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Salamander Mating Behaviors and Their Consequences for Individuals and Populations

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SALAMANDER MATING BEHAVIORS AND THEIR CONSEQUENCES FOR
INDIVIDUALS AND POPULATIONS

A Dissertation

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Abstract

I studied female mating behavior, sexual selection, and the consequences of polyandry for individuals and populations of the marbled salamander (Ambystoma opacum). I also compared the performance of several statistical approaches for analyzing genetic mating system data.

The first chapter summarizes the characteristics of several novel microsatellite DNA loci as well as cross-amplified loci for marbled salamanders and mole salamanders.

In the second chapter, I report estimates of sire number for 13 marbled salamander clutches based on microsatellite data from 32 hatchlings per clutch. Females mated with as many as three different males as indicated by conservative techniques. Less than half of females mated with multiple males. Based on comparative analyses, I recommend the parental reconstruction approach with the computer program GERUD for assessing multiple paternity.

The third chapter describes an experiment designed to study sexual selection. As expected, the potential for sexual selection was much higher for males than for females. Body size was unrelated to variance in male reproductive fitness. Only opportunity for selection and Morisita's index conformed to theoretical expectations of the relationship between operational sex ratio and the potential for sexual selection among males. Because opportunity for selection has intuitive links to formal sexual selection theory, I recommend its continued use.

In the fourth chapter, I compared offspring of polyandrous and monandrous females to explore the potential fitness consequences of multi-male mating. Polyandrous clutches had significantly higher survival to metamorphosis. No fitness-related measure at the egg or hatchling stage (clutch size, hatching success, hatchling size, etc.) differed between the two types of clutches.

In the fifth chapter, I analyzed effects of increased polyandry and male availability on genetic diversity, effective population size (N_e), and fitness of experimental populations. Although no effects were statistically significant, some effects were moderate to high in size. N_e was higher when estimated from hatchlings than with metamorphs.

Preface

This dissertation consists of five chapters, each written for a specific journal. Each chapter is independent and stands alone.

Chapter 1

Isolation and characterization of microsatellite DNA loci from *Ambystoma* salamanders

Amphibians are experiencing worldwide population declines and many species are becoming extinct, endangered, or threatened (Pechmann and Wake 1997; Alford and Richards 1999). Destruction, alteration, and fragmentation of suitable habitat are likely major factors contributing to these declines (e.g., Petranka et al. 1993; Delis et al. 1996; Hecnar and M'Closkey 1996; Semlitsch and Bodie 1998; Vos and Chardon 1998). Assessing the impacts of such changes in landscape ecology requires knowledge of population genetic structure and metapopulation dynamics at a small geographic scale. For example, it is important for conservation managers to understand patterns of gene flow among populations and their relationship to landscape-level habitat heterogeneity. Genetic markers may be used to detect population subdivision and interconnection and can provide useful information for theoretical population biologists and applied conservationists (e.g., Newman and Squire 2001).

Salamanders of the genus *Ambystoma* are especially important to amphibian conservation biology in North America. These animals depend on the terrestrial habitat that surrounds the wetlands in which they breed, a habitat type that is generally not well protected (Gibbons 2003). Also, it is unclear how much of this terrestrial habitat salamander populations require (Semlitsch 1998). Several species in this genus (e.g., *A. cingulatum* and *A. californiense*) are especially threatened, likely because of terrestrial habitat destruction (Barry and Shaffer 1994; Means et al. 1996; Petranka 1998). Detailed studies of population genetic structure and concomitant estimates of gene flow will augment our ability to manage the remaining populations of these and other ambystomatid salamanders. In this paper, we characterize 17 new polymorphic

microsatellite markers from marbled salamanders (*Ambystoma opacum*) and mole salamanders (*A. talpoideum*) that may be used for this purpose. These two species are locally uncommon in parts of their ranges. Marbled salamanders are listed as threatened in the state of Massachusetts and endangered in New Hampshire. Specifically, we report eight loci (five tetranucleotide, three dinucleotide) from the marbled salamander and nine (two tetranucleotide, six dinucleotide, one mixed) from the mole salamander. In addition, we successfully amplified several loci developed by Julian et al. (2003a,b) for other *Ambystoma* species in our focal species (seven for *A. opacum* and two for *A. talpoideum*).

We used Qiagen DNeasy kits to extract DNA from tail clips and enriched for microsatellites with a modified version of a technique used by Hamilton *et al.* (1999) (see Glenn & Schable 2005). Briefly, we digested DNA from one individual of each species with *RsaI* (New England Biolabs). The fragments were ligated to double stranded SuperSNX24 linkers (forward 5'-GTTTAAGGCCTAGCTAGCAGAATC-3', reverse 5'-GATTCTGCTAGCTAGGCCTTAAACAAAA-3'; modified from Hamilton *et al.* 1999). We then hybridized microsatellite-containing fragments to biotinylated oligonucleotide repeat probes, used magnetic streptavidin beads (Dynal) to capture the probes and microsatellites, and finally discarded unhybridized DNA. We enriched with the following oligonucleotide probes: $(TG)_{12}$, $(AG)_{12}$, $(AAG)_8$, $(ATC)_8$, $(AAC)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACCT)_6$, $(ACAG)_6$, $(ACTC)_6$, and $(ACTG)_6$. The enriched DNA was amplified via PCR as follows: 10 mM Tris-HCl pH 8.3, 50 mM KCL, 2.0 mM $MgCl_2$, 25.0 μg/mL BSA, 0.5 μM SuperSNX24 forward primer, and 0.5 units JumpStart Taq DNA polymerase (Sigma). We ligated this PCR product into PCR 2.1-TOPO vector (Invitrogen) before transforming One Shot Top10 Chemically Competent *E. coli* cells (Invitrogen). We screened for successful insertion

with the β-galactosidase gene before amplifying inserts from these positive colonies with M13

primers. Initially, we screened some clones (96 of each species) for microsatellites by spotting

the products on nitrocellulose membranes and hybridizing to oligonucleotide probes, resulting in

58 positives for *A. opacum* and 67 for *A. talpoideum*. Later, we bypassed this step and screened

for the presence of repeats by sequencing the inserts. In all, we sequenced a total of 242 unique

clones for *A. opacum* and 247 for *A. talpoideum* with Big Dye (version 3.0, Applied Biosystems)

chemistry and an ABI 377-96 sequencer. We edited sequences with Sequencher 4.1

(Genecodes) and then used Ephemeris 1.0 (available at

http://www.uga.edu/srel/DNA_Lab/programs.htm) to search for microsatellites. With Oligo 6.67

(Molecular Biology Insights), we designed 40 and 34 PCR primer pairs from 99 and 102 clones

that contained repeats for *A. opacum* and *A. talpoideum*, respectively. We modified one primer

of each pair with a tag at the 5' end (either 5'-GGAAACAGCTATGACCATG-3' or 5'-

CAGTCGGGCGTCATCA-3') allowing the binding of a fluorescently labeled oligonucleotide to

the PCR product for detection of polymorphism on the ABI 377 sequencer (cf. Boutin-Ganache

et al. 2001). One primer of each heterospecific pair was directly labeled with a commercially

available fluorescent dye and no tag was needed.

For all loci, we optimized PCR conditions using genomic DNA from 11-17 individuals

collected from Okie's Bay (*A. opacum*) and Flamingo Bay (*A. talpoideum*), Carolina bays on the

Savannah River Site in Aiken County, South Carolina. The reactions were performed in 25-μL

volume with an Eppendorf Mastercycler Gradient thermal cycler. Concentrations of the

reactions were10 mM Tris-HCl pH=8.3, 50 mM KCL, 1.5 mM MgCl$_2$, 25.0 μg/mL BSA, 0.4

μM unlabeled primer, 0.04 μM tag labeled primer, 0.36 μM dye labeled primer (HEX or 6-

FAM), 0.15 mM dNTPs, 0.25 units JumpStart Taq DNA Polymerase (Sigma), and 30-50 ng

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DNA template. Primers were optimized with three different touchdown PCR thermal cycling programs (Don *et al.* 1991). The programs test a range of annealing temperatures, 65-55ºC, 60-50ºC, or 55-45ºC. They consist of 5 cycles of 96ºC for 20 s, the highest annealing temperature for 30 s, and 72ºC for 1 min followed by 21 cycles of 30s of 96ºC, highest annealing temperature minus 0.5ºC each cycle for 30 s, and 72ºC for 1 min; and finally 10 cycles of 96ºC for 30 s, the lowest annealing temperature for 30 s, and 72ºC for 1 min. We scored alleles with Gensize Rox 500 ladder (Genetix) or CXR ladder (Promega) and Genescan 3.1.2 and Genotyper 2.5 software (PE Applied Biosystems) on the ABI 377 sequencer. For heterospecific primers we followed the same optimization procedures, except that primer concentrations were 0.4 µM for both labeled and unlabeled primers, and the third primer was not needed. Finally, we used the optimized PCR conditions to test each of our *A. opacum* primer sets for cross-amplification in *A. talpoideum* and vice versa. Observed and expected heterozygosity were calculated for each locus with Cervus 2.0 (Marshall *et al.* 1998). We used Genepop 3.3 (Raymond and Rousset 1995) to test for Hardy-Weinberg equilibrium (HWE) and genotypic linkage disequilibrium.

Of the 40 *A. opacum* primer pairs, 14 amplified consistently and eight were polymorphic (Table 1). Additionally, seven heterospecific loci yielded polymorphic product (Table 1). Of 13 consistently amplifying *A. talpoideum* loci, nine were polymorphic (Table 2). Three primer sets from Julian et al. (2003a,b) amplified *A. talpoideum* DNA consistently but only two were polymorphic (Table 2). Three of our novel loci cross-amplified monomorphic products (Table1,2). Among the polymorphic loci, we scored two to 15 alleles and heterozygosity was moderate to high ($\geq$ 0.40 for 22 of 24 loci). No pair of loci showed evidence for physical linkage (p>0.05 in all cases). After a Bonferroni correction, AjeD422 and AmaD23 did not meet the expectations of HWE in the *A. opacum* population (p<0.002 in both cases), most likely because

Table 1. Properties of 24 microsatellite loci and amplification results with *Ambystoma opacum* individuals collected from one population in Aiken County, South Carolina, USA.

Primer	Sequence 5'-3'	Dye	GenBank Accession Number	Touchdown Temperature	Repeat(s) in cloned allele	Size of cloned allele (bp)	No. of alleles	Size range (bp)	N	H_O	H_E
Aop2 F	*GGAAACAGCTATGACCATG* TCC TGA TAT TAC TGT ATG TTA T	HEX	AY667611	55	$(AAAC)_7$	186	3	202-210	11	0.55	0.45
Aop2 R	AAG AAT GCT CAA ATC TAT AC										
Aop6 F	CTT GGG AAA ATG AGA TAC	6-FAM	AY667612	60	$(ACAG)_5$	289	4	226-310	11	0.46	0.46
Aop6 R	*CAGTCGGGCGTCATCA* TCA ATC CAT CTA CCT TTA TC										
Aop7 F	GCC TGT TTC CTG AGA C	HEX	AY667613	55	$(AG)_{24}$	158	3	164-176	14	0.71	0.68
Aop7 R	*GGAAACAGCTATGACCATG* CCC AGA GTT AGG ATC AC										
Aop25 F	AGC CAA AGA AAT CTA CTC	HEX	AY667614	60	$(GT)_7$	136	3	158-162	14	0.43	0.53
Aop25 R	*GGAAACAGCTATGACCATG* AAT CCT TTT TTG TAA CAC T										
Aop29 F	CAG CGC CCA TTT ACT C	HEX	AY667615	60	$(AAAC)_6$	167	3	182-198	14	0.43	0.36
Aop29 R	*GGAAACAGCTATGACCAT* GCAATTAGAGGCTTTCAGT										
Aop31 F	*CAGTCGGGCGTCATCA* GGC TTG TCT CCA GTT GT	6-FAM	AY667616	60	$(AAAG)_{11}$	153	10	168-232	15	0.73	0.86
Aop31 R	TGA TTT TTC CTC TTT CAC TAC										
Aop36 F	CCT TGT GTG GAC ATG AT	HEX	AY667617	60	$(GAGT)_{11}$	125	6	131-163	16	0.75	0.83
Aop36 R	*GGAAACAGCTATGACCAT* GAA CCA AAA TAA CAG GAG TT										
Aop37 F	*CAGTCGGGCGTCAT* CAG AGG GAA ATC TAA TAA C	6-FAM	AY733034	60	$(AG)_{21}$	253	5	259-278	11	0.64	0.67
Aop37 R	GAT AAG CAC ATA GAA ATA ATA C										
Ata7 F	TCA CCT TTG CCT GTA AC	HEX	AY667618	55	$(AAAC)_5$	187	ND				
Ata7 R	*CAGTCGGGCGTCATCA* GGT ATT GCT TCT CTC ACT GT										
Ata14 F	TTG GGA AAC TGC TGA G	6-FAM	AY667619	65	$(AC)_9$	120	ND				
Ata14 R	*CAGTCGGGCGTCATCA* GAG GCA GAA GTT CAT ATT A										
Ata15 F	*CAGTCGGGCGTCATCA* GTT CCT TCT CCT CTT CTA T	6-FAM	AY667620	65	$(CT)_{13}$	229	ND				
Ata15 R	GGA AAG AAA GCA TGT AGA										
Ata17 F	*CAGTCGGGCGTCATC* AAC AGA TCA CCT CAG ACA	6-FAM	AY667621	60	$(AC)_8$	146	1	275	8	0.0	0.0
Ata17 R	GAT GAA TAC TAT GTA ACC AGA TAA										
Ata18 F	GCA TAG GAT ATT GGA CAC	HEX	AY667622	60	$(CT)_{21}$	148	1	126	8	0.0	0.0
Ata18 R	*GGAAACAGCTATGACCA* TGA TTT ACC AGA ACT TTC TA										

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Ata19 F	*CAGTCGGGCGTCATCA* GGA TCT TTT TGT GTA TCT AC	6-FAM	AY733035	60	$(AG)_{10}$*	266	ND				
Ata19 R	TTC CGT CAA TAC AAT TAC										
Ata29 F	*GGAAACAGCTATGACCATG* ACC TCT GCG AGA ACT AC	NED	AY733036	55	$(CTTT)_9$…$(CTTT)_{18}$	286	ND				
Ata29 R	CCA GTG ACC TTA GAT AGA C										
Ata32 F	*CAGTCGGGCGTCAT* CAG AAA AAC AGA AAA AGT CA	6-FAM	AY733037	65	$(AG)_{22}$	238	ND				
Ata32 R	GGC AAC AAA TAA TCA ACT C										
Ata34 F	*CAGTCGGGCGTCATCA* TGG GTG TGT TTC TTA GTG	HEX	AY733038	55	$(AG)_{33}$	191	ND				
Ata34 R	CCA ACT ATC AAC CAA CTA TG										
AmaD42 F	GAT GGA AAA TCA ATC AAG TGT G	NED	AF520749	60	$(TAGA)_4$…$(TAGA)_3$	ND	8	140-200	17	0.82	0.84
AmaD42 R	TAA CTA GCT GTC AAT CGC TCT C										
AmaD95 F	AGC GCT TAG ATA CCT CTC GG	NED	AF520751	60	$(TAGA)_8$…$(TAGA)_{14}$	ND	10	162-210	15	0.60	0.87
AmaD95 R	TAT TGC ATG TGA ATA TCG ATG G										
AmaD321 F	GAT GCC TTG AAA CTT GTT CTT C	6-FAM	AF520758	60	$(TATC)_{14}$	ND	7	128-176	14	1.00	0.84
AmaD321R	TGG TGC ATC TAT ATT CCT CAA G										
AmaD328F	CCC CAG TTT GTT TGT TTT GTA G	6-FAM	AF520759	60	$(TAGA)_{15}$	ND	10	313-357	15	0.93	0.91
AmaD328R	ATG ACC CTT CCA GCT AAT ACA G										
AjeD23 F	AAA ACC TCT GGA GAA ACA TGA G	NED	AY091795	60	$(TAGA)_{14}$	ND	8	180-260	15	0.73	0.83
AjeD23 R	GAA CAC AGG CTA CTA ACA ACA GG										
AjeD162 F	AAA TGT TCC AAC CAG TCA CAA C	NED	AY091802	60	$(TAGA)_{13}$	ND	12	165-221	16	1.00	0.91
AjeD162 R	GAT TAA GCT AGA GGG CTT GTC C										
AjeD422 F	CAA GGT GCT CAA GTT ACT GTT C	6-FAM	AY091811	60	$(TAGA)_{12}$	ND	5	247-263	16	0.31	0.78
AjeD422 R	CAA ATT CTG TAC CTG ACT GCT G										

Loci with blanks in the last four columns did not amplify *A. opacum* DNA consistently.

Tags for universal fluorescent primer binding are in italics.

Aop denotes primers designed for *Ambystoma opacum*, Ata for *A. talpoideum*, Ama for *A. maculatum*, and Aje for *A. jeffersonianum*.

Touchdown Temperature - initial annealing temperature of the thermal regime (see text).

Size of cloned allele - length in bp of PCR product amplified from the initial bacterial culture.

H_0 - observed heterozygosity.

H_E - expected heterozygosity.

* All repeats in clone: $(AG)_6…(AG)_7…(AG)_{10}…(AG)_8…(TGAG)_4(AG)_7…(AAAT)_5$.

ND – not determined.

Table 2. Properties of 24 microsatellite loci and amplification results with *Ambystoma talpoideum* individuals collected from one population in Aiken County, South Carolina, USA.

Primer	Sequence 5'-3'	Dye	GenBank Accession Number	Touchdown Temperature	Repeat(s) in cloned allele	Size of cloned allele (bp)	No. of alleles	Size range (bp)	N	H_O	H_E
Aop2 F	*GGAAACAGCTATGACCATG* TCC TGA TAT TAC TGT ATG TTA T	HEX	AY667611	55	(AAAC)$_7$	186	1	282	16	0.0	0.0
Aop2 R	AAG AAT GCT CAA ATC TAT AC										
Aop6 F	CTT GGG AAA ATG AGA TAC	6-FAM	AY667612	60	(ACAG)$_5$	289	ND				
Aop6 R	*CAGTCGGGCGTCATCA* TCA ATC CAT CTA CCT TTA TC										
Aop7 F	GCC TGT TTC CTG AGA C	HEX	AY667613	55	(AG)$_{24}$	158	ND				
Aop7 R	*GGAAACAGCTATGACCATG* CCC AGA GTT AGG ATC AC										
Aop25 F	AGC CAA AGA AAT CTA CTC	HEX	AY667614	60	(GT)$_7$	136	ND				
Aop25 R	*GGAAACAGCTATGACCATG* AAT CCT TTT TTG TAA CAC T										
Aop29 F	CAG CGC CCA TTT ACT C	HEX	AY667615	60	(AAAC)$_6$	167	ND				
Aop29 R	*GGAAACAGCTATGACCAT* GCAATTAGAGGCTTTCAGT										
Aop31 F	*CAGTCGGGCGTCATCA* GGC TTG TCT CCA GTT GT	6-FAM	AY667616	60	(AAAG)$_{11}$	153	ND				
Aop31 R	TGA TTT TTC CTC TTT CAC TAC										
Aop36 F	CCT TGT GTG GAC ATG AT	HEX	AY667617	60	(GAGT)$_{11}$	125	ND				
Aop36 R	*GGAAACAGCTATGACCAT* GAA CCA AAA TAA CAG GAG TT										
Aop37 F	*CAGTCGGGCGTCAT* CAG AGG GAA ATC TAA TAA C	6-FAM	AY733034	60	(AG)$_{21}$	253	ND				
Aop37 R	GAT AAG CAC ATA GAA ATA ATA C										
Ata7 F	TCA CCT TTG CCT GTA AC	HEX	AY667618	55	(AAAC)$_5$	187	2	188-192	15	0.13	0.33
Ata7 R	*CAGTCGGGCGTCATCA* GGT ATT GCT TCT CTC ACT GT										
Ata14 F	TTG GGA AAC TGC TGA G	6-FAM	AY667619	60	(AC)$_9$	120	5[†]	130-142	11	0.46	0.72
Ata14 R	*CAGTCGGGCGTCATCA* GAG GCA GAA GTT CAT ATT A										
Ata15 F	*CAGTCGGGCGTCATCA* GTT CCT TCT CCT CTT CTA T	6-FAM	AY667620	60	(CT)$_{13}$	229	5	238-278	15	0.40	0.66
Ata15 R	GGA AAG AAA GCA TGT AGA										
Ata17 F	*CAGTCGGGCGTCATC* AAC AGA TCA CCT CAG ACA	6-FAM	AY667621	60	(AC)$_8$	146	5	155-185	11	0.55	0.72
Ata17 R	GAT GAA TAC TAT GTA ACC AGA TAA										
Ata18 F	GCA TAG GAT ATT GGA CAC	HEX	AY667622	60	(CT)$_{21}$	148	6	153-175	11	0.64	0.73
Ata18 R	*GGAAACAGCTATGACCA* TGA TTT ACC AGA ACT TTC TA										

Primer	Sequence										
Ata19 F	*CAGTCGGGCGTCATCA* GGA TCT TTT TGT GTA TCT AC	6-FAM	AY733035	60	$(AG)_{10}$*	266	12	227-318	16	0.81	0.93
Ata19 R	TTC CGT CAA TAC AAT TAC										
Ata29 F	*GGAAACAGCTATGACCATG* ACC TCT GCG AGA ACT AC	NED	AY733036	55	$(CTTT)_9$…$(CTTT)_{18}$	286	15	108-306	16	0.56	0.91
Ata29 R	CCA GTG ACC TTA GAT AGA C										
Ata32 F	*CAGTCGGGCGTCAT* CAG AAA AAC AGA AAA AGT CA	6-FAM	AY733037	65	$(AG)_{22}$	238	11	162-260	16	0.75	0.87
Ata32 R	GGC AAC AAA TAA TCA ACT C										
Ata34 F	*CAGTCGGGCGTCATCA* TGG TGT GTT TTC TTA GTG	HEX	AY733038	55	$(AG)_{33}$	191	8	152-232	15	0.60	0.76
Ata34 R	CCA ACT ATC AAC CAA CTA TG										
AmaD42 F	GAT GGA AAA TCA ATC AAG TGT G	NED	AF520749	60	$(TAGA)_4$…$(TAGA)_3$	ND	7	129-161	15	0.40	0.82
AmaD42R	TAA CTA GCT GTC AAT CGC TCT C										
AmaD95 F	AGC GCT AGA TAC CTC TC GG	NED	AF520751	60	$(TAGA)_8$…$(TAGA)_{14}$	ND	ND				
AmaD95 R	TAT TGC ATG TGA ATA TCG ATG G										
AmaD321 F	GAT GCC TTG AAA CTT GTT CTT C	6-FAM	AF520758	60	$(TATC)_{14}$	ND	ND				
AmaD321R	TGG TGC ATC TAT ATT CCT CAA G										
AmaD328F	CCC CAG TTT GTT TGT TTT GTA G	6-FAM	AF520759	60	$(TAGA)_{15}$	ND	ND				
AmaD328R	ATG ACC CTT CCA GCT AAT ACA G										
AjeD23 F	AAA ACC TCT GGA GAA ACA TGA G	NED	AY091795	60	$(TAGA)_{14}$	ND	7	198-258	15	0.80	0.78
AjeD23 R	GAA CAC AGG CTA CTA ACA ACA GG										
AjeD162 F	AAA TGT TCC AAC CAG TCA CAA C	NED	AY091802	60	$(TAGA)_{13}$	ND	ND				
AjeD162 R	GAT TAA GCT AGA GGG CTT GTC C										
AjeD422 F	CAA GGT GCT CAA GTT ACT GTT C	6-FAM	AY091811	60	$(TAGA)_{12}$	ND	ND				
AjeD422 R	CAA ATT CTG TAC CTG ACT GCT G										

Loci with blanks in the last four columns did not amplify *A. talpoideum* DNA consistently.

Tags for universal fluorescent primer binding are in italics.

Aop denotes primers designed for *Ambystoma opacum*, Ata for *A. talpoideum*, Ama for *A. maculatum*, and Aje for *A. jeffersonianum*.

Touchdown Temperature - initial annealing temperature of the thermal regime (see text).

Size of cloned allele - length in bp of PCR product amplified from the initial bacterial culture.

H_0 - observed heterozygosity.

H_E - expected heterozygosity.

* All repeats in clone: $(AG)_6\ldots(AG)_7\ldots(AG)_{10}\ldots(AG)_8\ldots(TGAG)_4(AG)_7\ldots(AAAT)_5$.

ND – not determined.

† A non-specific band appears at 116 bp in each individual.

of null alleles. Two other loci (AmaD42 and Ata29) were nearly deviant from HWE in the *A. talpoideum* population (p<0.008 in each). These microsatellite markers will likely prove useful in future research concerning the population and evolutionary ecology of these species.

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Chapter 2

Low frequency of multiple paternity in a terrestrially breeding salamander and a

comparison of analytical techniques for half-sib progeny arrays

Abstract

The prevalence of female multiple mating in natural populations is important for many questions

in mating system evolution. Several statistical techniques use genetic data to estimate the

number of fathers represented in broods, but they have not been widely compared to assess the

magnitude of differences in their estimates. We used four microsatellite loci to investigate the

extent and frequency of multiple paternity in an unmanipulated marbled salamander (*Ambystoma

opacum*) population in South Carolina, USA. Marbled salamanders offer a good system for such

a study because females attend their egg clutches during the embryonic period, allowing

confident assignment of maternal genotype. We assayed 32 hatchlings and the attendant female

from each of 13 clutches and used three analytical approaches to estimate the number of males

contributing to broods: (1) allele counting, (2) parental reconstruction, and (3) computer

simulations based on population allele frequencies. By reanalyzing data from three recent

mating system studies with salamanders, we show that choice of analysis technique can

drastically affect estimates of sire number. Some computer simulation approaches yield

estimates nearly double those of parental reconstruction, which conformed exactly to results

obtained from strict paternity exclusion of potential sires in an experimental context. In contrast

to other salamander species, most clutches (54%) were not sired by multiple males, but most

multiply sired clutches had at least three fathers (67%). We discuss potential ecological

explanations for the low frequency of multiple paternity in marbled salamanders relative to other salamander species.

Introduction

Assessing the degree of multiple paternity in populations is important in behavioral ecology and evolution, population genetics, and conservation biology (Chesser & Baker 1996; Jennions & Petrie 2000; Bretman & Tregenza 2005). Female promiscuity may lower extinction risk via its influence on effective population size (Sugg & Chesser 1994; Newman & Pilson 1997; Martinez *et al*. 2000), cause extreme sexual selection among males, even in species with socially monogamous mating systems (Griffith *et al*. 2002), and lead to speciation via sexually anatagonistic coevolution (Arnqvist *et al*. 2000). Differences in the extent of polyandry, both within and among species, can help elucidate the complex relationship between ecological factors and mating system evolution (e.g. Emlen & Oring 1977). Many such investigations require accurate estimates of the number of males that contributed to progeny arrays (i.e. broods, clutches, or litters). For example, studies of sexual selection, sperm competition, and cryptic female choice require specific information about the number of males with which females mate (Birkhead & Moller 1998; Jones *et al*. 2002b, 2004).

Studying female mating behavior has been simplified by the use of genetic data. Genetic analysis of sibling arrays generally seeks to answer two main questions. First, what is the frequency of multiple mating among females? Second, how many males fathered each clutch? The former question is easier to answer and can be done so without complex statistics. For example, older studies classified broods as multiply sired if any locus yielded five or more alleles (e.g. Kellogg *et al*. 1998). However, allele sharing and/or homozygosity among fathers can

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potentially obscure multiple paternity, even when markers are highly polymorphic. To

ameliorate this problem, Neff *et al*. (2002) developed a Bayesian model and a computer program

for estimating the frequency of multiple mating that incorporates population genetic parameters.

Also, Kichler *et al*. (1999) created a likelihood-based computer program to estimate the

frequency of multiple paternity, designed especially for instances when sample sizes are low.

Although the identification of broods or clutches of offspring that were sired by more

than one male can be relatively simple and accurate when marker polymorphism and sample

sizes are high, assessing the number of males that contributed to a sibship is analytically more

challenging. This is especially true when sampling effort is limited and the distribution of

paternity among competing males is highly skewed. Several different statistical approaches have

been used to estimate the number of sires that produced a clutch. The easiest method, known as

allele counting (Bretman & Tregenza 2005) or the single-locus minimum method (Myers &

Zamudio 2004), is very conservative in its estimates because it does not consider multilocus

allele associations or population allele frequencies. Basically, the paternal alleles are tallied at

each locus and the highest number is divided by two and rounded up. The related multilocus

minimum method (e.g. Fiumera *et al*. 2001) is less conservative but also does not incorporate the

probability of allele-sharing and can require prohibitively large sample sizes when many loci are

used (Jones 2005). Emery *et al*. (2001) developed a more sophisticated technique that uses a

Bayesian approach of modeling the probability that the data resulted from a range of sire

numbers, given specified information about the population and mating system. Neff *et al*.

(2000a) independently developed probability models for estimating sire number, but they require

knowledge of the genotypes of some putative parents. DeWoody *et al*. (2000a) wrote computer

programs that simulate progeny arrays resulting from known parent combinations, allowing

statistical evaluation of the estimates. Jones (2001, 2005) developed programs that reconstruct parental genotypes from progeny data. Bretman and Tregenza (2005) calculated relatedness coefficients among offspring which allowed an estimation of the effective number of mates of adult females.

Each of the numerous statistical approaches has unique strengths, weaknesses, and limitations. Although some studies have evaluated the performance of certain techniques (e.g. DeWoody *et al*. 2000b; Fiumera *et al*. 2001; Jones 2005), rarely has this been done by independent researchers and very few have reported comparisons of results yielded by different statistical approaches to the same data. Using seven cricket broods, Bretman and Tregenza (2005) did compare the estimates of sire number yielded by the conservative single-locus allele counting method, parental reconstruction, and Bayesian probability models used in the program PARENTAGE. They found that allele counting yielded an estimate of 1-4 fathers lower than parental reconstruction and probability models for five multiply sired clutches. Parental reconstruction estimated 1-2 fewer fathers than the modeling approach. In a simulation study, Fiumera *et al*. (2001) showed that the multilocus allele counting method underestimated the true number of sires by 1-4 and that the estimates of their programs were more accurate. Clearly, different statistical analyses have the potential to profoundly impact our interpretation of data.

Salamanders offer several advantages in studying the mating system evolution. Because females completely control spermatophore transfer, the potentially confounding influence of male sexual coercion is nullified (Clutton-Brock & Parker 1995). Most groups have internal fertilization and females possess spermathecae for storage of sperm from several males (Sever 2002). We already know that several salamander species are polyandrous (e.g. Gabor & Halliday 1997; Gabor *et al*. 2000; Garner & Schmidt 2002; Myers & Zamudio 2004; Adams *et*

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al. 2005; Gopurenko *et al.* in press; J.D. Krenz, pers. comm.), but many of these studies were performed in the laboratory and unable to assess natural patterns of multiple mating. No detailed comparisons have been made among species.

The purpose of this study is to report female mating patterns in a previously unstudied salamander species and compare several techniques for analyzing half-sib progeny arrays. We collected marbled salamander (*Ambystoma opacum*) egg clutches and attendant females from a natural population and genotyped females and a sample of their offspring with microsatellite DNA loci. We report estimates of the frequency of multiple paternity, the number of sires contributing to each clutch, and the reproductive skew among competing fathers. To assess the importance of choice of statistical technique for analyzing such data, we performed four analyses: (1) allele counting, (2) parental reconstruction, (3) a Bayesian probability model, and (4) computer simulations based on population allele frequencies. We also present new analyses of previously published data from other salamander species and discuss potential explanations for the comparatively low multiple mating rate we observed among marbled salamander females.

Materials and methods

Study species

Marbled salamanders (*Ambystoma opacum*) breed in the fall at temporary wetlands. Courtship is terrestrial and often occurs away from the breeding site (Krenz & Scott 1994). After mating, females construct terrestrial nests under vegetative cover and remain with their eggs after oviposition for variable time periods. When nests are flooded during seasonal rains, hatchlings emerge from eggs and develop aquatically until metamorphosis. Because marbled salamanders sometimes breed on only a few nights during the fall season (D.A. Croshaw,

unpublished data), they can be considered explosive breeders. Males court females and deposit

spermatophores on the ground. Females are free to choose whether they participate in courtship

or accept spermatophores.

Sample collection

In November 2002, we collected 15 egg clutches and their mothers at Okie's Bay, a

wetland on the U.S. Department of Energy's Savannah River Site in Aiken County, South

Carolina. We held eggs in the laboratory for two to three weeks while embryos developed to the

point of hatching. We then hatched the eggs by submersing them in well water and collected a

sample of hatchlings from each clutch for subsequent microsatellite genotyping.

Microsatellite genotyping

We genotyped 32 hatchlings and the attendant females from each of the 15 clutches at

four microsatellite loci from Chapter 1. The overall exclusion probability, with one parent

known, and other basic properties of the loci were calculated with Cervus 2.0 (Table 1; Marshall

et al. 1998). Our PCR and genotyping protocols were described in detail in Chapter 1. Products

from each of the four loci were run together in a single lane on an ABI 377-96 automated DNA

sequencer. We scored alleles using Gensize Rox 500 ladder (Genetix) or CXR ladder (Promega)

and Genescan 3.1.2 and Genotyper 2.5 software (PE Applied Biosystems).

We visually inspected each of the progeny arrays to determine their compatibility

with the attendant female. In two of the 15 clutches, not all hatchlings were consistent with the

genotype of the attendant, indicating that they were most likely communal nests with offspring

from one or more unsampled females present. Because of the difficulty of analyzing such

Table 1. Basic properties of four microsatellite DNA loci for *Ambystoma opacum* used in this study (Chapter 1). Data are based on a sample of 110 adults. Exclusion probabilities are with one parent known (total is 0.994).

Locus name	Number of alleles	Observed heterozygosity	Expected heterozygosity	Exclusion probability
Aop31	16	0.87	0.83	0.65
AjeD162	15	0.89	0.90	0.78
AmaD321	11	0.85	0.84	0.67
AmaD328	13	0.91	0.88	0.75

broods, we did not consider these clutches further. For the other 13 clutches that were entirely full or half siblings, we tallied the frequency of each paternal allele. To minimize the impact of potential scoring errors on our interpretation of the data, individuals that contained rare paternal alleles were assayed again for confirmation. Many scoring errors were corrected in this way.

Statistical analysis of marbled salamander clutches

We analyzed the data in four ways (Table 2). First, we used single-locus allele counting, the most conservative technique for estimating the number of sires. Maternal alleles were first excluded before counting the number of remaining paternal alleles at each locus. Although identifying maternal alleles is problematic when offspring and mothers have identical heterozygous genotypes (Fiumera & Asmussen 2001), we employed the most conservative interpretation of such individuals, only counting one unique paternal allele even when identical individuals were common in an array. In this method, the number of sires is equal to half the number of paternal alleles at the most polymorphic locus, rounded up. Although this analytical approach provides the minimum number of males that sired a brood, it does not consider the probability of allele sharing among sires, nor does it incorporate informative multilocus allele associations. Thus, the allele counting method probably often underestimates the true extent of polyandry, especially when loci are not extraordinarily polymorphic.

We also analyzed the data with parental reconstruction which employs the multilocus genotypes of offspring in a brood to determine the genotypes of the parents. Although this technique is conservative in that it does not incorporate the probability of allele-sharing among fathers of a clutch in its estimates of sire number, it is less so than the single-locus allele counting method because it uses associations of alleles across loci. Jones (2001, 2005) created

Table 2. Summary of the four analysis techniques used in this study.

Method	Reference(s)	Computer program(s)	Advantages	Disadvantages
Allele counting	Bretman & Tregenza 2005	None	Easy to perform Minimizes false positives	Often underestimates polyandry Does not consider allele sharing among sires, allele associations, or failure to sample offspring from all sires
Parental reconstruction	Jones 2001, 2005	GERUD 1.0, 2.0	User friendly Conservative, but considers allele associations	Does not consider allele sharing among sires or failure to sample offspring from all sires
Computer simulations	Dewoody *et al.* 2000a	HAPLOTYPES GAMETES COUNT(s)	Considers probability of allele sharing among sires and failure to sample offspring from all sires	Extremely liberal estimates when sampling effort is low Arbitrary choices cause excessive variation
Bayesian probability model	Neff *et al.* 2002	f_{mm}	Incorporates mutations Uses prior probability distribution for multiple mating	Only estimates frequency of multiple mating Inflexible

computer programs that use an exhaustive algorithm for parental reconstruction when one (GERUD 1.0) or neither (GERUD 2.0) of the parents is assayed. We used GERUD 1.0. Each version of GERUD provides an estimate of the number of offspring sired by individual males and ranks potential solutions by their probability based on the rules of Mendelian inheritance and population allele frequencies. GERUD indicated many potential combinations of paternal genotypes for each of our multiply sired clutches. For estimates of reproductive skew within clutches, we averaged the parental contribution estimates of the most likely paternal combinations up to the arbitrary cutoff of priority scores that were one order of magnitude larger than the lowest priority score, i.e. the most likely paternal combination. We report averages of these estimates for all double-sire and triple-sire clutches.

We also used the program of Neff *et al*. (2002) to estimate the frequency of multiple paternity in our clutches. This method uses a Bayesian model that incorporates the number of loci in the analysis, their allele frequencies, the maternal genotype, the number of paternal alleles in each sibship, and the prior probability of multiple mating. It is limited by the need for assaying the shared parent of each brood, and it only calculates the overall frequency of multiple mating (f_{mm}), not the number of individuals contributing genetically to each array. However, this method calculates confidence intervals of f_{mm} and uniquely incorporates independent information about the probability of female polyandry. Because we had no such information available, we used a uniform prior probability distribution. The program also requires information about the number of sires contributing to polyandrous broods and their relative fertilization success. Because it does not consider the potential for multiple numbers of sires, we analyzed the data separately with two and three sires and the observed skew estimates from GERUD (Table 3).

Table 3. Parameters used for simulations in HAPLOTYPES and GAMETES. We used exact sample sizes for all clutches, exact clutch sizes for *D. ocoee*, and average clutch sizes for the other species. Skew numbers are in percent, rounded to the nearest integer. Standard deviations of observed skew are in parentheses, calculated from Figure 2 in Adams *et al.* (2005) for *D. ocoee*. We modeled skew as a geometric distribution in which the most successful male sires a proportion of total offspring (α) and each subsequent male sires the same proportion of the remaining progeny.

Species	Reference	Pop. size	No. of clutches	Clutch size	Sample size	Max. # sires	Observed skew	Modeled skew
Ambystoma opacum	This study	520[a]	13	70	32	6	2 sires: 78 (5), 22 (5)	6 sires: $\alpha = 0.67$
							3 sires: 61 (10), 26 (7), 13 (4)	
Ambystoma maculatum	Myers & Zamudio 2004	1700	6	50-100	7-51	8	2 sires: 62 (6), 38 (6)	8 sires: $\alpha = 0.48$
							4 sires: 41 (10), 29 (4), 18 (7), 12 (7)	
Taricha granulosa	Jones *et al.* 2004	14[b]	57[d]	170	11-49	8	2 sires: 82 (10), 18 (10)	8 sires: $\alpha = 0.79$
							3 sires: 71 (11), 19 (10), 10 (4)	
Desmognathus ocoee	Adams *et al.* 2005	5000[c]	26[d]	8-31	8-31	6	2 sires: 80 (9), 20 (9)	6 sires: $\alpha = 0.70$
							3 sires: 50 (8), 33 (9), 17 (4)	
							4 sires: 47, 26, 16, 11[e]	

a estimated from unpublished drift fence data

b average size of breeding populations in experimental tanks

c within the range of population size estimates of salamander species of similar size in the same region and habitat type

d did not analyze 1-2 clutches that were unintelligible in data files provided by original authors

e only data for one clutch reported

DeWoody *et al.* (2000a) developed a more sophisticated technique for analyzing half-sib

progeny arrays that incorporates computer simulations based on parameters specific to the

sampling regime and population under study. Researchers can specify the number of loci, the

allele frequencies at each locus, breeding population size, maximum number of unshared fathers

in a single brood, clutch size, sample size, and parentage distribution among the unshared

fathers. The computer programs GAMETES and HAPLOTYPES randomly select mating

combinations from a simulated population based on these user-defined parameters and Hardy-

Weinberg expectations. They then generate clutches of offspring based on Mendelian

inheritance and sample randomly from them before tallying the number of unique gametotypes

represented in the sample. GAMETES uses the single most polymorphic locus; HAPLOTYPES

uses multilocus data. The number of unique gametes from the most informative locus or

multilocus haplotypes is counted for each simulated clutch of known numbers of parents.

Repeated sampling from both the simulated parental population and each simulated mating

combination results in a statistical distribution of haplotypes or gametes detected for each

number of unshared parents. The programs then invert this process, creating a statistical

distribution for the number of parents that is associated with the number of gametes or

haplotypes detected. Output from the programs includes standard descriptive statistics such as

mean, mode, standard deviation, range, and confidence intervals of the true number of unshared

parents. Finally, researchers are able to match the number of haplotypes or gametes detected in

their real clutches to data from these simulated arrays.

To determine the number of haplotypes or gametes represented in our marbled

salamander clutches, we used three programs written by Fiumera *et al.* (2001). Because this task

can be ambiguous when the mother and some offspring have identical heterozygous genotypes

(Fiumera & Asmussen 2001), they created three programs, COUNT_LOW, COUNT_MED, and COUNT_HIGH in order of most to least conservative. COUNT_LOW treats these problematic cases as missing data, COUNT_MED randomly chooses one of the two potential alleles to be contributed by the unshared parent, and COUNT_HIGH assigns whichever allele is not detected in other offspring to the unshared parent. We used each of these three options and show that they sometimes provide very different estimates of the true number of fathers.

Simulations in HAPLOTYPES and GAMETES require the user to define the maximum number of sires per clutch, which we somewhat arbitrarily defined as six, double the highest minimum number observed in this study (see results). Six is fewer than reported as a likely maximum sire number in a congener (Myers & Zamudio 2004). Because unequal sharing of paternity among these competing sires can impact estimates of sire number (Myers & Zamudio 2004), we incorporated reproductive skew in our simulations. Observed inequality estimates from the parental reconstruction analysis (Table 3) indicated that skew roughly fit that expected from a geometric distribution defined by the following equation:

$$\text{proportion of progeny sired by } j\text{th father} = \alpha(1-\alpha)^{j-1},$$

where α is the fraction sired by the first, i.e. most successful, male. For α in computer simulations, we used the weighted average among all clutches of the most successful sire's fertilization success (Table 3).

Reanalysis of other salamander data

We used these same four analysis techniques on data reported for spotted salamanders (*Ambystoma maculatum*) in Myers & Zamudio (2004), rough-skinned newts (*Taricha granulosa*) in Jones *et al*. (2004), and Ocoee salamanders (*Desmognathus ocoee*) in Adams *et al*. (2005).

The data from Jones *et al*. (2004) offer a good opportunity to evaluate the performance of different types of analyses because their clutches were produced by females from breeding replicates that had access to only eight males. They assigned paternity to each offspring in their sample, thereby allowing an extremely accurate estimate of the true number of males that sired each brood.

The computer algorithm used by GERUD becomes so computationally intensive with more than four loci that even modern computers often have trouble performing the task. Because the data from Myers & Zamudio (2004) consisted of 10 loci, we were unable to use all the data in any single analysis employing parental reconstruction. Because GERUD does not accept loci with null alleles, missing data, or individuals that are incompatible with a single shared parent of the brood, we deleted data that violated these constraints. Of the remaining loci, we chose the two or three most polymorphic in each clutch to use in parental reconstruction. We included these markers in all analyses along with all combinations of the remaining, less polymorphic loci for a total never to exceed four. Exchanging equally polymorphic loci never resulted in a change in the estimate of sire number. Of their seven clutches, Myers & Zamudio (2004) reported that one was the product of two different females, and we did not reanalyze this clutch.

The computer program written by Neff *et al.* (2002) only allows 25 alleles per locus. To decrease the number of alleles for especially polymorphic loci in our new analyses, we were forced to group the lowest frequency alleles with alleles of similar size. As for the marbled salamander data, we analyzed the data separately with all numbers of sires and their relative success that we observed from the parental reconstruction analyses (Table 3). Because this program requires specific knowledge of the shared parent's genotype, we did not use it to analyze the spotted salamander data from Myers & Zamudio (2004).

We relied on observed data for specifying the parameters in HAPLOTYPES and

GAMETES (Table 3). For estimates of the distribution of paternity among competing sires, we

used the GERUD output for *Ambystoma maculatum*. Because mothers were not sampled, we

used GERUD 2.0 and randomly chose one of the combinations of loci to use in estimates.

Again, we averaged paternity contributions of all potential solutions that had priority scores

within one order of magnitude of the most likely solution and averaged these estimates for

clutches sired by two and four males. For the *Taricha granulosa* data, we used paternity

assignment based on strict exclusion to obtain estimates of skew.

We followed Myers & Zamudio (2004) who simulated large (100) and small (50) clutch

sizes in their analysis and deleted all loci containing null alleles for the *Ambystoma maculatum*

data. To choose the maximum number of sires possible in a clutch, we simply increased the

highest observed number in *T. granulosa* (five; Jones *et al*. 2002b) and *D. ocoee* (four) by a few

sires. We conformed to the choice of Myers & Zamudio (2004) of eight possible sires for *A.*

maculatum.

Results

Of the 13 marbled salamander clutches analyzed, only five (38%) were definitely sired by

multiple males as indicated by the allele-counting minimum method (Figure 1). Analysis with

parental reconstruction added one multiply sired clutch for six total (46%). The conservative

COUNT_LOW method of tallying the number of unique haplotypes or gametes yielded similar

results with computer simulations, either four (31%) or seven (54%) multiply sired clutches,

depending on whether the mode or mean number of sires is used (data not shown). Using the

COUNT_MED (Figure 1) or COUNT-HIGH method greatly increased the frequency of multiple
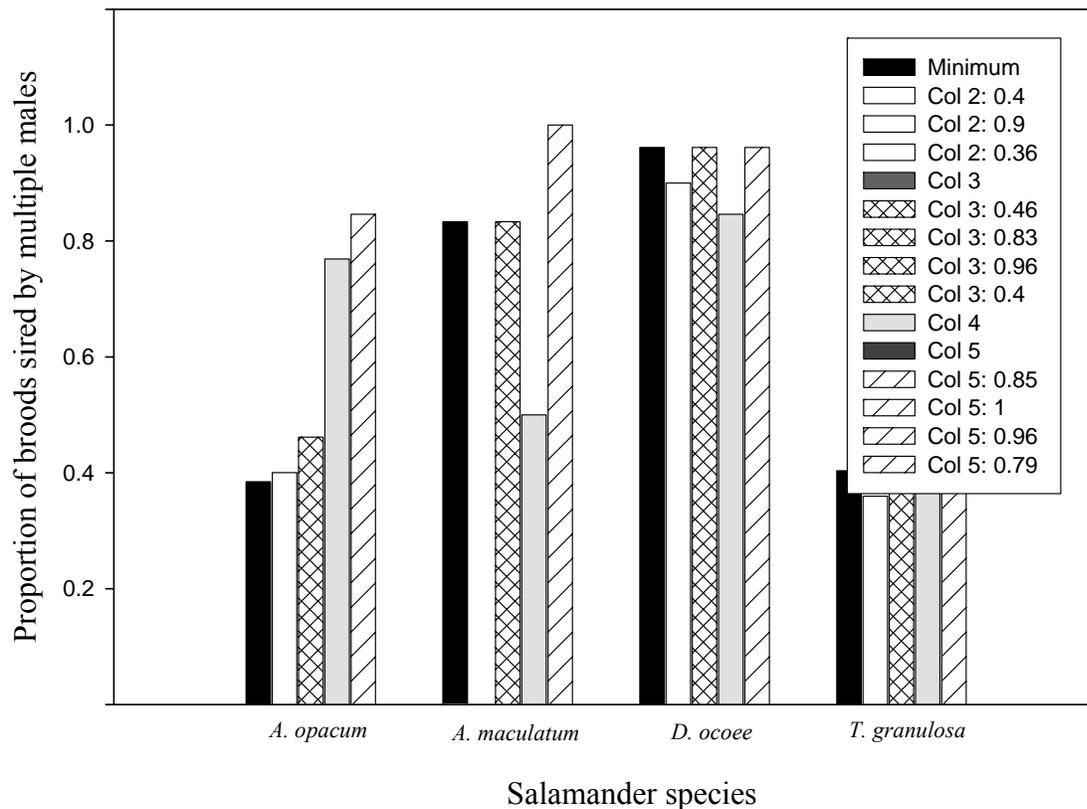
Figure 1. Frequencies of multiple mating for four salamander species estimated with five different analytical techniques: 1) allele-counting minimum method, 2) Bayesian probability models (f_{mm}), 3) parental reconstruction with GERUD 1.0 or 2.0, 4) computer simulations with HAPLOTYPES, and 5) computer simulations with GAMETES. Data are the number of broods classified as multiply sired divided by the total number of broods analyzed. For HAPLOTYPES and GAMETES, estimates are based on the COUNT_MED method of determining the number of unique haplotypes and gametes. The mode number of sires from computer simulations was used to categorize clutches as having single or multiple paternity. *Taricha granulosa* clutches were from a mating experiment in which animals were allowed access to a limited number of potential mates (Jones *et al*. 2004).

mating to 69-92%.

The minimum allele-counting method, parental reconstruction, and Neff et al.'s (2002) Bayesian probability model (but Bayesian not done for *A. maculatum*) all gave estimates of the frequency of multiple mating that were much higher in *A. maculatum* (83-100%) and *D. ocoee* (90-96%) than in *A. opacum* (38-46%). Estimates for *T. granulosa* were similar to those for *A. opacum* (36-40%), although the data were collected from experimental breeding tanks in which adults had limited mating opportunities.

Using the minimum number of sires for each clutch determined by parental reconstruction (Figure 2), marbled salamander females mated with an average of 1.77 males, fewer than did spotted salamanders (2.83), Ocoee salamanders (2.58), and rough-skinned newts (2.10) in natural habitat. The difference was not apparent when only considering multiple paternity clutches (*A. opacum*: 2.67, *A. maculatum*: 3.2, *D. ocoee*: 2.64, *T. granulosa*: 2.5), indicating that this was likely due to lower frequency of polyandry in marbled salamanders. We used the FREQ procedure in SAS (SAS Institute 2003) to determine if the four salamander species were different in the extent and frequency of polyandrous mating. The frequency distributions of sire numbers were significantly different among the four species ($\chi^2 = 26.1$, DF = 9, P = 0.002). Clutches with few sires were significantly more frequent in *Ambystoma opacum* than in *A. maculatum* ($\chi^2 = 9.8$, DF = 9, P = 0.02) and *D. ocoee* ($\chi^2 = 14.8$, DF = 9, P = 0.002) but not *Taricha granulosa* (data from Figure 1 in Jones *et al.* 2002b; $\chi^2 = 3.8$, DF = 9, P = 0.28).

Parental reconstruction analysis provided the best means for estimating reproductive skew among competing sires. Relative fertilization success was significantly different from unity in doubly sired clutches of marbled salamanders (Chi-square goodness of fit tests: P < 0.0001), spotted salamanders (P = 0.02), and rough-skinned newts (P < 0.0001; Figure 3; Table 3).
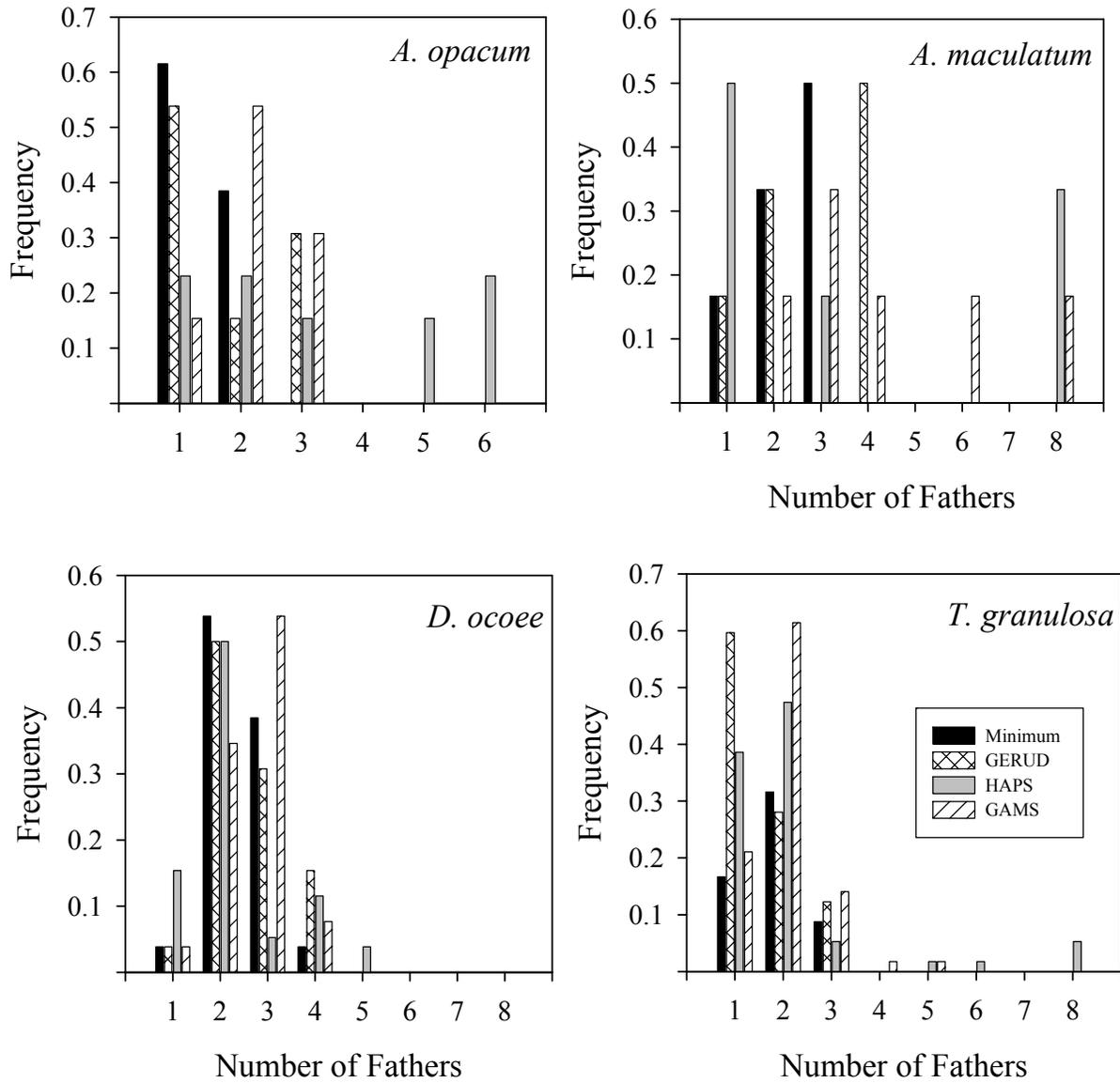
Figure 2. Frequency histogram of sire number for four salamander species estimated with four different analytical techniques: 1) allele counting minimum method, 2) parental reconstruction with GERUD 1.0 or 2.0, 3) computer simulations with HAPLOTYPES, and 4) computer simulations with GAMETES. The mode number of sires from computer simulations was used with HAPLOTYPES and GAMETES, and COUNT_MED was used to determine the number of unique haplotypes and gametes.

Clutches sired by three males also showed significant skew in *Ambystoma opacum* (P < 0.0001) and *Taricha granulosa* (P < 0.0001), and those sired by four clutches were skewed in *A. maculatum* (P = 0.0002). Patterns of fertilization success among competing males within three- and four-sire clutches of *A. opacum* and *A. maculatum* (Figure 3) conformed to a geometric distribution in which the most successful male sires a certain proportion of the brood and each subsequent male sires the same proportion of the remaining progeny (P = 0.36 and P = 0.84, respectively). Similar analyses in *T. granulosa* (P = 0.21) and *D. ocoee* (Adams *et al.* 2005) were also consistent with this model (Table 3). However, there was considerable intra- and interspecific variation in the value of α, or the proportion of the clutch sired by the most successful male.

We used ANOVA contrasts in SAS to test for within-species differences in estimated sire number among some analysis techniques (minimum allele-counting, GERUD, mode sire number from HAPLOTYPES with COUNT_LOW, MED, and HI, and mean sire number with LOW, Figure 4). We did a repeated measures analysis because each clutch was analyzed multiple times with different techniques. For *T. granulosa*, results from GERUD agreed exactly with paternity assignment of all sampled hatchlings with strict exclusion. In general, HAPS_MED and HI yielded extremely high estimates. HAPS_LOW, both with mode and mean, GERUD, and allele-counting gave lower estimates. The different analysis techniques yielded relatively uniform estimates when using the *D. ocoee* data, which was not the case for the other three species. HAPLOTYPES (Figure 4) and GAMETES both calculated extremely wide 95% confidence intervals (CIs), although GAMETES generally resulted in more narrow CIs than HAPLOTYPES. The upper limit of CIs was frequently equal to the defined maximum number of sires (i.e. 6 or 8), sometimes when the other analyses indicated single paternity.
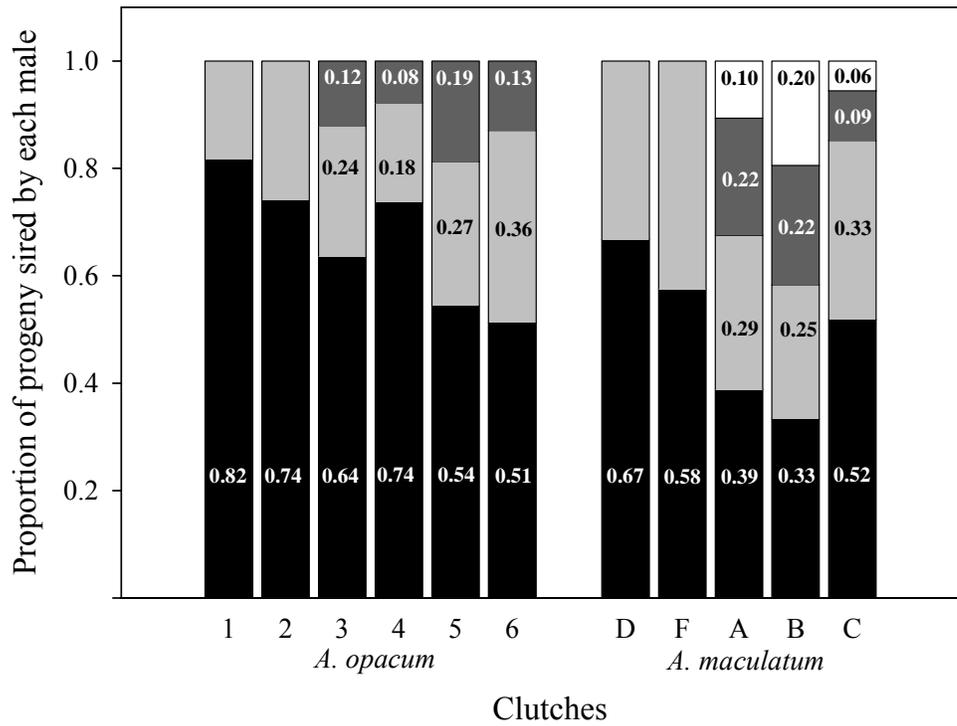
Figure 3. Observed paternity distributions for six *Ambystoma opacum* clutches and five *A. maculatum* clutches with multiple fathers. Estimates were obtained with either GERUD 1.0 (*A. opacum*) or GERUD 2.0 (*A. maculatum*), which report potential parental genotypes and the number of progeny compatible with each sire.
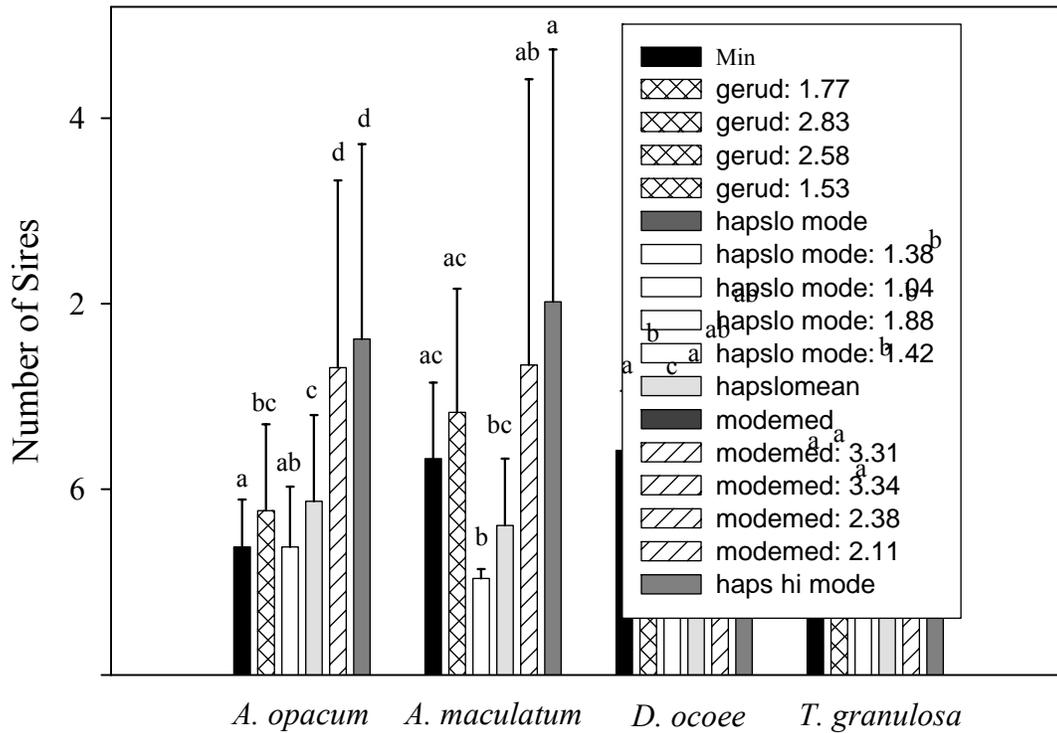
Figure 4. Mean (± SD) number of sires per clutch estimated by six analysis techniques for four species of salamander. Min: minimum allele-counting method, GERUD: parental reconstruction with GERUD 1.0 or 2.0, HAPS_LO: mode number of sires estimated in HAPLOTYPES combined with COUNT_LOW, HAPS_LO mean: mean number of sires estimated in HAPLOTYPES combined with COUNT_LOW, HAPS_MED: mode number of sires estimated in HAPLOTYPES combined with COUNT_MED, HAPS_HI: mode number of sires estimated in HAPLOTYPES combined with COUNT_HI. Letters above each bar indicate which estimates are significantly different at the 0.05 level in within-species analyses.

The COUNT programs prompt the user to define the maternal genotype when tallying the number of gametotypes found in broods. When mothers are not sampled, as in our analysis with spotted salamander data, numerous maternal genotypes are sometimes compatible with the data set. To determine the magnitude of differences yielded by these competing solutions, we ran the COUNT programs with all possible maternal genotypes (for five clutches) or with nine of 72 possible genotypes (one clutch). We found considerable within-clutch variation in mean and mode sire number when different maternal genotypes are chosen with HAPLOTYPES and GAMETES (data not shown). Ranges of sire number estimates for single clutches were as high as 1.6 (mean) and 7 (mode).

Discussion

This study shows that choice of statistical technique can have major impacts on the interpretation of data from half-sib progeny arrays. Some of the techniques are consistently conservative or liberal in their estimates of the number of fathers that contributed to salamander clutches. Significant differences in estimated sire number were given by these analytical methods, even though the same data were used for all analyses. Had the original researchers simply used another analytical technique, their papers would have made drastically different conclusions. Our analyses underscore the importance of carefully choosing analytical methods and the need for further statistical and empirical evaluation of existing techniques.

As expected, the minimum allele-counting method was the most conservative approach. Estimates made with parental reconstruction performed by the computer program GERUD were not much greater than those from allele-counting. DeWoody *et al.*'s (2000a) computer simulation package was generally very liberal, often giving numbers twice as large as the most

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conservative method. The 95% confidence intervals calculated by these programs tended to be very wide and likely would have been even wider had we modeled a greater maximum number of unshared parents. Much of the uncertainty reflected in these estimates is generated by the probability that progeny from some males were unsampled, which is not modeled by allele-counting or parental reconstruction. In three of the species considered in this study, clutch sizes are very large and complete assays of broods are impractical. To measure the true numbers of fathers that produce clutches, exhaustive sampling of clutches with extremely polymorphic markers is necessary.

Because we cannot know for certain the true number of males that contributed to these clutches, it is impossible to definitively rank the statistical approaches by accuracy. Analysis of the rough-skinned newt data, however, suggests that parental reconstruction is the best choice because GERUD agreed exactly with paternity assignment. Females only had access to eight males and each offspring was assigned to a father via strict exclusion. Although we cannot rule out the possibility that offspring from some males went unsampled, such an outcome is unlikely to equalize parental reconstruction with the much more liberal computer simulation programs. Therefore, of the techniques we used, we recommend GERUD, especially when a large proportion of progeny in clutches are sampled. Computer simulations in BROOD can estimate the sample size necessary for specific studies (DeWoody *et al.* 2000a).

GERUD is also the best program available for estimating relative fertilization success among competing sires, when paternity assignment via strict exclusion is impossible. It is very easy to use, provides unique output of possible parental genotypes, and ranks them by probability. It also offers simulations, in GERUDSIM 1.0 or 2.0, to assess the probability of reconstructing the correct solution. GERUD is not without its weaknesses, however. It cannot

accommodate mutations, scoring errors, null alleles, or missing data. None of the programs considered herein have good solutions to these problems. GERUD also does not perform well with more than four loci, which may preclude its utility when markers are not very polymorphic.

There were no consistent differences between the estimates of HAPLOTYPES and GAMETES, although there were a few occasions where the estimates of one were significantly greater than those of the other (results of analyses not shown). Choice of COUNT_LOW, MED, or HI is extremely important. MED and HI are usually not much different from one another but LOW tends to be much more conservative, either about the same as GERUD or considerably more so. The mean number of sires in computer simulation programs was consistently higher than mode. Estimates gained with LOW and mode sire number were closest to the number of sires resulting from strict exclusion with rough-skinned newts, although they were much lower than parental reconstruction estimates in the other species. Using COUNT_LOW and the mean number of sires may be a reasonable compromise, although we feel that this simulation approach is generally too imprecise for widespread use. Seemingly trivial and arbitrary issues, such as choosing among potential maternal genotypes when the mother is unsampled, the three count programs, or between GAMETES and HAPLOTYPES, have the potential to impact estimates to an unreasonable degree. In the one species that had very high sampling effort (*Desmognathus ocoee*), computer simulation results were not extremely higher than the other techniques, suggesting that choice of technique may not be as influential in cases where clutch sizes are small and sample sizes are large.

Neff *et al.*'s (2002) program for estimating the frequency of multiple mating is limited because it does not estimate the number of sires that contributed to clutches, can only simultaneously consider one potential number of males for multiply sired clutches, cannot

accommodate extraordinarily polymorphic loci, and does not easily incorporate missing data. The estimates of this program are similar to those of GERUD. We do not recommend it for most applications because GERUD can usually accomplish the same goals and is easier to use. However, in the rare case that researchers know the prior probability of multiple mating, this program is the only one which can use that information.

Comparing salamander mating systems

Our study is the first to investigate the extent and frequency of multiple mating by female marbled salamanders, although J.D. Krenz (pers. comm.) observed multiple paternity using allozyme markers. Because marbled salamanders do not have long-term sperm storage (Sever *et al.* 1995), the results demonstrate polyandrous mating by females during a single breeding season. Our data show that marbled salamanders were polyandrous to a significantly lesser extent than were spotted salamanders and Ocoee salamanders in natural mating situations (Myers & Zamudio 2004; Adams *et al.* 2005). Most analysis methods indicated that the extent of multiple paternity was also low in marbled salamanders. Clutches were very rarely, if at all, sired by more than three males, unlike the other species that have been studied. Although our sampling regime may have been unable to detect paternity by more than four sires in a clutch, the same is true for that of *A. maculatum* and *T. granulosa* (Myers & Zamudio 2004; computer simulations in BROOD, DeWoody *et al.* 2000a). Only the complete clutch sampling of Adams *et al.* (2005) was definitely sufficient to detect more than four sires. Thus, although differences in the extent of polyandry between marbled salamanders and Ocoee salamanders may reflect discrepancies in ability to detect large numbers of sires, the same explanation cannot account for differences among *A. opacum* and the other two species.

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Marbled salamanders and Ocoee salamanders mate and oviposit in terrestrial habitats, whereas spotted salamanders and rough-skinned newts do so at aquatic sites. Marbled salamanders breed only on rainy nights in autumn, which can be few in parts of their range; Ocoee salamanders breed over several months during the warm seasons of the year. Although marbled salamanders do migrate to temporarily dry wetlands for breeding, unlike Ocoee salamanders which breed in entirely terrestrial habitat, some mating occurs before they arrive (Krenz & Scott 1994; D.A. Croshaw, unpubl. data). Females may not encounter many males during their receptive periods, especially if they occur in terrestrial woodland habitat surrounding the natal pond.

Although Ocoee salamanders do not breed in mating aggregations, females probably encounter numerous males because populations can be incredibly dense (as much as 25 animals per square meter, Huheey & Brandon 1973). In contrast, our breeding population at Okie's Bay is very sparsely populated, probably not more than 0.05 breeding adults per square meter of wetland. The rough-skinned newt population studied by Jones *et al.* (2002b) contains approximately 0.79 breeders per square meter (based on pond size estimates provided by Jones *pers. comm.*). The population studied by Myers & Zamudio (2004) was estimated to be 1700 individuals at a 0.09 ha (N. Ostman pers. comm.) pond for a density of 1.83 breeders per square meter. Our marbled salamander population at Okie's Bay is probably at least 15 times more sparse than in any of the other three studies. These potential differences in encounter rate resulting from details of breeding ecology may explain the low incidence of multiple mating by females in our study population. However, only one population of each of these species has been studied and there are numerous other potential explanations. For example, marbled salamanders may limit their mating activity because of larger predation risk in terrestrial habitats. Further

work assessing multiple paternity in many populations of these and other species of salamander will enhance our understanding of the ecological factors that contribute to intra- and interspecific variation in female polyandry.

Reproductive skew in salamander clutches

This study is the first to report estimates of relative fertilization success among competing males within large salamander clutches. Adams *et al.* (2005) presented skew estimates based on a similar analysis of complete sampling of Ocoee salamander clutches which are small (9-31 eggs) in size. All four species that have been studied (*Ambystoma opacum*, *A. maculatum*, *Taricha granulosa*, and *Desmognathus ocoee*) fit a model of skew denoted by a geometric distribution in which the most successful male sires a proportion of the total progeny (α), and each subsequent male fertilizes the same proportion of the remaining eggs.

This similar pattern probably does not reflect common means of storing sperm from different males within the spermathecae of females. There are differences among salamander species in patterns of sperm precedence, and mating order is probably important in determining fertilization success of competing males. Evidence for first-male advantage exists for both *T. granulosa* and *A. maculatum* (Jones *et al.* 2002a; Tennessen & Zamudio 2003). However, *D. ocoee* and *Notophthalmus viridescens* have been reported to have mixed paternity with respect to mating order and members of the genus *Triturus*, closely related to *Taricha*, may have last-male paternity (Houck *et al.* 1985; Gabor *et al.* 2000; Sever 2002). Sever (2002) suggested that species with simple spermathecae, as presumably occurs in *Taricha* and *Ambystoma*, should have last male paternity. *Desmognathus* have complex spermathecae, facilitating interactions among

ejaculates from different males and potentially confounding order effects. No explanations exist to account for the considerable variation in α that occurs both within and among species.

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Chapter 3

Measuring sexual selection: a comparison of competing indices with mating system data

from a terrestrially breeding salamander

Abstract

Calculations for quantifying the potential for sexual selection remain controversial. Many indices have been suggestted in the literature, but each has its own unique advantages and disadvantages. Using marbled salamanders, we evaluated the performance of several measures by manipulating the strength of sexual selection in experimental breeding replicates of varying operational sex ratios. Theory predicts that sexual selection on males will be higher when the sex ratio is male-biased and lower when female-biased. We used microsatellite data to assign hatchling parentage, estimate adult fitness, and calculate several indices of inequality for quantifying sexual selection. Of five well-supported indices, opportunity for selection and Morisita's index were most likely to detect differences and always conformed to theoretical expectations. We conclude that using opportunity for selection is advantageous in sexual selection studies, but it should be tested against the null hypothesis of random variation in ambiguous cases. We used traditional statistical techniques related to Bateman's principles to study the mating system of marbled salamanders. Although opportunity for selection was higher in males than females, fitness was not related to any of six measured size traits. Nevertheless, variation in reproductive success was significantly greater than random expectations, although this was not the case for mating success. Sexual selection appears to be important in this system, but the specific traits that determine fitness variance have not been identified.

Introduction

　　Although sexual selection was first discussed over a century ago (Darwin 1871) and has been a major topic in the evolution literature for decades, there has still not been a consensus on how we should quantify its action. Most often, people have used a calculation (opportunity for selection, *I*) related to formal sexual selection theory that grew out of what came to be known as Bateman's principles (Arnold 1994), first put forth when Bateman published his pioneering research that described the mating system of *Drosophila* fruit flies (Bateman 1948). His ideas were based on the importance of fitness variance in determining the strength of sexual selection. Specifically, Bateman's principles suggest that, in any mating system, the sex that has higher variance in mating success (number of mates) and reproductive success (number of progeny) experiences more sexual selection. Also, a steep relationship between mating success and reproductive success (now commonly known as the Bateman gradient, Andersson & Iwasa 1996) is present in the sex with higher fitness variation and is essential for sexual selection to occur via traditional mechanisms of male-male competition and/or female mate choice (Arnold & Duvall 1994).

　　A fundamental quantity expressing the potential for sexual selection, the opportunity for selection (*I*), was later defined as the standardized variance in fitness and represents the upper limit of the intensity of sexual selection (Arnold & Wade 1984; Arnold 1986). *I* has come under attack over the last few decades for three main reasons. First, it does not incorporate random variation that is expected to occur even in the absence of selection. For example, Sutherland (1985) showed that Bateman's data, so important in shaping sexual selection theory during the twentieth century, can be explained by random chance alone and may not have reflected the action of sexual selection. He further showed that differences in fitness variance between the

sexes may result from simple randomness paired with sex differences in the time required for mating (i.e., gamete production, mate searching, mate handling time, etc.). Second, I is biased by differences in mean fitness (Downhower *et al.* 1987), measured as the number of mates acquired and/or the number of offspring produced. When mean fitness increases, I tends to decrease such that its expected value is the reciprocal of the mean. Such a situation is expected for any random variable with a Poisson distribution for which mean and variance are equal. Third, I is also biased by differences in group size, and larger groups tend to yield higher I (Fairbairn & Wilby 2001). In fact, the upper bound of I is equal to the number of individuals in the group.

Because of these problems, comparisons among studies and/or populations with I may be difficult and, as some have argued, perhaps invalid. Because sexual selection studies are often most useful in a broad context, several have suggested that we use other less biased measures so that comparisons can be more insightful. A litany of indices of inequality has been derived, used in various studies, and promoted as the universal best options (Ruzzante *et al.* 1996; Tsuji & Tsuji 1998; Nonacs 2000; Jones *et al.* 2002b). Generally, the new indices that are best supported deal with these problems better than I. All express fitness variation relative to random expectations, some are restricted to values below one to ameliorate group size dependence, and mean dependence may not be as severe as in I. Each of the proposed indices, however, has unique properties, and they vary considerably in their ability to resolve the problems presented by random variance and dependence on group size and mean fitness. There is no clear reason to choose any particular measure as a replacement for I, and compelling arguments for the continued use of I have been made (Jones *et al.* 2004, 2005). Most notably, it is the only potential measure that has a formal tie to mathematical sexual selection theory.

Attempts to evaluate some of these measures, both empirically (Fairbairn & Wilby 2001; Jones *et al.* 2004, 2005) and via computer simulation (Kokko *et al.* 1999; Nonacs 2003) found mixed results. All failed to include at least one index that has support in the literature. More work is needed to assess the strengths and weaknesses of these competing measures. Resolution of this disagreement is needed before a broad characterization of sexual selection in nature will be possible.

We can use parentage information gathered from genetic data to help resolve the controversy. Two recent studies experimentally manipulated the strength of sexual selection by varying sex ratios, quantified it with different indices, and compared index performance, especially whether they conformed to theoretical expectations and/or were biased by group size and mean fitness. Although Jones *et al.* (2004) found that opportunity for selection (*I*) and Morisita's index (*I*$_\delta$, Morisita 1962) yielded similar and expected patterns, they strongly argued in favor of *I* because it is tightly linked to sexual selection theory, an advantage that none of the other measures can claim. In a similar experiment, Jones *et al.* (2005) showed that *I* also performed well in a sex-role-reversed pipefish. Fairbairn & Wilby (2001), on the other hand, recommended the use of *I*$_\delta$ because it was least affected by changes in mean fitness and number of competitors. Morisita's index is also scaled to random expectations of variance whereas *I* is not. None of the comparative papers included the binomial skew index (Nonacs 2000) in their comparisons, even though it has been promoted in the literature as a good measure for all inequalities and performs well in simulation studies (Nonacs 2003).

Theoretically, changes in operational sex ratio (OSR) should alter the strength of sexual selection (Emlen & Oring 1977). When sex ratio is male-biased, sexual selection is expected to be higher among males than when it is female-biased. The same is true for females, although

they are less likely to experience sexual selection at all, in which case the pattern should not be pronounced. Some studies have shown that differences in OSR can result in changes in sexual selection (Hoglund 1989; Souroukis & Cade 1993; Grant *et al.* 1995; Fairbairn & Wilby 2001; Jones *et al.* 2004). Thus, in principle, the potential for sexual selection can be experimentally controlled with sex ratio, allowing researchers to compare the performance of different measures with empirical data. We used this approach with marbled salamanders.

Among vertebrates, salamanders offer good study systems for investigating sexual selection. In many species, adults aggregate in large numbers at pond breeding sites during a short period of the year. Females completely control spermatophore transfer so that the importance of male-male competition and male sexual coercion (Clutton-Brock & Parker 1995) are lessened relative to other animal groups. Nevertheless, because most salamander species are small in size, secretive, and difficult to observe during mating, sexual selection studies of them are rare. Laboratory and semi-natural mating experiments have investigated fitness variance (e.g. Houck *et al.* 1985), but only one study has used genetic markers to assign parentage of offspring at a natural breeding site (Jones *et al.* 2002b).

Body size is the most commonly measured trait in sexual selection studies with salamanders. Jones *et al.* (2002b) showed that male rough-skinned newts with long bodies and high tails tended to acquire more mates and sire more hatchlings than smaller individuals. A semi-natural experiment was generally consistent with these results (Jones *et al.* 2004). Although some observational studies with other salamander species have similarly suggested that large males tend to be favored by sexual selection (Houck 1988; Mathis 1991; Howard *et al.* 1997; Gabor *et al.* 2000), genetic techniques using other species have yielded inconsistent results (Garner & Schmidt 2003; Whiteman *et al. in press*). Post-copulatory processes such as sperm

competition and cryptic female choice, though rarely investigated, have the potential to severely affect the direction of sexual selection (Jones *et al.* 2002a), which is also likely to vary among diverse salamander lineages. Species that breed explosively in large aggregations may not exhibit male size advantage because of decreased ability of males to exclude others from mating opportunities. Female choice of large males may not occur in species without much sexual size dimorphism or secondary sexual characters.

The purposes of this study were twofold. First, we compared and evaluated five indices of inequality for quantifying the potential for sexual selection: opportunity for selection (I), Morisita's index (I_δ), standardized Morisita's index (I_p), index of resource monopolization (Q), and binomial skew index (B). Second, we explored the potential importance of sexual selection in marbled salamanders.

Materials and methods

Study species

Marbled salamanders (*Ambystoma opacum*) breed terrestrially in temporarily dry wetland basins of the eastern United States. Reproductive adults migrate from surrounding terrestrial habitats to breeding sites during warm, rainy nights in autumn. After mating, females oviposit beneath logs, vegetation, and other debris and attend their egg clutches during embryonic development. When seasonal rains inundate nest sites, hatchlings emerge and develop in the aquatic habitat until metamorphosis as in other *Ambystoma* species. About half of females mate with more than one male during a breeding season (Chapter 2). Because males contribute only gametes to reproduction, sexual selection is expected to be higher among males than females. Sexual dimorphism is minimal, although males tend to have brighter white bands on the dorsum

and breeding female immigrants tend to be slightly larger than males (D.A. Croshaw, unpubl. data). There are no secondary sexual characters, other than the swollen cloacae of males during the breeding season.

Experimental breeding replicates

We used galvanized steel cattle tanks (1.5 m diameter), coated on the inside with pool paint, as breeding arenas. Because marbled salamanders nest in terrestrial habitat, the tanks contained soil, logs, transplanted vegetation, cover boards, and leaf litter. Previous work confirmed that these experimental conditions are conducive to mating and nesting (D.A. Croshaw, unpubl. data). We placed screen covers over the tanks to exclude predators. Adult salamanders were added to the tanks on 22 October 2004. All animals were collected from terrestrial habitat surrounding Ginger's Bay, a small Carolina bay (Sharitz 2003) on the U.S. Department of Energy's Savannah River Site in Aiken County, South Carolina, USA. Because some females mate before arriving at wetland breeding sites (Krenz & Scott 1994), we attempted to minimize the number of prior matings by collecting all female salamanders at least 60 m from Ginger's Bay. Collecting sites included terrestrial drift fences with bucket traps (Gibbons & Semlitsch 1981) and a paved highway. Animals used in this experiment were held in the laboratory for no more than 10 days before the beginning of the experiment.

We set up two treatments (N = 5 tanks in each) of differing operational sex ratio, male-biased and female-biased. We randomly assigned six gravid females to each tank. Tanks in the male-biased treatment received eight males; those in the female-biased treatment received two. Although males were randomly assigned to tanks in the female-biased treatment, we stocked the male-biased tanks with four small and four large males to investigate the potential effects of

male size on fitness. Our stock of males was divided by sight into groups of small and large

individuals before randomly assigning animals from these groups to each tank. This procedure

was successful in producing size variation within male-biased tanks that was comparable to that

observed among breeding males at Ginger's Bay (D.A. Croshaw, unpubl. data).

We allowed the salamanders to mate and nest over several weeks, and collected eggs

from the tanks in late November 2004 by removing all nesting cover. Once all tanks had been

excavated, we submerged clutches of eggs in well water to induce hatching. We counted the

number of hatchlings produced by each clutch, euthanized a sample in a lethal dose of MS-222,

and preserved them in pure ethanol for subsequent microsatellite DNA genotyping. Adult

salamanders were weighed and measured before we took small tail clips that were similarly

preserved. We recorded wet mass to the nearest hundredth of a gram and snout-vent length

(SVL), tail length (TL), tail height (TH), and head width (HW) to the nearest half millimeter

before releasing all individuals.

Parentage assignment

We generated microsatellite genotypes at four loci (Aop31, AjeD162, AmaD321, and

AmaD328) for all adults and a sample of 16 hatchlings from each clutch using procedures

similar to Chapter 1. PCR products were run on an ABI 3130xl automated DNA sequencer with

CXR ladder (Promega), and alleles were scored with GENEMAPPER software (version 3.7).

We used the computer program CERVUS 2.0 (Marshall *et al.* 1998) to assign maternity and

paternity to all hatchlings in the sample. Because of female nest attendance, in many cases we

already were confident about maternity but used the microsatellite data as confirmation. After

parentage assignment, we manually compared each hatchling's genotype with those of the adults

in the replicate. We rescored individuals with potential scoring errors, identified when an allele

was present only once in a hatchling sample or when there was a single discrepancy between an

offspring and a putative parent. Our group of four loci was sufficient to allow strict exclusion of

all but the true parents in all cases, except when females had mated before the experiment

resulting in offspring from unsampled males.

Statistical analyses

Several female salamanders did not mate and nest. We performed the analyses with and

without these non-nesters included, and the conclusions were not altered. Throughout this paper,

we report the results when these females are included with zero fitness because this biases

interpretations toward the generally conservative idea that sexual selection was not different

between males and females. Females that mated prior to the experiment ($N = 16$) were excluded

from all analyses, leaving a total of 44 females. Mating success was estimated as the number of

genetic mates or the number of adults with which each salamander produced progeny. To

estimate reproductive success, we multiplied the proportion of each clutch's hatchlings that were

sired by competing males with the total number of hatchlings produced from that clutch.

We used our estimates of fitness based on both mating success and reproductive success

to calculate five measures (I, I_δ, I_p, Q, and B) of the potential for sexual selection, separately for

the two sexes in the two sex ratio treatments. We used the computer program SKEW

CALCULATOR 2003 (available at http://www.eeb.ucla.edu/Faculty/Nonacs/) to calculate the

latter four. Opportunity for selection (I) was calculated as, simply, the variance in fitness divided

by the square of mean fitness. To test for sex differences in mating frequency, we used

contingency table analysis and the Chi square test in the FREQ procedure in SAS (SAS Institute

2003). We used standard ANOVA techniques in GLM to test for mean differences between treatments and sexes in the potential for sexual selection as measured by the five indices, when of interest.

We quantified the relationships between size traits and fitness with the techniques of Lande & Arnold (1983). All measured size traits were used in addition to body condition index (CI), which we defined as the residuals obtained from a linear regression of mass on SVL in the SAS REG procedure (Jakob *et al.* 1996). We log transformed all body size data and standardized them to have a mean of 0 and variance of 1. We divided mating and reproductive success for each individual by their means to express the data as relative fitness. Standardized selection differentials were estimated as the covariance between the phenotypic traits and relative fitness. Standardized selection gradients were estimated by regression coefficients yielded by multiple regression analysis of size traits on relative fitness, also performed in the REG procedure.

Results

Frequency distributions of number of mates were different between the two treatments for males ($p = 0.026$) but not for females ($p = 0.314$, figure 1). Males had more mates when sex ratio was female-biased (mean = 1.8) than when it was male-biased (mean = 0.5). No male produced offspring with more than two females in the male-biased treatment, but two individuals did so with four and five females, respectively, when sex ratio was female-biased. Although males and females were not different when the male-biased ($p = 0.125$) and female-biased ($p = 0.140$) treatments were analyzed separately, males had significantly fewer mates (mean = 0.8) than females (mean = 1.2) when all data were pooled ($p = 0.039$) and more males than females completely failed to produce offspring.
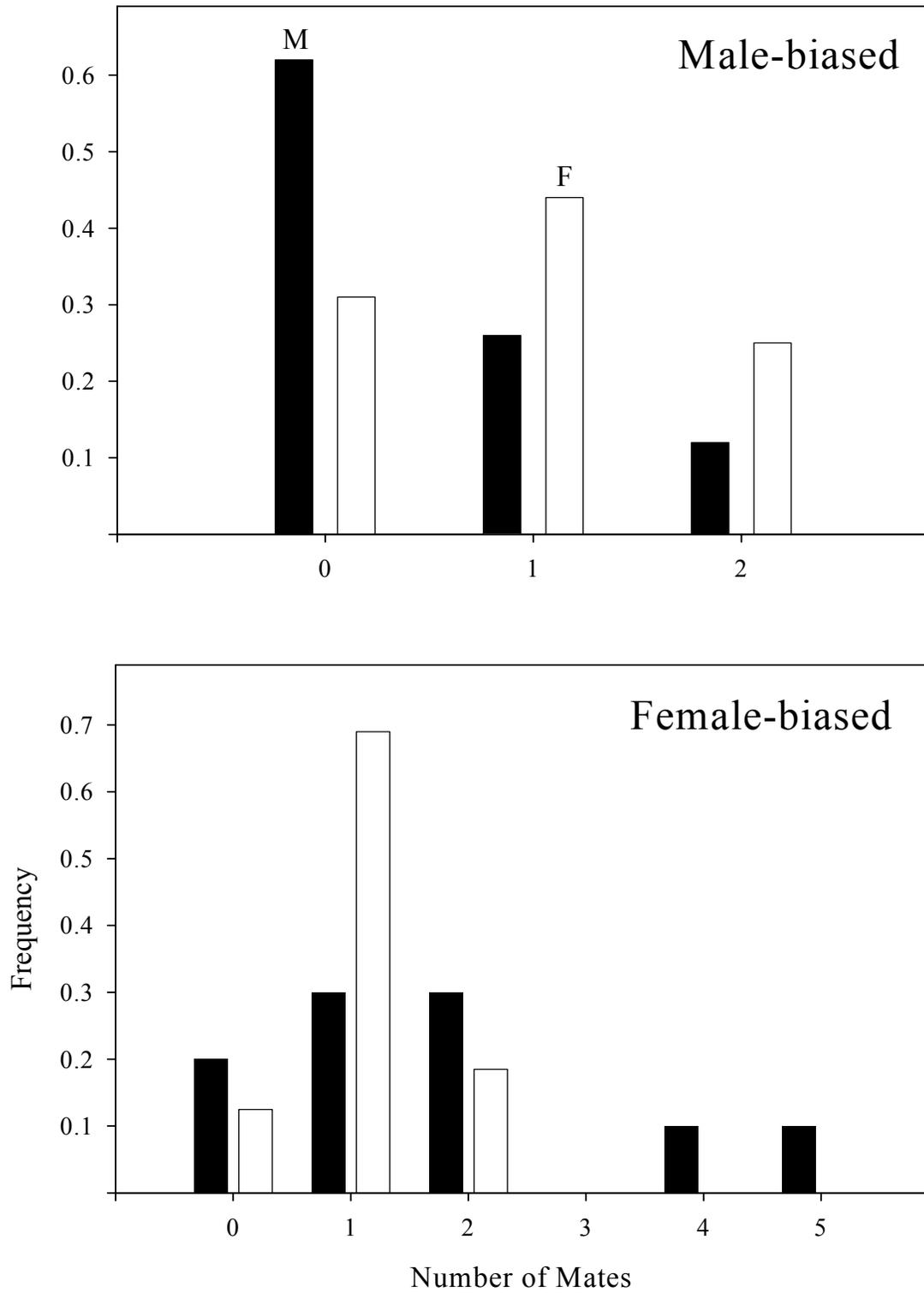
Figure 1. Frequency histograms of number of partners with which male (solid bars) and female (open bars) marbled salamanders produced offspring in the two sex ratio treatments.

Sex ratio treatment affected the potential for sexual selection among males (figure 2). When measured with opportunity for selection (I) and Morisita's index (I_δ), strength of sexual selection was usually significantly higher among males when sex ratio was male-biased than when it was female-biased. Means of these indices were as much as 3.2 times higher in the male-biased treatment than in the female-biased treatment. Only I and I_δ always conformed to theoretical expectations of differences in the potential for sexual selection among males based on changes in sex ratio (figure 2). All other indices yielded higher measures in the female-biased treatment than in the male-biased treatment for either mating success, reproductive success, or both.

Strength of sexual selection, measured by I and I_δ, was significantly higher among males (means as much as 5 times greater) than among females (figure 3), a result that was generally supported by the other three indices, although their ability to detect it was reduced. There was no statistically detectable effect of sex ratio treatment on sexual selection among females. As expected, Bateman gradients were significantly steeper for males than for females overall (p = 0.03, data not shown). When sexual selection was measured with I_p, fitness variance among males was significantly greater than expected to occur by random chance alone for reproductive success, but not for mating success (i.e., it did not reach the significance threshold of 0.5, figure 2).

There was no evidence that any of the six measured size traits was important in determining fitness of males or females in either treatment (table 1). This was the case when females that did not nest were included with zero fitness and when they were excluded from the analysis. Nearly all selection differentials and selection gradients were not significantly different from zero, and the direction of the relationships between size and fitness (indicated by signs) was

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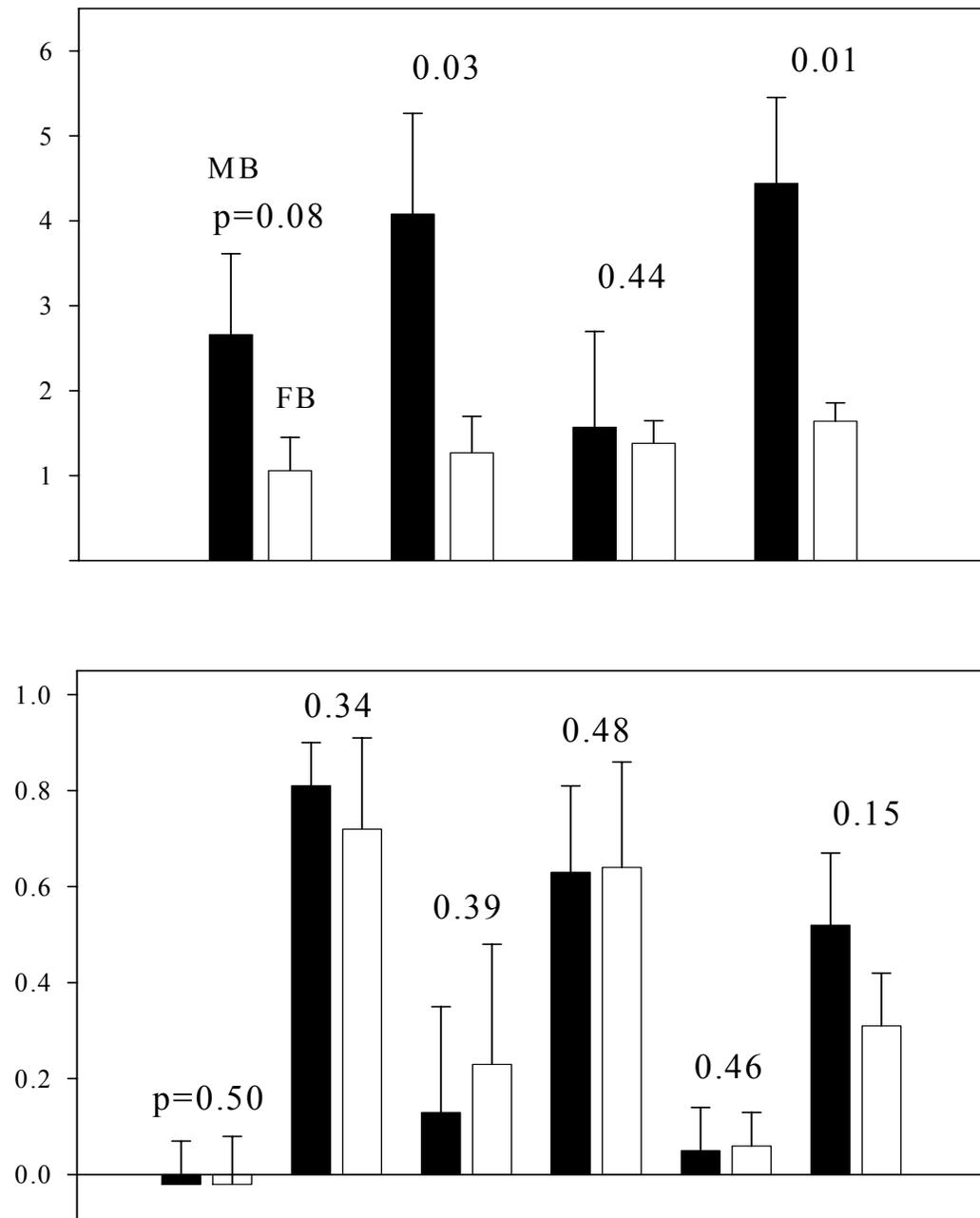
Figure 2. Mean indices of inequality (± SE) for male marbled salamanders in breeding replicates, separated by treatment. Solid bars are the male-biased (MB) sex ratio treatment; open bars are the female-biased (FB) treatment. MS, mating success; RS, reproductive success; I, opportunity for selection; I_δ, Morisita's index; I_p, standardized Morisita's index; Q, index of resource monopolization; B, binomial skew index. Numbers above each pair of bars are one-tailed p-values from simple statistical tests for differences between treatments.
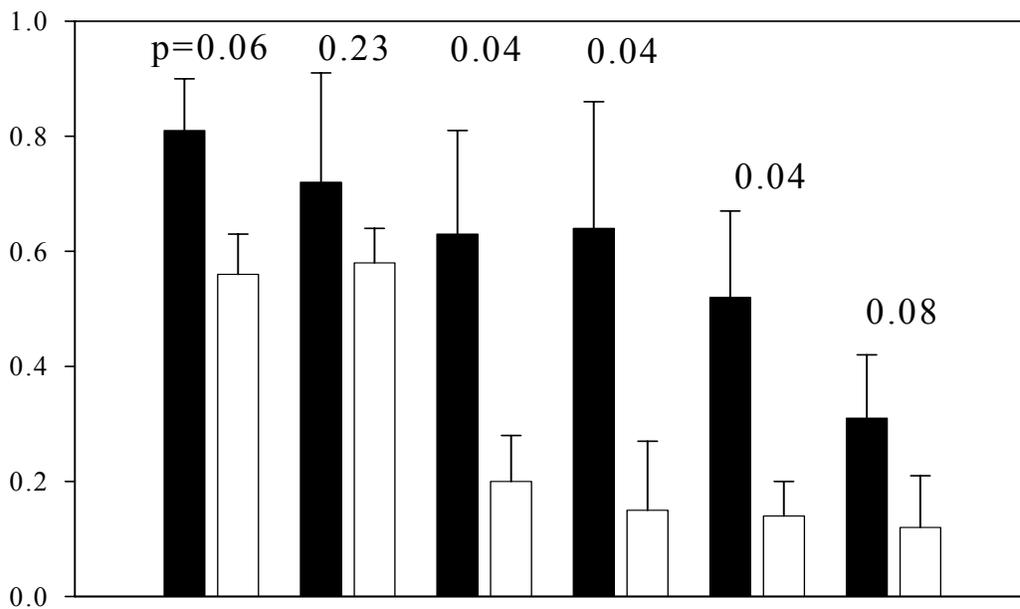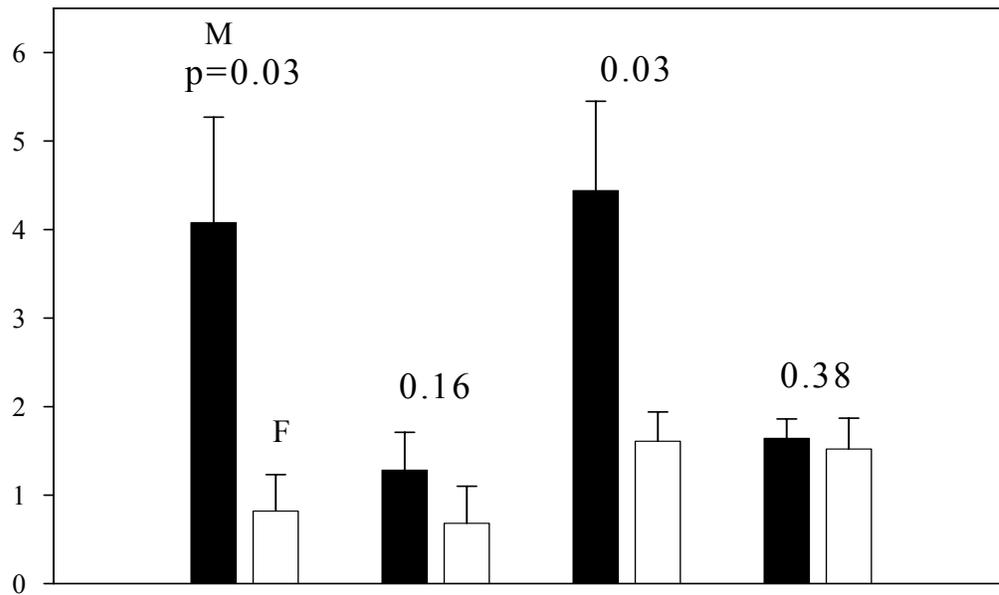
Figure 3. Mean indices of inequality (± SE) for reproductive success of male (solid bars) and female (open bars) marbled salamanders in breeding replicates, separated by treatment. MB, male-biased sex ratio treatment; FB, female-biased sex ratio treatment; MS, mating success; RS, reproductive success; *I*, opportunity for selection; I_δ, Morisita's index; I_p, standardized Morisita's index; *Q*, index of resource monopolization; *B*, binomial skew index. Numbers above each pair of bars are one-tailed p-values from simple statistical tests for differences between the sexes.

Table 1. Selection coefficients on size traits of marbled salamanders in experimental breeding replicates based on traditional analysis of selection on correlated characters (see text). Numbers are given for mating success and reproductive success data for males and females in each sex ratio treatment. We indicate p-values for tests that selection differentials (s') and selection gradients (β') are significantly different from zero.

	Mating success				Reproductive success			
	s'	p	β'	p	s'	p	β'	p
Males, male-biased								
Snout-vent length	-0.02	0.94	-2.56	0.26	0.10	0.76	-3.49	0.22
Mass	-0.11	0.66	3.22	0.29	-0.03	0.93	4.46	0.25
Body condition	-0.21	0.41	-2.15	0.23	-0.25	0.43	-2.99	0.18
Tail length	0.09	0.73	0.25	0.47	0.13	0.70	0.20	0.65
Tail height	-0.06	0.80	-0.13	0.79	0.04	0.90	-0.05	0.94
Head width	-0.14	0.59	-0.12	0.72	-0.04	0.91	-0.03	0.94
Females, male-biased								
Snout-vent length	0.37	0.24	1.23	0.79	0.29	0.41	1.93	0.66
Mass	0.44	0.16	-1.48	0.78	0.39	0.26	-2.50	0.61
Body condition	0.21	0.51	0.73	0.82	0.22	0.52	1.14	0.71
Tail length	0.40	0.21	-0.39	0.75	0.42	0.22	-0.32	0.78
Tail height	0.55	0.07	1.14	0.34	0.62	0.05	1.66	0.16
Head width	0.28	0.38	-0.23	0.82	0.12	0.72	-0.64	0.50
Males, female-biased								
Snout-vent length	0.01	0.97	0.73	0.92	-0.06	0.89	-1.14	0.92
Mass	0.43	0.16	-1.48	0.92	0.46	0.28	1.86	0.93
Body condition	0.51	0.08	1.21	0.92	0.59	0.15	-1.74	0.92
Tail length	0.29	0.37	0.02	0.97	0.26	0.55	-0.04	0.96
Tail height	0.51	0.09	0.60	0.56	0.49	0.25	0.81	0.60
Head width	0.41	0.46	0.40	0.56	0.41	0.34	0.65	0.53
Females, female-								
Snout-vent length	0.04	0.78	-1.29	0.22	0.13	0.42	-0.59	0.55
Mass	-0.11	0.48	2.78	0.09	-0.15	0.34	0.55	0.71
Body condition	-0.22	0.16	-1.76	0.13	-0.34	0.02	-0.96	0.37
Tail length	-0.11	0.49	-0.37	0.14	0.05	0.74	0.14	0.55
Tail height	-0.21	0.18	-0.57	0.08	-0.12	0.44	0.05	0.85
Head width	-0.09	0.58	0.02	0.96	0.02	0.89	0.32	0.27

not consistent for some traits. There was only one occasion, body condition index among females in the female-biased treatment, when fitness and size may have been related, and this is explained by the excess weight of females that did not oviposit. Thus, although the size traits show significant fitness variance, especially among males, we found no evidence that they are under sexual selection in this population.

Discussion

In accordance with theoretical expectations, sexual selection was consistently higher among males in the male-biased sex ratio tanks than those with female-biased sex ratios when measured with opportunity for selection (I) and Morisita's index (I_δ). Sexual selection was also higher among males than females, no matter which index was used. These results reflect true differences in the potential for sexual selection and are not artifacts of discrepancies in group size. First, although the male-biased treatment had more males than the female-biased treatment, our numbers are strikingly similar to those reported by Jones *et al.* (2004) whose treatments had equal numbers of males. Second, when all males and females in the experiment are considered together, so that there are two groups of similar size to compare (50 males, 60 females), the values of I (reproductive success: 3.02 for males, 0.61 for females; mating success: 1.89 males, 0.45 females) are similar to the means in the male-biased treatment, suggesting that group size is not prohibitively confounding. Finally, as we argue later in this section, paired increases in I and group size only reflect real differences in fitness variance.

Discrepancies in mean fitness alone also cannot account for the differences in the potential for sexual selection between treatments and sexes. Most compelling, I_δ conformed to expectations even though it has been shown to have no mean dependence analytically (Tsuji &

Kasuya 2001) and to have extremely weak negative mean dependence in a simulation study

(Nonacs 2003). Further, I_p, Q, and B all indicate that the potential for sexual selection was

higher among males than females and they each have very weak mean dependence (Nonacs

2003). Finally, we also show later that I continues to measure biologically relevant changes in

relative fitness variance despite mean differences. Our experimental approach of manipulating

operational sex ratio was undoubtedly sufficient to elicit real changes in the potential for sexual

selection among male marbled salamanders, and it also revealed that sexual selection is stronger

in males than females.

Recommended measures of the potential for sexual selection

Of the five indices studied, I and I_δ were the only ones that conformed to the theoretical

expectation that sexual selection among males should be stronger when operational sex ratio is

male-biased than when it is female-biased. As Jones *et al.* (2004) argued, I is advantageous over

all other potential measures, including I_δ, because of its formal relationship with quantitative

sexual selection theory. Opportunity for selection is useful and unique because it represents the

maximum strength of sexual selection, i.e. its intensity when fitness and phenotype are perfectly

correlated. Therefore, we recommend its continued use in sexual selection studies with the

addition of one important caveat. Because I does not control for expectations of random

variation, we believe that other indices which explicitly address this concern (e.g. each of the

other four measures considered herein) should be used to verify the presence of significant

fitness variance in certain situations. In our experiment, I was high among males when

calculated with either mating success or reproductive success data even though fitness was

unrelated to any of the phenotypic traits that we measured. Further analysis with other indices of

inequality revealed that the observed mating success variance was not significantly greater than expected to occur by chance alone, although reproductive success did yield significant fitness variance. Because fitness was not correlated with phenotype, we could not rule out the possibility that mating success was simply random. Had we relied solely upon I, we likely would have concluded that mating success data *did* indicate strong sexual selection among males. When researchers are unable to statistically relate fitness variance to phenotype, we recommend that they test observed variance against a null model of randomness, which is easily done with each of the other indices we studied. Because I_δ performed well by conforming to theory and detecting differences between groups, we feel that it is a good alternative to I in such cases. Tsuji and Tsuji (1998) proposed a simple statistical test for comparing observed values of I_δ to the expected value of 1 when fitness is random.

Whereas we believe that the inability of I to control for random expectations is a serious concern, its dependence on group size is not nearly as worrisome and may even be advantageous (A.G. Jones, pers. comm.). Selection should be higher when one or a few males gain all matings in a large group than if the same situation occurs in a small group. Opportunity for selection is not expected to experience artifactual increases along with increases in group size, which can only result if these differences cause decreases in mean fitness or increases in fitness variance, as indicated by a standard method of calculating I via dividing variance by the square of the mean. Because there are often only a limited number of mates to go around, it is intuitive that elevated numbers of competing males can both lower mean fitness and increase fitness variance. But such changes logically lead to stronger sexual selection and are precisely what researchers hope to measure with their chosen indices. Furthermore, in a strict statistical sense, increased sample size generally results in decreased variance, actually yielding a lower I if all else is equal.

Tsuji & Kasuya (2001) used a similar argument to discount criticisms of I_δ because of its group size dependence. They used a simple case involving groups of only breeders and non-breeders to illustrate that the value of I_δ remains unchanged, even when group size increases tenfold, as long as the proportion of breeders is not increased. The same point is true for I, although it actually *decreases* slightly when the size of the group increases (from 1.14 for a group of 8 individuals in which half have fitness of 1 and the other half have fitness of 0 to 1.01 for a group of 80 with the same proportion of breeders). Thus, as for I_δ, I only increases along with increases in group size when the potential for sexual selection is augmented in a real biological way.

Tsuji & Kasuya's (2001) proportion argument also applies to the dependence of I on mean fitness. When the proportion of total fitness units, whether mates or offspring, acquired by each individual in a group remains unchanged, so does I. Even if mean fitness is orders of magnitude higher, I stays the same as long as the relative fitness of the individuals is also unchanged, as indicated in one of the standard definitions of I, the variance in relative fitness. Thus, I may decrease with the mean if fitness conforms to a Poisson distribution (Downhower *et al.* 1987), but this will only happen in practice if paired with decreases in relative fitness. Again, the general purpose of using these indices in sexual selection studies is to convey the intuitive quantity of fitness variance, and I does just that even in the face of tremendous differences in mean fitness.

The statistical power of several of these indices has been examined before in simulation studies. Nonacs (2000) showed that B has higher power than Q and I_p, but I_δ was not included. Data reported by Kokko *et al.* (1999) may suggest that I_δ is more powerful than Q and I_p, but explicit comparisons were not made. Because the power of all potential indices has never been

compared in a rigorous simulation study, we believe that such an analysis is warranted because of the need for a measure that can detect discrepancies in the potential for sexual selection.

Sexual selection in marbled salamanders

In our breeding replicates, opportunity for selection was high for male marbled salamanders when calculated with mating success and reproductive success data. Reproductive success yielded high indices of inequality, no matter which measure was used. Because operational sex ratio at Ginger's Bay is typically much more male-biased than in our breeding replicates (1.33 male to female ratio in male-biased treatment, 1.96 among breeding immigrants in 2000, unpublished data), sexual selection is likely to be a formidable evolutionary force in this population. However, this study does not provide evidence about what traits explain reproductive fitness variation among males. None of six measured size traits affected fitness in males or females. This lack of an effect cannot be explained by low size variance within tanks because we purposely chose a range of male sizes to stock in each male-biased sex ratio replicate. Size ranges and standard deviations were comparable to those observed in males present at the breeding site for all traits.

Size advantages among males have been observed in observational studies of mating in other salamanders (e.g. Houck 1988; Howard *et al.* 1997; Able 1999; Gabor *et al.* 2000), and the only comparable work that used genetic techniques to assign paternity did so as well (Jones *et al.* 2002, 2004). But Garner & Schmidt (2003) found no evidence that size was a factor in determining fertilization success of males in 13 breeding trios of alpine newts. Marbled salamanders may be expected to experience little or no sexual selection on male morphology because there is minimal sexual dimorphism, no secondary sexual characters, and males are

slightly smaller than females, unlike the newt species in which paternity was previously studied. Generally, males are larger and/or have well developed secondary sexual characters in species that reportedly have a large-male advantage. However, in red-backed salamanders, males are smaller than females and size is apparently important in competition among males for access to females (Mathis 1991). The mixed results offered by the few available data highlight the need for more sexual selection studies to determine the factors influencing fitness variance in salamander species.

Currently, we can only speculate about the traits that may potentially explain our observations of variance in male reproductive fitness. Individual salamanders may respond differently to conditions in experimental breeding replicate, causing variance in motivation to breed. Only further studies at natural breeding sites can evaluate this idea. Male salamanders are limited in their sperm stores during the breeding season (Verrell 1986), and some individuals may be able to deposit more spermatophores than others. Sexual selection may favor males that are simply vigorous in courtship and spermatophore deposition. Alternate behaviors such as female mimicry and courtship interference by males (Arnold 1976) may also vary and influence mating success. Finally, it is possible that females choose males for mates based on some unmeasured size trait, genetic compatibility, or behavioral features.

Fitness variance that was significantly greater than expected due to random chance occurred among males when we analyzed reproductive success data but not for mating success. This cannot be explained simply by differences in the fitness currency because most of the indices have little or no mean dependence (Tsuji & Kasuya 2001; Nonacs 2003). If postcopulatory processes, either sperm competition or cryptic female choice, are particularly important in determining fertilization success, we might expect variation in the number of

offspring produced to be much greater than variation in the number of mates acquired. This result invites more work to uncover the postcopulatory mechanisms that govern fertilization in salamanders and suggests that future sexual selection studies with this group should incorporate genetic paternity analysis for a full understanding of fitness variance.

Conclusions

We advocate the use of opportunity for selection (I) and/or Morisita's index (I_δ) to quantify the potential for sexual selection because they were the only indices that conformed consistently to theoretical expectations. Although the inability of I to control for random processes is a legitimate disadvantage compared to I_δ, it is the only competing measure that has an intuitive and formal connection to sexual selection theory. However, when fitness variance is statistically unrelated to phenotypic variance, one of the other indices should be used to test whether the observed variance is greater than expectations of randomness. Our study strongly suggests that sexual selection is important in natural marbled salamander populations, although size does not explain fitness variation. Identification of the phenotypic traits that are sexually selected in this species awaits further work.

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Chapter 4

Fitness consequences of polyandrous mating for female marbled salamanders

Abstract

Female polyandry occurs to some extent in a very high proportion of species that have been

studied, even in socially monogamous mating systems. Because mating likely involves

considerable fitness costs to individual females, theory predicts that polyandrous females gain

fitness benefits that outweigh the costs, allowing the behavior to be maintained. Potential

benefits are non-genetic (or material) and genetic benefits, with only the latter likely to occur in

species where males provide females with sperm only. Genetic benefits could involve increased

genetic compatibility between parents, genetic variation among offspring, quality of paternal

genes, and quality or attractiveness of sperm. We report the first study of fitness consequences

of multi-male mating in an ambystomatid salamander. We compared fitness of monandrous and

polyandrous marbled salamander clutches, determined by paternity assignment with

microsatellite markers, from semi-natural breeding arenas at the egg, hatchling, and metamorph

stages. Larvae from polyandrous and monandrous clutches developed together in high density

field enclosures until metamorphosis. Clutch size, hatching success, hatchling size, and parental

female size were not significantly different between polyandrous and monandrous clutches. We

measured survival to metamorphosis and size at metamorphosis. Survival to metamorphosis, but

not size of metamorphic salamanders, was significantly greater for polyandrous clutches than

monandrous clutches (44% versus 40%). Our study provides the first evidence of survival

advantages of offspring produced by multi-male mating in an amphibian. We discuss the

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potential nature of these genetic benefits in terms of competing hypotheses to explain the
evolution of polyandry.

Introduction

Traditionally, females were thought to mate only with as many males as needed to
fertilize their entire lifetime egg production, typically only one or a few matings per breeding
season (e.g., Trivers 1972). However, recent evidence shows that females mate more often and
with more males than is seemingly necessary in most species, even those with socially
monogamous mating systems (Jennions & Petrie 2000 and references therein). This polyandrous
behavior is generally costly to females for several reasons, including decreased life expectancy
caused by physical injury or harmful chemicals in ejaculates, increased predation risk, deferred
foraging time, lowered fertility, and risk of contracting sexually transmitted diseases
(summarized in Stockley 1997). Theoretically, females must gain fitness benefits to offset these
mating costs and allow polyandrous mating to be adaptively maintained in extant populations.
Females can probably maximize their fitness by adopting an optimally promiscuous mating rate
(Arnqvist & Nilsson 2000). Because the relationship between number of mates and offspring
production (i.e., the Bateman gradient) is generally thought to be weak among females (Bateman
1948), the nature of these fitness benefits is far from obvious and understanding them will
require careful experimentation in a wide range of species.

Although polyandrous mating may not necessarily require an adaptive explanation
because of forced copulations, lack of fitness costs (e.g., Gould & Lewontin 1979), or correlated
evolution with other adaptive traits (Halliday & Arnold 1987), females of some species actively

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mate with many males despite clear costs (e.g., Orsetti & Rutowski 2003). The plethora of possible benefits enjoyed by polyandrous females can be divided into two broad categories: 1) non-genetic benefits (sometimes denoted as material benefits because most, but not all, involve material resources) and 2) genetic benefits. The former category is not controversial, and many such benefits have been demonstrated in a wide range of taxa. Non-genetic benefits include, but are not limited to, extra sperm, use of breeding resources, parental care, nuptial gifts, increased foraging rates, nutritive ejaculates, decreased harassment and infanticide, and promotion of egg maturation and ovulation (Jennions & Petrie 2000 and references therein).

In many species, males provide no obvious non-genetic resources to polyandrous females other than sperm, leading many to invoke genetic benefits hypotheses to explain female mating rates. Even when non-genetic benefits are present, polyandrous females are likely also to enjoy concomitant genetic benefits because of the presumed ubiquity of sexual conflict over control of reproduction via sperm competition and/or cryptic female choice (Eberhard 1996; Birkhead & Moller 1998; Jennions & Petrie 2000; Rice 2000). In general, we can classify the various proposed manifestations of genetic benefits into four categories: 1) genetic compatibility, 2) genetic diversity, 3) good genes or intrinsic male quality, and 4) good and/or sexy sperm.

Genetic compatibility hypotheses claim that multiply mating females produce a higher proportion of their offspring with genetically compatible sires, most likely via biased parental investment or sperm usage through sperm competition or cryptic female choice (Zeh & Zeh 1996, 1997; Tregenza & Wedell 2000). Varying genetic compatibility of mating pairs could influence female fitness through a variety of mechanisms, including inbreeding depression, genomic conflict, immune function, and fetomaternal interactions. Assessment of compatibility could occur via physiological interactions among ejaculates and offspring of competing sires and

the female reproductive system (Jennions & Petrie 2000). Although some studies have uncovered evidence for this hypothesis (Tregenza & Wedell 1998, 2002; Foerster et al. 2003), others could not (Simmons 2001; Garcia-Gonzalez & Simmons 2005).

The genetic diversity hypothesis, also known as genetic bet-hedging (Watson 1991), contends that polyandrous females produce broods with greater genetic variation than do monandrous females, perhaps allowing the progeny to respond better as a group to unpredictable environmental conditions. Genetic diversity due to polyandry in social insect colonies may promote resistance to parasites (Sherman et al. 1998; Baer & Schmid-Hempel 1999). However, simple crossing over during meiosis generates genetic variation in the absence of polyandry, Yasui (1998) argued that it is unlikely to result in the evolution of polyandry, and experimental evidence for this idea is scant in animals that are not eusocial (Jennions & Petrie 2000).

Females may remate in an effort to acquire genes for their offspring that confer attractiveness, viability, or low mutation load (Jennions & Petrie 2000; Radwan 2003), an idea sometimes known as the good genes or intrinsic male quality hypothesis. This idea predicts that females 'trade up' for higher quality males than their previous or social mates. For example, female smooth newts become choosier as they mate with additional males (Gabor & Halliday 1997), and socially monogamous birds are likely to engage in extra-pair copulations with more attractive or dominant males than their social mates (Hasselquist et al. 1996; Dickinson 2001). The good genes idea requires the presence of strong additive genetic variation for viability among males (e.g., Garcia-Gonzalez & Simmons 2005) which is not always apparent (Tregenza & Wedell 1998).

Polyandrous females would benefit if success in sperm competition is related to their sons' reproductive success (sexy sperm, Keller & Reeve 1995) or offspring viability (good

sperm, Yasui 1997). Few experimental studies have tested these hypotheses. Some evidence suggests that sperm competition success is heritable (Radwan 1998), and polyandry sometimes increases sons reproductive success (Bernasconi & Keller 2001; Pai & Yan 2002), although other competing hypotheses could not be excluded. One controlled experiment did not support the good sperm idea (Simmons 2001).

Each of the potential explanations for polyandry has received support in some species but not in others. Their predictions are clearly not mutually exclusive, so designing experiments to separate their potential effects is problematic, and several mechanisms may act simultaneously (e.g., Evans & Magurran 2000). Conflicting results often occur among careful experimental studies with the same model organism (see Arnqvist et al. 2005; Ivy & Sakaluk 2005), and benefits may be evident only under certain conditions. For example, nearly all experiments have been performed in the laboratory without subjecting offspring to stressful environments. Field experiments that place offspring from monandrous and polyandrous clutches in intense competition with one another have the potential to provide new and valuable insights. In general, exploring the relationships between diverse ecological factors, reproductive modes, and fitness consequences of mating behavior should further elucidate the importance of polyandry (Emlen & Oring 1977; Zeh & Zeh 2001). For a broad understanding of the evolution of polyandry, we need more work with diverse taxa under a range of experimental conditions.

Most of the previous experimental work investigating the fitness consequences of polyandry has involved invertebrates that have short generation times, mate readily in unnatural conditions, and are easily cultured in the laboratory. Understandably, the adaptiveness of polyandry in vertebrates, especially tetrapods, has remained relatively obscure, and no studies have experimentally measured performance of offspring from polyandrous and monandrous

clutches or broods in direct competition. Among tetrapods, salamanders offer good model systems for studying polyandry. In most species, females control sperm transfer during mating which is sometimes highly explosive, and males are unable to monopolize females and restrict their access to other males. Thus, interpretations of experimental results are not confounded by male-male interactions and male sexual coercion (see Clutton-Brock & Parker 1995). All studied species exhibit polyandry, and female anatomy and physiology allow the concurrent storage of sperm from several males before fertilization (Sever 2002). Finally, because many salamanders aggregate at breeding sites, produce large clutches, and have a defined larval period, they are relatively tractable experimental subjects.

Conflicting results were obtained by the only two studies investigating the potential fitness benefits of female polyandry in salamanders. Osikowski and Rafinski (2001) suggested that females remate only to obtain sperm, but Garner and Schmidt (2003) found support for the genetic compatibility hypothesis with another species. Neither study followed the fate of clutches beyond hatching, and the former did not verify that multiply mated females produced offspring with more than one male.

The purpose of this study was to compare the performance of polyandrous and monandrous marbled salamander (*Ambystoma opacum*) clutches from oviposition through metamorphosis under semi-natural conditions to determine if polyandrous females enjoy fitness benefits. We relate our results to the various non-genetic and genetic benefits that may explain the adaptiveness of polyandry.

Materials and methods

Study Species

Marbled salamanders (*Ambystoma opacum*) are terrestrial breeders of the eastern United States. Adults migrate to temporarily dry breeding ponds in autumn, and females nest under vegetation or debris after mating. Females remain with their egg clutches for variable durations. When seasonal rains flood nests, hatchlings emerge from the eggs and develop in aquatic habitat. Larvae metamorphose in spring and migrate to terrestrial habitat surrounding the natal site. About half of the clutches exhibit multiple paternity. It is probably very rare for greater than three different males to sire a single clutch (Chapter 2).

Field Experiment

We set up experimental breeding habitat in galvanized steel cattle tanks (1.5 m diameter) containing soil, leaf litter, transplanted vegetation, cover boards, and other debris. Each tank was secured from predators with screen covers. We stocked adult salamanders in the tanks on 22 October 2004. They were obtained from wooded terrestrial areas surrounding Ginger's Bay, a Carolina Bay (Sharitz 2003) on the Savannah River Site (SRS) in Aiken County, South Carolina. We attempted to minimize the proportion of females that mated prior to the beginning of the experiment by collecting salamanders only from distances at least 60 m from the wetland (Krenz & Scott 1994). We caught salamanders with drift fences and bucket traps or from a paved road near the bay. Animals were held in the laboratory for up to 10 days before being released into experimental breeding tanks.

The breeding replicates were also part of a companion study (Chapter 3) in which we assessed the effects of sex ratio variation on sexual selection. There were two sex ratio

treatments, male-biased and female-biased. Tanks in the former treatment contained eight males; those in the latter contained two. All tanks were stocked with six gravid females. We left salamanders undisturbed in the tanks for mating and nesting. In late November 2004, we excavated nesting cover and collected all adult salamanders and egg clutches. In early December, eggs were counted and submerged in well water for hatching, after which we counted living normal hatchlings, deformed hatchlings, dead hatchlings, and dead eggs before preserving a sample from each clutch in ethanol. Often, hatchlings die extremely quickly after hatching or in the process of hatching. Deformed hatchlings were variable but all were clearly morphologically aberrant to the human eye and exhibited slow, wandering locomotion. Hatching success was defined as the number of living normal hatchlings divided by the sum of all categories (i.e., living normal hatchlings, deformed hatchlings, dead hatchlings, and dead eggs). We set 50 hatchlings aside from four clutches of each of 10 tanks, five male-biased and five female-biased. Clutches were selected randomly from the set of clutches out of each tank that produced 50 or more viable hatchlings. For parentage analysis, we took small tail clips from adult salamanders after weighing and measuring them. Wet mass to the nearest hundredth of a gram and snout-vent length (SVL), tail length (TL), tail height (TH), and head width (HW) to the nearest half millimeter were recorded before releasing adults and extra hatchlings.

We held hatchlings in the laboratory with minimal but uniform amounts of food (zooplankton collected from the natal pond) for as long as three weeks. We did this while paedomorphic *Ambystoma talpoideum* were removed from enclosures. All individuals completely used their yolk sacs prior to the beginning of the experiment, but very little growth occurred. On 31 December 2004, we stocked 40 randomly selected hatchlings from each clutch in field enclosures (10 total) at Ellenton Bay, a large Carolina bay (~ 10 ha) also located on the

SRS in South Carolina. Ellenton Bay supports populations of marbled salamanders and congeneric mole salamanders (Gibbons et al. *in press*). We chose Ellenton Bay because it has a long hydroperiod and is relatively free of emergent vegetation, allowing easy addition of enclosures. Each enclosure received 160 total hatchlings from one of the breeding tanks, 40 from each of the four clutches. Seven of the 10 enclosures received a mixture of multiple and single paternity clutches. The rectangular enclosures (4.3 by 3.0 m), equipped with a bottom but not a top, were made of fiberglass window screen. They were supported by PVC pipe and aluminum poles and the screen bottoms were forced to the bay substrate with weights.

Salamander larvae were left to feed and develop during their normal larval period. At the beginning of April 2005, when marbled salamanders start to metamorphose in South Carolina, we suspended minnow traps at the water surface in enclosures to collect metamorphosing individuals. We checked the traps daily and also collected metamorphs at night as they came to the bay surface and climbed onto traps. Salamanders metamorphosed throughout April and into the third week of May. We took tail clips from metamorphs for parentage analysis and weighed and measured metamorphs using the same protocol as for adults before their release at Ginger's Bay.

We measured the dry mass of 16 hatchlings from our preserved samples or as many as were available from each clutch. We did this by freeze drying them for 6-12 hours, which was long enough to extract all moisture. Hatchlings were weighed with a microbalance to the nearest mg.

Parentage Assignment

We genotyped all adults and metamorphs along with a sample of 16 hatchlings from each clutch at four microsatellite DNA loci (Aop31, AjeD162, AmaD321, and AmaD328, Chapter 1). The laboratory procedures we used were similar to Chapter 1. Briefly, we ran the amplified microsatellites with CXR ladder (Promega) on an ABI 3130xl automated sequencer and scored alleles with GENEMAPPER software (version 3.7). We assigned maternity and paternity of hatchlings with CERVUS 2.0 (Marshall et al. 1998). Except for those produced by prior matings, all but one female and male were excluded from parentage of each hatchling by at least one mismatched allele. We were aided in this assignment process by the fact that hatchlings were grouped into discrete sibship units, i.e. clutches. First, we excluded females from maternity of each clutch before subsequently excluding males from paternity of individuals, which was extremely successful, in part, because every hatchling's mother was known.

Metamorphs could not be easily grouped into discrete sibship units, making strict exclusion of individual parentage more difficult. Therefore, we used a clutch exclusion approach whereby we excluded clutches that did not contain metamorph genotypes in at least one of the 16 hatchlings from the sample for all four loci. This method was extremely successful in assigning metamorphs to clutches and, thus, mothers. To test the accuracy of this method, we genotyped eight metamorphs from each of the 10 enclosures at four additional loci from Chapter 1 (Aop36, Aop37, AjeD23, and AmaD42). We used this suite of eight loci to attempt strict exclusion of potential mothers. Although we still could not assign a mother to several of the metamorphs, there was not a single case in which the clutch exclusion method contradicted the strict exclusion method with additional loci. Thus, we are extremely confident that the clutch exclusion method accurately and reliably assigned metamorphs to mothers. A small proportion of metamorphs

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could not be assigned to a clutch with the original four loci. We also genotyped these unresolved individuals at the additional four loci which revealed the identity of most mothers. However, even with the additional data, about 2% of metamorphs could not be assigned to a clutch via clutch exclusion and were not included in the analyses. A majority of these had missing data because they died in the process of collection from the enclosures and yielded DNA of low quality. Once metamorphs were assigned to a mother, we were able to assign paternity with strict exclusion easily, although we needed to collect data from the additional loci in a few cases.

Statistical Analyses

In all analyses, we used each clutch as the unit of observation. Data were either clutch averages or single measurements from each clutch. All clutches from the cattle tanks that produced living hatchlings, not just those used in the field enclosures, were classified as either single or multiple paternity based on the sample of 16 hatchlings from each (Chapter 3). All clutches used in the field experiment produced metamorphs, and we used the additional parentage assignments to check our classification. Additional sires were not detected in any of these clutches, which is to be expected. Among 13 field-collected clutches, the maximum representation of the most successful male in polyandrous clutches was 0.82, indicating that the probability that a multiple paternity clutch was misclassified as a single paternity clutch was about 0.04 (= 0.82^{16}). Because a great majority of all polyandrous clutches were sired by two males (> 95%), we did not attempt to categorize clutches further. Some clutches were excluded from analyses because data were unavailable. For example, hatching success was not observed in some clutches that experienced early hatching when heavy rains temporarily inundated the nest site. No data were used from clutches that did not produce more than one living hatchling

(N = 3) because paternity status was impossible to assess. We excluded the few metamorphs (N =15) that could not be assigned to a clutch with our suite of markers and a very small number (N = 5) that produced low quality DNA because of death and decomposition before collection. For some clutches that did not produce many living hatchlings, there were few or no individuals left to weigh after the initial genotyping to determine paternity status (N = 13 clutches only had data from five hatchlings or less).

We expressed survival and hatching success data in proportions which were square-root arcsine transformed before analysis. We were mainly interested in the effect of paternity status (single or multiple) on clutch performance. We analyzed the data using a mixed model ANOVA approach with random and fixed effects in the MIXED procedure in SAS (SAS Institute 2003). For the larval field experiment, we considered the enclosure effect to be random; sex ratio treatment and paternity status effects were fixed. Indeed, individual enclosures were expected to provide somewhat unique environments for larval development leading to correlated data and significant enclosure effects. Each community was likely somewhat singular in its predation and competitive environments. The enclosure effect was nested within the treatment effect. We analyzed the proportion of clutches that survived to metamorphosis and each of six size traits separately, the five that we measured directly along with body condition index (CI). CI was defined as the residuals obtained from a linear regression of mass on SVL in the REG procedure (Jakob et al. 1996). We took the same approach for the hatchling stage fitness data, although most measures were of clutches rather than individual offspring. The tank effect was random and nested within the treatment effect. The following dependent variables were analyzed separately: clutch size, hatching success, egg mortality, early hatchling mortality, hatchling deformity, hatchling mass, and the size traits that were measured in mothers.

We were also interested in testing for differences in variance between multiple and single paternity clutches to test the hypothesis that greater genetic variation within polyandrous sibships results in higher variation in performance. We used the GLM procedure with paternity status as the independent variable and Levene's test of homogeneity of variances for variables that had a single measure per clutch. For the size variables, we used the coefficient of variation within each clutch as a dependent variable in standard GLM models (see Byrne & Roberts 2000).

Results

Seven of the 10 enclosures had a mix of single and multiple paternity clutches present. In all analyses, there was never an instance where sex ratio treatment was statistically significant and we do not consider it further. Tank and enclosure effects were also uninteresting and included only as random effects in the design. For these reasons, we exclusively report paternity status effects here and do not separate the data based on tank, enclosure, or treatment. Of all the variables that we compared at the egg, hatchling, and metamorph stages, only survival to metamorphosis showed evidence of advantages of polyandrous mating (Table 1). Metamorphs from polyandrous clutches were slightly larger than those from monandrous clutches for all six size traits analyzed. However, the differences were very small in magnitude and not statistically significant (Figure 1). In the field enclosures, polyandrous clutches had higher survival to metamorphosis (N = 15, 44%) than monandrous clutches (N = 25, 40%; Figure 2). None of the fitness-related traits at the egg and hatchling stages differed between single and multiple paternity clutches, although all except hatchling deformity rate exhibited higher fitness in polyandrous clutches (Table 1, Figure 3).

Table 1. Comparison of single and multiple paternity clutches in means and variances of several potential fitness-related traits at the egg and metamorph stage. Mixed ANOVA model statistics are tests of the paternity status effect on means. SVL, snout-vent length; CI, body condition index; TL, tail length; TH, tail height; HW, head width. Variables marked with an * were tested for variance differences by using the coefficient of variation for each clutch in GLM models; others were tested with Levene's test for heteroscedasticity. The only p-value < 0.05 is in bold.

| | Mixed ANOVA Model | | | Variance |
	F	DF	P	P
Clutch size	0.60	34,1	0.44	0.45
Hatchling mass*	0.29	27,1	0.60	0.76
Hatching success	0.37	34,1	0.55	0.31
Egg mortality	0.22	34,1	0.64	0.43
Hatchling mortality	0.89	34,1	0.35	0.35
Hatchling deformity	0.52	34,1	0.48	0.79
Survival to metamorphosis	4.63	29,1	**0.04**	1.00
SVL at metamorphosis*	1.77	29,1	0.11	0.64
Mass at metamorphosis*	1.42	29,1	0.24	0.18
CI at metamorphosis*	0.02	29,1	0.90	0.10
TL at metamorphosis*	1.86	29,1	0.18	0.35
TH at metamorphosis*	0.21	29,1	0.65	0.51
HW at metamorphosis*	0.00	29,1	0.97	0.30
Female SVL	0.36	29,1	0.55	0.86
Female mass	0.15	29,1	0.70	0.09
Female CI	0.01	29,1	0.93	0.15
Female TL	0.36	29,1	0.55	0.34
Female TH	0.11	29,1	0.75	0.56
Female HW	0.07	29,1	0.79	0.17
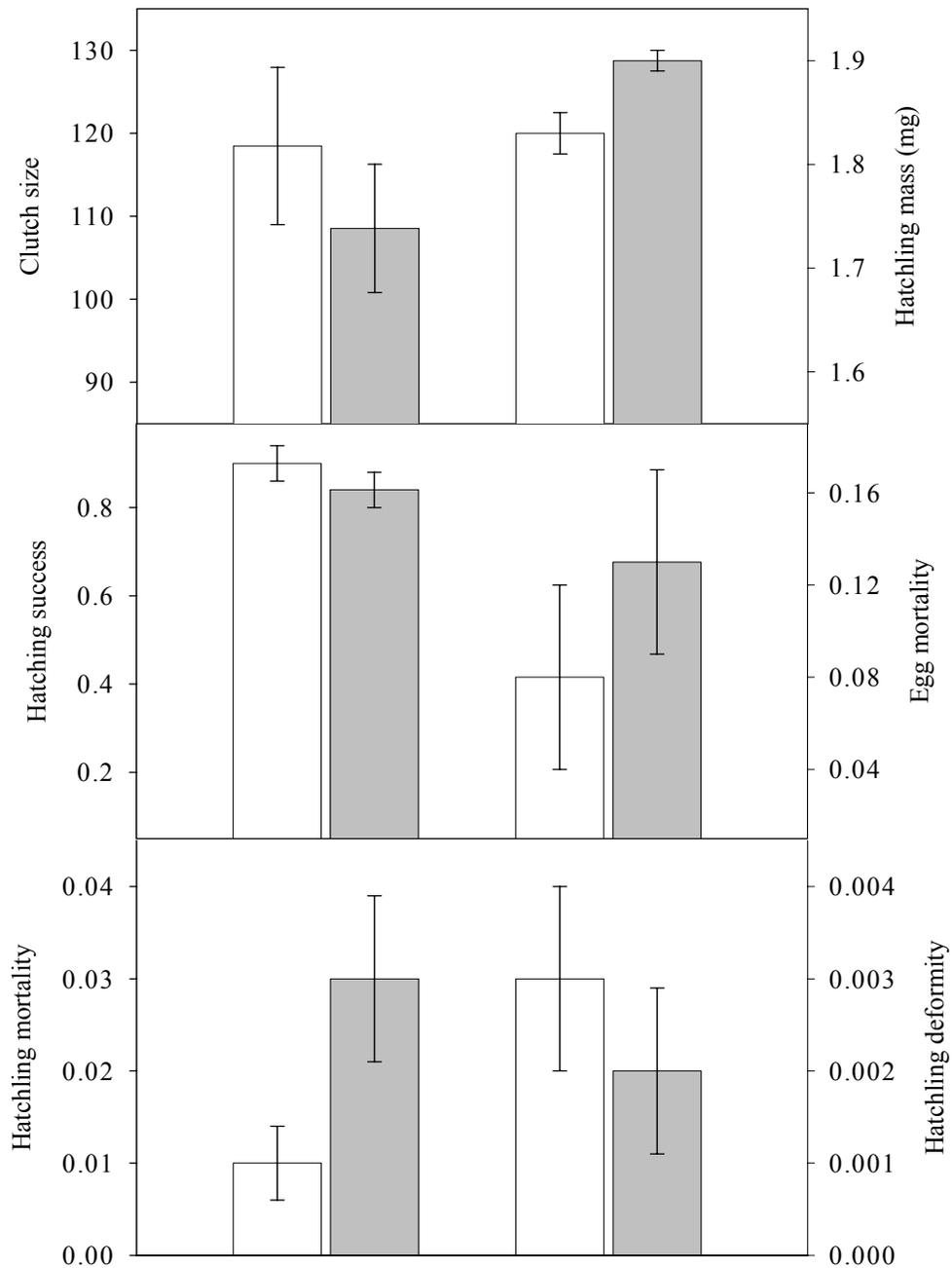
Figure 1. Mean (± SE) of six fitness-related measures of polyandrous and monandrous clutches at the egg and hatchling stages. Unfilled bars are polyandrous clutches; filled bars are monandrous. Each of the three panels has a right and left axis at different scales. The bars on the left correspond with the axis on the left and vice versa.
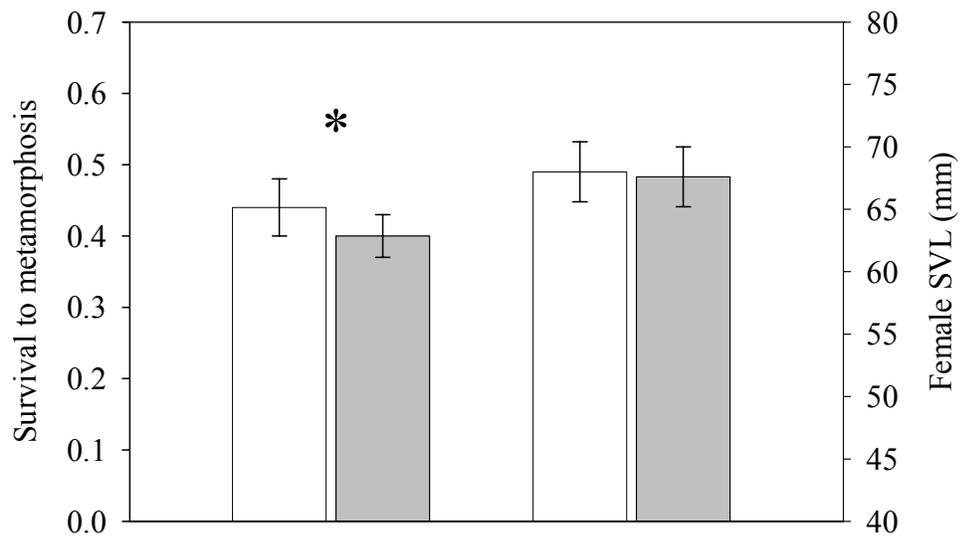
Figure 2. Mean (± SE) of parental female size and survival to metamorphosis of polyandrous and monandrous clutches. Unfilled bars are polyandrous clutches; filled bars are monandrous. The asterisk indicates a difference between the two clutch types at the 0.05 level. The bars on the left correspond with the axis on the left and vice versa.
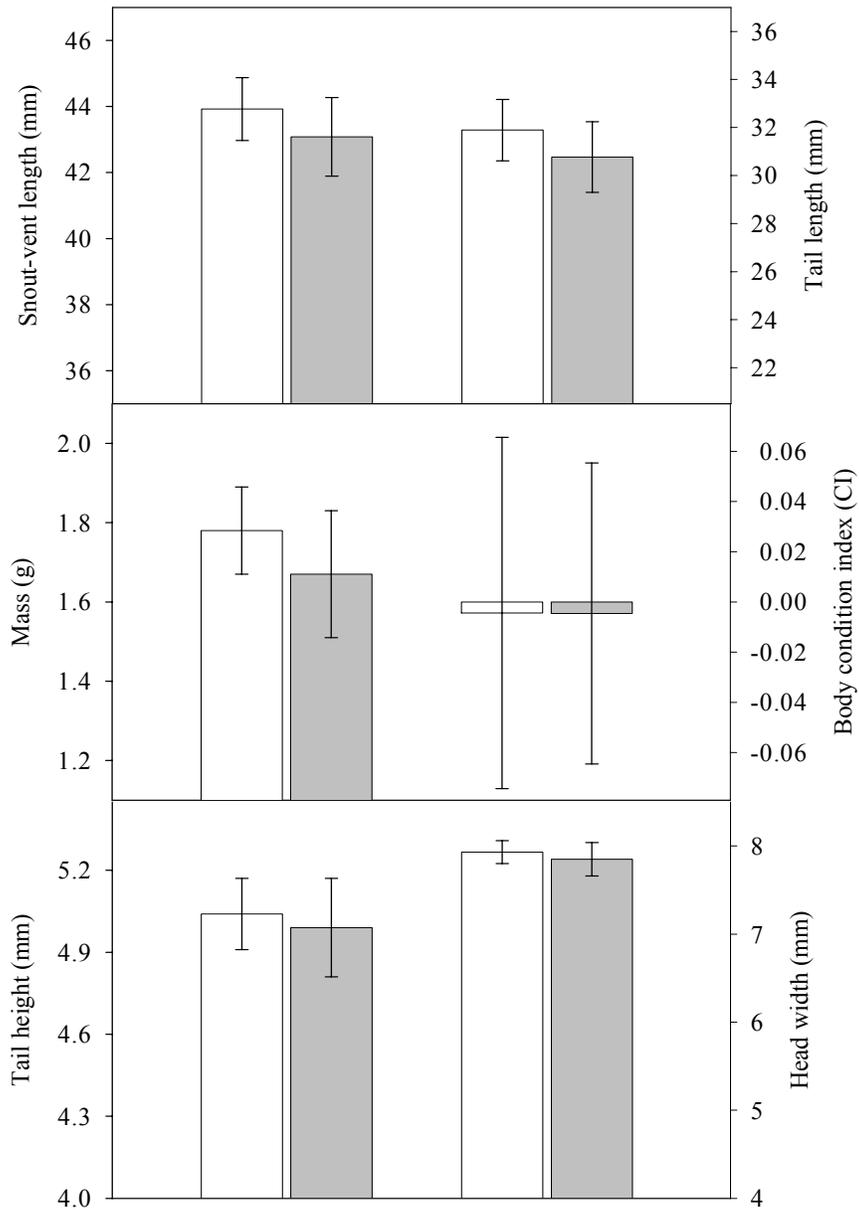
Figure 3. Mean (± SD) of six size traits of metamorphs from polyandrous and monandrous clutches. Unfilled bars are polyandrous clutches; filled bars are monandrous. Each of the three panels has a right and left axis at different scales. The bars on the left correspond with the axis on the left and vice versa.

Multiple and single paternity clutches did not differ in variance for any of the fitness traits measured in the entire study (Table 1). Maternal size was also not significantly different, although polyandrous females were slightly larger than monandrous females for all traits except head width. Very little of the variance in hatchling and metamorph size was explained by maternal size and none of the traits were significant predictors in multiple regression (data not shown).

Discussion

Our study is the first to uncover evidence for a fitness advantage of multi-male mating in an ambystomatid salamander. Although a vast majority of the potential effects we measured were not different between single and multiple paternity clutches, survival to metamorphosis was greater when females mated polyandrously. None of the fitness traits we measured at the egg or hatchling stages showed any evidence for differences between the two types of clutches. We discuss the importance of our results with respect to the major hypotheses of genetic and non-genetic benefits of polyandrous mating that may outweigh the fitness costs of mating and maintain the behavior.

Polyandrous clutches did produce metamorphs that were slightly, though not significantly, larger than those from monandrous clutches. Size differences may be evident at other stages of offspring development (e.g., at sexual maturity) or when animals experience more stressful environmental conditions. The effect of paternity status on survival to metamorphosis had a p-value (0.04) that would not be significant if corrected for multiple comparisons. We acknowledge that this may be a Type I error because of the large number of statistical tests performed in this study. However, because of the exploratory nature of this paper, we choose to

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interpret the result as a potentially important difference in fitness between polyandrous and monandrous females that should be examined further.

As in most studies of this kind in vertebrates (e.g., Olsson & Shine 1997; Hoogland 1998; Pearse et al. 2002; Prosser et al. 2002; Barber et al. 2005; Blouin-Demers et al. 2005), we were unable to randomly assign our experimental females to single and multiple paternity treatments because of the difficulty of generating a large number of mated females under controlled observations. Whether females chose to mate with several males in our breeding replicates may have been related to other traits such as age or size that could have resulted in spurious effects of paternity status on fitness. For example, if females of high genetic quality are more likely to mate polyandrously, any fitness differences we observed could result from this discrepancy. However, female size was completely unrelated to sire number in this experiment and in another salamander (Shillington & Verrell 1996), suggesting that female quality did not affect the paternity status of our clutches. We did not measure any other potential quality indicators (e.g., age, parasite resistance) but nevertheless believe it unlikely that quality relates strongly to mating frequency. Explosive salamander mating systems, such as in the marbled salamander, involve very few social dynamics among females that could translate quality variation into differences in courtship frequency. In fact, males indiscriminately court any salamander they encounter, including other males (D.A. Croshaw, pers. obs.). Of course, future experiments with random assignment are needed to address these concerns.

In mating systems in which males provide material benefits such as nuptial gifts during mating (Sakaluk 1984; Andrade 1996), it is not surprising that females mate polyandrously. When males provide nothing but sperm, as in salamanders, the simplest hypothesis is that females mate often because they need more sperm (e.g., Worden & Parker 2001). Our data do

not support this hypothesis because hatching success and the proportion of dead or unfertilized eggs were not significantly lower in monandrous clutches. Alternatively, if there is high variance in the amount of sperm transferred per sperm cap or sperm viability in female spermathecae, those females that have fewer sperm available for fertilizations may have been more likely to mate with several males. Although we cannot rule out this possibility, we believe it to be unlikely because each spermatophore contains many more sperm than are needed to fertilize all eggs in a clutch (Waights 1998). Females tend to mate and oviposit very quickly, leaving little time for sperm degradation. Sperm do not start to become inviable until at least a few weeks after mating (Sever et al. 1995). Thus, any female who mates at all should receive more than enough sperm for a single breeding event.

Instead, our results suggest that polyandrous females receive indirect genetic benefits potentially consistent with the genetic compatibility, good sperm, and intrinsic male quality hypotheses. Unfortunately, the design of this study cannot distinguish among these competing ideas. The genetic variation hypothesis was not supported because there was no evidence that variance in body size of metamorphs was higher within polyandrous clutches. Our study did not test the sexy sperm hypothesis because we did not follow the metamorphs to adulthood.

Only three previous papers relate to the potential benefits of polyandry in amphibians. Osikowski and Rafinski (2001) suggested that female Montandon's newts mate multiply because they need to replenish sperm supplies. Although they showed that multiply inseminated females produced more eggs and fewer non-developing eggs than did singly inseminated females, their data could be explained by other genetic benefit hypotheses including genetic compatibility, good sperm, and intrinsic male quality. Our results contrast with this paper because we did not find evidence to support the idea that female marbled salamanders mate multiply to receive more

sperm. Garner and Schmidt (2003) found that fecundity and hatching success were not different between multiple and single paternity clutches in alpine newts. They provided evidence that more fertilizations were gained by the male with lower relatedness to the female in multiple paternity clutches, suggesting the genetic compatibility hypothesis. Byrne and Roberts (2000), using an Australian frog, found no evidence that mating with several males increased fitness of females when their eggs were exposed to high desiccation risk and larvae were exposed to fluctuating water depths. This result is not surprising because female frogs with external fertilization generally cannot control which and how many males shed sperm onto their eggs. In such a mating system, females may be forced to mate with multiple males, even when the costs of mating are high. Such females may be engaging in so-called 'convenience polyandry' in which they are forced to acquiesce to male advances because of high costs of resistance (see also Lee & Hays 2004).

The potential fitness benefits of polyandry reported here are admittedly very small and may not be ecologically important. However, these results do not preclude the presence of additional large fitness effects in other important variables or in life stages not studied here. We did not measure juvenile survival, growth, or time to maturity nor did we follow our young salamanders into adulthood to quantify their reproductive success. It is possible that fitness consequences are realized at these later stages of development. In particular, the sexy sperm hypothesis predicts that male offspring from polyandrous clutches will be relatively more successful at acquiring mates and fertilizations as adults because of inherited traits (Wedell & Tregenza 1999; Bernasconi & Keller 2001; Pai & Yan 2002). The good sperm, intrinsic male quality, and genetic compatibility hypotheses are also consistent with later fitness effects, though to differing extents.

We only tested one set of field conditions. Our field experiment may not have captured the proper environment for eliciting large differences between polyandrous and monandrous clutches. In particular, we only manipulated larval density to be very high, presumably creating a stressful high competition environment (D.E. Scott, pers. comm.). However, we do not know if predation of young larvae was frequent which could have lowered density in the enclosures early in the experiment. Larval survival to metamorphosis was actually very high (42%), higher than expected, perhaps indicating that the enclosures were less stressful for the salamanders than we had hoped. We did not manipulate predation risk, food availability, parasite presence, or pond drying time, all of which could uniquely affect larval fitness in ways that create advantages for polyandrous females. Future experiments should use more complex environmental controls to explore potential benefits in more detail (e.g., Sakaluk et al. 2002).

Of course, the costs of mating are the most crucial part of the evolutionary paradox created by widespread universal female polyandry. Although most authors assume that mating is costly because of the ubiquity of sexual conflict, we actually know very little about mating costs in most systems. There are no studies available that have quantified the negative fitness consequences of the act of mating for female salamanders. If costs are low, we do not necessarily have to assume that there are substantial benefits of polyandry beyond the minimal effects reported here. Indeed, multiple studies that found no or very little advantages of polyandry have been published (e.g., Byrne & Roberts 2000; Prosser et al. 2002; Garner & Schmidt 2003; Orsetti & Rutowski 2003; Brown et al. 2004). Although costs undoubtedly accrue when the number of mates increases to very high numbers, we believe that cost differences may be relatively low when females mate with a small number of males. In our study of natural mating patterns in this species, females never clearly mated with more than three

males (Chapter 2). We badly need data to quantify the costs of mating, not only in salamanders, but in a variety of mating systems.

In this study, we showed that polyandrous marbled salamander females enjoyed small fitness benefits over monandrous individuals because a higher proportion survived to metamorphosis. Our data do not support the sperm replenishment or genetic variation hypotheses, but are consistent with the genetic compatibility, intrinsic male quality, and good sperm ideas. Future work should continue to explore the potential costs and benefits of mating in female salamanders with experiments that manipulate environmental conditions and follow offspring into adulthood.

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Chapter 5

The effect of female polyandry and male breeding numbers on population genetic diversity

and fitness correlates of metamorphic cohorts in marbled salamanders

Abstract

Mating systems are especially important in population viability because they affect

reproduction, individual fitness, population genetic diversity, and effective population size (N_e),

all of which can impact extinction risk. Promiscuous mating systems in which females mate

with several males in a single breeding season are expected to result in high N_e and relatively

low extinction risk compared to other mating systems. By varying the ratio of males to females

(8:6 or 2:6), we manipulated frequency of female polyandry in small breeding groups of marbled

salamanders (*Ambystoma opacum*). We then allowed larvae to compete at high density in field

enclosures and compared population parameters in the two mating treatments and at two stages

of offspring development, hatching and metamorphosis. With highly polymorphic microsatellite

DNA markers, we assigned maternity and paternity of an entire sample of hatchlings and every

metamorph that emerged from the field enclosures. We calculated survival to metamorphosis,

principal component scores of size at metamorphosis, allelic richness, gene diversity, variance N_e

(N_{ev}), sex ratio N_e, number of males successfully producing recruits, and opportunity for

selection (I) at both developmental stages. Breeding groups with many available males had

significantly more polyandrous clutches and marginally more total males that produced offspring

than those with low male availability. Effect sizes of the differences in N_{ev} and I between

hatchlings and metamorphs were moderate to high in magnitude, with higher N_{ev} and lower I for

hatchlings. This study provides a starting point for future experiments investigating the effects of mating on population ecology in vertebrates of conservation interest.

Introduction

The importance of behavior to population viability and management is increasingly being recognized (Curio 1996; Caro 1998; Sutherland 1998; Anthony & Blumstein 2000; Gosling & Sutherland 2000). Reproductive and mating behaviors are obviously crucial for long-term persistence of populations because new individuals must be produced to replace mortality losses. But aside from this intuitive connection between mating and population ecology, many additional, more inconspicuous relationships may exist (Wedekind 2002; Rowe & Hutchings 2003; Quader 2005). For example, mating systems can affect population genetics, dispersal and interconnection of subpopulations, and individual fitness of offspring. Supportive breeding programs (Ebenhard 1995; Wiese et al. 1996) will especially benefit from more knowledge of mating behaviors and their consequences for population ecology in threatened and endangered taxa (Grahn et al. 1998).

Natural mating systems are likely to become disrupted by ever-escalating anthropogenic influences (Rowe & Hutchings 2003; Quader 2005). Mating can be altered by decreases in population size and density, changes in population age- and sex-structure, degradation and fragmentation of habitat, or simply disturbance of the animals. Very small populations may have reduced population growth rates because females are unable to find mates at all or their mate choice is limited by lack of available males (Allee effects; Stephens & Sutherland 1999). Evidence suggests that choice in mating increases individual fitness (Crocker & Day 1987; Simmons 1987; von Schantz et al. 1994; Moller & Alatalo 1999; Drickamer et al. 2000, 2003;

but see Schaeffer et al. 1984). Females that do not get the opportunity to pair with a preferred male may refrain from mating, invest less in their offspring, or alter the primary sex ratio, each of which could exacerbate mating disruptions (Burley 1981; Moller & Thornhill 1998; Sheldon 2000; Kolm 2002). Selective removal of one sex or size class from harvested populations can also influence mate choice and social dynamics, especially in highly social species and those with sexual dimorphism that renders one sex more vulnerable. Increased patchiness of habitat can change the spatial distribution of females, and hence the mating system, especially when relatively rare resources are necessary for reproduction (e.g., Zabel & Taggart 1989). Finally, disturbance from human activities may result in less time spent on mating activity because of a perceived high risk of predation by animals (Forsgren 1992; Fuller & Berglund 1996; Gong & Gibson 1996; G. Jones et al. 2002). Thus, via each of these mechanisms, mating system disruption could be an additional and previously unrecognized factor that augments the dangers of population depletion, increasing the local extinction risk of affected populations.

Mating systems are important not only for producing more individuals but also for determining levels of genetic diversity in populations. Theoretical and empirical studies have shown that increased genetic diversity is beneficial to population persistence (Frankham 2005), presumably because genetically heterogeneous populations are likely to contain some individuals that are adapted to new environmental conditions, allowing populations to persevere in the face of stochastic environmental fluctuations and human influences. In short, genetic variance allows for future adaptation of populations. Moreover, genetic heterozygosity is sometimes correlated with several fitness traits, including growth rate, disease resistance, and survival (Samollow & Soule 1983; Mitton & Grant 1984; Danzmann et al. 1988; Ferguson & Drahushchak 1990; Jimenez et al. 1994). Population genetic diversity can decrease due to the loss of alleles by

genetic drift, which is expected to be stronger when populations are small. The concept of effective population size (N_e) is extremely important in conservation biology because it is negatively correlated with inbreeding and the rate of genetic diversity loss (Frankham 1995). Effective population size may be thought of as the size of an ideal population (i.e., one that meets several simplifying assumptions) that would lose genetic diversity at a rate equal to the reference population (Wright 1931). Experimental evidence suggests that lowered N_e increases the probability of population extinction when controlling for population size (Newman & Pilson 1997; Saccheri et al. 1998).

Mating systems can be important factors influencing N_e because of their influence on fitness variance (Nunney 1993; Anthony & Blumstein 2000; Wedekind 2002). According to Wright's (1940) definition of N_e, anything that increases variance in lifetime reproductive success results in a decreased N_e:

$$N_e = (NF - 1)/[F + (s^2/F)-1]$$

where N is the population size, F is the mean lifetime reproductive success, and s^2 is the variance in lifetime reproductive success. Promiscuous mating systems are generally expected to have the highest N_e (Fiumera et al. 2004; Kaeuffer et al. 2004). Strictly monogamous systems should be higher than strictly polygynous and polyandrous systems (Anthony & Blumstein 2000). Thus, multiple mating by both males and females is likely to increase N_e and decrease genetic diversity loss relative to strict monogamy. In particular, female polyandry should result in multiple paternity and allow more males to successfully produce recruits, lowering variance in male fitness (Sugg & Chesser 1994). Storz et al. (2001) suggested that polygyny results in lowered effective population size in a bat species, probably because of high variance in male reproductive success. Other examples of associations between N_e and mating systems have been reported

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(e.g., Robbins et al. 1987; Martinez et al. 2000) but experimental data are scarce. One study by Briton et al. (1994) did show that laboratory populations of *Drosophila* that were forced to be polygynous lost more genetic diversity than those forced to be monogamous. The relationship between mating system and N_e, especially in natural populations and species of conservation interest, remains poorly understood.

Both simulation and empirical studies suggest that risk of population extinction and vulnerability can be related to the type of mating system. Legendre et al. (1999) and Saether et al. (2004) used stochastic demographic population models to show that monogamy may result in a higher extinction risk than polygyny. Brashares (2003) found that monogamous mammals and polygynous ones with small harem sizes were more likely to experience local extinction than strongly polygynous species in Africa. Reed and Shine (2002) showed that elapid snakes without direct male-male competition were more likely to be endangered. Several studies have found evidence that populations experiencing strong sexual selection, which is mediated by mating systems, are more likely to go extinct. In North American birds, species that have sexual color dimorphism, presumably indicating the influence of sexual selection, had higher local extinction rates than monochromatic species (Doherty et al. 2003). Of birds introduced to islands, dichromatic species have had lower rates of successful establishment (McLain et al. 1995; Sorci et al. 1998). Although it is clear that mating systems can affect extinction risk, results of these few available studies concerning the relationships among N_e, mating system, and extinction are not entirely consistent. Moreover, the numerous other factors involved in determining N_e could be more important and often obscure the effects of mating system (Frankham 1995). The extremely complex relationship between mating system and N_e has not yet been elucidated and likely depends on other ecological and evolutionary factors.

Mating behaviors have profound impacts on individual fitness of animals which can also influence population persistence. Mean fitness is important for population growth rates, extinction risk, and colonization of new habitat. Because mating behavior is often an important factor in determining individual fitness (Jennions & Petrie 2000), it should also affect population viability. Mating with multiple males can influence female fitness in several ways (Jennions & Petrie 2000). For example, in some species, mating females receive direct benefits such as nuptial gifts. They may profit from producing groups of progeny with higher genetic variation or fertilizing a majority of their eggs with sperm from genetically compatible males. They could produce offspring that are good competitors if sperm competition ability is correlated with heritable fitness traits. Finally, by trading up genetically, polyandrous females may simply produce offspring with higher quality males on average.

Amphibian populations are especially important in conservation biology because they are currently experiencing declines (Beebee & Griffiths 2005). Unfortunately, extremely little information is available about N_e in amphibians and how it is affected by mating behavior. Amphibians generally have promiscuous or polygynous mating systems, although there is considerable variation among species. In salamanders, females of all species that have been studied mate with multiple males in a single breeding season (e.g., Gabor & Halliday 1997; Gabor et al. 2000; Myers & Zamudio 2004; Adams et al. 2005; Gopurenko et al. in press). Males also often mate with several females (A.G. Jones et al. 2002; Chapter 3), resulting in promiscuous systems. However, some females are monandrous and the frequency of single-male mating varies among species (Chapter 2). Effective population size could be affected by interspecific differences in the extent of female polyandry.

In the few studies that have estimated it in amphibians, N_e seems to be relatively small in general, around 100 or less (Merrell 1968; Easteal 1985; Berven & Grudzien 1990; Driscoll 1999; Funk et al. 1999; Jehle et al. 2001; Rowe & Beebee 2004). Some of these populations had extremely small N_e to adult census size (N) ratios, but others were large. Such variance could be related to the mating system and/or intense competition at the larval stage. Because amphibians often have very high fecundity, variance in reproductive success can be substantial and is only exacerbated by typically male-biased sex ratios (Pough et al. 1998) and postmating mechanisms that determine fertilization success of competing males (Garner & Schmidt 2003). High fecundity creates dense larval populations in ponds, and survival of hatchlings to metamorphosis can be very low (e.g., Stangel 1988). Due to the difficulty of determining parentage of metamorphic amphibians, assessments of individual variance in recruit production have been few. It is possible that only a few clutches produce a majority of recruits each year and, within single clutches, one of many males monopolizes most of the paternity. Each of these sources of variance, in addition to population fluctuations, could dramatically decrease N_e, and reduce the positive effects of multiple paternity. No previous work with amphibians has attempted to relate mating behavior to N_e, and only a few (Byrne & Roberts 1999; Osikowski & Rafinski 2000) have looked at mating and individual fitness-related traits that could affect population ecology (e.g., survival to metamorphosis, size at metamorphosis, Scott 1994).

The purpose of this study was to investigate the population level effects of male availability and polyandrous mating by female marbled salamanders (*Ambystoma opacum*). To manipulate the extent of polyandry and number of males that produced offspring, we varied the number of males available to females in breeding groups housed in experimental nesting habitat. Because the manipulation also resulted in differences in N_e and breeding population density, the

design was unable to distinguish among the effects of these variables and the number of available males. We compared parental N_e, genetic diversity, size at metamorphosis, and survival to metamorphosis of cohorts recruited from these breeding populations and the total number of males producing recruits, all variables that are potentially important in population viability. Specifically, we predicted that higher levels of polyandry and the number of males available to females would cause increases in one or more of these factors. We also were interested in whether N_e and genetic diversity changed during the larval period, so we compared estimates made at hatching and metamorphosis. Because variance in reproductive success, especially among males, influences N_e, we also calculated opportunity for selection (I), which is expected to vary with sex ratio, at both stages for comparison.

Materials and methods

Field Experiment

Our methods are presented in detail elsewhere (Chapter 4). Briefly, we set up two treatments of cattle tanks with breeding groups of marbled salamanders (*Ambystoma opacum*). Because *A. opacum* breed and nest terrestrially, the tanks contained soil, cover boards, vegetation, leaf litter, and other debris. Each tank contained six females and either two (low polyandry, N = 5) or eight males (high polyandry, N = 5). Breeding populations in the female-biased sex ratio treatment were expected to produce multiple paternity clutches less frequently and cohorts of young sired by fewer total males than the populations in the male-biased sex ratio treatment because of the difference in available male breeders. However, although polyandrous clutches were more common in the high polyandry group, the number of males that produced

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hatchlings was only marginally higher because some females (N = 12 out of 40 total whose offspring were used) mated before the beginning of the experiment (see Chapter 3).

Salamanders used in the tanks were collected from a drift fence completely encircling the Ginger's Bay, a small temporary wetland where *A. opacum* breed, as well as partial drift fences and a road that were located at least 60 m from the wetland. Because females are known to mate before arriving at the bay (Krenz & Scott 1994), we used only females collected from outer sites in an attempt to minimize mating prior to the initiation of the experiment. Males from all collecting sites were used in the tanks. Females were assigned randomly to tanks, but males were chosen to span a range of sizes in the high polyandry tanks for the purposes of a related study (Chapter 3). Males in the low polyandry tanks were randomly assigned. After breeding, nests were collected and eggs were submersed in well water to induce hatching. We selected egg clutches with many viable hatchlings for use in field enclosures. In December 2004, we stocked 40 hatchlings from each of four clutches in large field enclosures (N = 10) located at Ellenton Bay on the Savannah River Site in Aiken County, South Carolina. Each of the enclosures received hatchlings from a single tank. Enclosures were rectangular (3 by 4.3 m) and made of fiberglass window screen, with bottoms but not tops. Larvae were left in the enclosures to feed and develop until metamorphosis began in April 2005. We retrieved metamorphs with minnow traps and dip nets, measured them, and collected tail clips for subsequent genetic analysis. Size traits measured were snout-vent length, tail length, tail height, head width (all to the nearest half millimeter), mass (to the nearest hundredth of a gram), and body condition index (defined as the residuals from a linear regression of mass on snout-vent length).

Parentage Assignment

We used four microsatellite DNA markers from Chapter 1 to assign parentage of hatchlings and metamorphs (Chapter 4). Because marbled salamanders lay clutches of eggs in terrestrial nests, we were able to collect groups of sibling progeny. We genotyped 16 hatchlings from each of the 40 clutches used in the experiment. The microsatellite data were sufficient to assign maternity and paternity of every hatchling in the sample using strict exclusion. For metamorphs, we were unable to always use strict exclusion and resorted to an alternative, albeit equally rigorous, approach. Briefly, we compared each individual's genotype to those of the four sibling arrays that were used to stock each enclosure. We excluded individuals from clutch membership when a genotype at any of the four loci was not present at least once in the hatchling data set. Once a clutch was assigned, we could easily determine the mother first and the father subsequently in most instances. In the few cases where individuals could not be assigned to parents with the core loci, we used four additional markers. We also selected a sample of 8 metamorphs from each enclosure (total N = 80) to genotype at these extra loci to test the accuracy of our clutch exclusion approach. Clutch assignment never conflicted with strict parentage exclusion. A very small proportion of the metamorphs could not be assigned to parents with the data from the complete set of eight loci and were excluded from all statistical analyses.

With the parentage information, we were able to classify each clutch in the enclosure experiment as either polyandrous (having multiple paternity) or monandrous (having single paternity) with extremely high confidence. We could determine the exact number of males that produced metamorphs from each enclosure and an estimate of the number of males that produced

hatchlings in the clutches used for the field experiment. The microsatellite data provided further information about the genetic diversity of the cohorts recruited from the enclosures.

Statistical Analysis

There are several different formulations of the quantity N_e (inbreeding N_e, variance N_e, etc.) which model the same underlying processes of genetic diversity loss but refer to different mechanisms. There are also a number of ways to calculate and/or estimate each of the N_es. In this paper, we focus on the variance N_e which relates to the strength of genetic drift and can be calculated using sex ratio of breeders and variance in individual reproductive success. We performed analyses with both variance N_e (N_{ev}, calculated using the number of male and female breeders and their variance in fitness) and sex ratio Ne (N_{er}, calculated with only the census number of breeders). We used equations from Kimura and Crow (1963) for calculations. N_{ev} is given by

$$N_{ev} = 4 \, N_{em} \, N_{ef}/(N_{em} + N_{ef}),$$

where N_{em} and N_{ef} are the effective number of breeding males and females. The effective number of females was calculated as

$$N_{ef} = (N_f \mu_f - 1)/(\mu_f - 1 + \sigma^2_{kf}/\mu_f),$$

where N_f is the number of breeding females, μ_f is the mean reproductive success among females, and σ^2_{kf} is the variance in reproductive success. N_{em} was calculated in the same way, but with data from males. We calculated N_{er} with the same equation but used the census number of breeding males and females. To look at variance in relative fitness, we calculated opportunity for selection (*I*) for each breeding group as the variance in fitness divided by the square of mean fitness (Jones et al. 2004).

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To determine if the manipulation of male availability successfully influenced frequency of polyandry, we used the FREQ procedure in SAS (SAS Institute, 2003) to do a contingency table analysis. We used the TTEST procedure to test for differences in total sire number and report the one-tailed p-value to increase power. Our a priori hypothesis was that the high polyandry treatment would have greater total sires represented in the progeny. We then used repeated measures ANOVA and MANOVA in the GLM procedure to test for differences between the hatchling and metamorph stages as well as group differences in mean metamorph size and survival, N_{ev}, N_{er}, I, and genetic diversity. To compare genetic diversity of the two treatments, we calculated gene diversity (i.e., unbiased expected heterozygosity based on Hardy-Weinberg expectations, Nei 1973) and allelic richness, adjusted for sample size, of offspring at the hatchling and metamorph stages in FSTAT (v. 2.9.3.2; Goudet 1995). We averaged these two measures across our core group of four loci and square-root arcsine transformed gene diversity to meet the assumptions of parametric statistics. Treating each breeding group as a replicate, we paired quantities at the hatchling and metamorph stages using the REPEATED statement and tested for group differences as well as within-replicate (stage) effects. Differences between the two stages would indicate the importance of larval competition in determining population genetic parameters. We also compared the two groups of enclosures in survival to and size at metamorphosis. Survival data were expressed as the proportion of larvae that metamorphosed and were normalized with a square-root arcsine transformation. Because six different size measures of each metamorph were available, we reduced the dimensionality of the data by performing a principal component analysis in the SAS PRINCOMP procedure. Principal component mean scores for each enclosure were used in the analysis. We tested for group differences in transformed survival and the first two principal components using standard

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MANOVA in the GLM procedure. Because larval survival was considerably lower in the high

polyandry treatment, we inspected univariate ANOVA tests of treatment differences when the

number of surviving metamorphs was included as a covariate. We were also interested in the

effects of increased polyandry and total sire number on fitness variance, so we used Levene's test

of heteroscedasticity to compare variation in fitness correlates in the two groups of enclosures.

Because of the low number of replicates (N = 5 enclosures in each group), we calculated effect

sizes (Cohen's d, Cohen 1988) for all tests with p values below 0.2. This allowed us to assess

the potential for treatment effects in the absence of statistical significance and evaluate some

factors that may deserve further exploration.

Results

Polyandrous clutches were more frequent in the high polyandry treatment than in the low

polyandry treatment (Table 1, χ^2 = 5.23, df = 1, p = 0.02). The number of males that actually

sired offspring was also marginally higher in the high polyandry group (Figure 1, t = 1.55, df =

8, one-tailed p = 0.08). Our efforts to use only females that were unmated at the start of the

experiment were unsuccessful (N = 12 females mated prior to the experiment), causing the

number of sires represented in the offspring to be higher than expected in the low polyandry

group.

The number of males producing offspring was exactly the same for the hatchling sample

and the metamorphs from the enclosures (Figure 1); no males that sired hatchlings failed to

produce metamorphs and no new males were represented in the metamorphs (i.e., limited lethal

sampling of hatchlings revealed all males with offspring). In polyandrous clutches, the

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Table 1. Number of polyandrous and monandrous clutches selected from tanks in the high polyandry and low polyandry treatment groups for use in experimental field enclosures.

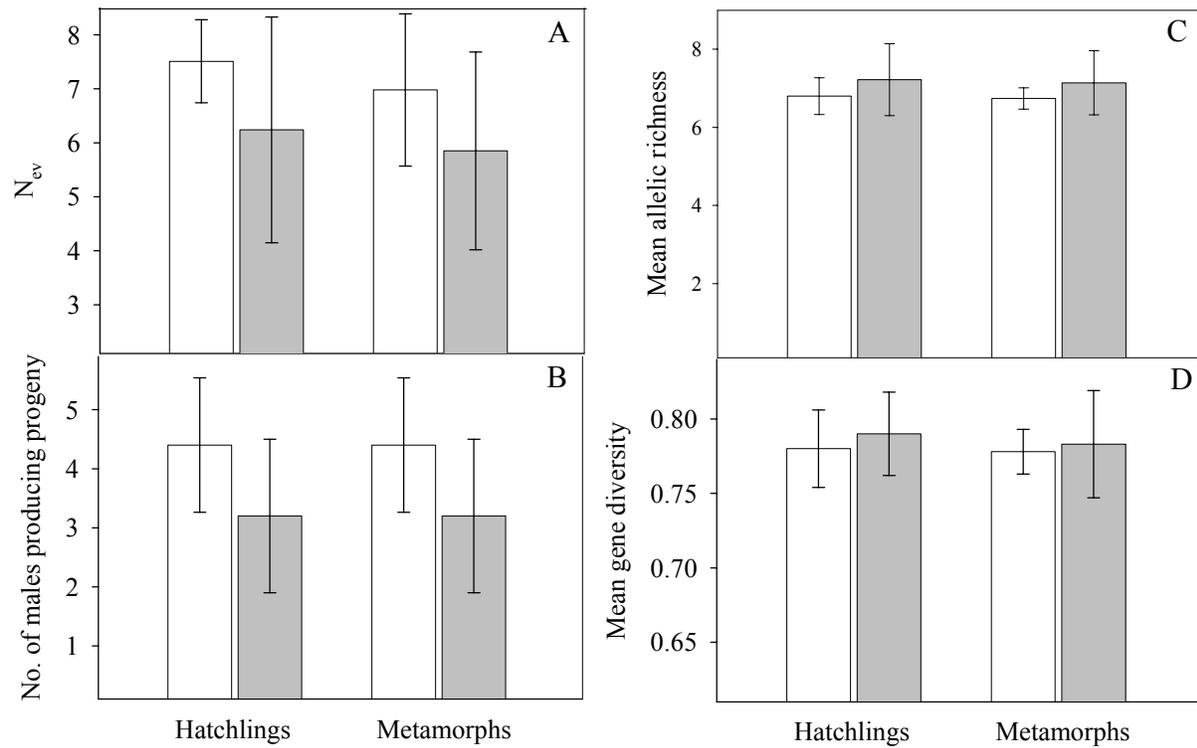|	Multiple paternity clutches	Single paternity clutches
High polyandry	11	9
Low polyandry	4	16

Figure 1. Mean (± SD) of several population quantities in the high and low polyandry treatments at the hatchling and metamorph stages. Unfilled bars are the high polyandry treatment; filled bars are the low polyandry.

proportion of young produced by the two competing males stayed relatively constant in the samples from the two stages of development (Table 2). Variances in size at and survival to metamorphosis were not different between the two groups (results of statistical analyses not shown).

The first two principal components explained 80% of the size variation among metamorphs (Table 3). Every size variable loaded heavily on the first component, but the second component was highly positively correlated with body condition index and negatively correlated with snout-vent length and tail length.

None of the analyses were statistically significant at the 0.05 level when corrected for multiple comparisons (Table 4). Effect sizes were small to moderate for comparisons between the two developmental stages in N_{ev} and I (Figure1; Figure 2). There was higher opportunity for selection and lower N_{ev} in the metamorphic cohort. Effect sizes were large for the first two principal components and survival (Table 4). The high polyandry treatment produced metamorphs that were larger overall, especially in length, than the low polyandry treatment (Figure 3). Animals from the low polyandry treatment tended to score higher on the second principal component, indicating that they were a bit shorter and stouter than those from the high polyandry treatment. The low treatment produced more surviving metamorphs than did the high treatment. When the number of surviving metamorphs was included in the model, univariate tests showed that principal component scores were not different between the two treatments (PC1: F = 1.83, df = 1, p = 0.22; PC2: F = 5.83, df = 1, p = 0.05).

Table 2. Paternity share enjoyed by competing males within polyandrous clutches from estimates at the hatchling stage and exact proportions at the metamorph stage.

Clutch	Hatchlings: sire A	Hatchlings: sire B	Metamorphs: sire A	Metamorphs: sire B
1	0.69	0.31	0.84	0.16
2	0.88	0.12	0.63	0.37
3	0.94	0.06	0.93	0.07
4	0.81	0.19	0.93	0.07
5	0.73	0.27	0.80	0.20
6	0.69	0.31	0.50	0.50
7	0.69	0.31	0.50	0.50
8	0.93	0.07	0.92	0.08
9	0.81	0.19	0.65	0.35
10	0.81	0.19	0.90	0.10
11	0.69	0.31	0.94	0.06
12	0.69	0.31	0.83	0.17
13	0.88	0.12	0.69	0.31
14	0.81	0.19	0.67	0.33
15	0.80	0.20	0.90	0.10

Table 3. Results of principal component analysis on six size traits in metamorphic marbled salamanders. PC1 through PC5 are the ordered principal components. Loadings are indicated for each size variable. Cumulative variance is expressed as a proportion.

	PC1	PC2	PC3	PC4	PC5
Snout-vent length	0.41	-0.52	-0.097	-0.067	-0.48
Mass	0.50	0.056	0.044	0.093	-0.49
Tail length	0.40	-0.29	0.65	0.31	0.49
Tail height	0.41	0.18	0.018	-0.85	0.28
Head width	0.42	0.035	-0.73	0.34	0.43
Body condition index	0.27	0.78	0.20	0.24	-0.18
Cumulative variance explained	0.62	0.80	0.88	0.95	1.00

Table 4. Results of repeated measures ANOVA and MANOVA for four separate analyses. Stage refers to within-population effects and stage by treatment is the interaction. We only calculated effect sizes for effects with p-values less than 0.2. NA, not applicable; PC1, first principal component of metamorph body size; PC2, second principal component of metamorph body size; N_{ev}, variance effective population size; I, opportunity for selection. PC1, PC2, and larval survival were analyzed together. Other variables were analyzed separately.

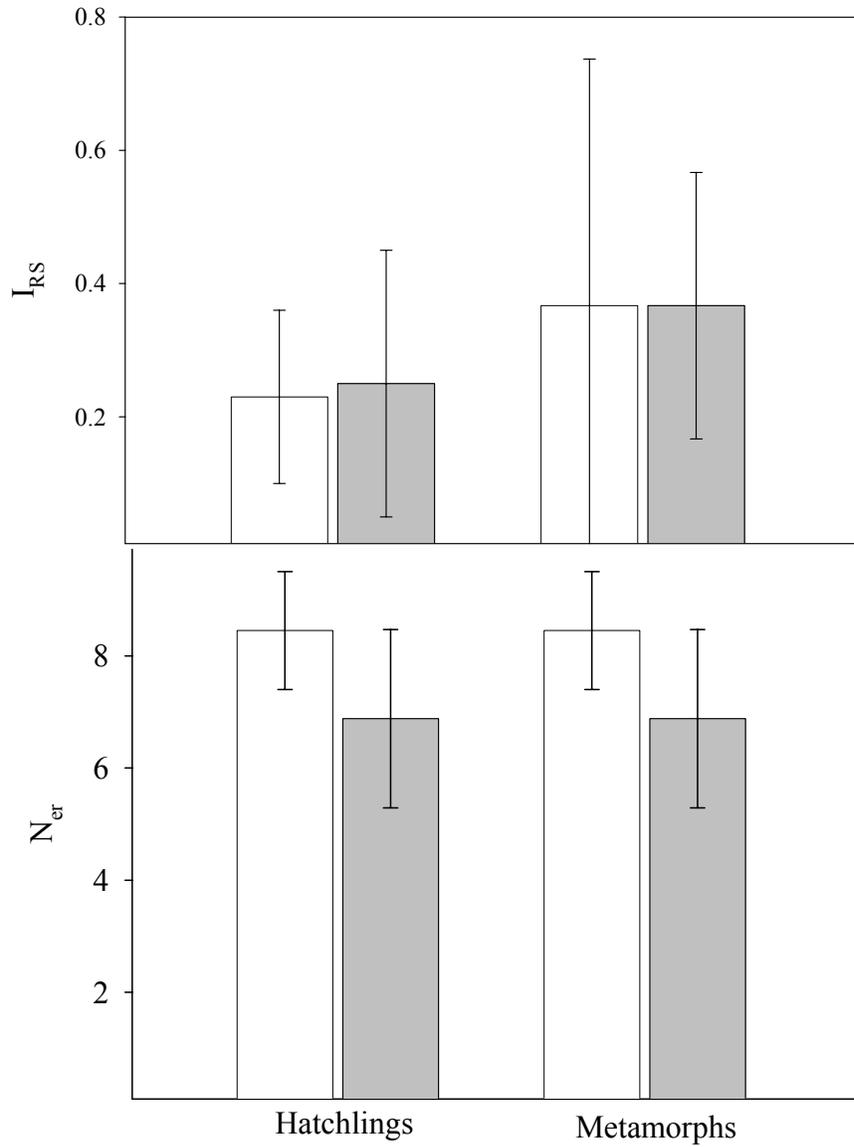| Variables	Treatment effect			Stage effect			Interaction			Univariate treatment effect			Cohen's D
	F	DF	P	F	DF	P	F	DF	P	F	DF	P	
Allelic richness	0.56	7,2	0.59	0.11	8,1	0.75	0.00	8,1	0.98	NA			NA
Gene diversity	0.19	7,2	0.83	0.46	8,1	0.51	0.07	8,1	0.80	NA			NA
PC1	2.75	6,3	0.13	NA			NA			2.35	8,1	0.16	0.97
PC2										9.45	8,1	0.02	1.95
Survival										2.24	8,1	0.17	0.99
N_{ev}	0.71	7,2	0.53	3.09	8,1	0.12	0.07	8,1	0.80	NA			0.29
I	0.02	7,2	0.98	3.25	8,1	0.11	0.02	8,1	0.90	NA			0.57

Figure 2. Mean (± SD) of sex ratio effective population size (N_{er}) and opportunity for selection calculated from reproductive success in the high and low polyandry treatments at the hatchling and metamorph stages. Unfilled bars are the high polyandry treatment; filled bars are low polyandry.
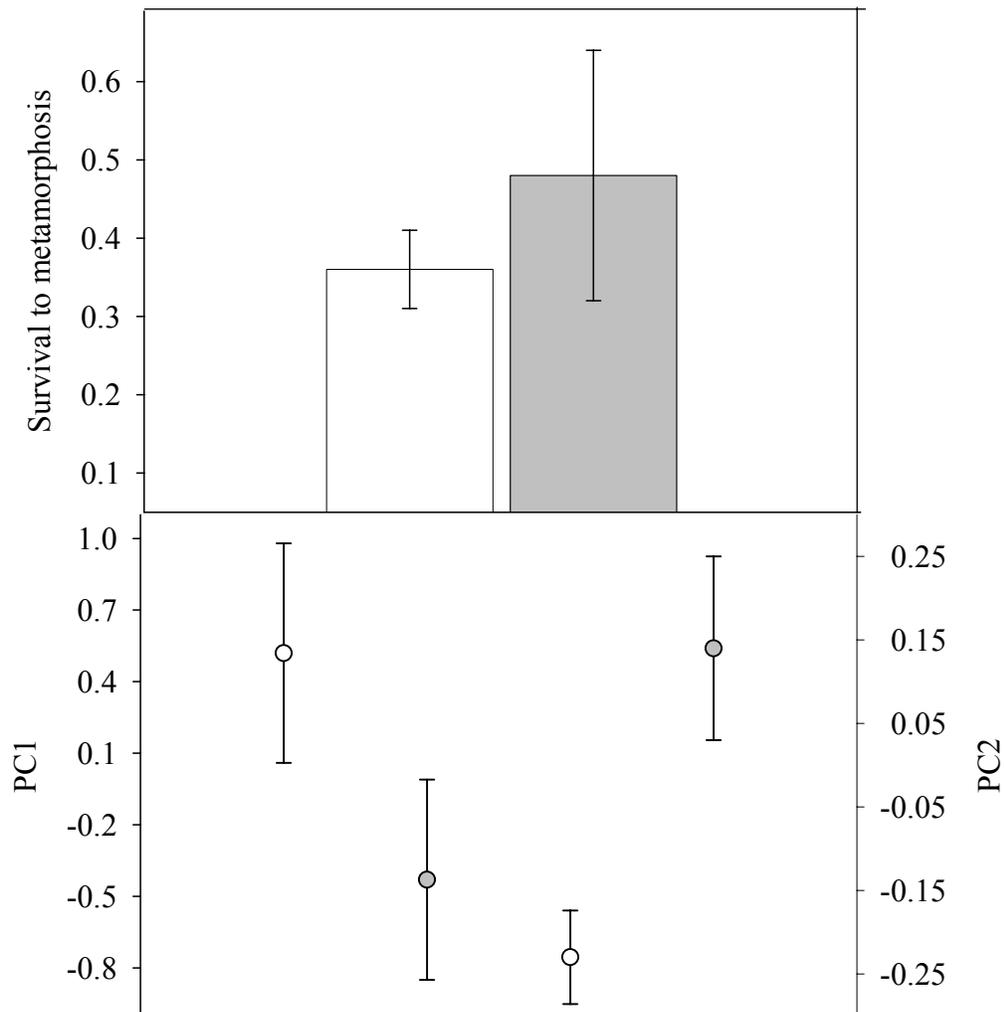
Figure 3. Mean (± SD) survival to metamorphosis and principal component scores of size at metamorphosis for the two groups of enclosures. Mean principal component scores were calculated for each enclosure and numbers reported are from these means. Symbols on the left correspond with the axis on the left and vice versa. Unfilled symbols are the high polyandry treatment; filled bars are low polyandry.

Discussion

In this study, we found no significant differences in population parameters between the high polyandry and low polyandry treatment or between the hatchling and metamorph stages of offspring development. This did not reflect similarity between treatments in N_e and the level of polyandry. We showed that polyandry was significantly more frequent in the high polyandry treatment and that marginally more males produced offspring. Thus, our attempt to produce two experimental groups of differing mating system and effective population size (N_e) was successful, despite the fact that some females had already mated with other males before the beginning of the experiment. However, because of the experimental necessities of working with salamanders in the field, our statistical tests did not enjoy extremely high power. We calculated effect sizes for the statistical tests that were marginally significant and identified some potentially interesting patterns in the data. We discuss these effects because we feel they are worthy of consideration and may be biologically important. Later in this section, we also discuss the possibility that other influential factors mask any effects of mating behavior on population genetic diversity and N_e.

First, size at and survival to metamorphosis had very large effect sizes (0.97 to 1.95). Animals produced from the high polyandry treatment were larger overall than those from the low polyandry treatment. Those from the low polyandry treatment scored significantly higher on the second principal component which was positively correlated with body condition index and negatively correlated with snout-vent length and tail length. However, because this principal component was much less important in explaining size variation (62% vs. 18%), the results still indicate that larger animals were produced from the high polyandry treatment. The larger size of metamorphs from the high polyandry treatment may be due to the higher larval survival in the

low polyandry treatment. If larval mortality occurred early in the experiment, which seems likely, overall density in the low polyandry enclosures was likely higher for most of the larval period. In fact, principal component scores were not different between the two treatments when the number of surviving metamorphs was included as a covariate. Also, although polyandrous clutches produced slightly larger metamorphs than did monandrous clutches, size differences were not significant in this experiment (Chapter 3). The most likely explanation is that the size difference we detected was a spurious association made evident by the disparity in population density which resulted in lower growth rates in the low polyandry enclosures.

The higher survival in the low polyandry group is difficult to explain, especially in light of our previous analysis showing that individual polyandrous clutches produced more metamorphs than did monandrous clutches (Chapter 4). The most likely explanation is that the difference is due to singular environmental conditions within the individual enclosures. Indeed, we noticed potentially important differences among enclosures because predatory snakes (e.g., *Seminatrix pygaea* and *Nerodia* sp.) invaded some but not others. Paedomorphic mole salamander (*Ambystoma talpoideum*) predators also were present in variable numbers, although little of the survival variance was explained by mole salamander density (data not shown). The survival difference was likely due to random chance, with more of the low polyandry enclosures just happening to produce many metamorphs.

Effect sizes of developmental stage were small to moderate for variance effective population size (N_{ev}, 0.29) and opportunity for selection (I, 0.57) but were greater than 0.2, Cohen's (1988) threshold for a small effect. Parental N_{ev} was greater at the hatchling stage and I was greater at the metamorph stage. This pattern makes sense because both quantities are strongly related to variance in reproductive success, albeit in opposite directions. If there is

genetic variance in competitive ability in high density larval environments, we should expect some parents to produce more metamorphs than others, creating these differences. Opportunity for selection was much greater in the breeding groups when all males, even those that sired very few or no hatchlings, were included in the analysis (Chapter 3). Thus, although fitness variance at the fertilization and hatchling stages likely results in a very small N_e/N ratio, as in most other amphibians (e.g., Scribner et al. 1997; Jehle et al. 2001), our results suggest that N_e is often further reduced during the larval aquatic stage. These decreases often may be density-dependent, as most ponds can produce only a limited number of metamorphs. High competition probably increases variance among adults in production of both metamorphs and new breeding individuals, which of course would exacerbate the small N_e. Larval survival in this experiment was relatively high (42%) in comparison to most other studies in *Ambystoma* (Scott 1990 and references therein). In more typical situations, where larval survival can be as low as 1%, N_e must be drastically different if measured at the two stages of progeny development. We believe that further work in experimental and natural populations would frequently show greater changes in these parameters from hatching to metamorphosis.

Somewhat surprisingly, despite the fact that more clutches were polyandrous and more males were represented in the enclosures of the high polyandry group, neither measure of genetic diversity showed even a marginally significant difference. In fact, allelic richness and gene diversity were both slightly higher in the low polyandry group at both stages. This result probably reflects the substantial overlap in the number of sires that produced offspring in the two groups. Of course, genetic diversity and N_e are very closely related quantities, and extreme fitness variance has the potential to decrease overall genetic diversity in the same way. Both allelic richness and gene diversity may have been lessened in this experiment by competition

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among males for fertilizations and among larvae in the high density enclosures. These variances can be substantial in ambystomatid salamanders (this study; Adams et al. 2005; Chapter 3).

We can contrast our results with those from a similar experiment that used *Drosophila* fruit flies (Briton et al. 1994). In their study, experimental populations with female-biased sex ratios had much lower gene diversity, N_e, and reproductive fitness than those with an equal sex ratio. Our results are in direct opposition to their findings about fitness and gene diversity because our low polyandry, or female-biased sex ratio, treatment did not have lower genetic variation or produce fewer metamorphs. In fact, they actually were higher than the high polyandry, or male-biased sex ratio, treatment for both of these measures. The probable explanation for the discrepancy is that they used a much more female-biased sex ratio (7 to 1, females to males versus 3 to 1 in this study). They also allowed the differences to accumulate over eight generations and did not experience the outside mating problem. The study of Briton et al. (1994) showed that mating patterns and sex ratio can potentially influence important population parameters. More experiments like the one reported herein, using natural or semi-natural settings and species of conservation interest, should further our understanding of these complex relationships.

Several empirical studies have reported that mating patterns affect N_e, but they were not experimental and, therefore, cannot completely rule out other causal agents. For example, Martinez et al. (2000) found a higher N_e and rate of multiple paternity were associated in a single Atlantic salmon population compared to another population. Of course, other factors such as habitat quality or level of disturbance could be confounding the association. Dobson et al. (2004) estimated N_e to be higher than N in a colony of black-tailed prairie dogs and hypothesized that their tendency to form social breeding groups caused the unexpectedly high N_e. Kaeuffer et

al. (2004) showed that a feral domestic cat population with promiscuous mating had higher N_e than one with a polygynous mating system. Most of this correlational evidence supports the idea that mating in general, and female polyandry in particular, can increase N_e. Although we find the available information compelling, only an experimental approach like ours can provide definitive answers about the importance of mating for N_e.

This study provides a starting point for continued field experiments investigating the effects of mating on population ecology, especially in vertebrate animals of conservation concern. Further effects of the level of polyandry and correlated variables may have been masked by unusually high larval survival and mating of females prior to capture. Mating may influence the population parameters studied here, but a complete understanding of its importance relative to other portions of animal life history (e.g., larval competition in aquatic habitats and juvenile competition in uplands) awaits more experimental research.

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Appendix

Institutional Animal Care and Use Committee
UNIVERSITY OF NEW ORLEANS

Date: January 16, 2006

To: Dean Croshaw
                Joseph H. K. Peschman, Ph.D.

From: Gerald J. LaHoste, Ph.D.
                Chair, Institutional Animal Care and Use Committee

Re: IACUC Application # UNO-083

Dear Mr. Croshaw,

Your application for the use of animals in research entitled "Multiple paternity and estimating reproductive skew among competing sires in marbled salamanders" has been approved. The (retroactive) start and expiration dates for this approval are: October 1, 2002 – September 30, 2005.

UNIVERSITY OF NEW ORLEANS Institutional Animal Care and Use Committee

Institutional Animal Care and Use Committee

UNIVERSITY OF NEW ORLEANS

Date: January 16, 2006

To: Dean Croshaw
             Joseph H. K. Peschman, Ph.D.

From: Gerald J. LaHoste, Ph.D.
             Chair, Institutional Animal Care and Use Committee

Re: IACUC Application # UNO-084

Dear Mr. Croshaw,

Your application for the use of animals in research entitled "Investigating sexual selection and the benefits of multiple mating in marbled salamanders" has been approved. The (retroactive) start and expiration dates for this approval are: October 1, 2002 – September 30, 2005.

Vita

Dean A. Croshaw grew up in a farming community in central Kentucky. He earned his undergraduate degree from Earlham College in Richmond, Indiana. His interests in ecology and evolution led him to graduate school where he first gained a master's degree and later a doctorate studying reproductive ecology and mating system evolution at the Savannah River Ecology Laboratory on the U.S. Department of Energy's Savannah River Site in Aiken County, South Carolina.