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Insights into the mating habits of the tiger salamander (*Ambystoma tigrinum tigrinum*) as revealed by genetic parentage analyses

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Abstract

Among urodeles, ambystomatid salamanders are particularly amenable to genetic parentage analyses because they are explosive aggregate breeders that typically have large progeny arrays. Such analyses can lead to direct inferences about otherwise cryptic aspects of salamander natural history, including the rate of multiple mating, individual reproductive success, and the spatial distribution of clutches. In 2002, we collected eastern tiger salamander (*Ambystoma tigrinum tigrinum*) egg masses (> 1000 embryos) from a ~80 m linear transect in Indiana, USA. Embryos were genotyped at four variable microsatellite loci and the resulting progeny array data were used to reconstruct multilocus genotypes of the parental dams and sires for each egg mass. UPGMA analysis of genetic distances among embryos resolved four instances of egg mass admixture, where two or more females had oviposited at exactly the same site resulting in the mixing of independent cohorts. In total, 41 discrete egg masses were available for parentage analyses. Twenty-three egg masses (56%) consisted exclusively of full-siblings (i.e. were singly sired) and 18 (44%) were multiply sired (mean 2.6 males/clutch). Parentage could be genetically assigned to one of 17 distinct parent pairs involving at least 15 females and 14 different males. Reproductive skew was evident among males who sired multiply sired clutches. Additional evidence of the effects of sexual selection on male reproductive success was apparent via significant positive correlations between male mating and reproductive success. Females frequently partitioned their clutches into multiple discrete egg masses that were separated from one another by as many as 43 m. Collectively, these data provide the first direct evidence for polygynandry in a wild population of tiger salamanders.

Keywords: ambystomatid, microsatellites, multiple paternity, oviposition, reproductive skew, sexual selection

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Introduction

Sexual selection theory predicts that the variance in reproductive success should be greater in males than in females because male reproductive success is limited only by partner availability whereas female success is limited by resource availability (Bateman 1948). Estimating the variance in individual reproductive success is nearly impossible using conventional ecological approaches, but molecular genetic parentage analyses allow new insight into the breeding biology of species where direct observation of courtship and fertilization is difficult (DeWoody *et al*. 1998; Fiumera *et al*. 2002). By establishing genetic identities of parents and progeny, one can quantify variation in individual fertilization success, evaluate the relative reproductive success of alternative mating tactics, and determine the predominant breeding system in the population (Avise *et al*. 2002; Jones & Ardren 2003). Furthermore, molecular assessments of reproductive success often lead to surprising conclusions and new biological insights (DeWoody *et al*. 2000a; Griffith *et al*. 2002).

To date, most such breeding appraisals have been conducted in mammals, birds, and fishes, but many aspects of amphibian natural history make them ideal for studies of sexual selection. For example, the breeding strategies

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employed by eastern tiger salamanders (*Ambystoma tigrinum tigrinum*) should exacerbate the variance in individual reproductive success for several reasons. First, breeding is explosive and generally occurs within aggregated swarms of adults, so intrasexual fitness differences may be exaggerated by competition (Arnold 1976; Wells 1977; Harris & Lucas 2002). Second, the operational sex ratio (OSR) in these aggregations is often skewed towards males because female receptivity is not perfectly synchronized (Halliday & Verrell 1984). Thus, the OSR may vary considerably within a breeding season and may affect male sperm allocation tactics in response to mate availability (Harris & Lucas 2002). Third, receptive females produce large numbers of eggs and males vie for mating success by courting as many females as possible. Males also compete for access to courting females by sexual interference or defence (Arnold 1976; Howard *et al*. 1997). Females are led towards spermatophores deposited on the substrate by courting males, and these are either inducted for internal fertilization or rejected. Multiple mating by females (i.e. the induction of spermatophores deposited by different males) may result in sperm competition, either by sperm precedence (Jones *et al*. 2002) or cryptic female choice (Eberhard 1996). Therefore, the variance in reproductive success is expected to be highly skewed among males, both within and among egg masses (i.e. progeny arrays) that contain as many as 50 embryos.

Herein, we utilized genetic parentage analyses of field-collected egg masses to characterize the breeding biology of the eastern tiger salamander. In particular, we used a suite of microsatellite markers to (i) test for the presence of multiple mating and the degree of reproductive skew, and (ii) describe the predominant breeding system in this species. By virtue of our sampling design, we were also able to examine the spatial patterns of female oviposition in a wild population — a hitherto unexplored facet of salamander breeding behaviour. Our analyses of parental reproductive patterns in the absence of sampled adults, while unconventional, proved fruitful and the procedures used here to infer parental adult genotypes should be useful in any species where large progeny arrays are available but potential breeders are not.

Methods

Sampling and laboratory analysis

Following a period of adult salamander breeding events in April 2002, 41 tiger salamander egg masses were sampled from an 80 m linear transect located within a shallow water-filled ditch along the north fence of the Purdue Wildlife Area, Tippecanoe County, Indiana, USA. Egg masses were labelled according to their positions relative to a series of 17 fence-posts separated by intervals of 4.7 m from west to east

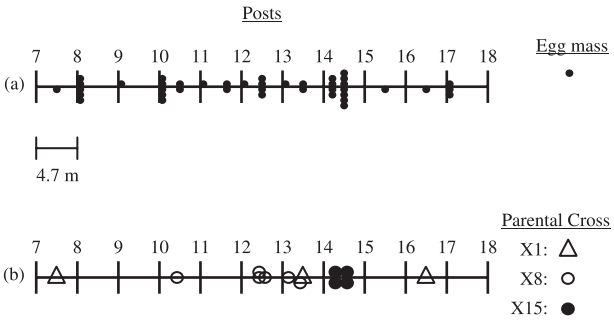


Fig. 1 (a) The distribution of 41 tiger salamander egg masses alongside a roadside ditch. The actual transect encompassed fence-posts 1–18, but no embryos were located between posts 1–7. (b) An example of how three of 17 female salamanders partitioned their clutches along the transect. Three egg masses from clutch X1 were discovered 14–42.5 m apart. Egg masses from clutch X8 ranged from < 1–14 m apart, and four egg masses from clutch X15 were all found within a meter of one another. For clarity, oviposition sites for 14 of the 17 clutches are omitted.

within the transect (see Fig. 1). Thus, egg masses 12 and 13 were nearest to posts 12 and 13 (respectively) whereas egg mass 12.5 lay intermediate to these posts. Several egg masses were spatially clustered (i.e. in close proximity to one another), and these egg masses were further labelled to define identity. For example, egg masses 17a and 17b were near to and west of post 17 but within 30 cm of one another. Egg masses were temporarily stored in plastic bags until arrival in the laboratory later the same day, when individual embryos were dissected. Embryo cohorts (i.e. those from the same egg mass) were collectively stored at room temperature in 50-mL conical tubes containing 100% ethanol; the ethanol was replaced after 1–3 days to ensure that the concentration remained near 100%.

In preparation for DNA extraction, each embryo was rinsed with distilled water and then incubated overnight (with rotation) at 55 °C in 500 μL of SNET buffer (5 mM EDTA, 400 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1% SDS) containing 5 μL proteinase K (10 mg/mL). Total genomic DNA was isolated by a single extraction using 500 μL of PCI (phenol:chloroform:isoamyl alcohol in a 25 : 24 : 1 ratio) followed by an isopropanol precipitation and 70% ethanol wash. Pellets were resuspended in 100 μL of purified water. Four polymorphic microsatellite primer pairs designed from a variety of ambystomatids were used in polymerase chain reaction (PCR) to amplify the following loci: Atex 65 (Williams & DeWoody 2004), ATS5-7 (Mech *et al*. 2003), 52.34 (F primer 5′-TGTACAGACAGGCA-AGAGGTATTGACAGT-3′, R primer 5′-GTCTCCCAC-TTTAATTTCCCTCAGTTTTT-3′), and 60.9 (F primer 5′-TATTTATTGAACGTGAACCTACTGCTGAGAA-3′, R primer 5′-AAAGTAACATTAGATTGGGGGAGGGAT-AGA-3′). Primer sequences of 52.34 and 60.9 are courtesy of K. Zamudio, Cornell University.

All four microsatellite loci were amplified in a single 10-μL PCR containing ~100 ng of template DNA; 0.25 U of *Taq* DNA polymerase (New England Biolabs); 0.25 mM of dNTPs; 2.5 mM $MgCl_2$; and 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.9, 0.05 mg/mL BSA). Final primer concentrations for PCR were 0.18 μM, 0.2 μM, 0.2 μM and 0.08 μM, respectively, as above and each forward primer was end-labelled with a fluorescent dye. Temperature profiles for PCR consisted of a 2-min 95 °C denaturation step followed by 30 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s. A final 5-min extension step followed and PCR products were stored at 4 °C. Amplicons from each embryo were scored for size via electrophoresis on an ABI 377 sequencer (Applied Biosystems) using associated GENESCAN 3.1.2 and GENOTYPER 2.5.2 software.

Parental multilocus genotype assignment

To estimate the minimum number of parents contributing gametes to each egg mass and to reconstruct putative parental genotypes, embryo genotypes were analysed collectively. Egg masses were identified as containing either full-sib (single sired) or half-sib (multiply sired) relationships among embryos, using a deductive approach similar to that of DeWoody *et al*. (1998); see also Jones & Ardren (2003). Thus, we use the term 'egg mass' to refer to physically distinct cohorts of embryos collected in the field, and the term 'clutch' to represent an array of full- or half-sib embryos that (by genetic deduction) share a dam. Thus, each dam has exactly one clutch but may produce multiple egg masses. In some cases, we use the term 'progeny array' to collectively describe a group of embryos because egg masses must be genetically analysed before clutches can be identified (and in some cases a clutch is equivalent to an egg mass). Hence, a single female's clutch may be distributed among several egg masses and an egg mass may occasionally contain clutches from one or more females via ovipositional admixture. Full-sibling clutches had no more than four alleles at any given locus distributed among embryos in a manner consistent with expectations of biparental Mendelian inheritance. In contrast, a clutch contained half-siblings if more than four alleles were present at any of the four loci but a distinct maternal genotype was identified.

Parental genotypes for each egg mass were inferred across loci using patterns and frequencies of allelic associations among embryos (Jones & Ardren 2003; Jones 2005). Each unique maternal and paternal multilocus genotype was assigned an arbitrary identifier (F#, M#). Likewise, inferred parental crosses (e.g. F4 crossed with M11) were assigned an arbitrary identifier (X#). Maternal alleles were identified by the presence of one or two distinct homozygotes per locus and/or either of two distinct alleles distributed among all embryos in a mass. In cases of single maternity/multiple paternity, assignment of maternal multilocus genotype was by simple subtraction (DeWoody *et al*. 1998; Jones & Ardren 2003). The program GERUD 2.0 (Jones 2005) was used to verify the paternal genotypes associated with multiply sired progeny arrays. As well as identifying the minimum number of sires and their genotypes for each progeny array, GERUD 2.0 also provided estimates of juvenile numbers sired by each father. In contrast to half-sib progeny arrays, parental multilocus genotypes cannot be inferred directly from full-sib masses (DeWoody *et al*. 2000a) and thus our assignments were provisional and relied upon those genotypes inferred directly from the half-sib progeny arrays (Fig. 2a). In instances where both sire and dam genotypes of a full-sibling clutch could not be assigned to one of the parental genotypes derived from the half-sib clutches, the assignment of multilocus genotype to either sex was arbitrary.

Further demonstration of our genotyping procedures is presented in Fig. 2b, where 54 embryos are illustrated from egg masses 7.5, 13.5b and 16.5. Parentage analyses (Fig. 2a) reveal that four different parents contributed gametes to these three egg masses. We assume these four parents are a single female (F1) and three different males (M1, M2 and M3) based upon two primary lines of evidence. First, multiple paternity has been documented previously in other ambystomatid salamanders (Tennessen & Zamudio 2003). Second, the spatial distribution of the three egg masses is inconsistent with multiple maternity. That is, we find it implausible that a single male mated with three females, each of whom happened to have deposited her gametes in direct association with those of two other females, especially when the distance between egg masses 7.5 and 16.5 is over 40 m. It seems imminently more plausible that a single female (F1) inducted spermatophores from three (or more) males and then distributed her gametes among the three egg masses; we refer to this collective mating event as X1 (Table 1). The assumption of multiple paternity allows us to assign the female and each male a multilocus genotype which, over the course of the entire study, can then be used to polarize the gender of adults in the single-paternity masses (Fig. 2a). For example, all 56 embryos from egg masses 8a1 and 8b1 are consistent with parentage by a single female and male. The genotype of male M3, a sire of egg masses 7.5, 13.5b and 16.5, is also consistent with parentage of 8a1 and 8b1. Thus, we assume that M3 sired these embryos with a heretofore unsampled female (designated F2) in mating event X2 (Table 1). Female F2 oviposited her fertilized eggs in two distinct egg masses which were collected within 30 cm of one another. In this case, a single clutch was distributed among two egg masses. By similar logic, the genders of most 'single paternity' parents (Table 1) were ascertained.

Four egg masses had more than two distinct homozygotes at a locus. In theory, this could result from scoring errors, null alleles, allelic dropout, or admixture of distinct

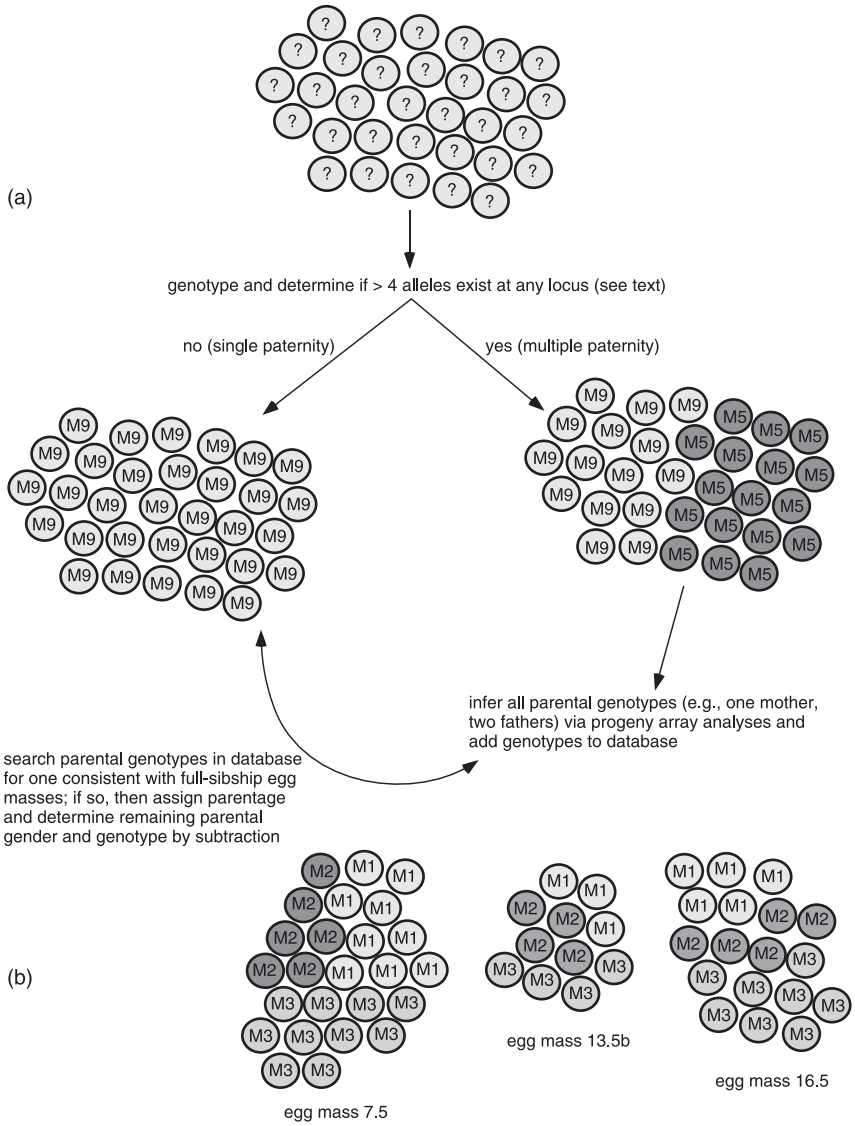


Fig. 2 (a) The assignment of parental genotypes was an iterative process that relied on strong inferences made from multiply sired egg masses. Paternal genotypes of the multiply sired egg masses were derived from GERUD 2.0 (Jones 2005) and, in conjunction with inferred maternal genotypes, were used in a candidate database as potential parents of the singly sired egg masses. In a few instances, no genotype in our database was consistent with parentage of a singly sired egg mass and in such cases the assignment of multilocus genotype to either sex was arbitrary. However, the arbitrarily-assigned genotypes/genders were not used as candidates in the database. 'M5' refers to male 5 and 'M9' to male 9. (b) Clutches may be partitioned among multiple egg masses. Here, parentage analyses reveal that three egg masses (7.5, 13.5b and 16.5) were partitioned from clutch X1 derived from female F2. Furthermore, each embryo is assigned one of three sires (M1, M2 and M3). In this figure, embryos fertilized by a given sire are shaded and grouped for sake of clarity. Refer to methods for more details.

clutches at the same oviposition site. After genotypic verification (i.e. rerunning gels and rescoring), suspect egg masses were examined for admixture by estimating multilocus genetic distance among all embryos within the mass and then clustering related embryos based on these distances. The program POPULATIONS 1.2.28 (Copyright 1999, O. Langella, www.pge.cnrs-gif.fr/bioinfo/accueil/index.php) was used to estimate minimum genetic distances (D_m, Takezaki & Nei 1996) among all individuals within an egg mass. These distances then were used to construct UPGMA trees to cluster individuals by genetic similarity using the program TREEVIEW (Page 1996). The clusters were examined by eye and, where necessary, clusters were further amalgamated if assignment of a single multilocus maternal genotype among individuals was possible. Average pairwise relatedness estimates (*r* coefficient of relatedness, Queller & Goodnight 1989) among individuals were estimated both within and between clusters to assess the validity of the clustering process. Additional relatedness estimates reported elsewhere in results used the same coefficient calculation.

Reproductive variance and oviposition

To determine if the variance in reproductive success was more skewed in males than females, we compared mating success (number of sexual partners) and reproductive success (number of progeny produced) between sexes. Furthermore, each multiply sired clutch was evaluated for paternal reproductive skew as measured by observed departures from uniform expectations using chi-squared tests.

We conducted computer simulations to evaluate whether egg masses partitioned from individual clutches were

Table 1 Clutch data associated with each egg mass, including sample size (*N*), putative female parents (♀), the inferred female–male cross (X), and putative male parents (♂). Also included are numbers of embryos sired by each male (Progeny) and their frequency (%) within each multiply sired cohort. Multilocus genotypes for putative female and male parents as per Appendix. An asterisk indicates that parental genotype reconstruction was incomplete, but inferred from Appendix

		Single paternity					Multiple paternity				
Egg mass	*N*	♀	X	♂	Egg mass	*N*	♀	X	♂	Progeny	%
8a1	32	F2	2	M3	7.5	25	F1	1	M1	9	36
8b1	24	F2	2	M3					M2	6	24
10f	16	F3	7	M4					M3	10	40
10.5a	23	F7	8	M7	8a2	40	F3	3	M3	9	22.5
11.75b2	26	F10	11	M11					M4	31	77.5
12	22	F11	12	M12	8b2	31	F3	3	M3	15	48.4
12.5a	27	F7	8	M7					M4	16	51.6
12.5b	24	F7	8	M7	8c	92	F4	4	M1	25	27.2
12.5c	36	F7	8	M7					M4	20	21.7
13	17	F7	8	M7					M5	26	28.3
13.5a	25	F7	8	M7					M6	21	22.8
14.25a	7	F12	13	M5	9a	36	F5	5	M3	9	25
14.25c	17	F11	12	M12					M7	21	58.3
14.5a	5	F14	15	M13					M8	6	16.7
14.5b1	4	F13*	16	M9*	10a	8	F6	6	M4	5	62.5
14.5b2	13	F14	15	M13					M8	2	25
14.5c	26	F14	15	M13					M9*	1	12.5
14.5d	33	F14	15	M13	10c	29	F6	6	M4	11	37.9
14.5f	10	F13	16	M9					M8	14	48.3
15.5	18	F11	12	M12					M9	4	13.8
17a	31	F15	17	M14	10d	22	F5	5	M3	7	31.8
17b	16	F11	12	M12					M7	12	54.5
17c	31	F11	12	M12					M8*	3	13.6
					10e	29	F3	3	M3	11	37.9
									M4	18	62.1
					10.5b	31	F8	9	M4	9	29
									M9	22	71
					11a	20	F9	10	M10	7	35
									M11	13	65
					11.75b1	18	F9	10	M10	7	38.9
									M11	11	61.1
					12.5d	30	F5	5	M3	8	26.7
									M7	19	63.3
									M8	3	10.0
					13.5b	11	F1	1	M1	3	27.2
									M2	4	36.4
									M3	4	36.4
					14.25b	9	F13	14	M9	7	77.8
									M12*	4	21.1
					14.5e	16	F13	14	M9	13	81.2
									M12	3	18.8
					16.5	18	F1	1	M1	5	27.8
									M2*	5	27.2
									M3	8	44.4

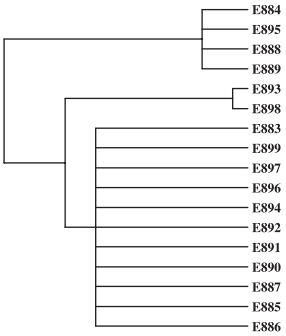
physically clustered or widely distributed. Thus, we compared the mean geographic distance among egg masses derived from a single clutch ($\bar{d}_{obs}$) to the expected distance assuming random oviposition along the transect. We generated the distribution expected under random oviposition by re-sampling (10 000 replicates) the average distance among several egg masses that were randomly sampled from a matrix of pairwise distances between all egg masses. For each clutch that was partitioned into multiple egg masses, $\bar{d}_{obs}$ was compared to the simulated random distance distribution.

Results

From 1051 genotyped embryos 983 (94%) were confidently scored for at least three of the four loci. Genetic diversity among embryos was high (a mean of 12.3 alleles per locus), with the maximum number of alleles observed at Atex 65 (n = 23), followed by 52.34 (n = 16), 60.9 (n = 7) and ATS5-7 (n = 3). Null alleles were infrequent, but observed at locus 60.9 (4 egg masses derived from two females) and at locus Atex 65 (3 egg masses associated with a single female). Using Cervus 2.0 (Marshall *et al*. 1998), estimates of expected null allele frequency based on reconstructed adult genotypes ranged from –0.008 to 0.026 for the three most polymorphic loci, whereas the estimate for locus ATS5-7 was 0.268. The high frequency null allele(s) at this locus resulted in a nonsignificant deficiency of observed heterozygotes, but did not impede parentage analyses because this locus was rarely informative (i.e. parentage was resolved with the three hypervariable loci). A single putative mutation was observed at locus 52.34 and six were identified at locus Atex 65. At Atex 65, five of the six *de novo* mutations were independent, but one was a clustered mutation (Woodruff *et al*. 2004) seen in two spatially separate egg masses of the same clutch. The small proportion (< 5%) of embryos with null alleles and/or mutations were removed from progeny arrays prior to GERUD 2.0 analysis.

Four of the original 41 field collected egg masses were not analysed due to small sample size. An additional four egg masses were each identified as admixed (i.e. containing two distinct clutches) and thus were each partitioned as two egg masses using the phenetic clustering procedure. Egg masses partitioned by this approach were renamed by adding a suffix to their identifier (e.g. 14.5b was divided into 14.5b1 and 14.5b2, Fig. 3). After these procedures, we were able to confidently score 967 embryos among 41 identified egg masses (Table 1). Of these, 23 egg masses (56%) consisted of only full-siblings whereas 18 (44%) were multiply sired and thus consisted of a mixture of full- and half-siblings. Reconstruction of the parental genotypes from the progeny arrays indicated that 29 parents contributed gametes to the embryos collected from the transect (Table 1 and Appendix). Of these 29 parents, 15 were female and 14 were male (Appendix). The inferred genotype of each presumptive parent was unique, with an average probability of identity of 3.2×10^{-6} (Appendix). Among these 29 parents, we identified 17 distinct matings or 'crosses' designated X1–X17 (Table 1), each of which produced a distinct clutch.

Embryos from crosses X1–X17 were distributed among the 41 collected egg masses, indicating that females often partition their clutches spatially as multiple egg masses. For example, clutch X8 was a cross between F7 and M7 and was distributed among 6 egg masses (Table 1). Out of a total of 967 embryos, 483 resulted from nine singly sired clutches partitioned among 23 egg masses. The remaining 484 embryos resulted from eight multiply sired clutches distributed among 18 egg masses. Mean (± SE) numbers of embryos per clutch and egg mass was 53.7 (± 16.3) and 21.2 (± 1.9) for singly sired cohorts compared to 60.5 (± 10) and 26.9 (± 4.4) for multiply sired cohorts. Average clutch and egg mass sizes did not differ between the multiple and single paternity groups (all t-test; $P > 0.1$). Average pairwise relatedness (r) among all parents contributing to clutches was –0.048. Average relatedness between parents within



Embryo	Egg mass	Microsatellite locus 52.34	Atex 65	ATS5-7	60.9
E895	14.5B1	382/430	344/420	240/244	No Data
E888	14.5B1	388/430	348/352	240/244	No Data
E884	14.5B1	388/408	344/352	244/246	No Data
E889	14.5B1	382/408	348/352	244/246	No Data
Female type: F13		408/430	352/420	240/246	
Male type: M9		382/388	344/348	244/244	
E883	14.5B2	384/390	332/400	240/246	No Data
E885	14.5B2	380/390	332/400	240/246	No Data
E886	14.5B2	384/390	332/400	240/246	No Data
E887	14.5B2	380/390	332/400	240/246	No Data
E890	14.5B2	380/390	332/400	240/246	No Data
E891	14.5B2	380/390	332/400	240/246	No Data
E892	14.5B2	380/390	332/400	240/246	No Data
E893	14.5B2	380/390	332/344	240/246	No Data
E894	14.5B2	380/390	332/400	240/246	224/234
E896	14.5B2	380/390	332/400	240/246	224/236
E897	14.5B2	380/390	332/400	240/246	224/236
E898	14.5B2	380/390	**328**/400	240/246	No Data
E899	14.5B2	380/390	332/400	240/246	No Data
Female type F14:		390/390	332/332	240/240	224/236
Male type M13:		380/384	344/400	246/246	234/236

Fig. 3 Embryos from distinct clutches within an admixed egg mass (14.5b) can be distinguished via UPGMA analysis of minimum genetic distances (D_m, Takezaki & Nei 1996). Multi-locus genotypes for each embryo and the inferred parents are shown below the dendrogram. The relatedness coefficient of the entire admixed mass (–0.070 ± 0.008) is low compared to the two constituent egg masses (0.274 ± 0.029 & 0.609 ± 0.081). Bold-type allele indicates a presumptive *de novo* mutation relative to the inferred parental genotype. Note that no data was available at locus 60.9 at egg mass 14.5b1. In such instances, parental genotypes were inferred from other egg masses that shared the same parents.

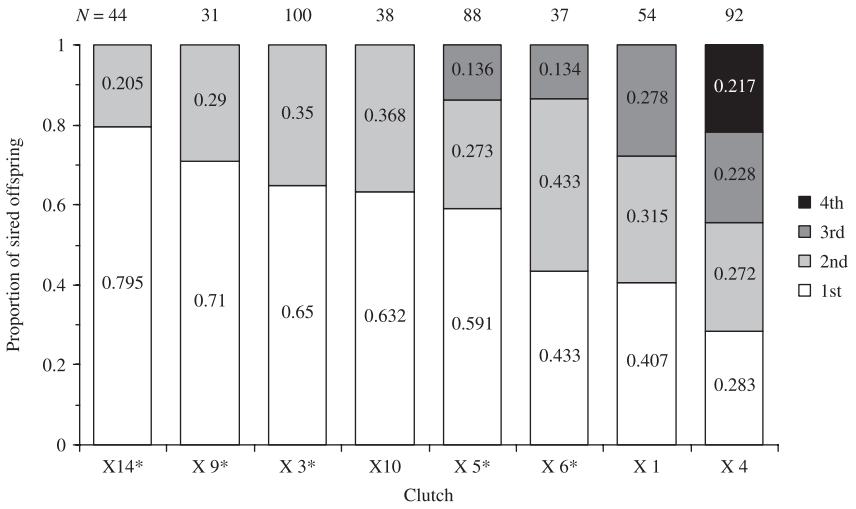


Fig. 4 Reproductive skew within eight clutches sired by two or more males. Clutches marked with * are significantly skewed from equal paternal contributions ($P < 0.05$). The number of embryos within each clutch (N) is indicated above the bars, whereas the proportion of paternity by each male is indicated within bars. Males labelled as 1st to 4th most successful contributor within a clutch are not necessarily of the same genotypic identity among clutches.

a clutch was –0.067 for singly sired clutches and –0.050 for multiply sired clutches, suggesting that the most matings occurred between unrelated adults.

Polygyny was common in this population. For example, four clutches distributed among 11 egg masses (7.5, 8a1, 8a2, 8b1, 8b2, 9a, 10d, 10e, 13.5b, 16.5) each contained male genotype M3 crossed with one of four females: F1, F2, F3 and F5 either in combination with other M types or as a full-sibling clutch (Table 1).

Polyandry was employed by eight females and the average number of sires per multiply mated female was 2.6. The proportion of embryos fertilized by the most successful father ranged from 0.632 to 0.795 among clutches sired by two males, from 0.407 to 0.591 in clutches sired by three males, and was 0.283 for the single clutch with four sires (Fig. 4). Male reproductive skew within each multiply sired clutch was evaluated by comparing the observed distribution of within-clutch reproductive success against the null hypothesis of a uniform distribution; five of the eight multiply sired clutches (X3, X5, X9, X6 and X14) were significantly skewed (χ^2 tests, all $P < 0.05$). This trend was generally evident among partitioned egg masses, where a particular male consistently contributed to the majority of progeny (Table 1).

Mating success (number of partners) was not significantly different (t-test; $P > 0.05$) among the sexes: females mated with a mean of 1.87 males, whereas males mated with a mean of 2.14 females. Variance in male mating success was almost twice that for females (1.67 vs. 0.98). Similarly, mean reproductive success of adult females and males (as measured by number of progeny produced in the entire sample) did not significantly differ. The variance in male reproductive success ($\sigma_m = 3223.3$) was at least twice that of females ($\sigma_c = 157.0$) and probably much greater, as some adult males in the population — those who were unsuccessful breeders — are presumably unrepresented in our survey. Male mating success was significantly correlated to reproductive success (Spearman's rank correlation $r_s = 0.648$, $P < 0.006$, $N = 14$), indicating that males mated to numerous partners generally also sire a greater number of progeny. The correlation between female reproductive success and mating success was not significant ($r_s = 0.311$, $P > 0.150$, $N = 8$), indicating that clutch size was not correlated with a female's mating success.

Frequency and distribution of oviposition within the transect varied among females. Six of the 17 clutches were each observed as single egg masses, but 11 females partitioned their clutches among multiple egg masses (mean 3.2). Clutches partitioned among multiple sites did not appear to be biased towards single or multiple paternities (ratio 5:6); similarly four of the six nonpartitioned clutches were singly sired. Pairwise spatial distribution of egg masses varied from clustered (within a 1-meter radius) to those broadly separated by distances up to 42 m (Fig. 1). Mean oviposition distance among multiple egg masses partitioned from a single clutch was 7.2 m. Roughly one-third (32%) of all within-clutch pairwise oviposition distances were within 1 m and over 55% were within 5 m, although egg masses from one partitioned clutch (X1) were separated by up to 42 m. The average distance among egg masses from three (X12, X5 and X3) of the 11 partitioned clutches was consistent with that expected for a random pattern of oviposition (i.e. they were neither clustered nor overdispersed). In contrast, seven partitioned clutches (X8, X10, X14, X15, X16, X2, X6) were clustered at distances significantly less than that expected by chance, whereas the egg masses from one clutch (X1) were significantly dispersed (Table 2).

Discussion

Analytical methods of parentage analyses in breeding aggregations

Although genetic parentage analyses can provide powerful direct tests of behavioural observations, the analysis of

Table 2 The geographic distribution of egg masses partitioned from single clutches along the transect. Eleven females partitioned their clutches into multiple egg masses, and the mean pairwise distance among egg masses partitioned from each clutch is shown ($\bar{d}_{obs}$, in meters). The probability that $\bar{d}_{obs}$ was consistent with that expected from a simulated distribution of average distances assuming random oviposition is also shown (*P*). A simulated distribution for each clutch was generated by resampling (10 000 replicates) mean pairwise distances among randomly chosen egg masses. The no. of egg masses refers to the number of empirically observed egg masses from a clutch, and serves as the number of egg masses compared during each iteration of the resampling procedure

Clutch	No. of egg masses	$\bar{d}_{obs}$	*P*
X1	3	28.2	0.009
X12	5	12.1	0.271
X5	3	11.0	0.237
X3	3	6.9	0.074
X8	6	6.2	0.005
X10	2	3.5	0.011
X14	3	< 1	< 0.001
X15	4	< 1	< 0.001
X16	2	< 1	< 0.001
X2	2	< 1	< 0.001
X6	2	< 1	< 0.001

aggregate breeders can be problematic for a variety of reasons. Specifically, the identification of paternal genotypes solely from full-sib progeny arrays is inferential, and thus parental genotype reconstruction from progeny array data is impossible when monogamy predominates (DeWoody *et al*. 2000a). In this study, we could reconstruct parental genotypes because nearly half (44%) of the progeny arrays were multiply sired and thus their paternal genotypes could be reconstructed confidently. Once known, many of these sires could also be assigned to the 'monogamous' (i.e. single male/single female) pairings and thus parental genotype inference was an iterative process (Fig. 2a). Our approach is conservative in that numerical estimates of sires and dams (i.e. 15 dams and 14 sires; Appendix) represents the minimum number of parents required to explain the progeny array data (see DeWoody *et al*. 2000b, c).

The confounding effects of egg mass admixture were resolved by adopting a phenetic approach of clustering genetically similar individuals and thus disentangling progeny of two or more distinct breeding efforts (Fig. 3). The computationally simple approach of deriving minimum genetic distances among individuals and then clustering individuals using the UPGMA algorithm was adequate for resolving admixture within this study. This approach has seldom been adopted for genetic parentage analyses (although see Valenzuela 2000), but can work well if a sufficient number of loci are typed, if they are sufficiently variable, and if the progeny arrays are of sufficient size. The results of the phenetic clustering analyses, in conjunction with our iterative approach of parental genotype reconstruction, allowed us to identify egg masses that contained embryos oviposited by two females. A subset of the data was re-analysed using Queller & Goodnight (1989) relatedness-based metric (*r*) in the same UPGMA framework, and in each case *r* corroborated the genetic distance results.

Multiple mating and courtship behaviours

Our data are the first genetic evidence that multiple mating within an aggregation of breeding *Ambystoma tigrinum tigrinum* results in clutches of mixed paternity. Clutches consisted of first-degree relatives (i.e. full-siblings) or second-degree relatives (half-siblings) related by shared maternity. Results also indicated both sexes engaged in multiple mating with evidence of both polygyny and polyandry within our data set. These findings are in accord with the behavioural observations of male and female promiscuity during *A. t. tigrinum* breeding aggregations (Arnold 1976; Howard *et al*. 1997). Genetic evidence for multiple mating now has been documented in various urodele lineages (Gabor *et al*. 2000; Jones *et al*. 2002; Garner & Schmidt 2003; Tennessen & Zamudio 2003; Adams *et al*. 2005) suggesting this behaviour may be widespread among salamanders.

For males, mating with multiple partners provides an obvious benefit by increasing their likelihood of siring offspring among a vast field of competitors — potentially increasing an individual's overall reproductive success. Reasons for polyandry are not as obvious, as females may, in theory, be assured of reproductive success by a single mating event (Halliday 1998). Direct benefits from multiple mating in the form of nuptial gifts from males are not available to females; on the contrary, courtship with multiple partners may be costly to the female in terms of fitness and energy allocation (Garner & Schmidt 2003). Similarly, sexual coercion by males to induce females to engage in unwanted mating (Lee & Hays 2004) is unlikely to occur in tiger salamanders as spermatophore induction is controlled by females. Female *A. t. tigrinum* frequently reject spermatophores following courtship (Howard *et al*. 1997), suggesting a strong active role for female choice in spermataphore induction. Multiple mating may provide indirect benefits to a female by fertilization assurance or by long-term benefits to the genetic diversity of her progeny, in which case females may actively mate with various partners (Halliday & Arnold 1987; Stockley *et al*. 1993; Reynolds 1996; Birkhead & Parker 1997).

On the other hand, multiply sired clutches may be a product not of female choice or male coercion, but of anatomy. Specialized sperm-storage organs called spermatheca are common among salamanders (Sever & Brizzi 1998; Sever 2002; Adams *et al*. 2005). Sperm storage has been documented in *A. t. tigrinum*, but the duration of storage

is contentious. Rose & Armentrout (1976) suggested sperm can be stored and used over seasons, but Sever (1995) argued that sperm is flushed out of the spermatheca if not used within 2 days. If the former is correct, sperm storage across seasons could account for multiply sired clutches. Controlled breeding experiments in association with paternity analyses are required to determine the duration of viable sperm storage in tiger salamanders.

The incidence of multiple paternity reported herein for tiger salamanders (~43% of clutches) contrasts with that reported for the spotted salamander, *Ambystoma maculatum* (> 70%; Myers & Zamudio 2004). This disparity may simply be due to sampling bias or may result from courtship/mating differences between the species. Both species employ male–female nudging to initiate contact and induce spermatophore deposition (Petranka 1998), but in contrast to *A. maculatum*, tiger salamanders engage in additional bouts of tactile courting prior to deposition that could allow greater opportunity for female assessment of a potential mate's fitness (Kumpf 1934; Houck & Arnold 2003). Thus, the more elaborate courtship behaviours in tiger salamanders could reduce the need for bet hedging. If true, we predict that full-sib clutches would be more common in salamanders with complex courting behaviours.

Variance in reproductive success

Variance in reproductive success among breeding individuals was observed both within and between the sexes. For example, some sires fathered more than 10 times as many offspring as other males; similar, albeit less extreme, patterns were apparent among females. The overall variance in female reproductive success was roughly half that for males, consistent with the expected effects of sexual selection acting on males. Myers & Zamudio (2004) proposed that high levels of male/male competition in aggregate breeding systems would manifest as pronounced reproductive skew among the paternal genetic contributions to multiply sired clutches. In our data set, significant reproductive skew was apparent in five of the eight multiply sired clutches where the most successful father sired > 50% of the embryos (Fig. 4). In addition, the correlation between male mating success and reproductive success was significant. Thus, male *A. t. tigrinum* increase their reproductive success by mating with numerous partners.

Insemination and fertilization are temporally decoupled in urodeles (Houck & Arnold 2003), so variation in reproductive success among males contributing to a clutch may be associated with the order, quality, and/or quantity of spermatophores inducted by the female. Alternatively, variation in male reproductive success may result from cryptic female choice among collected sperm (Eberhard 1996). Tennessen & Zamudio (2003) found evidence for sperm precedence in controlled breeding experiments of spotted salamanders, where the first male to mate with a female sired more offspring than subsequent males (i.e. first-male advantage). Similar results have been reported for rough-skinned newts (*Taricha granulosa*), where the relative proportion of eggs sired did not differ among successively deposited egg masses. To explain this pattern, Jones *et al*. (2002) proposed a simple model of 'topping off' for urodeles, whereby females engage multiple partners if the quantity of sperm inducted from the first sire is insufficient to fill her spermatheca; presumably this is followed by random admixture of sperm prior to fertilization. Under this model, female assessment of male quality during courtship and their complete control over sperm induction negates a need to invoke postinduction cryptic female sperm choice. The mating order among sires was unknown in the current study and thus we cannot comment directly on sperm precedence for this species. Nevertheless, our data from egg masses distributed among multiple sites along the transect indicate that within multiply deposited clutches (i.e. within each partitioned element of a female's clutch) each sire's contribution was proportionally equivalent among sites. For example, the clutch resulting from cross X5 was oviposited at three sites distributed over 18 linear meters. Paternity by the most successful sire (M7) varied little — from 58.3% to 63.3% — among these three sites. Similar patterns were observed for crosses X3, X10, and X14 (Table 1). These data are concordant with the contention of Jones *et al*. (2002) that sperm from multiple males are admixed within the spermatheca prior to fertilization.

Spatial oviposition patterns

Gravid *A. t. tigrinum* females are thought to oviposit within 24 h of spermatophore induction (R. Howard, personal communication), but spatial aspects of oviposition have not been studied, presumably because the molecular tools to do so have heretofore been unavailable. For example, a female might distribute fertilized egg masses in no apparent pattern (random deposition), deposit her egg masses near one another (clustering), or females may utilize communal oviposition sites (hot spots). Furthermore, we might expect competition among females for access to desirable oviposition sites.

Our molecular data clearly document that gravid females routinely partitioned clutches as discrete egg masses, with only 6 of the 17 clutches observed as a single egg mass (i.e. 11 of 17 clutches were partitioned among multiple egg masses). We documented four instances of admixture of genetically discrete clutches, whereby two unrelated mothers oviposited at the same site and their spawn coagulated into a single jellified egg mass. Many of the spatially clustered egg masses deposited by different females shared at least one sire (Table 1), suggesting a female tendency to oviposit

at or near sites of sperm induction. Roughly one-third (32%) of the egg masses derived from partitioned clutches were deposited within a meter of one another, and 55% were sampled within 5 m. Thus, most females partitioned their clutches over short geographic distances, and these distances often deviated from that expected under a random deposition model (Table 2). However, there was no evidence that individual females monopolized oviposition sites; if so, we would not expect to see admixture (see above) or egg masses derived from different females deposited immediately adjacent to one another. This lack of strong geographic structuring among oviposition sites is concordant with the general absence of postovipositional parental care among salamanders in lentic environments (Nussbaum 2003).

Thus, our data contradict the random deposition (null) hypothesis and support the idea that females cluster multiple egg masses from the same clutch. We find no empirical support for the hot spot hypothesis despite its theoretical appeal (e.g. predation dilution; see Alexander 1974).

Conclusions

Our reconstruction of breeding and oviposition in *Ambystoma tigrinum tigrinum* is one where females often induct spermatophores from two or more males and then partition their clutches among several egg masses that are most often found within 5 m of one another. Variance in reproductive success is greater in males than in females, and males increase their reproductive success as they mate with more females. We make these strong inferences about tiger salamander reproduction by virtue of the large progeny arrays produced by females, a powerful suite of microsatellites, and novel analytical approaches to parentage analyses — despite not having directly captured any of the parents.

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The authors and other members of the DeWoody laboratory use a variety of genetic markers to address questions pertinent to parentage, mate choice, and molecular evolution. Most of the time, organisms are sampled from populations in their natural settings, but in some instances controlled breeding experiments are conducted to test specific hypotheses.

Appendix

Inferred (un-sampled) maternal and paternal genotypes at four microsatellite loci (52.34, 60.9, Atex 65 and ATS5-7) for 15 female (♀) and 14 male (♂) parents. P_{ID}, probability of identity

	Maternal genotype at locus:						Paternal genotype at locus:				
♀	52.34	60.9	Atex 65	ATS5-7	P_{ID}	♂	52.34	60.9	Atex 65	ATS5-7	P_{ID}
F1	384/384	224/224	284/336	240/246	1.21×10^{-7}	M1	380/426	226/226	284/360	244/246	1.28×10^{-8}
F2	376/380	234/246	388/424	244/244	4.44×10^{-6}	M2	376/386	226/234	388/420	244/244	6.16×10^{-6}
F3	376/430	224/226	332/344	244/246	1.19×10^{-6}	M3	384/390	234/236	372/400	246/246	3.12×10^{-7}
F4	384/384	236/236	408/424	244/244	4.87×10^{-7}	M4	378/408	224/224	384/408	244/246	7.87×10^{-8}
F5	380/398	234/234	412/424	244/244	6.90×10^{-8}	M5	384/426	240/246	372/412	244/244	4.52×10^{-9}
F6	386/396	224/228	408/420	244/244	1.56×10^{-7}	M6	376/408	224/240	332/380	244/246	8.86×10^{-7}
F7	380/386	228/234	332/396	246/246	1.77×10^{-7}	M7	384/390	224/226	332/412	246/246	8.40×10^{-7}
F8	384/386	224/228	352/416	246/246	1.63×10^{-7}	M8	376/380	224/240	388/404	240/244	1.56×10^{-6}
F9	376/408	234/246	404/424	244/244	1.29×10^{-6}	M9	382/388	224/226	344/348	244/244	2.71×10^{-10}
F10	376/384	226/234	332/412	244/244	4.86×10^{-6}	M10	384/386	224/246	392/408	240/244	1.28×10^{-6}
F11	380/408	224/228	276/336	240/244	4.41×10^{-7}	M11	376/400	224/226	368/388	244/244	1.82×10^{-7}
F12	380/390	226/240	352/388	246/246	5.70×10^{-7}	M12	384/396	228/234	352/388	244/246	9.52×10^{-7}
F13	408/430	224/226	352/420	240/246	9.33×10^{-7}	M13	380/384	234/236	344/400	246/246	3.12×10^{-7}
F14	390/390	224/236	332/332	240/240	3.97×10^{-8}	M14	386/402	224/224	388/400	246/246	6.50×10^{-5}
F15	384/394	224/224	376/416	244/244	1.65×10^{-7}						