energy available for reproduction in the winter. Laboratory experiments conducted during the winter showed that the mating behavior of paedomorphic males occurred at a much lower frequency than that of metamorphic males. In

contrast, field experiments that best mimicked natural conditions revealed symmetric intermorph breeding and included multiple paternity shared among males of each morph. This and other studies suggest that there is little evidence of sexual isolation among morphs based on behavioral interactions alone. However, the potential for partial isolation still occurs because of temporal and spatial differences in the frequencies of each morph in nature. Our results suggest that further studies on this system, and other similar polyphenisms, may provide valuable insight into the mechanisms that underlie the evolution of reproductive isolation.

**Keywords** Reproduction isolation · Reproductive success · Polyphenism · Facultative paedomorphosis · Salamander

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H. H. Whiteman  
Department of Biological Sciences,  
Murray State University,  
Murray, KY 42071, USA

H. H. Whiteman (✉) · J. D. Krenz · R. D. Semlitsch  
Savannah River Ecology Laboratory,  
Drawer E,  
Aiken, SC 29802, USA  
e-mail: howard.whiteman@murraystate.edu  
Tel.: +1-270-7626753  
Fax: +1-270-7622788

J. D. Krenz  
Department of Biological Sciences,  
Minnesota State University,  
Mankato, MN 56001, USA

J. D. Krenz · R. D. Semlitsch  
Division of Biological Sciences,  
University of Missouri,  
Columbia, MO 65211, USA

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### Introduction

Most studies testing models of speciation have used extant species and looked for variation in reproductive isolation based on ecological or evolutionary separation (Otte and Endler 1989; Panhuis et al. 2001; Schluter 2001; Via 2001). Very few researchers have studied systems in which speciation has yet to occur but has the potential to do so (e.g., Grant and Grant 1989; Johannesson et al. 1995; Hendry et al. 2000; Jones et al. 2003; Whiteman and Semlitsch 2005). Focusing on these rare systems, however, may provide needed insight into speciation mechanisms, particularly those occurring in sympatry (Barton and Hewitt 1985; Butlin 1987, 1989; Grant and Grant 1989; Via 2001).

Polymorphisms provide a model system for studying reproductive isolation (Maynard-Smith 1966; Felsenstein 1981; Rice 1984; Diehl and Bush 1989). Because polymorphic species exhibit phenotypic variation that is often dramatically different between morphs, polymorphisms functionally reduce the study of speciation to the study of reproductive isolation (West-Eberhard 1986, 1989, 2003; Wilson 1989; Kondrashov et al. 1998). Polyphenisms

(environmentally cued polymorphisms) might be especially important, in that many current models of sympatric speciation rely on a genotype X environment interaction (Via 2001). Indeed, West-Eberhard (1986, 1989, 2003) suggested that polyphenic morphs can evolve as alternatives to established morphs, allowing a species to develop a new specialization (and perhaps species) without abandoning the established form.

Although polyphenic phenotypes exist in a wide range of taxa (Roff 1996), facultative paedomorphosis in salamanders provides a unique vertebrate system in which to address these questions. Facultative paedomorphosis occurs when individuals either transform into terrestrial, “metamorphic adults” or become branchiate, “paedomorphic adults” depending on the environmental conditions experienced during larval development (Whiteman 1994; Denoel et al. 2005). This polyphenism appears to be a response to the individual’s expected success in the aquatic vs terrestrial environment (Wilbur and Collins 1973; Whiteman 1994).

There is considerable support for the hypothesis that the origin and maintenance of facultative paedomorphosis are results of selection. However, only recently has differential fitness between the two morphs been studied in earnest (Whiteman 1994; Denoel et al. 2005), particularly in regard to breeding behavior and mating success (Bovero et al. 1997; Whiteman et al. 1999; Denoel et al. 2001; Denoel 2002, 2003; Krenz and Verrell 2002; Whiteman and Semlitsch 2005). Such data are critical, because the importance of polyphenism as a mechanism leading to speciation is dependent on its influence on both assortative mating and subsequent reproductive isolation (West-Eberhard 1986, 1989, 2003).

Few studies have directly tested for intermorph breeding among facultatively paedomorphic species, and most have been conducted under laboratory conditions. Whiteman et al. (1999) and Krenz and Verrell (2002) observed successful intermorph breeding that led to viable embryos in *Ambystoma tigrinum nebulosum* and *Ambystoma talpoideum*, respectively. Both studies revealed few differences in courtship behavior between morphs; however, Krenz and Verrell (2002) found that courtship in heteromorphic pairings occurred less frequently, proceeded more slowly, and was more likely to end before sperm transfer. Denoel et al. (2001) and Denoel (2002) utilized a paired design in *Triturus alpestris* and found complete sexual compatibility between morphs. Denoel (2003) compared the effects of competition between males of each morph in triadic encounters vs pairs of one male of each morph with a female. He found no evidence for assortative mating, although competition between males of each morph reduced the sperm transfer rate in comparison to paired encounters.

The results of this previous research suggest little evidence for reproductive isolation between morphs. However, only two of the above studies were conducted using encounters in which males of each morph must compete for access to females and in which females can exhibit mate choice (Whiteman et al. 1999; Denoel 2003). In addition, none of the previous studies has directly measured reproductive success (RS) but rather has used proxies

such as the number of spermatophores deposited by males (Krenz and Verrell 2002) or picked up by females (Bovero et al. 1997; Whiteman et al. 1999; Denoel et al. 2001; Denoel 2002, 2003).

The facultatively paedomorphic mole salamander, *A. talpoideum*, may be an ideal candidate for studies of reproductive isolation between morphs. Mole salamanders exhibit complex courtship behavior (Shoop 1960; Verrell and Krenz 1998), and the two morphs can successfully interbreed (Krenz and Verrell 2002), although the extent to which they do so in natural populations is unclear. The degree of intermorph breeding may be influenced by a variety of factors, including temporal, spatial, and behavioral separation among morphs (Whiteman and Semlitsch 2005). For example, paedomorphic adults of this species begin to breed earlier in the season than metamorphic adults in the same population (Scott 1993; Krenz and Sever 1995; Whiteman and Semlitsch 2005). In addition, because the immigration of metamorphic adults from terrestrial habitats varies over the season (Semlitsch 1985b), different numbers of the two morphs are present in breeding ponds at any point in time (Whiteman and Semlitsch 2005). Spatial separation of morphs within the pond can also occur, depending on environmental conditions, with paedomorphs concentrating in deeper habitats (Whiteman and Semlitsch 2005). These factors should lead to reduced intermorph breeding and thus partial premating isolation.

Ecotypic variation could also have a significant impact on intermorph breeding. In particular, female mate choice might be influenced by previous selection for paedomorphosis, as measured by the hydroperiod of the locality (Semlitsch et al. 1990). Ponds that are temporary and thus produce only metamorphic adults might show strong female choice against paedomorphic males. This would occur because mating with a paedomorph might lead to decreased fitness among offspring, if the genetic component of paedomorphosis led to longer larval periods or a reduced propensity to metamorphose, both of which have negative consequences in temporary ponds. Alternatively, lack of discrimination among female metamorphic adults from such a population would suggest that females do not have the ability to discriminate by morph either because discrimination has not been selected in the past or because little heritable variation in this behavior exists.

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 (Whiteman and Semlitsch 2005; Semlitsch, unpublished data). Body size also varies within years, because smaller metamorphic adults tend to arrive at breeding ponds before larger individuals (Semlitsch 1981; Semlitsch et al. 1993; Whiteman and Semlitsch 2005). Size-specific male mating success has been previously observed in some species of salamanders in which males compete for mates (Janzen and Brodie 1989; Houck 1988; Mathis 1991; Howard et al. 1997), thus body size could strongly influence relative male mating success of each morph and subsequently impact the degree of intermorph breeding.

These hypotheses must be tested to determine how environmental, morphological, and behavioral differences among morphs influence the degree of assortative mating. In this paper, we utilized a series of laboratory and field experiments to evaluate the potential for intermorph breeding between morphs in *A. talpoideum*. Our experiments were designed to test the null hypothesis of no assortative mating between morphs by assessing the impacts of temporal (time of year), spatial (locality, size of experimental arena), and morphological (body size) variation on intermorph breeding.

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## Methods

### Study sites and collecting procedures

All experiments were conducted during September–March 1994–1997 at the Department of Energy’s Savannah River Site in Aiken County, SC. We collected *A. talpoideum* from three 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, while Karen’s Pond (0.25 ha) is temporary and contains only metamorphic adults (Semlitsch 1985a; Semlitsch and Gibbons 1985; Semlitsch et al. 1990).

All metamorphic adults were captured with drift fences and pitfall traps at each study site, while paedomorphic adults were captured using unbaited plastic minnow traps (Whiteman and Semlitsch 2005). All individuals were used in experiments as soon as possible after capture (except paedomorphic females, as described below). *A. talpoideum* do not store sperm across years (Trauth et al. 1994), hence virgin females were obtained by capturing individuals before that year’s breeding event. For metamorphic adults, unmated females were obtained using pitfalls and by capturing individuals along drift fences during migrations. During the 1994–1995 field season, paedomorphic females were captured throughout the fall and determined to be gravid based on body condition and cloacal inspection (Whiteman and Semlitsch 2005). Beginning in fall of 1995, unmated paedomorphic females were captured before the breeding season (early September) using minnow traps and placed at low density in screen cages (six to eight females per 1.5 m<sup>3</sup> cage) within their natal pond. Salamanders within cages could feed freely and remained in good condition for months. For short-term storage (<1 month), unmated females and males were separated by sex and kept in plastic boxes (20–25 animals per 45×25×20 cm box) with moistened paper towels (metamorphic adults) or well water (paedomorphic adults) at 4°C in an environmental chamber. Salamanders showed no negative effects from these conditions and exhibited normal breeding behavior once provided with warmer temperature (14°C).

### Laboratory experiments

In these experiments, we observed breeding interactions between one paedomorphic male and one metamorphic male with either a paedomorphic or metamorphic female (a trio). During the 1994–1995 breeding season, all experiments took place between January 17 and March 1, 1995, with all animals collected from Ellenton Bay. Preliminary experiments revealed that paedomorphic females were not receptive, perhaps because unmated females were not readily available in the populations during this time of year, hence all experiments were conducted with metamorphic females. During the 1995–1996 season, experiments were conducted between January 19 and March 15, 1996 using males and paedomorphic females from Ellenton Bay and metamorphic females from Ellenton Bay, Flamingo Bay, and Karen’s Pond. The Flamingo Bay population served as a control for differences in behavior based on locality and not hydroperiod. Timing of experiments was dependent primarily on the phenology of breeding migrations at each study site and the limited availability of salamanders for other experiments (see below).

Experiments were conducted in 38-L aquaria with well water and outdoor carpet for substrate. Two aquaria were housed in a walk-in environmental chamber set at 14°C, with one red incandescent 60-W bulb providing light for each tank. The aquaria were cleaned, filled with 10 cm of well water, and allowed to acclimate. At the same time, adults were moved from storage into the environmental chamber in individual containers and measured for snout–vent length (SVL, mm) and mass (g). After 2 h of temperature acclimation, one male of each morph was added to each aquarium. Males were matched for equal body size. After males had acclimated to the tank for at least 30 min, one female was added to each aquarium.

Once courtship behavior was initiated, we concentrated on recording three behaviors: spermatophore deposition without a female directly following the male, spermatophore deposition with a female directly following the male, and spermatophore pickups by the female. Depositions without a female provide some indicator of the mating readiness of males, but spermatophore depositions with a female is more indicative of male mating success. Pickups provide the best indicator of male mating success and female choice as well as confirmation of intra- vs intermorph breeding.

After a female was added to each tank, courtship behaviors often began quickly. We observed courtship until behaviors waned for more than 15 min, as preliminary experiments suggested that little relevant courtship would occur within the subsequent 3 h. In tanks where courtship did not begin immediately, we monitored trios for at least 1 h before removing the individuals. After each experimental trial, individuals were returned to environmental chambers and then released to the appropriate source population.

## Field experiments

### *Genetic analysis*

For each of the field experiments, we assigned adults to replicates using morph, sex, body size, and a three-locus genotype inferred from protein electrophoresis. Genotypic variation at the three loci [6-phosphoglucose dehydrogenase (PGD), lactate dehydrogenase-1 (LDH-1), and malate dehydrogenase-1 (MDH-1)] was used to assemble breeding groups of adult males and females that would allow the determination of paternity for each offspring. Allele frequencies in the Ellenton Bay population (estimated from 228 adults screened for potential use in the Trio 1 mating experiment described below) were 0.43, 0.23, 0.10, and 0.24 (alleles 1 to 4, respectively; PGD); 0.55, 0.10, and 0.35 (MDH-1); and 0.34 and 0.66 (LDH-1). For each locus, all possible genotypes were observed naturally and in frequencies not significantly different than that expected for a population in Hardy–Weinberg equilibrium. Mendelian inheritance patterns for each allele for each locus were observed independently in offspring sets with known parents.

Adult tail tips were excised and subjected to electrophoresis, while the adults were stored in individual containers at 4°C. Once genotyped, males and females were assigned such that each male had one or more unique alleles within the replicate to enable the determination of paternity of offspring by comparing adult male genotypes to the female and offspring genotypes. Following the appearance of embryos in the tanks, adults were removed with unbaited plastic minnow traps and through visual inspection. Resulting larvae were fed ad libitum until being harvested and genotyped whole. All adults and larvae were successfully genotyped.

We calculated the probability of failing to detect paternity by a father siring only 10% of the sampled offspring as  $(\alpha\beta)^\chi$ , where  $\alpha$  is the probability that his offspring received a unique paternal allele,  $\beta$  is the probability of sampling an offspring sired by another male ( $\beta$  equals 0.9 by assumption), and  $\chi$  is the number of offspring in the sample. For most experiments,  $\alpha$  equaled 1, but in some experiments,  $\alpha$  was less than 1 because males shared some alleles. The probability of failure was less than 5% for every tank in each of the field experiments.

### *Trio experiments*

These experiments were designed to simultaneously determine mating success among trios in similar fashion to those in the laboratory breeding experiments while obtaining a better estimate of RS of each morph through genetic analysis, because spermatophore pickups do not exclude the potential for sperm choice or competition (e.g., Birkhead and Moller 1998; Gabor et al. 2000; Jones et al. 2002). In Trio experiment I, single females of each morph were provided access to two similar-sized males, one of each morph, in a 30×30 cm tank filled with 20 cm of well

water and natural leaf substrate. Tanks were housed in 1,000-L metal stock tanks with 20 cm of well water to provide thermal buffering, and ten tanks randomly assigned to each of two ponds. We created 20 replicates of this experiment on 22 January 1996. In Trio experiment II, we repeated the Trio I experiment in large artificial ponds (1,000-L polyethylene stock tanks). Ponds were filled to a depth of 30 cm with water and contained 1 kg of a pine straw/deciduous leaf mixture to mimic the natural bottom of the source populations as well as provide a complex environment for breeding. We created 24 replicates of this experiment on 29 October 1996.

On 13 April 1996, we sampled ten hatchlings from each Trio experiment I replicate with larvae; we collected the remaining individuals on 2 May. For Trio II, all remaining larvae from each tank were collected on 25–26 May 1997. Each larva was placed in an individually labeled Eppendorf tube and frozen at  $-70^\circ\text{C}$ , where it remained until electrophoretic analysis was conducted.

### *Quint experiments*

These experiments were designed to measure RS in a complex natural environment where females were allowed to choose among a variety of different males. Single females of both morphs were presented with four males (two of each morph plus a female—quint) in large artificial ponds (1,000-L polyethylene stock tanks) that were created in identical fashion to the Trio II experiments described above. One male of each morph was large (largest 10% of the natural distribution), and one was small (smallest 10%; Semlitsch 1985a). The first Quint experiment ( $N=20$  tanks) was set up on 8 December 1994 and hatchlings collected on 10–12 April 1995; the second ( $N=20$  tanks) was set up on 15 January 1996 and hatchlings collected on 3–7 May 1996.

### *Statistical analyses*

All statistical analyses were conducted using StatView (SAS Institute 1999). We combined data across years for both laboratory and Quint experiments to increase sample sizes. For laboratory and Trio experiments, we compared differences between male morphs in mean body size with a paired  $t$  test and mean courtship or mating success using a Wilcoxon test because of heterogeneous variances and an abundance of zeros. For these and all tests outlined below with multiple response variables,  $\alpha$  was reduced appropriately (Rice 1989).

For Quint experiments, we used ANOVA to evaluate differences in body size among males of each morph and size class, and the effects of male morph and body size on mating success as measured by the proportion of offspring sired. We used  $\chi^2$  to test for departures from equal paternity when multiple paternity existed within a single female's clutch.

For Quint experiments, we also calculated Yule's  $V$ , an index of sexual isolation (Pielou 1977; Gilbert and Starmer 1985). Yule's  $V$  ranges from 1 (complete intermorph breeding) to  $-1$  (complete intramorph breeding). We used  $t$  tests to determine whether the resulting  $V$  statistics varied from the null hypothesis of nonassortative mating by morph (i.e.,  $V=0$ ). Yule's  $V$  could not be calculated for the other experiments because of incomplete designs or lack of data.

## Results

### Laboratory experiments

Metamorphic males were larger than paedomorphic males in both SVL and mass during experiments using females from Ellenton Bay and Karen's Pond (all paired  $t > 3.0$ , all  $p < 0.01$ , all  $N > 9$  pairs;  $\alpha = 0.025$ ), but did not differ in body size during experiments using females from Flamingo Bay (both paired  $t < 2.07$ , both  $p = 0.07$ , both  $N = 11$  pairs).

The number of spermatophore depositions and pickups varied between male morphs and, to a limited degree, among source populations for females. Experiments with metamorphic females from Ellenton Bay showed that metamorphic males had significantly more depositions with a female and pickups than paedomorphic males, which had no depositions with a female and thus no spermatophore pickups (Table 1). There was no significant difference in the number of depositions without a female among male morphs. Paedomorphic females from Ellenton Bay elicited spermatophore depositions in only one replicate, from a metamorphic male. Because of the lack

of response by males in trials with paedomorphic females, data were not analyzed statistically.

Experiments with metamorphic females from Karen's Pond showed similar trends to those using females from Ellenton Bay, with no depositions with a female by paedomorphic males and no pickups by females (Table 1); however, there was no significant difference between morphs in any of the response variables at  $\alpha = 0.0125$ . In contrast to the other localities, paedomorphic males from Flamingo Bay were observed depositing spermatophores with metamorphic females (in two replicates; Table 1), leading to no significant differences in the number of spermatophores deposited with a female. Flamingo Bay experiments were similar to those using animals from Karen's Pond in that males of each morph did not differ significantly in spermatophore pickups or depositions without a female ( $\alpha = 0.0125$ ).

### Field experiments

#### *Trio experiment I—winter 1995–1996*

Eight of the 20 experimental tanks (40%) produced at least ten larvae for analysis of RS (range 15–57), representing the choices of two paedomorphic females and six metamorphic females. There were no significant differences in body size (SVL or mass) between paedomorphic and metamorphic males within replicates (both paired  $t < 0.77$ , all  $p > 0.46$ ,  $N = 9$  pairs;  $\alpha = 0.025$ ). There was no evidence for multiple paternity, and paedomorphic males were completely excluded from paternity except for one offspring in one trio, an individual that either male could have sired. Two cases of intermorph reproduction were detected, both

**Table 1** Mean number of spermatophore depositions with a female following the male, without a female following the male, and pickups ( $\pm 1$  SE) during the laboratory experiments

	MM	PM	$z$	$P$
Ellenton Bay (MF; $n=28$ )				
Deposits with female	5.50 $\pm$ 0.93	0.00 $\pm$ 0.00	$-3.92$	$<0.0001$
Deposits without female	1.79 $\pm$ 0.46	0.54 $\pm$ 0.24	$-2.39$	0.02
Pickups	0.82 $\pm$ 0.20	0.00 $\pm$ 0.00	$-3.18$	0.002
Ellenton Bay (PF; $n=10$ )				
Deposits with female	0.60 $\pm$ 0.60	0.00 $\pm$ 0.00	–	–
Deposits without female	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	–	–
Pickups	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	–	–
Flamingo Bay (MF; $n=11$ )				
Deposits with female	6.46 $\pm$ 2.41	1.18 $\pm$ 0.82	$-2.37$	0.02
Deposits without female	1.46 $\pm$ 0.76	0.73 $\pm$ 0.51	$-0.41$	0.69
Pickups	0.91 $\pm$ 0.39	0.00 $\pm$ 0.00	$-2.04$	0.04
Karen's Pond (MF; $n=10$ )				
Deposits with female	5.70 $\pm$ 1.92	0.00 $\pm$ 0.00	$-2.20$	0.03
Deposits without female	1.10 $\pm$ 0.82	0.20 $\pm$ 0.20	$-1.07$	0.29
Pickups	0.70 $\pm$ 0.34	0.00 $\pm$ 0.00	$-1.84$	0.07

Data were not analyzed for paedomorphic females because of lack of response  $\alpha = 0.0125$  for each locality

MM metamorphic male, PM paedomorphic male, MF metamorphic female, PF paedomorphic female

involving metamorphic males with paedomorphic females. Metamorphic males functionally sired all of the scored offspring compared to paedomorphic males, which had no RS except for the one possible offspring described previously (Fig. 1; Wilcoxon paired sign test,  $z=-2.71$ ,  $p=0.007$ ). The single clutch with less than ten offspring exhibited complete RS by the metamorphic male and no intermorph breeding.

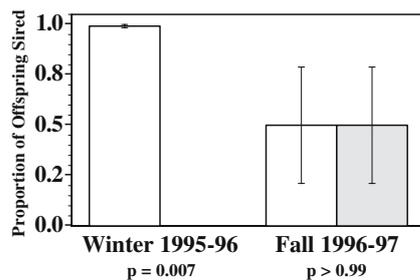
#### Trio experiment II—fall 1996–1997

Only four of the 24 tanks (16.7%) produced at least ten larvae for paternity exclusion analysis (range 21–85), representing the choices of three paedomorphic females and one metamorphic female. Metamorphic males were significantly larger than paedomorphic males in terms of SVL and mass (both paired  $t>7.7$ , both  $p<0.0003$ ,  $N=7$  pairs;  $\alpha=0.025$ ) during this experiment. Multiple paternity was not detected in any of the clutches, and intermorph breeding occurred in one of the four clutches, between a paedomorphic female and a metamorphic male. Each male morph sired two of the clutches, producing equivalent RS (Fig. 1;  $z=0.00$ ,  $p>0.99$ ).

Of the three tanks with less than ten offspring, two had evidence of intermorph breeding between a paedomorphic male and metamorphic female, and all three clutches were sired by paedomorphic males.

#### Quint experiments

During 1994–1995, five of the 20 tanks (25%) produced more than ten larvae (range 11–112), representing two paedomorphic and three metamorphic females. An additional tank with a metamorphic female produced sufficient offspring, but all experimental males were excluded from paternity. During 1995–1996, 11 of the 20 tanks (55%) provided sufficient larvae for paternity estimation (range 28–137 larvae per tank), representing five paedomorphic and six metamorphic females. Among the 16 analyzed tanks, there was a significant morph  $\times$  size class interaction in SVL, with the difference between large and small paedomorphic males greater than that between the two size



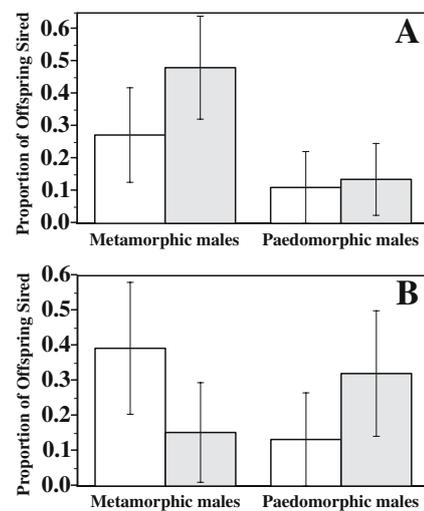
**Fig. 1** Mean proportion of offspring sired ( $\pm 1$  SE) for metamorphic (open bars) and paedomorphic (grey bars) males during the Trio field experiments. Data from replicates with each female morph are included. Sample sizes: winter 1995–1996,  $N=8$  replicates; fall 1996–1997,  $N=4$ . See text for statistics

classes of metamorphic males ( $F_{1,60}=6.4$ ,  $p=0.014$ ). Large males of each morph did not differ in SVL ( $56.5\pm 0.29$  vs  $56.1\pm 0.45$  for metamorphic and paedomorphic males, respectively).

Multiple paternity was detected in 4 of the 16 clutches, representing two females of each morph. Seventy-eight percent (39/50 genotyped larvae) of a metamorphic female's clutch was sired by a large metamorphic male, and the remainder (22%) was sired by a large paedomorphic male ( $\chi^2=15.7$ ,  $p<0.005$ ). Another metamorphic female's clutch (54.6%, 41/75) was sired by a large metamorphic male, with the remainder (45.4%) sired by a small metamorphic male ( $\chi^2=0.65$ ,  $p>0.30$ ). Approximately 93% (13/14) of a paedomorphic female's clutch was sired by a small paedomorphic male, with the remaining larvae sired by a large metamorphic male ( $\chi^2=10.3$ ,  $p<0.005$ ). Seventy-five percent of another paedomorphic female's offspring (24/32) was sired by a small metamorphic male, whereas the large paedomorphic male sired the remainder (25%;  $\chi^2=8.0$ ,  $p<0.005$ ).

Across all females, 8 of the 16 clutches resulted from intermorph matings, with five metamorphic male–paedomorphic female pairings and three paedomorphic male–metamorphic female pairs. Yule's  $V$  was calculated as 0.218, which was not significantly different from the null expectation of nonassortative mating by morph ( $V=0$ ;  $t=0.20$ ,  $p=0.58$ ,  $N=16$ ). Four clutches with less than ten offspring also showed evidence of intermorph breeding, with three paedomorphic females and one metamorphic female mating with large metamorphic males, and no evidence of multiple paternity.

There were no significant differences in the mean proportion of offspring sired between male morph, size class, or the interaction (all  $F_{1,60}<2.5$ , all  $p>0.11$ ; Fig. 2). Morph effects were not significant when size class was removed from the analysis ( $t=1.6$ ,  $p=0.11$ ,  $df=62$ ). When



**Fig. 2** Mean proportion of offspring sired ( $\pm 1$  SE) for small (open bars) and large (grey bars) males of each morph during the Quint field experiments. Results are separated by female morph. **a** Metamorphic females,  $N=9$  replicates. **b** Paedomorphic females,  $N=7$ . See text for statistics

analyzed by female morph, metamorphic males sired approximately three times the mean proportion of a metamorphic female's offspring that were sired by paedomorphic males ( $0.38 \pm 0.11$  vs  $0.12 \pm 0.08$ ; Fig. 2a), although this comparison was not significant ( $F_{1,32}=3.6$ ,  $p=0.07$ ). This result did not change with the removal of nonsignificant size effects. No other responses were significant for females of either morph (all  $F < 1.75$ , all  $p > 0.19$ ).

### Summary of field experiments

Although differences in year, season, and/or experimental conditions occurred across field experiments, we pooled the results to gain greater insight into the relationships described above. Of 36 clutches (including those with less than ten offspring), 16 (44.4%) were the result of intermorph breeding, which is not different from random expectation ( $\chi^2=0.44$ ,  $p=0.51$ ). Of the 28 clutches with ten or more offspring, four were sired by multiple males (14.3%). Taking multiple paternity into account, metamorphic males sired 20.6 (73.6%) of the clutches and 616 of the 825 hatchlings (74.7%; includes all hatchlings), both of which differ significantly from random expectation (clutches:  $\chi^2=6.2$ ,  $p < 0.013$ ; hatchlings:  $\chi^2=200.8$ ,  $p < 0.001$ ). Large males sired 8.87 of 16 clutches (55.4%) and 314 of 516 hatchlings (60.9%; includes all hatchlings). Only the latter result differed from random expectation (clutches:  $\chi^2=0.2$ ,  $p > 0.5$ ; hatchlings:  $\chi^2=24.3$ ,  $p < 0.001$ ).

## Discussion

The experiments reported here suggest that the potential for sexual isolation among morphs varies with environmental conditions (Table 2). During laboratory experiments, we never observed successful spermatophore transfer between paedomorphic males and metamorphic females, while we

commonly observed matings between metamorphic males and metamorphic females. Intermorph breeding was thus never observed in the laboratory. In field experiments, however, intermorph breeding was common, including paternity of clutches shared with males of both morphs. In the Trio experiments, intermorph breeding was asymmetric, with metamorphic male–paedomorphic female pairings the norm. In the more complex and realistic Quint experiments, intermorph breeding was relatively symmetric, and Yule's  $V$  suggested little evidence for sexual isolation between morphs. Metamorphic males had the highest RS in both laboratory experiments and the winter Trio experiments, while the autumn Trio and the Quint experiment suggested equal RS among male morphs across all females.

Thus, different experiments, even those within the same time of year, provide contrasting results. Laboratory experiments suggested that Trio encounters would not lead to intermorph breeding, yet field experiments with the exact same morph–sex complement showed considerable intermorph breeding, as did the more complex Quint experiments. One reason for these results was the lack of availability of sexually receptive paedomorphic females for laboratory experiments. Within Trio field experiments, paedomorphic females were more likely to mate with metamorphic males than paedomorphic males. Gravid paedomorphic females were not available for the first laboratory experiment, and during the second, they were sluggish and only a single spermatophore deposition occurred among ten replicates. Yet, it is clear that within field experiments, unmated paedomorphic females were able to successfully court with males. It is possible that had the laboratory conditions been changed, or had different females been used during laboratory experiments, more mating would have been observed between metamorphic males and paedomorphic females.

The timing of our experimental setups was limited, in part, by the migration and capture of sufficient numbers of metamorphic adults. This was especially true for the field

**Table 2** Summary results of the experiments conducted during this study

Experiment	Time of year	Intermorph breeding <sup>a</sup>	Symmetry	Yule's $V$	Multiple paternity <sup>b</sup>	Male RS advantage	Male body size differences
Laboratory	Winter	0/59	–	–	–	M	M > P
Trio I	Winter	2/9	No <sup>c</sup>	–	0/8	M	None
Trio II	Autumn	3/7	No <sup>c</sup>	–	0/4	Equal	M > P
Quint	Winter	11/16	Yes	0.218 <sup>d</sup>	4/16	Equal (overall) M(MF) <sup>e</sup>	Complex <sup>f</sup>

RS reproductive success, M metamorphic adult, P paedomorphic adult, MF metamorphic female

<sup>a</sup>Laboratory experiments: number of replicates with intermorph pickups/total number of replicates. Trio and Quint experiments: number of clutches with evidence of intermorph breeding/total number of clutches assessed

<sup>b</sup>Number of clutches with evidence of multiple paternity/total number of clutches assessed

<sup>c</sup>All intermorph breeding was between a metamorphic male and a paedomorphic female

<sup>d</sup>Not significant at  $\alpha=0.05$

<sup>e</sup>Although reproductive success was similar among males of each morph when analyzed across all females, metamorphic males sired three times more offspring with metamorphic females than large paedomorphic males ( $p=0.07$ ). See text for details

<sup>f</sup>The difference between large and small paedomorphic males was greater than that between the two size classes of metamorphic males. Large males of each morph did not differ in body size

experiments, in which we often had to genotype dozens of adults to identify sets of individuals for each replicate that would provide the genetic differences needed to identify sires for offspring. These constraints necessitated the late start of many winter experiments, when paedomorphic females would not naturally be breeding. Nonetheless, our experiments show that, if paedomorphic females did naturally breed in winter, they could be mated by metamorphic males. Thus, the environmental separation in breeding times between morphs, caused in part by the timing and amount of rain in the fall, may be a more important factor affecting sexual isolation among morphs in nature than mate-recognition behavior during courtship. This latter result is consistent with both field observations and breeding experiments on this species (Krenz and Verrell 2002; Whiteman and Semlitsch 2005) and other facultatively paedomorphic salamanders (Whiteman et al. 1999; Denoel et al. 2001; Denoel 2002, 2003). However, future experiments will be needed to directly address this hypothesis.

In the Laboratory experiment, we manipulated female locality to determine if female choice was affected by selection against the propensity for paedomorphosis under different hydroperiods at natal ponds. Because females from both permanent and temporary ponds were unresponsive to paedomorphic males, we cannot determine whether female behavior depends on the type of source pond, or whether females from temporary habitats do not recognize paedomorphic males as mates, given that it is unlikely that individuals from such ponds have ever interacted with paedomorphic adults.

The Quint experiment revealed no evidence for a large body size advantage among males in terms of mean proportion of offspring sired or number of clutches sired, but we did detect a large male advantage in terms of the number of hatchlings sired. These results suggest that body size may be less important to male mating success under complex environments and low-density conditions where finding a receptive mate may be paramount to winning male–male interactions. Some studies have shown clear large-male advantages, particularly during laboratory experiments (Janzen and Brodie 1989; Houck 1988; Mathis 1991; Howard et al. 1997). In contrast, another field experiment using seminatural enclosures in the congener *Ambystoma opacum* found no effects of male body size on mating or RS (D. Croshaw, unpublished data). Thus, further experiments are warranted, particularly under variable density treatments, to better test this hypothesis.

Male body size was an unavoidable confounding factor within some of our experiments (Table 2). Metamorphic males are often larger than paedomorphic males in nature (Whiteman and Semlitsch 2005), and thus experiments with such body size disparity are more realistic. Although we attempted to match males, it was impossible to complete the experiments without pairing individuals that varied in size. In most cases, however, differences in body size among males were slight. In addition, results of replicates when paedomorphic males were slightly larger than metamorphic males were not different in outcome than other replicates. The lack of substantial size effects during

the Quint experiments, in which we actively manipulated body size as a controlled variable, also suggests that the slight body size differences found during the Laboratory and Trio experiments were unlikely to affect our results. Nonetheless, the possibility remains that body size effects could have masked our ability to evaluate levels of intermorph breeding or morph-specific mating success among males.

Differences in the RS of males also varied with environmental conditions. Male RS was equal between morphs during the autumn Trio and the more complex Quint experiments (Table 2). During the Laboratory and winter Trio experiments, metamorphic males had higher RS than paedomorphic males in terms of pickups or the proportion of offspring sired. This result may be, in part, a reflection of the lack of intermorph breeding during laboratory experiments, when only metamorphic females were actively breeding. However, metamorphic males actively bred with paedomorphic females during other experiments.

Differential male RS among morphs has implications for the maintenance of this polyphenism. The increased RS of metamorphic males in some experiments suggests a fitness benefit for males that metamorphose, at least under some environmental conditions. In contrast, Whiteman and Semlitsch (2005) suggested that paedomorphic males should have higher mating success within natural breeding ponds because of numerical advantages, asymmetric reproductive isolation, and the benefits of early reproduction for offspring (see also Ryan and Plague 2004). Additionally, paedomorphic *A. talpoideum* mature earlier than metamorphic adults (Semlitsch 1985a; Ryan and Semlitsch 1998). The ability of metamorphic males to garner increased RS through breeding interactions may temper the benefits of paedomorphosis to males and thus help maintain facultative paedomorphosis, at least within this sex (Whiteman 1997; Denoel et al. 2005). Because our understanding of the fitness payoffs to each morph and sex is incomplete, our ability to test hypotheses about the maintenance of this and other polyphenisms will require further studies aimed at estimating lifetime RS (Whiteman 1994, 1997; Denoel et al. 2002, 2005).

This and other studies (Krenz and Verrell 2002) suggest that there is little evidence of sexual isolation among morphs in this species based on behavioral interactions alone, but rather that temporal, spatial, and other environmental variation may play a larger role in the degree to which the two morphs interbreed (Whiteman and Semlitsch 2005). Such differences are unique to *A. talpoideum*, because other facultatively paedomorphic species tend to have more constrained breeding seasons (Whiteman et al. 1999) or little evidence for spatial separation among morphs during breeding (Denoel and Joly 2001; Denoel, unpublished data). *A. talpoideum* might thus serve as a unique vertebrate model for studies of the evolution of reproductive isolation and particularly how environmental variation might impact isolation. Because most studies testing current speciation models have used extant species groups (Panhuis et al. 2001; Schluter 2001; Via 2001)

rather than model taxa in which speciation has yet to occur (Grant and Grant 1989; Johannesson et al. 1995; Hendry et al. 2000; Jones et al. 2003; Whiteman and Semlitsch 2005), future studies on this and other polyphenisms may provide unique opportunities to clarify the mechanisms underlying these questions.

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