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Cover Illustration

A spotted salamander (*Ambystoma maculatum*). In pages 1564–1576, Chandler & Zamudio show that male reproductive success in spotted salamanders is positively correlated with both body size and relatedness, and that selection acting on relatedness is facilitated by sperm storage, causing detectable effects on the genetic composition of offspring. Photo credits: H.W. Greene.

Close
Reproductive success by large, closely related males facilitated by sperm storage in an aggregate breeding amphibian

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Abstract

The outcome of sexual selection on males may depend on female mate choice and male–male competition as well as postcopulatory processes such as cryptic female choice and sperm competition. We studied the outcome of sexual selection in the spotted salamander (Ambystoma maculatum), specifically examining the role of body size and relatedness on male reproductive success. Using controlled mating experiments in the field, we gave females access to three males of different sizes. We used seven microsatellite loci to determine paternity in the resulting larvae, estimate relatedness (r) between females and their mates, and calculate $md^2$ (a measure of within-individual genomic divergence), heterozygosity, and standardized heterozygosity in the larvae. Both body size and relatedness to the female were significant predictors of male reproductive success. The relatedness of the males available to a female did not influence the amount of stored sperm she used to sire her larvae. Nonetheless, computer simulations showed that the average $md^2$, heterozygosity, and standardized heterozygosity of the offspring were lower than expected by random mating. These differences are due to the use of stored sperm to fertilize some eggs; $md^2$, heterozygosity, and standardized heterozygosity of larvae sired by stored sperm were significantly lower than those of larvae sired by the experimental males. These results suggest that relatedness may further influence a male's long-term reproductive success by determining whether his sperm is stored for later breeding seasons. Sexual selection in this salamander likely involves a complex interaction among many factors and may act over many seasons.

Keywords: explosive breeder, mating system, microsatellites, paternity, salamander

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Introduction

Competition for mates can intensify sexual selection and lead to dramatic and relatively rapid diversification (Masta & Maddison 2002). Sexual selection can act on traits via male–male competition and direct or cryptic female mate choice. These selective modes may act on different traits (Howard et al. 1997) or may even oppose one another (Moore & Moore 1999; Bonduriansky & Rowe 2003). For example, the victor in male–male competition may not be the optimal mate for a female, and in that case, female choice may limit the selective strength of this competition.

One trait commonly implicated in sexual selection is male body size. In many species, selection favours large males because they are more successful competitors (Howard et al. 1997). Likewise, females may prefer large males because they offer better resources (Houck 1988; Mathis 1991) or because body size may be an indicator of fitness or genetic quality (Cooper & Vitt 1993; Miyazaki & Waas 2003). However, genetic compatibility between females and their potential mates, not simply overall genetic quality, may be another important factor in female mate choice (Garner & Schmidt 2003).

In the spotted salamander (Ambystoma maculatum), both male–male competition and female choice are likely to
influence sexual selection because this species is a polyandrous aggregate breeder with a highly male-biased sex ratio (Husting 1965; Arnold 1976). Competition among males may favour large individuals if they deposit more spermatophores, increasing their chances of inseminating a female. They may also court more efficiently or vigorously, or engage more frequently in sexual interference (Arnold 1976). Previous characterizations of the mating system of this species have shown that males that arrive first at breeding aggregations sire more offspring than late-arriving males, multiple paternity is common in natural breeding aggregations, and the use of stored sperm by females contributes to the genetic diversity of offspring (Tennesen & Zamudio 2003; Myers & Zamudio 2004); however, selection on body size has never been tested in this species. Although spotted salamanders do not exhibit territorial defence during the short periods they are at breeding ponds, they do defend burrows from intraspecific competitors during most of the year (Ducey & Ritsema 1988). Therefore, male–male competition outside the breeding season may also favour large males indirectly by allowing them to monopolize the best territories and thus arrive at breeding aggregations before smaller males (Tennesen & Zamudio 2003).

Polyandrous mating systems such as the spotted salamander’s may be favoured when female mate or gamete choice is dependent on genetic compatibility (Tregenza & Wedell 2000). In that case, we would predict that genetic relatedness or similarity might play a role in female mate choice in spotted salamanders. Females may choose mates based on their genetic relatedness or compatibility to minimize the negative fitness effects of inbreeding (Pusey & Wolf 1996; Tregenza & Wedell 2000; Garner & Schmidt 2003). Outbreeding may significantly enhance the fitness of offspring, particularly in populations that are highly inbred (Madsen et al. 1999). On the other hand, females may also exhibit outbreeding avoidance (Harmsen & McKay 1985), to avoid reduced fitness that can result from genetic incompatibility and disrupting co-adapted gene complexes (Tregenza & Wedell 2000).

Female mate choice based on genetic compatibility may act directly, through chemical signals delivered during courtship (Houck & Arnold 2003), or it may be cryptic, relying on postcopulatory mechanisms (Birkhead & Pizzari 2002). Many species of salamanders have specialized spermathecae (Sever 2003) and may fertilize their eggs with stored sperm in naturally breeding populations, creating potential for long-term sperm competition or cryptic female choice (Houck & Schwenk 1984; Tennesen & Zamudio 2003). Therefore, if none of the males available for breeding are high-quality or compatible mates, they may choose to fertilize most or all of their eggs with sperm from previous matings. Here, we use controlled field breeding experiments and microsatellite paternity analyses to examine the outcome of sexual selection in the spotted salamander. Specifically, we address three questions: (i) is body size an important determinant of male reproductive success in this species? (ii) does relatedness play a role in reproductive success either through inbreeding or outbreeding avoidance and, if so, how does this process impact the genetic composition of the offspring?, and (iii) do females adjust their use of stored sperm according to mate availability? Combined, quantifying these determinants of reproductive success will contribute to our understanding of the costs and benefits of this aggregate mating system.

Materials and methods

Study species

The spotted salamander (*Ambystoma maculatum*) is an explosive breeder; individuals leave their terrestrial home ranges every spring and migrate to large breeding aggregations in vernal pools (Husting 1965). Competition for mates is intense because of highly male-skewed operational sex ratios (Hillis 1977). Males do not defend territories or resources in breeding aggregations, but instead form large aggregates or courting groups, resulting in a ‘polyandrous frenzy’ (Arnold 1976). Males deposit 20–40 spermatophores a night on the substrate and sometimes cover other males’ spermatophores with their own, in a form of sexual interference (Arnold 1976). However, unlike their congener the tiger salamander (*Ambystoma tigrinum*), spotted salamanders do not exhibit direct agonistic male–male competition; when multiple males court a single female they nudge each other and interact with the female but do not attempt to monopolize her (Arnold 1976).

As we would expect, multiple mating by females is common in this system. Females pick up 15–20 spermatophores in a night (Arnold 1976; Petranka 1998). Multiple paternity is also common, with approximately 70% of clutches being sired by two to eight males (Myers & Zamudio 2004). After mating, females store sperm and fertilize eggs internally, and lay their clutches within a few days (Savage & Zamudio 2005).

Field experiments and tissue collection

We conducted field experiments to test the relative effects of body size and relatedness on male reproductive success. On the nights of March 29, March 31, and April 9, 2002, we set up mating chambers modelled after those of Tennesen & Zamudio (2003) at Ringwood Pond, Tompkins County, New York, a vernal pool with a breeding population of several hundred spotted salamanders. The focal pond was
completely surrounded by a drift fence and 25 evenly spaced pitfall traps that allowed us to capture animals as they entered the pond to breed each season. In each chamber, we simultaneously enclosed one female and three males of different sizes, all captured entering the pond on the night of the experiment. We chose males according to the size distribution of males in this population: medium males were approximately 15 g in weight, the mean weight; large males were 18 g or larger (mean + 1 SD) and small males were smaller than 12 g (mean – 1 SD). We lined the bottom of each chamber with leaf litter and pond debris and partially submerged the chambers in the pond, allowing salamanders to mate normally overnight. The following morning, we removed all animals from the chambers and removed two to four toe clips, which were preserved in 100% ethanol. We immediately released the males at their location of capture and returned females to the laboratory, where they were kept until oviposition. We housed females in plastic shoeboxes with dechlorinated, deionized water in a climate-controlled room with a photoperiod of 12:12 h and temperature range of 12–19 °C. We placed all harvested clutches in breeding nets submerged in aquaria containing dechlorinated, deionized water with air pumps for oxygenation. Just before hatching, larvae were sacrificed and preserved in 100% ethanol for subsequent genotyping.

**Laboratory protocols**

We digested adult toe clips and whole larvae overnight in 450 μL lysis buffer (2.5 mM Tris, 8 mM NaCl, 0.1% SDS, 0.2 mM EDTA, 0.01% β-mercapto-ethanol) with proteinase K (0.20 mg for larvae and 0.38 mg for toe clips) and then treated the extract with RNase A (0.01 mg) for 1 h before purification. We purified genomic DNA using standard phenol–chloroform cleanup and ethanol precipitation (Sambrook & Russell 2001). Genomic extracts were diluted to 100 ng/μL and used as templates for the amplification of seven microsatellite loci (Julian et al. 2003). Polymerase chain reaction (PCR) products were diluted and electrophoresed on a 5% polyacrylamide gel on an ABI PRISM 377 DNA sequencer (Applied Biosystems). Genotypes were sized with 500 ROX size standard using GENESCAN version 3.1 and GENOTyper version 2.5 (Applied Biosystems).

**Paternity and relatedness analyses**

We used CERVUS version 2.0 (Marshall et al. 1998; Slate et al. 2000) to estimate allele frequencies and heterozygosities and to confirm that all loci were in Hardy–Weinberg equilibrium.

Paternity was determined using two exclusion methods, a strict one allowing no mismatches between offspring and assigned fathers, and a relaxed method that allowed one mismatch before a potential father was excluded, to account for possible null alleles and genotyping error. Exclusion paternity assignment was carried out using a script written in R version 2.4.1 (R Development Core Team 2004) (script available from C.H.C. upon request). For paternity analyses, we used only loci for which both the mother and larva were genotyped. For each locus, the maternal and paternal alleles were identified. Potential fathers were then checked for the presence of the paternal allele, if it could be identified. If the paternal allele could not be identified because larval and maternal genotypes were identical heterozygotes, the presence of either larval allele in a potential father was considered a match. If a potential father was not scored at a particular locus, he was not excluded as a potential sire because of incomplete genotyping. If all potential fathers were excluded after checking all loci, we concluded that the larva was fathered by stored sperm. If paternity could not be determined because multiple potential fathers had genotypes consistent with the larval genotype at all loci, that larva was excluded from subsequent analyses requiring known paternity. In some cases, the strict exclusion method attributed a larva to stored sperm because of genotyping error or mutation, while the relaxed exclusion method assigned paternity to one of the experimental males. In other cases, the strict exclusion method assigned paternity to one of the experimental males, but the relaxed exclusion method resulted in ambiguous paternity because multiple experimental males were consistent with the larva’s genotype when one mismatch was allowed. However, when both methods yielded an answer, the results were always consistent.

Relatedness (r) among adults was estimated using the program KINSHIP version 1.3.1 (Queller & Goodnight 1989). KINSHIP estimates values of r using codominant markers and takes into account population allele frequencies. Estimates of r range from –1.0 to 1.0, with a value of –1.0 being the most unrelated and a value of 1.0 meaning identical genotypes.

To examine the effects of mate choice on the genomic composition of the offspring, we calculated three within-individual measures of genomic diversity for all larvae: mean \( d^2 \) (Neff 2004), heterozygosity, and standardized heterozygosity. Mean \( d^2 \) assumes a stepwise mutation model and is a better measure of high levels of genomic divergence than heterozygosity, even in the presence of some multistep mutations (Neff 2004). For each individual, we calculated \( md^2 \) as the squared difference between the individual’s two alleles (measured in repeat units) averaged over all loci for which the individual was genotyped (Coulson et al. 1998). Heterozygosity was calculated as the proportion of scored loci for which an individual was heterozygous. Standardized heterozygosity was calculated as the individual’s heterozygosity, divided by the average of the population mean heterozygosities of
The scored loci; thus, standardized heterozygosity downweights loci that are highly polymorphic (Amos et al. 2001). We calculated \( m d^2 \), heterozygosity, and standardized heterozygosity for each larva, and then computed the average values over all larvae for comparison with null distributions generated by simulation. If the mother or any of the potential fathers of a given larva were not genotyped at a particular locus, that locus was omitted from the calculation of diversity values for that larva, to avoid biasing comparisons with simulated values for each clutch (described in detail below). We also calculated the average value of each diversity measure of all larvae sired by experimental males and those sired by stored sperm, and compared the two using a randomization test (described in detail below).

**Statistical analyses**

Because the reproductive success of a male in a mating chamber is not independent of the other two, we could not test directly for effects of body size and relatedness — using standard analyses treating each male as an independent data point. Therefore, we evaluated a hierarchical series of models of male reproductive success using likelihood-ratio tests. We modelled the number of larvae sired by each male as a multinomial process and ignored relatedness into our model, we defined a new function to predict a male’s success in terms of relatedness, derived from the beta density function:

\[
g(r) = \frac{\Gamma(\alpha + \beta r) r^{(\alpha - 1)} (1 - r)^{\beta - 1}}{\Gamma(\alpha) \Gamma(\beta)}
\]

where \( \Gamma(\cdot) \) is the gamma function; \( r \) is relatedness, linearly re-scaled to vary from 0 to 1 instead of –1 to 1 (as required by the beta density function); and \( \alpha \) and \( \beta \) are shape parameters. This function was chosen because it can take on a variety of shapes, depending on the values of \( \alpha \) and \( \beta \). Then, because the multinomial model requires probabilities that sum to 1, we calculated a male’s probability of siring a given larva in terms of his \( g \)-value as well as the \( g \)-values of the other two males in the chamber from the following equation:

\[
p_{ui} = \frac{S_{ui}}{S_{ui} + S_{u2} + S_{u3}}.
\]

This probability function is appropriate because if relatedness is a factor in female mate choice, it is likely to behave in relative terms, depending on the potential mates available to a female, not on an absolute scale. The log-likelihood for the ‘relatedness only’ model is thus:

\[
L = \sum_{i=1}^{m} \log \left( \frac{(x_i + y_i + z_i)!}{x_i!y_i!z_i!} \frac{1}{3^3} \frac{1}{3^3} \frac{1}{3^3} \right)
\]

where \( m \) is the number of clutches; and \( x_i, y_i, z_i \) are the numbers of larvae sired by the large, medium, and small males, respectively, in chamber \( i \). This is a valid null model because although reproductive skew is well-documented in this species and was indeed observed in our experiment, if skew is random with respect to size, the net effect across all clutches will be equal probability of success for the large, medium, and small males.

In the ‘size only’ model, we allowed a male’s probability of siring a given larva to vary with size. The log-likelihood for this model is given by the formula:

\[
L = \sum_{i=1}^{m} \log \left( \frac{(x_i + y_i + z_i)!}{x_i!y_i!z_i!} p_1^{x_i} p_2^{y_i} (1 - p_1 - p_2)^{z_i} \right)
\]

where \( p_1 \) is the probability of a large male siring a given larva and \( p_2 \) is the probability of a medium male siring a given larva. The null model is a special case of the ‘size only’ model in which the parameters \( p_1 \) and \( p_2 \) are constrained to equal 1/3.

In the ‘relatedness only’ model, we assumed that relatedness alone determines male reproductive success. To incorporate relatedness into our model, we defined a new function to predict a male’s success in terms of relatedness, derived from the beta density function:

\[
g(r) = \frac{\Gamma(\alpha + \beta r) r^{(\alpha - 1)} (1 - r)^{\beta - 1}}{\Gamma(\alpha) \Gamma(\beta)}
\]

where \( \Gamma(\cdot) \) is the gamma function; \( r \) is relatedness, linearly re-scaled to vary from 0 to 1 instead of –1 to 1 (as required by the beta density function); and \( \alpha \) and \( \beta \) are shape parameters. This function was chosen because it can take on a variety of shapes, depending on the values of \( \alpha \) and \( \beta \). Then, because the multinomial model requires probabilities that sum to 1, we calculated a male’s probability of siring a given larva in terms of his \( g \)-value as well as the \( g \)-values of the other two males in the chamber from the following equation:

\[
p_{ui} = \frac{S_{ui}}{S_{ui} + S_{u2} + S_{u3}}.
\]

This probability function is appropriate because if relatedness is a factor in female mate choice, it is likely to behave in relative terms, depending on the potential mates available to a female, not on an absolute scale. The log-likelihood for the ‘relatedness only’ model is thus:

\[
L = \sum_{i=1}^{m} \log \left( \frac{(x_i + y_i + z_i)!}{x_i!y_i!z_i!} p_1^{x_i} p_2^{y_i} (1 - p_1 - p_2)^{z_i} \right)
\]

where \( p_1 \) and \( p_2 \) are the fertilization probabilities for males 1 and 2, respectively, in clutch \( i \), calculated from the equations described above. It contains two parameters, \( \alpha \) and \( \beta \), used to calculate \( p_1 \) and \( p_2 \) from the relatedness values of the three males in chamber \( i \). The null model is a special case of this model in which \( \alpha \) and \( \beta \) are constrained to equal 1.

Finally, the ‘size+relatedness’ model considered the possibility that both size and relatedness influence a male’s reproductive success. This model was similar to the ‘relatedness only’ model but allowed large, medium, and small males to have different \( g \)-functions, each with different \( g \)-values for the parameters \( \alpha \) and \( \beta \), and an additional parameter \( \gamma \):

\[
\begin{align*}
S_1(r) &= \gamma_1 \frac{\Gamma(\alpha_i + \beta_i)}{\Gamma(\alpha_i) \Gamma(\beta_i)} r^{(\alpha_i - 1)} (1 - r)^{\beta_i - 1}, \\
S_2(r) &= \gamma_2 \frac{\Gamma(\alpha_i + \beta_i)}{\Gamma(\alpha_i) \Gamma(\beta_i)} r^{(\alpha_i - 1)} (1 - r)^{\beta_i - 1}, \\
S_3(r) &= (1 - \gamma_1 - \gamma_2) \frac{\Gamma(\alpha_i + \beta_i)}{\Gamma(\alpha_i) \Gamma(\beta_i)} r^{(\alpha_i - 1)} (1 - r)^{\beta_i - 1},
\end{align*}
\]
The fertilization probabilities \( (p_1, p_2) \) were calculated from the \( g \)-values in the same way as in the ‘relatedness only’ model, and the log-likelihood is given by the same formula. This model, then, has eight parameters \((\alpha_1, \beta_1, \gamma_1, \alpha_2, \beta_2, \gamma_2, \alpha_3, \beta_3)\). The null model is a special case of the ‘size+relatedness’ model in which \( \gamma_1 = \gamma_2 = 1/3 \) and \( \alpha_1 = \alpha_2 = \alpha_3 = 1 \) and \( \beta_1 = \beta_2 = \beta_3 = 1 \). The ‘size only’ model is also a special case of the ‘size + relatedness’ model with just parameters \( \gamma_2 \) because the other parameters are constrained such that \( \alpha_1 = \alpha_2 = \alpha_3 = 1 \) and \( \beta_1 = \beta_2 = \beta_3 = 1 \); while the ‘relatedness only’ model is another special case with \( \gamma_1 = \gamma_2 = 1/3 \), and \( \alpha_1 = \alpha_2 = \alpha_3 \) and \( \beta_1 = \beta_2 = \beta_3 \).

For each model, parameter values were optimized to maximize likelihood, and log-likelihood scores were computed from the optimized parameter estimates. Likelihood-ratio tests were then used to perform pairwise comparisons among models to determine which best explained the data. All calculations were performed in \textsf{R} \texttrademark\ version 2.4.1 (R Development Core Team 2004) (scripts available upon request). Tests were performed twice, once with each paternity exclusion method.

To assess qualitatively how body size affects male reproductive success, we constructed box plots of the proportion of larvae sired by large, medium, and small males, and examined parameter values. To examine how relatedness to females affects male reproductive success, we used our estimates of the parameters from the ‘relatedness only’ and ‘size+relatedness’ models to plot the \( g \)-functions for the both models.

We also tested for effects of relatedness between females and potential mates on the proportion of larvae sired by stored sperm, using simple linear regressions in \textsf{JMP} version 6.0.0 (SAS Institute). We used the proportion of larvae sired by stored sperm, estimated using the strict and relaxed exclusion methods, as the response variables, and the mean, maximum, and minimum \( r \) values as predictors, resulting in six tests.

To examine whether mate choice and sexual selection have detectable effects on the genetic composition of offspring, we used a series of simulations written in \textsf{R} \texttrademark\ version 2.4.1 (R Development Core Team 2004) (scripts available upon request) assuming random mating to generate null distributions for average \( md^2 \), heterozygosity, and standardized heterozygosity values. In the simulations, for each larva genotyped in our experiment, we generated a simulated counterpart by using the same mother, selecting a father, and randomly choosing one allele from each parent at each locus. If an experimental larva, its mother, or any of the potential fathers in that clutch were not genotyped at a particular locus, that locus was also omitted from the simulated larva. The simulated population-wide average \( md^2 \), heterozygosity, and standardized heterozygosity were then calculated, and the simulation was repeated 1000 times to generate null distributions for these values. The observed value for each of these parameters was compared to the null distributions to obtain a \( P \) value for the observed values; the null distributions were also used to generate 95% confidence intervals for expected values for each of the parameters.

In simulation #1, a simulated counterpart was generated for all genotyped larvae in each clutch, and fathers were randomly chosen from among the three males in the mating chamber. Simulation 1 therefore reveals whether the observed average \( md^2 \), heterozygosity, and standardized heterozygosity values are within the ranges expected by random mating within experimental chambers.

In simulation #2, a simulated counterpart was generated for all genotyped larvae not sired by stored sperm, and fathers were selected randomly from the three males in the mating chamber. This simulation was the same as simulation #1, but larvae sired by stored sperm were excluded. The null distributions were then compared to the average \( md^2 \), heterozygosity, and standardized heterozygosity of all larvae not sired by stored sperm. Thus, if observed and expected values of \( md^2 \) or heterozygosity are significantly different in simulation #1, simulation #2 reveals whether that difference is due to the genetic make up of larvae sired by stored sperm.

In simulation #3, only larvae with assigned paternity were included. In this simulation, fathers were randomly chosen, but we maintained overall differences in paternity success among the three experimental males in the simulations. Therefore, simulation #3 differs from #1 and #2 because it includes reproductive skew, rather than assuming equal mean reproductive success of males in each chamber. To incorporate reproductive skew, we chose fathers for simulated larvae based on the fathers of their experimentally observed counterparts, but we randomized the identities of the fathers within each chamber. For example, in one replicate, all observed larvae from a given clutch found to be sired by the large male in the chamber might have simulated counterparts fathered by the small male in the chamber, and vice versa. Simulation #3 was designed to determine whether any differences between observed and expected values in simulation #2 are due to differential reproductive success among competing males.

Simulation #4 was identical to simulation 3, except the identities of the fathers were not randomized: a simulated counterpart was generated for each observed larva using the same mother and father. Therefore, this simulation is a negative control. If a significant difference between the expected and observed values is detected in this simulation, it must be due to some factor other than stored sperm or reproductive skew, such as genotyping errors or preferential transmission of particular alleles. Simulations #2 through #4 were each run twice: once using paternity assignments from the strict exclusion method and once using the relaxed exclusion method.

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Finally, we compared the genomic diversity of larvae sired by stored sperm and experimental males. We compared average $md^2$, heterozygosity, and standardized heterozygosity of larvae sired by experimental males in all clutches with the average values of larvae sired by stored sperm using randomization tests with 1000 replicates, implemented with a script in R version 2.4.1 (R Development Core Team 2004). A $t$-test was not used because the number of larvae sired by stored sperm was smaller than the number of larvae sired by experimental males, and because the values were not normally distributed (data not shown).

In each replicate, we calculated the mean for each diversity measure of the experimentally sired larvae, the mean of the larvae sired by stored sperm, and used the difference between the means (experimental — stored) as the test statistic. Each set of randomization tests was carried out twice, once using the strict exclusion method to assess stored sperm use and once using relaxed exclusion, and we performed two sets of randomization tests, the first randomized within clutches, and the second randomized globally (across all clutches).

Results

Larvae and genotypes

We genotyped a total of 676 larvae from 14 different clutches at seven microsatellite loci. All loci were highly polymorphic, even within clutches, and contained on average 7.9 alleles (range 4–12) across the entire sample (Table 1). All loci were in Hardy–Weinberg equilibrium. The total exclusionary power of the seven loci combined was 0.997. In 183 cases (out of 4424 larval genotypes), neither larval allele matched the maternal genotype, either because of mutation, genotyping error, or the presence of a null allele. The mean observed maternal mismatch rate across all seven loci was 4.1%. Assuming genotyping errors are independent, this error rate suggests approximately 25% of all larvae genotyped at all seven loci may have at least one mistyped locus and thus may have incorrectly been assigned paternity by strict exclusion. However, only 3.1% of all larvae (21 out of 680) would be likely to have two or more mistyped loci; thus the relaxed exclusion method, which allows one mismatch, can correctly assign paternity to the vast majority of larvae, with only a slight decrease in exclusion power in most cases (Table 2). All clutches contained at least 35 genotyped larvae (Table 2).

Average pairwise male–female relatedness ($r$) within each experimental chamber was 0.019 ± 0.241 (SD) (range −0.400 to 0.678) (Fig. 1), suggesting that males and females randomly selected for the mating experiments are on average, unrelated.

Body size, relatedness, male success, and sperm storage

All experimental males but one were successful in fertilizing at least a few eggs (Table 2).

Our likelihood-ratio tests comparing paternity models indicate that both body size and relatedness play a role in influencing male reproductive success (Table 3). Regardless of paternity assignment method, the ‘size only’ model and ‘relatedness only’ models both significantly outperformed the null model of equal reproductive success, and the ‘size + relatedness’ model significantly outperformed all three in explaining the pattern of paternity.
Body size is positively correlated with paternity; thus large males, on average, sire more offspring than smaller ones (Fig. 2). The parameter estimates for the ‘size only’ model support this observation \((p_1 = 0.413, p_2 = 0.340\) for strict exclusion, \(p_1 = 0.394, p_2 = 0.349\) for relaxed exclusion, indicating that larger males have a higher probability of siring a particular larva).

Plotting the \(g\)-functions using the parameter estimates indicates that male reproductive success generally increases with increasing relatedness to female mates over the range of \(r\)-values found in our population (Fig. 3). In the ‘size+relatedness’ models, however, the success of small males follows the opposite pattern, decreasing with increasing relatedness. The results from the ‘size+relatedness’

<table>
<thead>
<tr>
<th>Clutch</th>
<th>Female weight (g)</th>
<th>N</th>
<th>Male</th>
<th>(r)</th>
<th>Larvae sired by each male</th>
<th>Larvae sired by stored sperm</th>
<th>Unassigned larvae</th>
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<td>Med</td>
<td>0.144</td>
<td>25</td>
<td>24</td>
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<td>Med</td>
<td>0.195</td>
<td>3</td>
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<td>Lg</td>
<td>-0.305</td>
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<td>17</td>
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<td>-0.400</td>
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<td>Lg</td>
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<td>7</td>
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<td>I</td>
<td>19.8</td>
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<td>Lg</td>
<td>-0.222</td>
<td>30</td>
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<td></td>
<td></td>
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<td>9</td>
<td></td>
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<tr>
<td>J</td>
<td>19.7</td>
<td>48</td>
<td>Lg</td>
<td>0.123</td>
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<td>10</td>
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<td>21.5</td>
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<td>-0.131</td>
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<td>1</td>
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<td>3</td>
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<tr>
<td>M</td>
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<td>36</td>
<td>Lg</td>
<td>-0.075</td>
<td>12</td>
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<td></td>
<td></td>
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<td>Med</td>
<td>0.678</td>
<td>12</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>22.6</td>
<td>48</td>
<td>Lg</td>
<td>0.147</td>
<td>31</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Med</td>
<td>-0.081</td>
<td>2</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Female weight, number of larvae genotyped in each clutch (N), relatedness (\(r\)) between females and males in each experimental chamber, and the number of larvae sired by each male, by stored sperm, and with unassigned paternity using both strict and relaxed exclusion methods.
model also support the finding that larger males have more offspring, as the \( g \)-function for large males is consistently higher than that of medium and small males over the range of \( r \)-values in our population.

Finally, genetic relatedness, measured by \( r \), was not directly correlated with stored sperm use in any case (Fig. 4).

**Genetic composition of offspring**

Simulation #1 indicated that the observed average \( md^2 \), heterozygosity, and standardized heterozygosity of offspring were significantly lower than the values expected by random mating within chambers (Table 4). Simulation #2 showed no difference between observed and expected mean heterozygosity and standardized heterozygosity values, but did show a difference for \( md^2 \). Simulation #3 also showed no difference between observed and expected heterozygosity and standardized heterozygosity, or for \( md^2 \) using strict exclusion, but the observed \( md^2 \) value was significantly lower than expected by chance when the relaxed exclusion method was used. These results indicate that the difference between the observed and expected heterozygosity and standardized heterozygosity values seen in simulation #1 is due to the inclusion of larvae sired by stored sperm, but that reproductive skew may be at least partially responsible for the difference between the observed and expected values of \( md^2 \). Simulation #4, a negative control, showed no differences between observed and expected values for all three diversity measures, as predicted.

Average \( md^2 \), heterozygosity, and standardized heterozygosity of larvae sired by stored sperm were significantly lower than the average values of larvae sired by experimental males, regardless of exclusion method or whether randomization tests were global or within clutches (Table 5).

**Discussion**

**Male body size**

Our results indicate that body size does play a role in sexual selection in spotted salamanders: larger males, on average, sire more offspring than smaller males (Table 3, Fig. 2). Our experimental design did not allow us to determine the mechanism producing this pattern,
but there are a few possible explanations. First, larger males may have a direct competitive advantage over smaller ones, monopolizing access to females, as in the tiger salamander (*Ambystoma tigrinum*) (Howard et al. 1997). Alternatively, larger males may deposit more spermatophores than smaller males, as in the small-mouthed salamander (*Ambystoma texanum*) (Harris & Lucas 2002), increasing the odds that a female will pick up one of their spermatophores. Indirect sexual interference in this species also occurs in the form of spermatophore capping, in which one male covers another male’s spermatophores with his own (Arnold 1976); a final explanation for the size advantage we have observed is that larger males, with a larger supply of energy reserves and sperm, may be more likely to cap their competitors’ spermatophores.

These data should be interpreted with caution, however, because the variance in the proportion of offspring sired by each male within each clutch is still relatively large (Fig. 2). Although larger males sire more offspring on average, in at least six of our 14 clutches, for example, the largest male sired fewer offspring than one or both of his smaller competitors (Table 2). Clearly, size is not the only, or even the most important, determinant of male reproductive success. In fact, other factors, such as a male’s arrival time at the breeding aggregation (Tennessee & Zamudio 2003) and relatedness (see below), are also important for male fitness.

**Relatedness, stored sperm, and offspring genomic divergence**

The degree of relatedness between males and females was also a significant predictor of the number of offspring sired by males (Table 3, Fig. 3). In most cases, males generally have greatest success when they are either very closely related to or more divergent from their mate (Fig. 3), producing a surprising pattern of disruptive selection. However, the $r$-values in our population span only a portion of this range. When restricting our findings to the range of $r$-values actually observed in our population, most of the left portion of the male success curves (Fig. 3) is truncated, leaving a pattern of directional selection in which males that are more closely related to females have the highest reproductive success. The one exception in which male success decreases with increasing relatedness (small males when using the relaxed exclusion method) suggests an interaction between body size and relatedness, underscoring the context-dependence of female choice (Fig. 3). In this instance, while males generally have higher success when they are not too divergent from females, very small males may be undesirable regardless of relatedness. However, further data are necessary to assess the biological significance of this pattern, since our analyses did not yield confidence intervals for the shape parameters used to generate the plots of our $g$-functions; it is conceivable that the interaction may simply result from a limited number of small males with large $r$-values.

Our analyses also revealed that the effect of relatedness on male reproductive success impacts the genetic composition of the next generation. Simulation #1 showed that the average genomic divergence ($\bar{md}^2$), heterozygosity, and standardized heterozygosity of the offspring were significantly lower than predicted by chance (Table 4). This difference is due to the use of stored sperm to fertilize a proportion of the eggs in each clutch. Two lines of evidence support this inference. First, there was a significant difference between the observed and expected average $\bar{md}^2$, heterozygosity, and standardized
heterozygosity values when larvae sired by stored sperm were included (simulation #1), but these differences disappeared for heterozygosity and standardized heterozygosity when larvae sired by stored sperm were excluded (simulation #2), suggesting that the larvae sired by stored sperm are at least partially responsible for the difference. Reproductive skew may have also played some role in causing this deviation, because $P$ values approached significance in simulation 2 when the relaxed exclusion method was used, but were not near significance when random reproductive skew was incorporated (simulation #3).

The second line of evidence that stored sperm bias the genetic composition of offspring is that average $md^2$, heterozygosity, and standardized heterozygosity of offspring to null expectations under a variety of mating scenarios: random mating (simulation #1), random mating with simulated larvae sired by stored sperm excluded (simulation #2), reproductive skew included (simulation #3), and a negative control with larvae sired by the same parents as the observed larvae (simulation #4). Values in the table indicate the mean observations for each measure of larval genetic diversity, 95% confidence interval, and two-tailed $P$ values. Asterisks represent values significantly different ($P < 0.05$) from the simulated null distribution. All three measures of diversity were significantly lower than predicted by simulation #1, and $md^2$ was lower than predicted by simulation #2, suggesting that the use of stored sperm and biases the genetic composition of the offspring.

<table>
<thead>
<tr>
<th>Simulation number</th>
<th>Null hypothesis</th>
<th>Paternity exclusion method</th>
<th>$md^2$</th>
<th>Heterozygosity</th>
<th>Standardized heterozygosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Random mating</td>
<td>N/A</td>
<td>7.56**</td>
<td>0.738*</td>
<td>0.972*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(7.96–8.68, $P = 0.001$)</td>
<td>(0.743–0.771, $P = 0.011$)</td>
<td>(0.978–1.01, $P = 0.011$)</td>
</tr>
<tr>
<td>2</td>
<td>Random mating excluding larvae sired by stored sperm</td>
<td>Strict</td>
<td>8.11*</td>
<td>0.748</td>
<td>0.985</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(8.16–8.98, $P = 0.031$)</td>
<td>(0.738–0.771, $P = 0.516$)</td>
<td>(0.971–1.01, $P = 0.539$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Relaxed</td>
<td>7.74**</td>
<td>0.743</td>
<td>0.978</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(8.01–8.76, $P = 0.003$)</td>
<td>(0.741–0.767, $P = 0.077$)</td>
<td>(0.976–1.01, $P = 0.085$)</td>
</tr>
<tr>
<td>3</td>
<td>Random mating with reproductive skew</td>
<td>Strict</td>
<td>8.08</td>
<td>0.747</td>
<td>0.983</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(7.99–9.15, $P = 0.112$)</td>
<td>(0.728–0.778, $P = 0.643$)</td>
<td>(0.958–1.02, $P = 0.663$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Relaxed</td>
<td>7.77*</td>
<td>0.752</td>
<td>0.990</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(7.81–9.03, $P = 0.035$)</td>
<td>(0.735–0.785, $P = 0.641$)</td>
<td>(0.966–1.03, $P = 0.655$)</td>
</tr>
<tr>
<td>4</td>
<td>Negative control</td>
<td>Strict</td>
<td>8.08</td>
<td>0.747</td>
<td>0.983</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.762–8.29, $P = 0.419$)</td>
<td>(0.735–0.760, $P = 0.942$)</td>
<td>(0.967–1.00, $P = 0.948$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Relaxed</td>
<td>7.77</td>
<td>0.752</td>
<td>0.990</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(7.47–8.14, $P = 0.868$)</td>
<td>(0.749–0.773, $P = 0.134$)</td>
<td>(0.986–1.02, $P = 0.148$)</td>
</tr>
</tbody>
</table>

Fig. 4 The proportion of larvae in a clutch sired by stored sperm is not correlated with the relatedness between a female and her available mates. Regardless of paternity assignment method and measure of relatedness between the female and her three available mates, there is no statistically significant correlation between proportion of larvae sired by stored sperm and male–female relatedness.
One possible explanation for this difference is that, with relaxed exclusion as the paternity assignment method, reproductive skew was taken into account (simulation #3) by stored sperm were excluded (simulation #2), and when different from chance expectations even when larvae sired by stored sperm and experimental males. Values in the table are differences between average measures of diversity for each group (experimental — stored), 95% CI estimated from randomization with 1000 replicates, and P values for two-tailed statistical comparisons. All three measures of diversity are significantly different between the two larval categories, independent of the paternity method or randomization scheme.

<table>
<thead>
<tr>
<th>Randomization method</th>
<th>Paternity exclusion method</th>
<th>Measure</th>
<th>Observed difference</th>
<th>95% CI lower bound</th>
<th>95% CI upper bound</th>
<th>P</th>
</tr>
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<tr>
<td>Within clutches</td>
<td>Strict</td>
<td>$m^2$</td>
<td>2.670</td>
<td>0.519</td>
<td>2.032</td>
<td>0.001**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heterozygosity</td>
<td>0.047</td>
<td>-0.034</td>
<td>0.022</td>
<td>0.001**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Standardized heterozygosity</td>
<td>0.062</td>
<td>-0.043</td>
<td>0.031</td>
<td>0.001**</td>
</tr>
<tr>
<td></td>
<td>Relaxed</td>
<td>$m^2$</td>
<td>2.567</td>
<td>-0.356</td>
<td>2.103</td>
<td>0.014*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heterozygosity</td>
<td>0.071</td>
<td>-0.068</td>
<td>0.023</td>
<td>0.001**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Standardized heterozygosity</td>
<td>0.089</td>
<td>-0.090</td>
<td>0.030</td>
<td>0.001**</td>
</tr>
<tr>
<td>Globally</td>
<td>Strict</td>
<td>$m^2$</td>
<td>2.670</td>
<td>-1.033</td>
<td>0.940</td>
<td>0.001**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heterozygosity</td>
<td>0.047</td>
<td>-0.037</td>
<td>0.036</td>
<td>0.014*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Standardized heterozygosity</td>
<td>0.062</td>
<td>-0.048</td>
<td>0.046</td>
<td>0.013*</td>
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<tr>
<td></td>
<td>Relaxed</td>
<td>$m^2$</td>
<td>2.567</td>
<td>-1.768</td>
<td>1.586</td>
<td>0.005**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heterozygosity</td>
<td>0.071</td>
<td>-0.058</td>
<td>0.065</td>
<td>0.025*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Standardized heterozygosity</td>
<td>0.089</td>
<td>-0.078</td>
<td>0.085</td>
<td>0.029*</td>
</tr>
</tbody>
</table>

Curiously, unlike heterozygosity and standardized heterozygosity, average $m^2$ values were significantly different from chance expectations even when larvae sired by stored sperm were excluded (simulation #2), and when reproductive skew was taken into account (simulation #3) with relaxed exclusion as the paternity assignment method. One possible explanation for this difference is that $m^2$ may be a better measure of inbreeding than heterozygosity, with greater power to detect patterns in certain situations (Neff 2004). The difference between observed and expected $m^2$ values, then, may reflect not only the consequences of differential use of stored sperm, but also differential use of the sperm from our experimental males, whereas heterozygosity and standardized heterozygosity could not detect the latter effect on the offspring because of these measures’ weaker signal strength.

In any case, these analyses indicate that sexual selection acts on male relatedness not only in the current breeding season, but also in the long term via sperm storage. Sperm storage and postcopulatory processes such as sperm competition and cryptic female choice are known to play an important role in sexual selection in other species (e.g. Newcomer et al. 1999; Zamudio & Sinervo 2000). In the spotted salamander, a high proportion of clutches contain larvae sired by stored sperm from previous breeding seasons (Table 2; Tennessen & Zamudio 2003). Between 6.5% (relaxed exclusion) and 20.1% (strict exclusion) of all larvae in our study, and 21% to 48% of all larvae in the previous study by Tennessen & Zamudio (2003) were sired by stored sperm. Therefore, if relatedness influences a female’s use of a male’s stored sperm, sexual selection on male relatedness has the potential to be a very important factor in the evolution and population genetics of this species.

Our findings raise several additional questions about the spotted salamander’s mating system. If females prefer more genetically similar males, why didn’t they increase their use of stored sperm when the males available to them may not have been ‘compatible’ mates (Fig. 4)? One potential explanation is that diverse offspring and therefore multiple paternity in itself may be advantageous (Newcomer et al. 1999), causing females to use some sperm from new mates regardless of how ‘good’ their stored sperm may be. Furthermore, there may be a limit on the quantity of stored sperm or length of time sperm are viable in female spermathecae. To date, no studies have specifically quantified the length of viability of stored sperm in the spotted salamander, although storage over more than one breeding season has been reported from paternity analyses of controlled mating experiments (Tennesen & Zamudio 2003) and is known in other salamanders (Baylis 1939). Future research therefore should examine the dynamics of sperm storage in this species and the mechanisms governing its use in fertilization.

Our results indicate that males are more successful if they are large and not too distantly related to their mates. Inbreeding avoidance, which would result in the opposite pattern of higher offspring $m^2$ and heterozygosity values than expected by chance, is a common phenomenon across
diverse taxa (e.g. Hoogland 1992; Waldman et al. 1992; Garner & Schmidt 2003), but in our study, outbreeding avoidance or inbreeding preference is the observed pattern. The presence of fine-scale population genetic structure but some migration among ponds in our study area (Zamudio & Wieczorek 2007) indicates that local adaptation and therefore outbreeding avoidance are at least possible in our system. Alternatively, stabilizing selection for optimal, intermediate genomic divergence, has been documented in other species (Neff 2004) and could produce a pattern mimicking outbreeding avoidance if closely related individuals are rare. This is a possible explanation in our focal population; the distribution of r-values among breeding adults in our population is biased towards 0 and negative values (Fig. 1). Moreover, inbreeding avoidance is not a universal phenomenon; this species may simply be one exception among a handful of others that have already been described (e.g. Cohen & Dearborn 2004; Jennions et al. 2004).

One factor still unknown in this system is the fitness consequences for offspring with differing genetic composition. Secondary effects of genetic composition have been shown to be important for individual fitness. For example, genetic diversity is correlated with aggressiveness in juvenile land-locked salmon (Salmo salar; Tiira et al. 2003), and with survival in horseshoe bats (Rhinolophus ferrumequinum; Rossiter et al. 2001). These secondary effects in turn may influence the long-term reproductive success of parents. Research into potential relationships between internal genomic diversity and offspring fitness in this species would further illuminate the factors driving sexual selection and mating system evolution.

We have shown that body size and relatedness are significant predictors of male fitness in spotted salamanders. Moreover, relatedness becomes even more important when stored sperm is used to fertilize eggs in future breeding seasons because sexual selection through sperm storage has significant detectable effects on the genetic composition of offspring. Reproductive success in this species is therefore highly complex, determined by an interplay of many factors, including the timing of mating, selection favouring increased male body size, the use of stored sperm, and cryptic female choice or sperm competition.

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References


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This research was conducted in partial fulfillment of Chris Chandler’s undergraduate honors thesis. Research in the Zamudio laboratory focuses on population differentiation and mating system evolution in amphibians and reptiles. We are particularly interested in the determinants of reproductive success in aggregate breeding species.