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An Investigation Into Possible Motivations Of Polyandry In The Spotted Salamander, *Ambystoma maculatum*

Jonathan Reynolds-Engel
The University of North Carolina Asheville
One University Heights
Asheville, North Carolina 28804 USA

Faculty Advisor: Dr. Rebecca Hale

Abstract

Spotted salamanders (*Ambystoma maculatum*) exhibit polyandry. The behavior has been well documented but the selective advantage of multiple matings have been insufficiently studied. Using a mesocosm experiment and microsatellite analysis, we determined if *A. maculatum* polyandry was driven by sperm-limitation, male quality, or genetic bet-hedging. We created a series of mesocosms using wild-caught salamanders to control the number of males accessible to the study females. Egg masses were harvested, and DNA was collected from all adults and embryos. Microsatellites of six previously characterized loci were used to determine paternity. Data were combined with data generated previously in the Hale Lab. Collectively, these data support that there is a combination of factors that influence the polyandrous behavior, most notably sperm limitation and male quality.

1. Introduction

Bateman's Principle is a long-standing idea that states a male's fitness is limited only by the number of females he can successfully inseminate whereas a female's fitness is usually independent of the number of copulative partners she has[1,2]. This principle tends to be supported in organisms with long gestation periods or smaller numbers of offspring, however, organisms with role reversals, cooperative breeding, or certain mating systems (e.g. polyandry) violate the principle[3].

Polyandry is a mating system that can oppose Bateman's Principle. Polyandry occurs when females use sperm from multiple males to fertilize their eggs[4]. Many studies of polyandry have examined insects because of the large sample sizes possible and financial ease of running experiments[2]. Using the models and ideas developed in these experiments, polyandry has been more widely studied and justified in species across all the animal kingdom. Polyandry can occur occasionally in some species; however, other species practice polyandry as the normal mating system[5]. The behavior can be explained by several different proposed hypotheses[6,2].

The intrinsic male-quality hypothesis suggests that females in polyandrous systems mate with the most viable male present. This viability is showcased physiologically or socially[7]. Under the intrinsic male-quality hypothesis, males advertise their quality with honest signals that females use to assess male quality[8]. The hypothesis is that males who can afford more elaborate or expensive displays are likely to have 'good genes' and allow the female to have a larger number of successful offspring than those sired by less fit males[2]. A female encountering and mating with a higher quality male after already mating leads to polyandry.

Genetic bet-hedging is another hypothetical explanation for polyandry. It postulates that females seek multiple males to ensure genetic diversity in their offspring and/or prevent genetic incompatibilities with the

fathers affecting the survivorship of their broods[9,6]. Discussions of bet-hedging need to account for both genetic incompatibilities and genetic diversity as driving factors.

The third common hypothesis for polyandry is sperm limitation. Sperm limitation states that if a single individual's sperm is not abundant enough to fully fertilize an entire clutch, a female who mates with more males will have more fertilized eggs in her clutch[10,11]. Male quality and sperm limitation can have some overlap. A male in better physical condition is more likely to be able to produce more viable sperm. For example, guppies with brighter and more orange coloration in their scales were found to have higher fertilization success than less attractive males when their sperm were artificially inseminated in equal quantities into female guppies[12].

The three hypotheses are not mutually exclusive and generate some similar predictions. For example, both the sperm limitation and the genetic bet-hedging hypotheses predict that a female with access to more males will mate with more males, resulting in more males fathering offspring in her clutch (i.e., greater multiple paternity). In contrast, access to more males will not always result in greater multiple paternity under the intrinsic male quality hypothesis; the number of males with whom a female mates depends on the order in which she encounters males of different quality. If females are sperm limited, then the proportion of fertilized eggs should be higher among females with greater access to males, whereas this may not be true under the other two hypotheses. Finally, where multiple paternity does occur, genetic bet-hedging should produce a clutch with paternity relatively equally distributed among males, whereas polyandry to find a higher quality mate should produce paternity skewed toward one father if the fertilization event is controlled by females rather than by sperm competition.

I examined these three hypotheses for polyandry in *Ambystoma maculatum*, the spotted salamander. *Ambystoma maculatum* are a salamander species of least concern with a range stretching from the eastern coast of North America to the Great Plains[13]. They are, like many salamander species, especially abundant in the Appalachian Mountains[14]. A large amount of wildlife biomass in streams and ponds in the Appalachian region consists of salamanders[15]. Understanding their reproductive motivations and systems could lead to success in maintaining a healthy population of this species while many amphibian species are in decline[16].

Spotted salamanders breed in fish-free habitats of standing water[14]. Male salamanders court multiple females simultaneously [17]. During courtship, the males deposit their nutrient-rich spermatophores into the environment. The spermatophores are nutrient-rich masses of proteins and spermatozoa. In many species, such as the rattlebox moth, the spermatophores contain both genetic material and nongenetic material[18]; this nongenetic material can sustain the zygotes or benefit the mother in some way. This can allow for females with multiple mates to experience increased fecundity. Females then collect the spermatophores through their cloaca. They store multiple spermatophores internally at any time and then lay a single large clutch (~350 clear or opaque eggs[19]) using a combination of the genetic material provided by the fathers[20]. There has been limited research into sperm precedence in salamanders. Ample research has been done on sperm layering in birds and arthropods, but information on spatiotemporal interactions of the eggs and spermatophores is limited for spotted salamanders[21,22]. Female spotted salamanders are capable of breeding in successive years[23].

The goal of this study was to tease apart these three hypotheses by addressing the following questions. First, does the number of males contributing to a clutch increase with the number of males that females encounter? Second, does egg fertilization success increase with the number of males contributing to a clutch? Third, in clutches with multiple paternity, what is the distribution of paternity across fathers? To address these questions, I continued work on a sex ratio manipulation experiment begun by Dr. Rebecca Hale and Undergraduate Researcher Jacob Boone (UNCA 2019).

2. Methods and Materials

2.1. Study System

In spring of 2019, Hale and Boone set up 12, 1.0m diameter circular wading pools in outdoor greenspace of the University of North Carolina Asheville. The pools were each filled with tap water and dried red oak (*Quercus falcata*) leaves. The filled pools were left covered with mesh screens but otherwise undisturbed for two weeks

to allow for temperature normalization and settling of the added read oak leaves. The settling allowed the wading pools to better mimic natural vernal pools.

Two female wild-caught spotted salamanders were placed in each pool. Sex ratios were manipulated in each pool so that there were four replicant of each of three treatments: two males per two females, four males per two females, and six males per two females. However, not enough gravid females were caught so the design was altered. Adults were only able to be added to seven pools. The 1:1 ratio was setup in triplicate, while the 2:1 and 3:1 ratios were set up in duplicate. Pools were left covered with mesh while the specimens bred. One egg mass from each of the seven pools was harvested for analyses.

2.2. Specimens

Egg masses and adults were harvested and brought to the laboratory. Using microscopy, embryos were taken from their egg masses and qualified as either non developing or developing. Non-developing embryos may represent either unfertilized eggs or genetic combinations between the sire and dam that are incompatible with life. After embryos hatched, hatchlings were euthanized using Tricaine-S nerve toxin overdoses. Adults suffered from a mysterious fungal infection and were similarly euthanized, then dissected to determine sex. tissue samples (~0.5cm) were obtained from the tails of adults. The tissue samples from the hatchlings and adults were stored at -80˚ C until DNA could be extracted.

2.3. DNA Extraction

DNA was extracted from thawed specimens using Qiagen DNEasy DNA extraction kits™ (Qiagen, Hilden, Germany). Six microsatellite loci were PCR-amplified from both the hatchlings and the potential parents: AmaD42, AmaD95, AmaD321, and AmaD328 described by Julian et al., and Ama 34 and Ama 61 first described by Wieczorek et al.[24,25]. The PCR recipe was modified from Julian et al (Table 1).

We used an M13 method, whereby reverse primers were designed with a19-bp M13 tag on the 5' end[26]. This allows for the use of a single fluorescent dye primer across multiple PCR reactions, which is far more economical than purchasing labeled primers for each locus.

Table 1 PCR Recipe Concentrations with M13 dye

Ingredient	Final [ ] in reaction	16 µl reaction
PCR Buffer	1x	1.6 µl
Taq	1.0 unit	0.2 µl
dNTPs 10mM	0.25 mM	0.4 µl

Forward primer 10mM	0.25 µM	0.4 µl
Reverse primer 10mM	0.5 µM	0.8 µl
M13 Dye	0.25 µM	0.4 µl
Water		10.2 µl
DNA	2.0 µl	2.0 µl
		16 µl

Jacob Boone focused on collecting PCR product the polar sex ratio pools, 1:1 and 1:3. I focused my extractions primarily on individuals from the 1:2 pools to build a more complete data set. Also, I re-extracted and prepared PCR products for individuals that had shown inconclusive allele-calling during Jacob's extractions.

2.4. Thermal Cycler Protocol

After obtaining low microsatellite yields from the Julian et al. protocol, we began using a touchdown protocol originally developed by R.G. Reynolds[24]. We used Applied Biosystems SimpliAmp™ PCR Thermal Cyclers (Applied Biosystems, Foster City, CA).

The touchdown protocol used the following conditions: denaturation at 95˚C for 5 min; 10 cycles at 95˚C for 20 s, 60-50˚C for 60 s, and 72˚C for 40 s. stepping down 1˚C each cycle from 60 to 50˚C; 20 cycles at 95˚C for 20 s, 48˚C for 20 s, and 72˚C for 40 s; and a final extension at 72˚C for 10 min[27].

2.5. Genotyping

PCR products were pooled into one of two groups and sent to North Carolina State University's Genomic Sciences Laboratory (GSL) where they underwent fragment analysis. In one group, Ama34, Ama61, AmaD42, and AmaD321 were pooled. In the second group, AmaD95 and AmaD328 were pooled. Samples sent to GSL contained 4ul pooled PCR product, 0.5 ul GeneScan™ 500 LIZ® size standard (Applied Biosystems, Foster City, CA), and 5.5 ul Hi-Di™ Formamide (Applied Biosystems, Foster City, CA).

Peak files were processed using the binning function of the microsatellite plug-in in Geneious Prime® 2020.1.2 (Biomatters Ltd.) to call genotypes. Bins were manually expanded to include nearby peaks outside of the range of automated binning. Data were cleaned manually when the binning function returned errors. Parentage analysis was conducted in GERUD 2.0[28]. Parental genotypes (n = 36) were used to calculate allele frequencies and to estimate exclusion probabilities, following Dodds et al. (1996). Half-sib groups of offspring were then processed assuming an unidentified mother. Because not all candidate parents were genotyped at all

loci, assuming unidentified mothers allowed me to include data for more loci in each analysis. Numerous loci provided unusable data, returning an error indicating multiple mothers were predicted. Such loci were excluded until a set of usable loci were identified. This process was repeated for each clutch of half-sibs. Predicted maternal and paternal genotypes were compared against known genotypes for candidate parents, though in many cases parents could not be identified with certainty. I report the minimum number of fathers predicted for each clutch.

3. Results

We found between six and thirteen alleles at the six microsatellites we amplified (Table 2). We had the most success with amplification of AmaD95 with 81% of individuals amplified. The least success was had with D328 with only 37% of individuals having successful amplifications at that locus. The gaps in reporting were caused by a combination of failures to amplify or low (<200) peaks.

Table 2 *Ambystoma maculatum* microsatellites examined and alleles found at those loci

Locus *Dye Used*	Number of alleles found at locus	Heterozygosity among candidate parents	Proportion of Microsatellite Markers Amplified	Repeat	Reference
Ama34 *VIC*	12	0.90 (30)	0.56	Dinucleotide	Wieczorek et al. 2002
Ama61 *VIC*	13	0.5 (22)	0.56	Dinucleotide	Wieczorek et al. 2002
AmaD42 *NED*	6	0.54 (26)	0.72	Tetranucleotide	Julian et al. 2003
AmaD95 *NED*	8	0.0 (6)	0.81	Tetranucleotide	Julian et al. 2003
AmaD321 *6-FAM*	12	0.33 (21)	0.64	Tetranucleotide	Julian et al. 2003
AmaD328 *6-FAM*	6	0.19 (16)	0.37	Tetranucleotide	Julian et al. 2003

A regression analysis of the relationship between the number of available males and the number of fathers represented in a clutch returned a non-significant ($t=1.57$, $P=0.18$), weak correlation of $r^2=0.3309$ $y = 0.2206x + 1.3235$ (Figure 1).

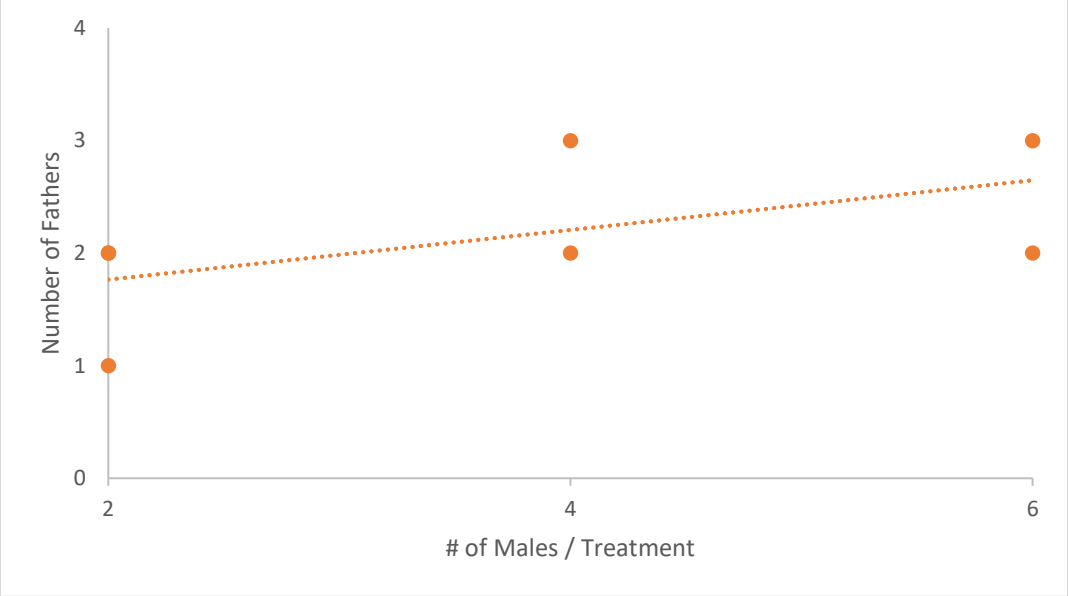

Figure 1 Relationship between the number of available males and the number of fathers (±SE) represented in a single clutch. $r^2 = 0.3309$, $P > 0.05$. Two points occur at (2,2).

A regression analysis of the impacts of the number of fathers on embryo survivorship showed even weaker ($r^2=0.0874$) and insignificant ($t = -0.69$, $P=0.54$) results $y = -0.0932x + 0.9274$. (Figure 2).

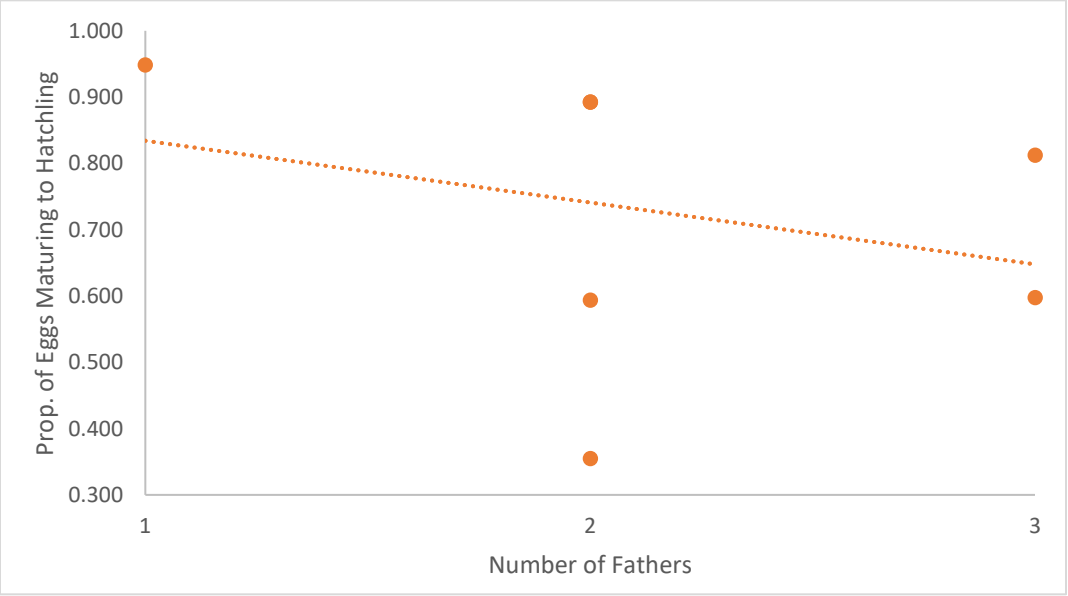

Figure 2 Relationship between the ratio of viable eggs maturing into hatchlings (±SE) and the number of fathers in the clutch. $r^2 = 0.0874$, $P>0.50$.

GERUD 2.0 prepared lists of the most likely 75-100 pairings of parents in each clutch. The mean number of offspring for each represented parent in these combinations was obtained using Microsoft Excel (Table 3). The

suggested parental pairings qualitatively showed heavy skewing of offspring numbers between fathers, with differences as large as 1:12 between two fathers. The top 20 most likely pairs or triads of paternal contributions to each clutch had Chi-squared goodness of fit tests performed on them. These data had mixed significance (Table 3).

Table 3 Average sampled hatchlings by father and total hatchling survivorship by clutch

Treatment	Fathers	Mean Sampled Offspring/Father	Sampled Offspring	$\chi 2$*	Hatched Offspring/Total Eggs	Ratio Survived
2	1	8.00	8	N/A	111 / 117	0.95
2	2	7.81	13	χ^2=0.598 P=0.439	75 / 84	0.89
2	2	4.74	9	χ^2=2.123 P=0.145	38 / 107	0.36
4	3	7.23	19	χ^2=3.01 P=0.223	147 / 242	0.61
4	2	2.32	3	χ^2=0.281 P=0.596	60 / 101	0.59
6	2	5.06	8	χ^2=0.921 P=0.337	143 / 176	0.81
6	2	6.15	11	χ^2=3.87 P=0.049	50 / 56	0.89

*χ^2 obtained using the 20 most likely paternal pairings provided by GERUD 2.0.

4. Discussion

Of the proposed hypotheses, our data best support male quality, sperm limitation, or a combination of both as the most likely factors influencing polyandrous behavior. The data suggest that there is no single selective advantage for the polyandrous behavior but rather a complex combination of causations. The weak decline in egg survivorship as the number of fathers increases may suggest that genetic bet-hedging is an unlikely influence, as we would have expected to see an increase in survivorship with increased diversity in each clutch. However, further research could be done to determine if there are benefits to diversity further along in the hatchlings' lifecycles that this experiment was not designed to account for. These same data suggest that male quality may be an important contributing factor to the polyandrous behavior; potentially higher-quality males tend to have a more dominant share of the brood, and some broods with fewer fathers have a higher survivorship. Also, the general upward trend in number of fathers, represented as the number of males in each treatment, increases suggests sperm limitation could play a role in *A. maculatum* polyandry.

Chi-squared goodness of fit tests showed significant skewed paternal representation in two of the egg masses (Table 3). Only one clutch had a single represented father. The skewed representations suggest that male-quality could be a motivation for the females' polyandrous behavior. However, the lack of consistently significant skew across the other clutches could indicate that these skewed clutches were the result of other factors that were unaccounted for. The variance could be the result of some factor of the polyandrous behavior, such as spatial/temporal location of the spermatophores, or just a result of random chance of fertilization events[8,9].

Interestingly, the results of our data analysis indicated a previously undocumented VIC-61 spike. Many sampled *A. maculatum* showed a VIC-61 spike at a lower length then previously documented[23]. The spike appeared consistently in offspring and in adults. Further investigation should be done to determine if it is a result of interspecies hybridization or some other cause.

While our data offers no significant, firm conclusions as to the motivation for spotted salamander polyandry, it does suggest an upper limit or cap to the number of individuals with which a female spotted salamander will mate. Further work could be done to learn if this is a physiological limitation caused by the anatomy or physiology of spermatophore storage, or if this is a result of the polyandric motivation itself.

The experiment aimed to qualify female motivation and paternal representation within clutches, but it is vital to discuss the health of the males in the experiment. Healthier males can devote more energy to the production and distribution of spermatophores. The mysterious fungal outbreak that resulted in the euthanizing of all of the adult specimens may have had varying levels of adverse effects on the specimens before the outbreak was identified and the specimens euthanized. Also, as all of the adults were procured from the wild, we had no available data on the health of the individuals before they were caught. Future refinements of this experiment could attempt to control for health of the specimens by potentially using laboratory bred males with equal access to resources.

Our data should be expanded to include more clutches. While the likely father calculations were made using 5-20 data points each, having a limited number of clutches to report on resulted in low statistical power. Some of the microsatellites consistently returned errors with the GERUD 2.0 software, forcing us to remove individuals from the analysis and reducing the available data points. Since every microsatellite did not return data for every individual, any incomplete individuals had to be excluded, further limiting our sample sizes, sometimes as drastically as a reduction from more than 20 individuals to only four. Increasing the number of analyzed clutches and individuals would improve both the accuracy and reliability of our results.

Also, while efforts were made to capture females before they had mated, some were captured in minnow traps and could have mated before entering the pools they were housed in. One of the clutches from the six-male treatment was suggested to have two fathers, but both suggested fathers were homozygous and there were three loci represented in the clutch's offspring, suggesting an additional, unanalyzed parent. It should be reemphasized that, even though these females may have had access to plenty more males than our experimental design provided for, there still appears to be a maximum of three males represented per clutch.

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