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Investigating the Motivations for Polyandrous Mating Behavior in *Ambystoma maculatum*

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Abstract

Female spotted salamanders (*Ambystoma maculatum)* engage in polyandrous behavior, and the motivation behind this behavior is currently unclear. Several studies suggest polyandrous mating may be driven by sperm limitation, efforts to seek higher quality mates, or instinctual drive for genetic diversity in offspring. These hypotheses make different predictions for how the probability of fatherhood should vary among males when females have varying numbers of mates. Research began with the design of a mesocosm experiment to manipulate the mating ratios of *Ambystoma maculatum*. Males and females were captured from the Sandy Bottom Nature Preserve (Buncombe County, North Carolina). Captured adults were divided among twelve miniature manufactured vernal pools, with each pool having one of four male:female ratios. After two weeks, adults and egg masses were collected. Adults were euthanized, as were offspring after hatching. Individuals were analyzed at 6 microsatellite loci and parentage was determined in Cervus™. Results suggest that sperm limitation is not a motivating factor for polyandry; mate quality and drive for genetic diversity are potential motivating factors; however, more data collection and analysis is needed.

1. Background

Spotted salamanders (*Ambystoma maculatum*) have a wide distribution throughout the Eastern United States [13]. Classified as aquatic breeders, mature spotted salamanders typically breed in seasonally ephemeral, fish-free habitats such as vernal ponds, swamps, and roadside ditches, but may occasionally use permanent ponds [13]. Copulation is achieved through females picking up spermatophores laid by males with their cloaca, and internally storing this sperm for later use in fertilization. During breeding events, males court multiple females and each male can deposit up to 40 spermatophores [15]. Additionally, females can mate with multiple males before laying a single egg mass; Adams [1] observed that most females use sperm from only two or three males to fertilize their eggs.

Polyandry, the practice of females mating with multiple males, is a mating strategy in which females use sperm from various males to fertilize eggs [13]. Current literature suggests that polyandry creates competition among males to fertilize egg masses; however, this competition does not provide any direct (material) benefit to females, particularly in species where males do not provide parental care or nuptial gifts[11]. Given that the female has nothing to gain as an individual through multiple mating, it is theorized that polyandrous behavior has a motivation that is not readily apparent.

Females may gain indirect benefits by mating with multiple males for a variety of reasons[2,3,4]. For example, females may need sperm from more than one male to fertilize all eggs in a clutch, a phenomenon called sperm limitation[12,16]. Sperm limitation has been observed in *Ambystoma maculatum* and other aggregate breeding species[15]. If sperm from one male is insufficient to fertilize all eggs, then females who have access to more males will have higher fertilization success (more offspring). Additionally, sperm precedence based on order of mating has been observed for a variety of taxa from insects to vertebrates [15]. This may support the sperm limitation hypothesis if the order in which a female mates functions as a selective pressure because this would suggest that males whose spermatophores are picked up first should have higher shares of paternity.

Alternatively, females may benefit by ensuring offspring have the greatest variance in sources of paternal DNA [16]. Essentially, females may employ a risk-spreading strategy in which they mate with multiple males to increase the probability that at least some of her offspring will have genotypes favorable in their environments. Using a simulation approach, Yasui & Garcia-Gonzalez [20] showed that this strategy, while producing lower mean fitness across females, is successful because it reduces fitness variance, particularly when parental genetic incompatibilities are common. So, if diverse paternal DNA is a priority, then the number of fathers in a clutch should increase with an increase in available males, and all males contributing to a clutch should have similar shares of paternity; this would ensure maximum genetic diversity among offspring.

Further, females may mate more than once if they encounter a higher quality mate after they have already mated [5]. Criteria for what classifies as higher quality is currently unclear; however, Crowshaw et al. [6] observed that in experimental breeding groups with males of ranging sizes, size had little impact on male mating and reproductive success. If male quality is a selective pressure, then the number of fathers in a clutch should remain constant despite an increase in available males because females will only mate with males they deem superior. In this scenario, male reproductive success within a clutch should be heavily skewed, presumably toward favored males having a considerably greater share of paternity.

The primary question we examined is: why do females engage in polyandrous behavior? To do this, we set up a mesocosm experiment. Twelve miniature manufactured vernal pools were filled with water and leaf litter; varying ratios of male and female adult *Ambystoma maculatum* were placed in each pool (2 females: 2 males; 2 females: 4 males; or 2 females: 6 males). Adults and resulting egg masses were harvested after a period of breeding (~2 weeks) to have DNA extracted and sequenced. The results of sequencing helped to assign parentage to males and females from the 4 pools we analyzed; this in turn enabled us to determine which hypotheses (sperm limitation, mate quality, or drive for genetic diversity) were motivating factors for polyandrous behavior and to what degree.

2. Methods

2.1 Study System

In the Spring of 2019, we designed a mesocosm experiment to have 4 replicates of 3 sex ratio treatments. All pools had 2 female spotted salamanders and either 2, 4, or 6 males in a 1 m diameter wading pool with 100 g dry red oak (*Quercus falcata*) leaves and filled with tap water, aged for 2 weeks before animals were introduced. Pools were covered with mesh screen. Although we were able to collect males for all pools, we were only able to place gravid females in 8 of the pools. Specimens were captured prior to breeding and produced 16 egg masses across the 8 pools.

2.2 Specimen Harvest

At the conclusion of our mesocosm experiment, egg masses and adults were collected and returned to the lab. Embryos were separated from egg masses and scored as developing or not developing. Non-developing embryos may represent unfertilized eggs. Developing embryos were retained until hatching; hatchling specimens were then euthanized using Tricaine-S nerve toxin overdoses. Adults succumbed to a fungal infection and had to be euthanized. Tissue samples subsequently were obtained from the tails of adults (~0.5cm of tissue), and each animal was dissected to confirm sex. Both hatchling and adult specimens were stored at-80 °C for later DNA extraction and analysis.

2.3 DNA Extraction

Qiagen DNEasy DNA extraction kits™ (Qiagen, Hilden, Germany) were used to extract DNA from adult and hatchling specimens. DNA extracted from hatchlings and putative parents were amplified using six microsatellite markers developed for *Ambystoma maculatum*: AmaD42, AmaD95, AmaD321, and AmaD328 from Julian et al. (2003), and Ama34 and Ama61 from Wieczorek et al. (2002). In order to consolidate the data, only PCR product from pools containing a low female:male ratio (2:2) or a high female:male ratio (2:6) had fragments analyzed. Partway through our experiment we noticed consistent lack of amplification from markers AmaD328 and AmaD95 and discontinued their use, focusing only on AmaD42, AmaD321, Ama34, and Ama61. After PCR product from high sex ratio pools and low sex ratio pools were gathered, they were sent off to the North Carolina State University Genomic Sciences Laboratory for fragment analysis. Protocol used for PCR as follows:

Table 1 PCR Recipe Concentrations

Ingredients	Final [ ] in rxn	Amt for one 30ul rxn	16ul rxn
PCR Buffer	1x	3 ul	1.6 ul
Taq	1.0 unit	0.4 ul (2 units)	0.2 ul
dNTPs 10mM	0.25 mM	0.75 ul	0.4 ul
Forward primer 10mM	0.5 uM	1.5 ul	0.8 ul
Reverse primer 10mM	0.5 uM	1.5 ul	0.8 ul
Water		20.85 ul	10.2 ul
DNA*		2 ul	2 ul
		30	16

*Add DNA separately to each tube

2.4 Thermal Cycler Protocol (touchdown)

Initial polymerase chain reactions (PCR) were conducted following the protocol of Julian et al. (2003), with low yield for microsatellites. In an attempt to improve yield, we used an unpublished touchdown protocol developed for boas by R.G. Reynolds (personal communication) which were performed on Applied Biosystems SimpliAmp™ PCR Thermal Cyclers (Applied Biosystems, Foster City, CA).

The PCR protocol was as follows: initial denaturing at 95 °C for 5 minutes; 10 cycles of: 95 °C for 20 seconds, 60 °C for 1 minute, 72°C for 40 seconds; 20 cycles of 95°C for 20 seconds, 48°C for 20 seconds, 72°C for 40 seconds; followed by 72°C for 10 minutes.

2.5 Data Analysis

Peak data returned from North Carolina State University Genomic Sciences Laboratory were analyzed using Geneious Prime© software (Geneious, Aukland, New Zealand) with the microsatellite add-in. All samples were manually trimmed and edited. Allele frequency data were analyzed in Cervus 3.0 [1]. Because all embryos from a sample were siblings, we first ran the maternity analysis to identify the most likely mother. We then assigned this mother as the known mother of all individuals from the sample and performed the paternity analysis. It is possible that females may have mated prior to collection and that some offspring may be fathered by a male outside of our sample. Therefore, we assumed a 0.5 probability that our sample included the actual father.

3. Results

3.1 Fertilization Rate

In many pools, more than two egg masses were deposited despite there being only two females. This strongly suggests that females parse their eggs into multiple masses. For the 31 egg masses deposited, we evaluated the relationship between the number of males in a pool and % embryos alive as a measure of fertilization rate so that we could determine whether sperm limitation reduces fertilization success when females mate with few males. The data suggest that there is no association between the proportion of developing embryos with the number of males available to mate with (Figure 1). These data do not support our hypothesis of sperm limitation being a motivation for polyandrous behavior; otherwise, each replicate (either 2, 4, or 6 males) would have an increase in the number of fertilized embryos. The genotype data from pools 2, 4, and 5 showed pool 2 had 13 out of 16 hatchlings that could be assigned parentage, with a skew towards female 2 (P2F2) for maternity; paternity could not be assigned. Pool 4 had 8 out of 11 hatchlings that could be assigned parentage, with a skew towards female 1 (P4F1) for maternity; all males had equal contributions to paternity. Pool 5 had 13 out of 13 hatchlings that could be assigned parentage, with a skew towards female 1 (P5F1) for maternity and male 2 (P5M2) for paternity (Figure 3).

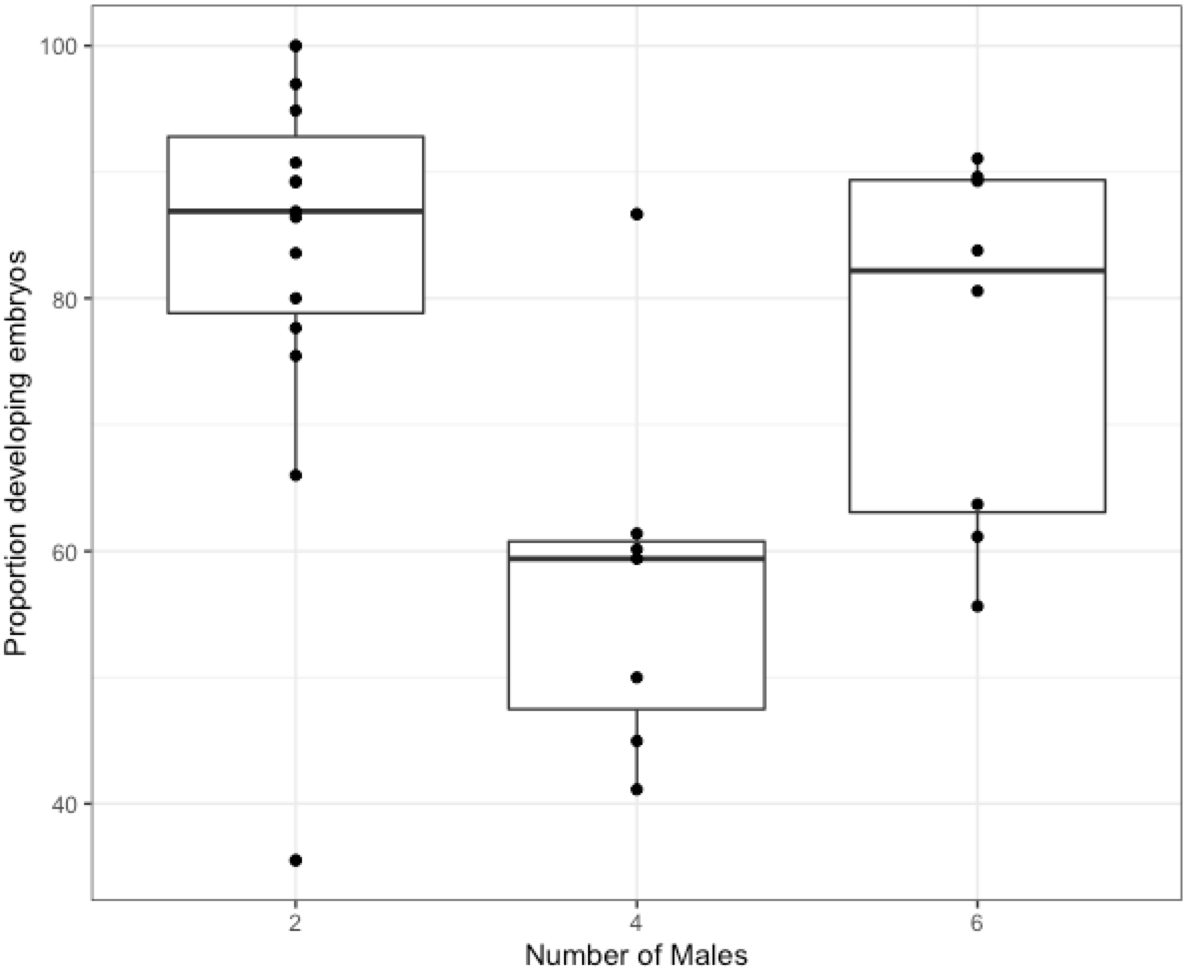

Figure 1. Number of developing embryos per number of available males

Figure 1 The proportion of developing embryos varied with numbers of males, but not in a consistent direction.

3.2 Electrophoresis And Analysis Of Allele Frequencies

We were only able to receive genotyping data from 3 pools (2, 4, and 5), each with a female:male ratios of 2:2, 2:6, and 2:6 respectively (Figure 2, Table 1). Heterozygosity (determined via Geneious Prime © fragment analysis) among the loci varied considerably, ranging from 47%-84% with large disparities between observed and expected values.

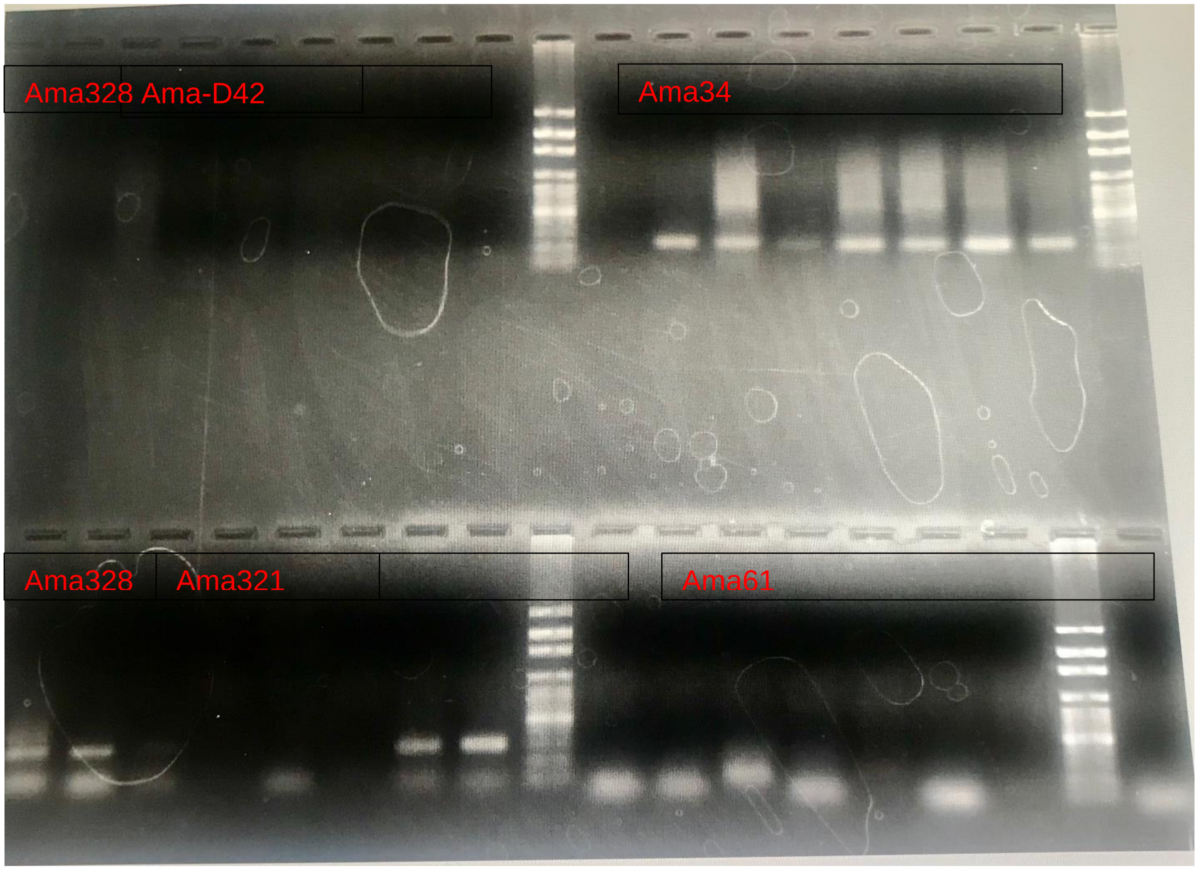

Figure 2. Gel image of microsatellite markers

Figure 2 Representative agarose gel electrophoresis of PCR products amplified using the protocol of Julian et al. (2003). 2 rows of 20 lanes (only lanes 1-18 for each row pictured). Gel imaging of hatchling DNA; top wells (lanes 1-20 from right to left): lanes 1, 10 = DNA ladder; lanes 2-9 = Ama34; lanes 11-18 = Ama-D42; lanes 19-20 = Ama61 (not pictured). Bottom wells (lanes 1-20 from right to left): lanes 2, 10 = DNA ladder; lane 1 and 3-7 = Ama61; lanes 8-9 and 11-16 = Ama321; lanes 17-20 = Ama328. Ama328 and Ama 95 were discontinued due to poor amplification success.

Table 2 Locus data for the four loci that successfully amplified with some consistency.

Locus	**Observed number of alleles (size range)**	**Size of repeat**	**% Individuals amplifying**	**H_{obs}**	**H_{exp}**
Ama34	10 (91-113 bp)	2 bp [18]	79%	.84	.65
Ama61	11 (249-273 bp)	2 bp [18]	69%	.47	.84
AmaD42	7 (144-168 bp)	4 bp [12]	84%	.68	.64
AmaD321	9 (136-176 bp)	4 bp [12]	63%	.63	.87

3.3 Multiple Mating And Paternity Skew

Although paternity could only be assigned in three of the four samples, the data suggest that when females have access to more males, they mate with and use sperm from more of them (Figure 3). The one egg mass collected from a pool with two males had both males contributing paternity, whereas three and six males contributed paternity to the egg masses collected from six-male pools. Pool 2 had 13 out of 16 hatchlings that could be assigned parentage, with a skew towards female 2 (P2F2) for maternity; paternity could not be assigned. Pool 4 had 8 out of 11 hatchlings that could be assigned parentage, with a skew towards female 1 (P4F1) for maternity; paternity had a relatively even distribution. Pool 5 had 13 out of 13 hatchlings that could be assigned parentage, with a skew towards female 1 (P5F1) for maternity and male 2 (P5M2) for paternity (Figure 4).

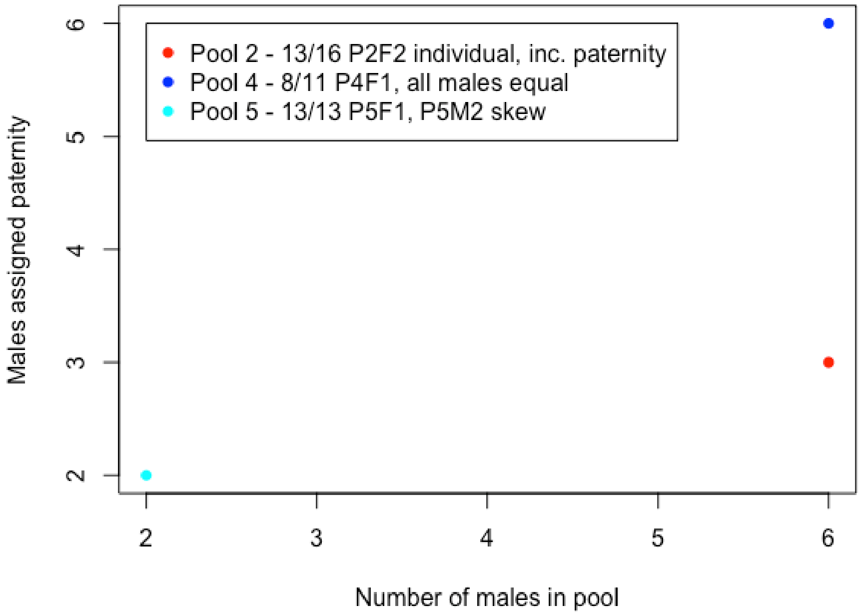

Figure 3. Number of males in pool assigned paternity

Figure 3 The number of males obtaining some paternity as a function of how many males females had opportunity to mate with. Males could be assigned paternity for pool 2 (2 female:6 male), pool 4 (2 female:6 male), and pool 5 (2 female: 2 male).

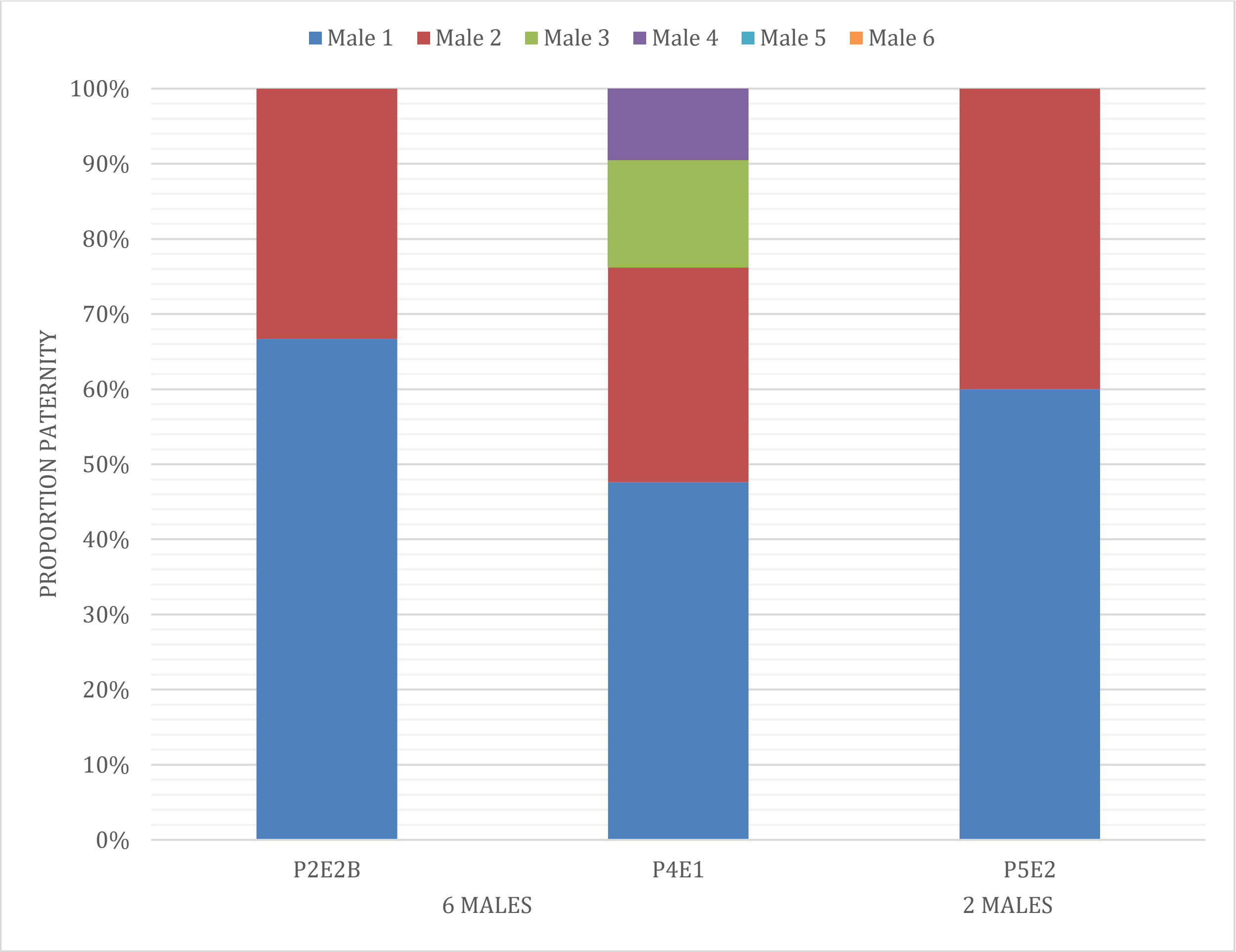

Figure 4. Skew analysis of paternity by pool

Figure 4 The percentage of paternity organized by number of males and which pools had either 2 or 6 males. Also displays which male had what proportion of paternity. Males could be assigned paternity for pool 2 (2 female:6 male), pool 4 (2 female:6 male), and pool 5 (2 female: 2 male).

4. Conclusions

We began our investigation by examining three hypotheses for the motivation behind female polyandry in *Ambystoma maculatum*: sperm limitation, efforts to seek the highest quality male, and instinctual drive for genetic diversity. Our first hypothesis postulated that sperm limitation, a need for multiple sperm donors to fertilize all eggs, was a motivation for polyandrous behavior. Results suggest that this hypothesis is incorrect. If more sperm is needed to fertilize all eggs, then as the number of available males increases, so should the number of developing embryos. Figure 2 demonstrates no such correlation, with pool 2 having the highest number of developing embryos with a female:male ratio of 2:2; whereas pools 4 and 5 have lower numbers of developing embryos despite having a higher number of putative males.

Our second hypothesis postulated that females' mate with multiple males in an effort to find the highest quality male. Our results suggest that the highest quality male hypothesis could potentially be a motivation for polyandrous behavior. Figure 3 displays pool 5 as having a paternity skew towards male 2 (P5M2) over male 1 (P5M1). These data could support our 'highest quality' hypothesis; however, pool 2 and pool 4 display data that contradicts this hypothesis with inconclusive paternity and equal paternity contributions respectively. More data should be examined to affirm or refute this hypothesis.

Our third hypothesis theorized that an instinctual drive for genetic diversity is the motivating factor behind polyandrous behavior. If this hypothesis is supported, then all putative males within a pool should have equal shares of paternity to ensure offspring have the highest genetic diversity possible. Figure 3 suggests this hypothesis may be supported, with pool 4 showing all males as having equal shares of paternity; however, pools 2 and 5 do not support this with inconclusive paternity assignment and a skew towards individual P5M2 respectively. More data should be examined to affirm or refute this hypothesis.

Thus far, our research suggests that sperm limitation is not a motivating factor for polyandrous behavior in female *Ambystoma maculatum*. More genetic data is needed to discern whether or not 'highest male quality' or 'instinctual drive for genetic diversity' hypotheses are true. Future research directions may cross examine polyandry among the *Ambystoma* genus, associations between polyandry and maternal care of egg masses, or other potential driving factors for polyandry such as genetics or brain morphology.

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