

1 **Multiple Paternity in an Aggregate Breeding Amphibian: the Effect of**
2 **Reproductive Skew on Estimates of Male Reproductive Success**

3

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15 **Abstract**

16 Aggregate or explosive breeding is widespread among vertebrates and likely
17 increases the probability of multiple paternity. We assessed paternity in seven field
18 collected clutches of the explosively breeding spotted salamander (*Ambystoma maculatum*)
19 using ten microsatellite loci to determine the frequency of multiple paternity and the
20 number of males contributing to a female's clutch. Using the Minimum Method of allele
21 counts, multiple paternity was evident in 70% of these egg masses. Simple allele counts
22 underestimate the number of contributing males because this method cannot distinguish
23 multiple fathers with common or similar alleles. Therefore, we used computer simulations
24 to estimate from the offspring genotypes the most likely number of contributing fathers
25 given the distributions of allele frequencies in this population. We determined that two to
26 eight males may contribute to *A. maculatum* clutches; therefore, multiple paternity is a
27 common strategy in this aggregate breeding species. In aggregate mating systems
28 competition for mates can be intense, thus, differential reproductive success (reproductive
29 skew) among males contributing to a female's clutch could be a likely outcome. We use
30 our data to evaluate the potential effect of reproductive skew on estimates of the number of
31 contributing males. We simulated varying scenarios of differential male reproductive
32 success ranging from equal contribution to high reproductive skew among contributing
33 sires in multiply-sired clutches. Our data suggest that even intermediate levels of
34 reproductive skew substantially decrease confidence in estimates of the number of
35 contributing sires when parental genotypes are unknown.

36 Introduction

37 Reproductive strategies in vertebrates fall along a continuum depending on the
38 degree of monopolization of mates, availability of resources, and duration of courtship
39 (Emlen & Oring 1977; Wells 1977). At one end of this continuum is territorial monogamy,
40 a system in which males defend areas from other males and/or guard females with whom
41 they mate; in these systems mating season and courtship usually take place over several
42 weeks or months (Wells 1977). At the other end of this spectrum is aggregate, or
43 explosive, breeding that occurs over a very short time period with little or no
44 monopolization of either space or individuals (Wells 1977). In aggregate mating systems,
45 males and females typically arrive together at a neutral location for a brief, intense bout of
46 mating. Under these circumstances a 'lottery system' is established where each competing
47 individual has a very small opportunity to mate, but few are actually successful, therefore
48 individuals vary dramatically in realized reproductive success (Nunney 1993).

49 Aggregate breeding is widespread across many vertebrate taxa including fish,
50 amphibians, and reptiles (including birds) (Roberts 1994; Willmott & Foster 1995;
51 Birkhead 2000). Aggregate breeding may have evolved in response to a number of
52 selective environments. For example, selection to breed in a narrow time window when
53 environmental conditions are optimal, or selection to reduce time and energy costs
54 associated with pair bonding may have favored the evolution and maintenance of a system
55 where courtship is rapid and limited. Regardless of the selective pressures promoting its
56 evolution, aggregate breeding always allows for large amounts of genetic mixing to take
57 place in a short time because it presents an opportunity for both males and females to mate
58 multiply and hence produce offspring with different genetic compositions. If genetic

59 mixing is an advantage of this mating system, we expect that aggregate-breeding taxa will
60 generally exhibit multiple paternity. Although we have estimates for patterns of male
61 reproductive success in vertebrate mating systems, particularly in birds and mammals
62 (MacWhirter 1991; Roff 2002), we know significantly less about reproductive patterns in
63 amphibians, despite their high diversity of mating systems and reproductive modes
64 (Haddad and Pombal 1998; Denoel *et al.* 2001). For example, despite ample opportunity
65 for multiple paternity in many amphibian mating systems (Roberts *et al.* 1999), few cases
66 of multiple paternity have been documented in frogs and salamanders (but see Jones *et al.*
67 2002), and the mating system of the more cryptic caecilians is virtually unknown.

68 Determination of paternity in amphibians (and ectotherms in general) has been
69 challenging due to limitations imposed by mating behavior and life history traits specific to
70 these lineages. Many amphibians and fishes have very large clutch or litter sizes, which
71 have necessitated the development of statistical and methodological methods for analyzing
72 subsamples of large broods (DeWoody *et al.* 2000). Likewise, most ectotherms show
73 limited parental care or association with their young, and it is not always possible to
74 sample or fingerprint potential parents, prompting the development of simulation
75 techniques for inference of contributing parents or reconstruction of half sib groups
76 (Blouin *ref*, DeWoody *et al.* 2000; Fiumera *et al.* 2001). Finally, in multiply-sired
77 clutches, unequal contribution by sires (reproductive skew) can affect paternity estimates,
78 although the degree to which reproductive skew biases estimates of the number of
79 contributing parents has not been rigorously evaluated.

80 The spotted salamander, *Ambystoma maculatum*, is an aggregate-breeding
81 amphibian, and thus an ideal species in which to characterize reproductive success and

82 multiple paternity as the first step in understanding the fitness consequences of this mating
83 system. Adult spotted salamanders migrate to vernal pools in the early spring for short,
84 ardent, 1- 3 day bouts of breeding (Hillis 1977; Petranka 1998), an extremely limited time
85 for interaction between the sexes. In *Ambystoma*, fertilization is internal although sperm
86 transfer is indirect via spermatophores (sperm packets atop a gelatinous base) that males
87 deposit on the pond floor (Halliday and Verrell 1984). Individuals of both sexes
88 congregate in large numbers, swimming, nudging and rubbing each other (Bishop 1941;
89 Petranka 1998). Males deposit, on average, 20-40 spermatophores on the pond bottom and
90 females pick up 15-20 of these in one breeding night (Arnold 1976; Petranka 1998).
91 Female spotted salamanders search for spermatophores with their hindlimbs while moving
92 along the substrate; upon encounter, a female picks up the sperm cap with her cloaca
93 leaving behind the gelatinous base, thus gaining no benefits aside from the sperm (Arnold
94 1976; Petranka 1998). Males do not limit female access to spermatophores making it
95 possible for a female to be inseminated by multiple males in the courting group. Shortly
96 after mating, females lay their eggs on submerged vegetation in the pond (Petranka 1998),
97 where larvae hatch and develop with no parental care.

98 Given our current knowledge of natural history and breeding behavior of this
99 species we have several expectations for the reproductive system of *Ambystoma*
100 *maculatum*. We predict that an aggregate-explosive breeder with multiple matings, such as
101 spotted salamanders (Arnold 1976), should exhibit high levels of multiple paternity in
102 nature. In this study, we conducted a paternity analysis to genetically characterize the
103 reproductive system in this species by assessing paternity of field-collected egg masses.
104 Using computer simulations that take into account the genetic structure of the focal

105 population, and assuming an equal contribution of sires to a female's clutch, we
106 determined the minimum sample size of eggs in a clutch needed for detecting multiple
107 sires and estimated the number of males that contribute to clutches using both direct
108 genotype counts and computer simulations. Given the intensity of male-male competition,
109 if multiple paternity is common it is possible that the contribution of males to offspring
110 within a clutch will be skewed. We use our dataset to quantify the potential impact of
111 reproductive skew on estimates of paternity. We simulate a range of skew scenarios (from
112 even paternal contribution to highly skewed clutches) and compare the estimates of
113 number of contributing males and their statistical confidence.

114 Characterizing the degree of multiple paternity and reproductive fitness in this
115 system increases our understanding of the evolution and maintenance of aggregate mating
116 systems. Likewise, quantifying the potential impact of reproductive skew on male paternity
117 estimates will help guide development of future theoretical approaches for quantifying
118 reproductive success in systems where parental alleles are not known as well as the design
119 and analysis of empirical data that address reproductive success in other systems where
120 reproductive skew is likely to occur.

121

122 **Materials & Methods**

123 *Field site and sample collections*

124 We collected seven spotted salamander egg masses from Ringwood Pond,
125 Tompkins County, New York, USA during the 2000 and 2001 breeding seasons. Clutches
126 A, B, and C were collected in spring 2000; clutches D, E, F, and G were collected in 2001.
127 All egg masses were collected three to four days after the commencement of the breeding

128 season and thus likely within 24 hours of oviposition. Egg masses were brought back to the
129 lab and maintained in individual containers (500 or 2000 ml) with dechlorinated water.
130 Clutches were monitored daily for development; larvae were allowed to hatch naturally
131 and were then preserved for later genetic analyses. Original clutch sizes ranged from 30 -
132 200 eggs and numbers of hatched larvae from these clutches ranged from 7-51. Consistent
133 with previous field and lab studies, egg mortality was approximately 50-90% of each
134 clutch, and was assumed to affect eggs with equal probability (Stenhouse 1987; Metts
135 2001; Tennessen & Zamudio 2003). All preserved larvae were used in subsequent
136 paternity analyses.

137

138 *Genotyping*

139 Whole preserved larvae were digested using Proteinase K (100 µg) in 500 µL lysis
140 buffer (2.5 mM Tris, 8 mM NaCl, 0.1%SDS, 0.2 mM EDTA, 0.01% β-mercapto-ethanol).
141 Samples collected in 2001 were additionally treated with 10 mg/mL RNaseA. Genomic
142 DNA was purified using standard phenol-chloroform organic cleanup and ethanol
143 precipitation protocols (Sambrook & Russell 2001). Extracts were diluted in distilled water
144 to a concentration of 100 ng/µl for use as templates for amplification of ten microsatellite
145 loci via the polymerase chain reaction (PCR) (Wieczorek *et al.* 2002). Amplified products
146 were combined in multiplex groups of 3 or 4 loci, and electrophoresed on 5%
147 polyacrylamide gels on an ABI prism 377 DNA Sequencer (Applied Biosystems, Costa
148 Mesa, CA). Peaks were sized using TAMRA 350 or 500 size standards. Genotype results
149 were analyzed using Genescan v.3.1 and Genotyper v.2.5 (Applied Biosystems, Costa

150 Mesa, CA). Fragment sizes were checked and assigned to pre-defined allele size-classes by
151 hand (Wieczorek *et al.* 2002).

152

153 *Multiple Paternity Estimation*

154 Multiple paternity was assessed using two estimators: 1) the Minimum Method and
155 2) a simulation estimate. The Minimum Method is more conservative and relies entirely on
156 counts of alleles represented within clutches. The Minimum Method assigns multiple
157 paternity within a clutch by using patterns of allelic distribution in offspring to determine
158 maternal genotypes and assumes that all alleles not accounted for by maternal genotype
159 were necessarily contributed by fathers. Therefore, a half-sib brood is evident if there are
160 more than four alleles present at a given locus, two of which are maternal and three or
161 more paternally contributed alleles. Multiple paternity is defined only in those cases in
162 which 1) a maternal genotype could be reconstructed and three or more alleles were
163 additionally present or 2) if maternal genotype could not be reconstructed but five or more
164 alleles were present.

165 The Minimum Method, although informative, is conservative in that it does not
166 account for multiple paternity by males with similar genotypes. We used the COUNTS,
167 BROOD, and HAPLOTYPES computer programs (Fiumera *et al.* 2001, DeWoody *et al.*
168 2000) to further analyze our data. These programs overcome the difficulties of assessing
169 exactly how many parents contributed to a progeny array in the absence of information on
170 parental genotypes by taking into account allele frequencies in the adult population.
171 BROOD determines the mean number of offspring that must be sampled to detect multiple
172 paternity in a clutch, COUNT estimates the number of parental haplotypes from the

173 empirical genotype data, and HAPLOTYPES uses that information to estimate the number
174 of contributing males given allele frequencies in the population.

175 We used the BROOD program to determine the mean number of offspring
176 necessary to detect all parental gametes in a brood. This program determines the mean
177 number of offspring that must be sampled (along with 95% confidence intervals) to
178 observe the same allele frequencies that are seen in the entire population and the number
179 needed to detect every gamete in the parental gene pool, including those which may be
180 shared amongst parents (N^*).

181 COUNTS determined the number of unique haplotypes of paternal origin evident in
182 the offspring genotype data. This program deduces the alleles contributed by a male
183 through subtraction, any allele in the progeny not displayed by the mother must have come
184 from a father (Fiumera *et al.* 2001). We used the COUNTS_MED program; in the case of
185 an offspring with the same heterozygous genotype as the mother, this program randomly
186 assigns one of the two possible alleles as the presumed contribution from a sire and tallies
187 the contribution only if it is unique (Fiumera *et al.* 2001). The number of haplotypes within
188 the clutch is then used to determine an expected number of unique fathers.

189 HAPLOTYPES simulates a breeding population based on empirical allele
190 frequencies in the study population (DeWoody *et al.* 2000). We used HAPLOTYPES to
191 simulate clutches that approximated our field collected egg masses in size and sampling
192 and to assess the number of haplotypes one would expect to find in clutches sired by
193 different numbers of males. Using the number of haplotypes determined from COUNTS
194 for each clutch, we then estimated the number of males that most frequently produced that
195 number of haplotypes in our simulated clutches.

196 We varied the parameters in the programs to simulate an array of conditions that
197 may exist in spotted salamander aggregations. We simulated clutch sizes of 50 and 100
198 eggs, consistent with field-collected clutch sizes that ranged from approximately 30-150
199 eggs each. We also varied the number of males potentially contributing to a clutch from
200 one to eight for BROOD and HAPLOTYPES. This range allows for single paternity and
201 encompasses previous estimates of multiple paternity from controlled mating experiments
202 (Tennesen & Zamudio 2003). The adult population at our study site was estimated at 1700
203 individuals (Zamudio, unpublished) and both parents and progeny were sampled 1000
204 times in all simulations. For the initial estimate of male reproductive success we assumed
205 equal contributions from each of the males contributing to a clutch (i.e. no within clutch
206 reproductive skew).

207 Both BROOD and HAPLOTYPES simulate breeding populations based on
208 empirical allele frequencies. Allele frequencies for each of the 10 microsatellite loci were
209 estimated from two more extensive microsatellite studies in our focal population and
210 surrounding ponds (Wieczorek & Zamudio, in prep; Tennesen & Zamudio 2003). Overall
211 frequencies were estimated by pooling genotypes for 118 individuals for 5 neighboring
212 ponds in the Ringwood watershed. These populations are panmictic (Wieczorek &
213 Zamudio, in prep) and represent a single breeding deme. For most loci, the alleles included
214 in our egg clutches were a subset of the pool of alleles found among adults in this breeding
215 deme; in those cases we used the allele frequencies from the previous studies to directly
216 estimate allele frequencies for our simulations. These loci have an expected heterozygosity
217 that ranges from 0.329 to 0.820 (Wieczorek *et al.* 2002). At loci Ama 5-1, Ama 9-4, Ama
218 12-7, Ama 34, and Ama A, a single allele that had not been detected in the original

219 watershed data set was represented in our clutches. For locus Ama 5-1 the rare allele was
220 also evident in a previous study assessing paternity at Ringwood (Tennesen & Zamudio
221 2003). To account for this allele, genotypes of 78 adults from Ringwood Pond (Tennesen
222 & Zamudio 2003) were combined with the more extensive population data from the
223 Ringwood watershed to recalculate overall allele frequencies for that locus. For the
224 remaining four loci, the novel allele we detected in our clutches had not been present in
225 either of the earlier studies. In these cases, we assigned an arbitrary but small allele
226 frequency of 0.002 to the rare allele and reduced the allele frequency of the most common
227 allele by the same margin. This value is the expected frequency of an allele seen only once
228 in the Ringwood watershed data set, therefore, this recalculation did not dramatically
229 change allele frequencies.

230

231 *Null Alleles*

232 A null allele is an allele that fails to amplify during PCR due to large increases in
233 the size of the product or mutations in the flanking primer sites (Dowling *et al.* 1996). The
234 presence of a null allele is indicated by genotype frequencies that do not conform to
235 Mendelian expectations due to an overabundance of an apparent shared allele (Dowling *et*
236 *al.* 1996; Fitzsimmons 1998). A maternal null allele was presumed present when three or
237 more different homozygous offspring were observed within a clutch. Offspring receiving
238 the null allele from the mother would appear homozygous for the paternal allele resulting
239 in an apparent overabundance of homozygous individuals. Using the Minimum Method,
240 we assigned “conditional multiple paternity” in situations where a null allele was
241 determined to be present as part of the maternal genotype and four additional alleles were

242 present among the offspring. None of the computer programs permit simulations of the
 243 occurrence of null alleles in the population, and the COUNTS program does not accept loci
 244 with null alleles. Thus, loci in which null alleles were evident were eliminated from
 245 subsequent analyses for that particular clutch.

246

247 *Quantifying the effects of reproductive skew on estimates of multiple paternity*

248 The original versions of BROOD and HAPLOTYPES only permit simulation of
 249 clutches with equal male representation within clutches, thus we modified those programs
 250 to permit simulations using different parameters that characterize reproductive skew.
 251 Modified programs are available from the first author (E.M.M) upon request. We
 252 simulated male reproductive skew within clutches in three ways. First, reproductive
 253 success among fathers is assumed to follow a geometric distribution based on the
 254 following equation

255 Proportion of offspring from j th father = $\alpha (1-\alpha)^{j-1}$

256 where α is a measure of increasing reproductive skew. Under this scenario of skew, the
 257 most successful male sires a fraction (α) of the offspring in a females clutch, and each
 258 successive male sires the same proportion of the remaining eggs. We simulated scenarios
 259 of skew where α was equal to 0.125 (where eight males sire even proportions of remaining
 260 offspring in a clutch), and where α ranged from 0.2 to 0.9 at 0.1 intervals. This geometric
 261 distribution of paternity could be expected in cases where male precedence influences
 262 paternity rates, such that later males have smaller and smaller reproductive success. In the
 263 other two skew analyses we assumed that one dominant male fathered most of the
 264 offspring of the larvae in a clutch and the remaining larvae are distributed equally among

265 remaining males. In the first of these, the most successful (dominant) male sires twice as
266 many offspring in the clutch than the other male or males (hereafter referred to as the 2X
267 scenario). Finally we also simulated the scenario where the dominant male fathers 60% of
268 all offspring and the remaining offspring are shared equally among the other males
269 (hereafter referred to as the 60% scenario). Results from each simulation were compared to
270 the simulations with no reproductive skew (i.e. all males contribute equally to clutches in
271 which they sire offspring). Combined these three scenarios of reproductive skew cover a
272 wide range of possibilities, some of which have been observed in previous studies focusing
273 on determinants of fitness among competing males (Jones et al. 2002; Tennessen &
274 Zamudio 2003).

275

276 **Results**

277 *Minimum Method*

278 We genotyped 175 larvae from seven clutches at ten polymorphic loci and used
279 these data to reconstruct patterns of allelic variation and paternity (Table 1). Using the
280 Minimum Method, multiple paternity was observed in five out of seven clutches (clutches
281 A, B, C, F and G). Multiple paternity was assigned unambiguously for at least one locus in
282 clutches A, B, C, and F. Conditional multiple paternity (when there was evidence of a null
283 allele) was evident in one locus of clutch G as well as in each of the other four clutches.
284 All loci were used to determine the number of contributing fathers using Minimum
285 Method; for clutches A, B, C, F, and G the minimum number of fathers represented was 3,
286 3, 2, 3, and 2, respectively (Table 1).

287 In multiply sired clutches, male genetic contribution was not shared equally. In
288 Table 1, loci highlighted as indicative of multiple paternity exhibit high frequencies of the
289 maternal alleles and paternal alleles from the most common sires. The remaining alleles,
290 contributed by other males, are lower in frequency in the clutch. Closer examination of the
291 most frequent alleles within a clutch and comparison with population allele frequencies
292 yields two results. In several of the clutches (e.g. Clutch C and E at locus Ama 61, Clutch
293 A at locus Ama 11-2B) the most frequent allele within the clutch was also one of the most
294 frequent alleles within the population (Table 1 and Appendix). This suggests that either 1)
295 reproductive skew is present and the most successful male coincidentally had a common
296 allele, or 2) skew is not high and several males with the most common allele contributed to
297 these clutches and are categorized as a single male by the Minimum Method. In other
298 instances the most common paternal allele in the clutch is relatively infrequent in the
299 population and is more likely to represent a contribution from a single male, and thus more
300 strongly supports high reproductive skew. For example, in clutch C, the most frequent
301 allele at locus Ama 4-10 is allele 241, because this allele is relatively rare in the population
302 with a frequency of only 0.052, it was most likely the contribution of one very successful
303 male. Although these data do not allow us to quantify the degree of reproductive skew,
304 they do suggest that not all males are contributing equally to egg clutches.

305 Multiple maternity was found in a single clutch (clutch E). Two loci, Ama 5-1 and
306 Ama 34 have offspring genotypes that are inconsistent with a single maternal genotype
307 (Table 1). In addition, this also exhibits a much higher frequency of loci with null alleles
308 (classified by three or more homozygous types) than the remaining clutches (0.7 compared
309 to an average of 0.283 for the others). These excess homozygote larvae are likely the result

310 of the second mother and her mate(s). Clutch E was an unusually large clutch with
311 approximately 175 eggs, significantly larger than the average clutch size in this region
312 (Bishop 1941). It is likely that two females deposited eggs on the same branch and the jelly
313 masses surrounding the eggs coalesced, making the egg masses appear as one clutch. This
314 clutch was eliminated from subsequent analyses. Additionally, three clutches showed
315 evidence of multiple maternity at loci Ama 5-1 (G and D) and Ama 34 (C). These loci
316 were highly polymorphic and the extra maternal allele was present in very few offspring.
317 In these cases we assumed that the additional maternal allele was not due to multiple
318 maternity, but resulted from a single mutation or genotyping error because the rare allele
319 appears in only 1-3 larvae. These clutches were retained for subsequent analyses (although
320 the individual loci were not).

321 Single paternity was found in a single clutch (clutch D). At all but one locus (Ama
322 5-1, discussed above) this clutch exhibited a single male genotype pattern. This clutch was
323 one of the smallest clutch included in the study and would be unlikely to demonstrate
324 multiple paternity based on allele counts.

325

326 *Null Alleles*

327 Null alleles were relatively common in our data producing a string of homozygous
328 offspring exhibiting alleles from only one parent (Figure 1). In analyzing these results one
329 can maximize the number of observed alleles by including all alleles irrespective of the
330 frequency with which they are seen. Alternatively, a more conservative approach excludes
331 alleles only seen once, under the assumption that they result from mutation or genotyping
332 error. This method necessarily produces lower numbers of null alleles; however, it also

333 decreases the chance of inferring multiple paternity associated with relatively rare alleles.
334 In this study we chose to maximize discrimination of multiple paternity at the expense of
335 including higher levels of potential null alleles. Previous studies in this system using the
336 same markers detected null alleles at a frequency of 1.8% (Tennesen and Zamudio, 2003).

337

338 *Computer Simulations*

339 We performed an initial set of simulations to test the effect of sampling regimes on
340 detection of multiple paternity in this population of *Ambystoma maculatum*. Not
341 surprisingly, BROOD simulations suggest that the number of sampled offspring necessary
342 to detect multiple paternity increases as the number of contributing males increases (e.g.
343 number of sampled offspring required increases from <10 larvae with one contributing
344 father to 65 when 8 males contribute to the clutch). We also simulated various sampling
345 conditions to evaluate our ability to detect various levels of multiple paternity in
346 HAPLOTYPES (Fig. 2). Within clutch sampling has a greater effect on haplotype numbers
347 than the size of the original clutch. Thus, small within clutch sampling limits the ability to
348 discern the number of contributing males especially when three or more males sire
349 offspring. Therefore the extent of multiple paternity could easily be underestimated when
350 within clutch sample size is small. **These results of subsampling simulations are in full**
351 **concordance with those reported in the original description of the programs (refs).**

352 Given our BROOD results, clutches A, B, C, E, and F are sufficiently large for the
353 detection of multiple paternity by at least two males, and clutches A, B, and C were large
354 enough to detect multiple paternity by up to four or six males. Not surprisingly, these are
355 the largest clutch sizes with 51, 32, and 46 genotyped offspring respectively. We chose

356 clutches A, B, and C for further analysis. Using the COUNTS_MED program we
357 determined the number of unique haplotypes from unshared parents. Haplotype counts do
358 not include loci with null alleles and therefore analysis was based on 7 loci for clutch A
359 (excluding Ama 4-10, Ama A, and Ama 34), 6 loci for clutch B (excluding Ama 5-1, Ama
360 A, Ama 12-7, and Ama 34), and 7 loci for clutch C (excluding Ama 61, Ama 4-10, and
361 Ama 34). The number of unique haplotypes in clutches A, B, and C estimated by
362 COUNTS were 44, 13, and 38, respectively. We used these numbers for simulations in the
363 HAPLOTYPES program.

364 First we performed simulations assuming no reproductive skew, by employing the
365 programs as they were originally developed. We simulated three clutches (A, B and C) in
366 HAPLOTYPES, each with the corresponding set of loci that were used in the COUNTS
367 program. For clutch B, we used an approximation of 50 eggs with 20 sampled and
368 estimated number of haplotypes and fathers for the simulated clutch. COUNTS determined
369 that clutch B contained 13 unique haplotypes based exclusively on the genotypes of the
370 offspring. Comparing this number to the simulated distribution of haplotypes, we can infer
371 that this clutch was sired by a mean of 1.7 male parents (Table 2). However, one sire
372 occurred twice as frequently as two sires in our simulations (Fig. 1); 95% confidence limits
373 suggest that anywhere between one and four males may have contributed to this clutch.
374 Thus, with only simulations, we cannot rule out single paternity or siring by two or more
375 males. However, when simulation data are combined with the results from the Minimum
376 Method, multiple paternity is confirmed and most likely consisted of two or three males
377 (Table 2).

378 We simulated clutches A and C using a 100-egg clutch of which 50 were sampled.
379 For clutch C, COUNTS determined 38 unique haplotypes. In the HAPLOTYPES
380 simulation this corresponds to a mean of 4.1 males and the most common number of
381 fathers for that haplotype count was 3 (Table 2; Fig. 1). Clutch A produced similar results.
382 COUNTS determined 44 haplotypes corresponding to a mean of 5.9 sires and the most
383 common number of males contributing to this clutch was 8 (Table 2; Fig. 1).

384 In a second set of simulations we incorporated scenarios of reproductive skew to
385 assess the impact of differential reproductive success on our estimates of multiple
386 paternity. In BROOD simulations, differential male reproductive success within a clutch
387 (increasing from no skew, to 2X, to 60%) dramatically increases N^* , especially when a
388 large number of males contribute to a clutch. For example, for a clutch with five
389 contributing sires, the number of sampled larval genotypes required to detect multiple
390 paternity are 40, 46, and 74 respectively for increasing skew scenarios. We also simulated
391 the probability of detecting multiple paternity using a geometric distribution for
392 contributions of multiple sires (Table 3) and in most instances our program was unable to
393 compute subsampling because it required more offspring to find N^* than were present in
394 the clutch. Thus, multiple paternity could easily be undetected if reproductive skew is
395 large.

396 Increasing reproductive skew from an even distribution, to 2X, to the 60% scenario,
397 generally increased the estimates of mean and most frequent number of contributing males
398 (Table 2); however, incorporating reproductive skew in simulations also dramatically
399 decreases the confidence in estimates of number of contributing males. Modeling
400 reproductive skew as a geometric allocation of paternity in a female's clutch results in a

401 less predictable pattern: the mean, and the most common number of simulated fathers
402 increases (as in clutch B) or decreases (as in clutches A and C). Differences between the
403 2x/60% results and the geometric distribution results are due in part to differences in their
404 fundamental approach. The geometric distribution approach allows fathers to sire a certain
405 proportion of available eggs in series (as would be expected in systems with serial mating)
406 while the other methods account for a proportion of total offspring sired which may be
407 more applicable in cases of sperm storage. Regardless of the model for reproductive skew,
408 changing male representation within a clutch in HAPLOTYPES simulations produced a
409 dramatic shift in the frequency curves resulting in a flatter or evenly parceled distribution
410 curves for the estimated number of males contributing to a clutch (Fig 2).

411

412 **Discussion**

413 *Multiple paternity*

414 Studies of a variety of taxa have demonstrated that multiple mating and multiple
415 paternity can be important components of species-specific reproductive strategies
416 (Birkhead 2000). In this study, we have confirmed that multiple paternity occurs
417 commonly in natural spotted salamander breeding aggregations. Multiple paternity by 2-3
418 sires was evident in more than 70% of egg masses using the conservative Minimum
419 Method; therefore, it is probable that multiple paternity may be even more common. Our
420 analyses also suggest that the number of sires contributing a clutch can be relatively high,
421 with as many as eight males contributing to a clutch. These results have important
422 implications for future studies of the costs and benefits of amphibian reproductive

423 strategies. Such a high prevalence of multiple paternity suggests a selective advantage for
424 multiple mating and increased male representation in a female's brood.

425 The spotted salamander is a good study species to address questions about the
426 benefits underlying the evolution and maintenance of this mating system. For females, the
427 benefits of multiple mating come in both material and genetic forms. However, it is
428 unlikely that material benefits are a factor influencing the evolution and maintenance of
429 this mating system; unlike insects (Thornhill 1983), female salamanders do not receive
430 nutrients from either the male's spermatophores (Arnold 1976; Petranka 1998) or from
431 nuptial gifts. In addition, females do not benefit from parental care of eggs or larvae
432 because clutches are left unattended after oviposition. Lastly, material benefits do not
433 include sperm quantity in this system. Studies of the reproductive biology of salamanders
434 suggest that females are not sperm limited and could fertilize and deposit a complete clutch
435 of eggs with a single or few spermatophores (Halliday & Verrell 1984; Sever *et al.* 1999).

436 A direct assessment of all the possible genetic benefits for multiple mating is
437 beyond the scope of this study. However, given our knowledge of the breeding behavior
438 and natural history of this species, we can briefly evaluate several of the proposed
439 hypotheses, including intrinsic male quality, offspring diversity, and the genetic
440 compatibility hypotheses (Newcomer *et al.* 1999). The 'intrinsic male quality' hypothesis
441 (Zeh & Zeh 2001) assumes that multiple mating enables sperm competition and cryptic
442 female choice, thus increasing the probability that the highest quality sperm fertilizes eggs.
443 This is consistent with ambystomatid breeding tactics where the female acquires many
444 spermatophores thus providing an opportunity for sperm mixing and competition. The
445 male reproductive skew we observe in our clutches lends support to this hypothesis; it is

446 possible the highest quality male fertilizes a majority of the clutch while secondary males,
447 with lower quality or poorly competing sperm, father fewer offspring. However, this
448 pattern of reproductive skew may simply be a result of male precedence rather than quality
449 differences in sperm from different males (Jones *et al.* 2002). Offspring diversity and
450 genetic compatibility may also play a role in this system. Genetic compatibility has
451 previously been shown to be a significant factor influencing mating success in salamanders
452 (Garner & Schmidt 2003). Variable environmental conditions leading to initial spring
453 breeding can be highly variable even at the local level so a diverse clutch of offspring may
454 be a way for females to hedge their bets (Thompson & Gates 1982, Skelly 2003).

455

456 *Detecting multiple paternity in natural populations*

457 In a highly fecund species with large numbers of offspring, it is rarely possible to
458 genotype all of the offspring from a single reproductive bout (DeWoody *et al.* 2000).
459 Consequently, computer simulations that take into account population allele frequencies
460 and reproductive skew are critical to estimate the number of offspring needed to detect
461 multiple paternity. In the spotted salamander, egg mortality can be high and this loss of
462 larvae further compromises sample sizes. BROOD determined that in the cases of highest
463 clutch mortality in our study, the remaining larvae, even if fully genotyped, were often
464 insufficient in number to establish multiple paternity. Methods to reduce embryo mortality
465 in the lab or extract DNA from early stage embryos would thus enhance our ability to
466 characterize reproductive patterns in this species.

467 Our results emphasize the importance of accounting for reproductive skew in
468 estimates of multiple paternity when parental genotypes are unknown. For most systems,

469 we rarely have *a priori* knowledge of the degree of reproductive skew among males
470 contributing to a clutch of eggs; in fact, this is often one of the parameters we wish to
471 estimate in natural populations. Our simulations emphasize that increases in reproductive
472 skew decrease our power to determine the number of males contributing to a clutch. Thus,
473 one fruitful strategy might be to complement paternity analyses of wild-collected clutches
474 with studies of clutches resulting from controlled matings where paternal contributions are
475 known (Tennessen & Zamudio 2003). This approach allows us to limit the number of
476 contributing males and more accurately estimate the differences in male reproductive
477 success. Likewise, when the possible range of reproductive skew is known, as was the case
478 in our study (Tennessen and Zamudio, 2003), it is still important to simulate various skew
479 scenarios to infer a range of potential contributing males rather than rely on the simplistic
480 assumptions of equal contribution by competing sires.

481

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601 **Author Information Box**

602 This research was conducted in partial fulfillment of Erin Myers' undergraduate
603 honors thesis. Research in the Zamudio lab focuses on population differentiation and
604 mating system evolution in amphibians and reptiles. We are particularly interested in the
605 determinants of reproductive success in aggregate breeding species.

606 **Figure Legends**

607 **Fig. 1.** Results of simulations in the HAPLOTYPES program for clutches A, B, and
 608 C, assuming equal contribution of multiple males to eggs in a clutch (no reproductive
 609 skew). Histograms on the left reflect the number of times that 44, 13, and 38 haplotypes
 610 (the empirical number for each of those clutches estimated by COUNTS) were seen in
 611 simulations assuming no reproductive skew. Curves on the right represent the number of
 612 contributing fathers inferred given increasing numbers of observed haplotypes among
 613 offspring. White circles indicate the mean estimate of male parents, black circles indicate
 614 the number of male parents most commonly recovered in simulations, and grey circles are
 615 cases where the mean and most common number of sires are equal. Black triangles above
 616 each curve represent the number of haplotypes estimated for each clutch by COUNTS.
 617 Error bars represent the 95% confidence intervals about the mean (rounded to the nearest
 618 integer). Note that the maximum number of haplotypes possible in a given simulation is
 619 the same as the number of eggs sampled, therefore the simulation for clutch B includes a
 620 smaller range of possible haplotypes.

621 **Fig. 2:** The effect of incorporating reproductive skew in HAPLOTYPES
 622 simulations on estimates of multiple paternity. For clutches A (top row), B (middle row),
 623 and C (bottom row) histograms reflect the number of times that 44, 13, and 38 haplotypes
 624 (the empirical number for each of those clutches estimated by COUNTS) were observed
 625 for varying numbers of sires in simulations including reproductive skew (2X, 60% and 4
 626 points along the geometric distribution, $\pi = 0.125, 0.3, 0.6, \text{ and } 0.9$). Overall counts for the
 627 histogram will vary because of differences in the number of times that a particular
 628 haplotype number was seen in 1000 simulations.

Clutch	N	Ama 61	Ama 5-1	Ama 9-4	Ama 11-2B	Ama 4-10	Ama A	Ama 3-3	Ama 2C2	Ama 12-7	Ama 34
D	7	251 (4)	384 (3)	221 (4)	243 (1)	243 (6)	133 (4)	169 (4)	207 (7)	304 (14)	102 (1)
		253 (10)	386 (3)	223 (8)	247 (9)	263 (2)	157 (2)	187 (1)	209 (2)		108 (5)
			388 (1)	235 (2)	257 (4)	267 (6)	169 (8)	193 (4)	213 (3)		118 (8)
			390 (1)					241 (3)	221 (2)		
			396 (2)								
		398 (4)									
E	14	229 (6)	386 (6)	191 (4)	243 (2)	237 (4)	157 (8)	169 (13)	207 (9)	294 (1)	90 (10)
		251 (5)	388 (1)	205 (10)	245 (2)	243 (9)	169 (12)	187 (5)	211 (2)	300 (7)	100 (1)
		253 (11)	390 (3)	217 (5)	247 (9)	265 (1)		193 (5)	213 (14)	304 (14)	102 (1)
			392 (2)	221 (6)	255 (8)	267 (2)		195 (1)	215 (2)	330 (2)	110 (1)
			394 (2)	223 (2)	257 (5)	221 (2)		237 (1)	221 (1)		118 (12)
			398 (8)					249 (2)			120 (1)
		400 (2)									
F	18	229 (5)	384 (1)	191 (13)	241 (1)	237 (2)	157 (2)	169 (12)	195 (2)	294 (4)	90 (4)
		251 (3)	388 (2)	211 (2)	243 (15)	243 (14)	167 (4)	187 (2)	207 (4)	304 (11)	98 (1)
		253 (8)	390 (6)	217 (2)	247 (8)	245 (2)	169 (3)	191 (1)	213 (15)	324 (2)	106 (1)
			392 (1)	219 (6)		263 (2)	175 (1)	193 (4)	221 (3)	334 (2)	108 (13)
			394 (2)	221 (10)				241 (1)	223 (4)		118 (3)
			398 (3)	227 (2)							
		400 (1)									
G	7	251 (6)	384 (2)	191 (1)	247 (10)	237 (6)	169 (4)	169 (5)	207 (4)	300 (1)	90 (5)
		253 (8)	386 (4)	205 (4)	243 (3)	243 (6)	167 (6)	187 (5)	213 (3)	304 (8)	98 (4)
			390 (6)	221 (7)	257 (1)				215 (2)	310 (3)	118 (5)
			392 (2)	223 (2)					221 (5)		

Legend: Non-informative locus (single or multiple paternity possible) Null allele detected Multiple paternity Multiple paternity with null allele Multiple maternity

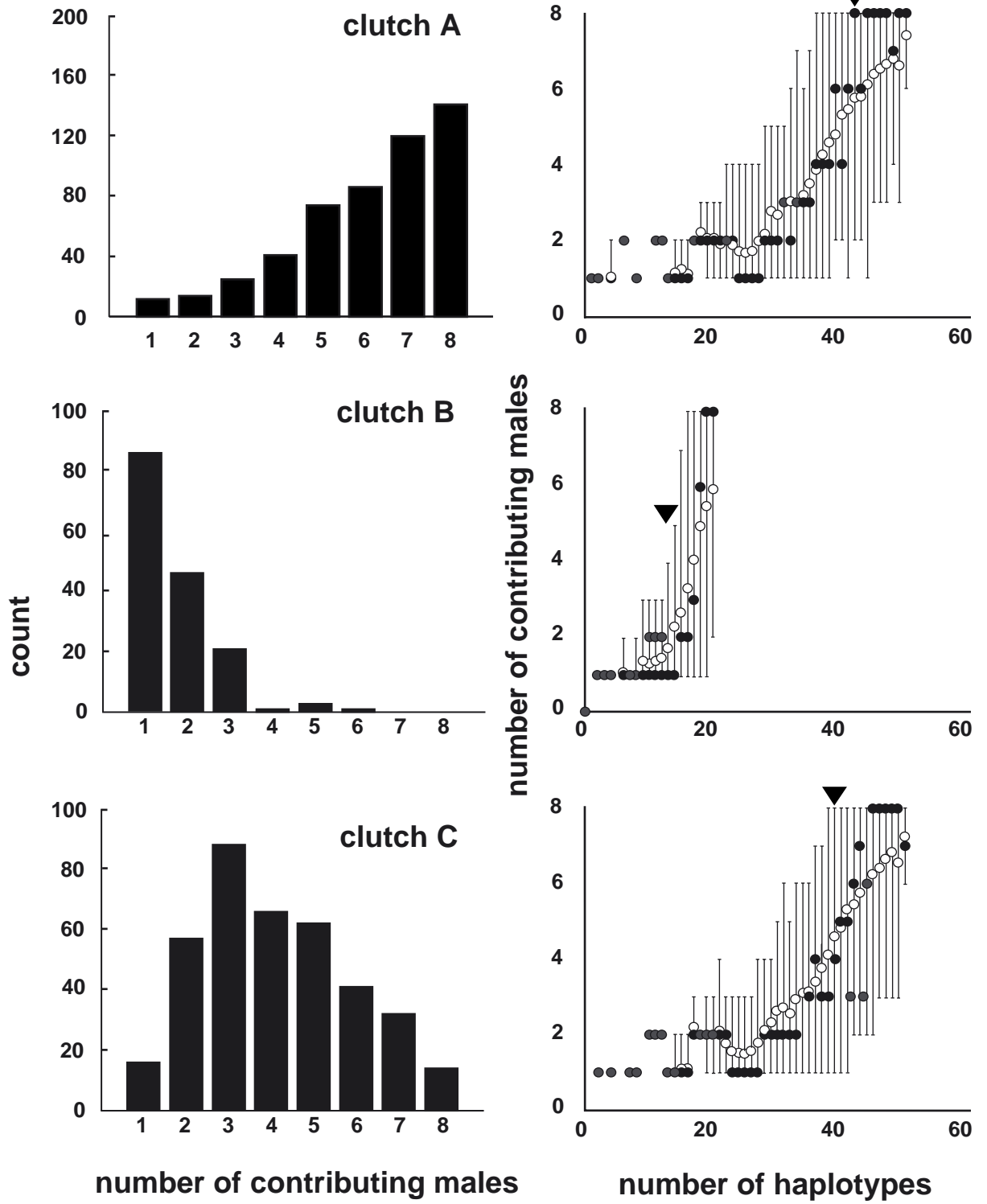


Figure 1

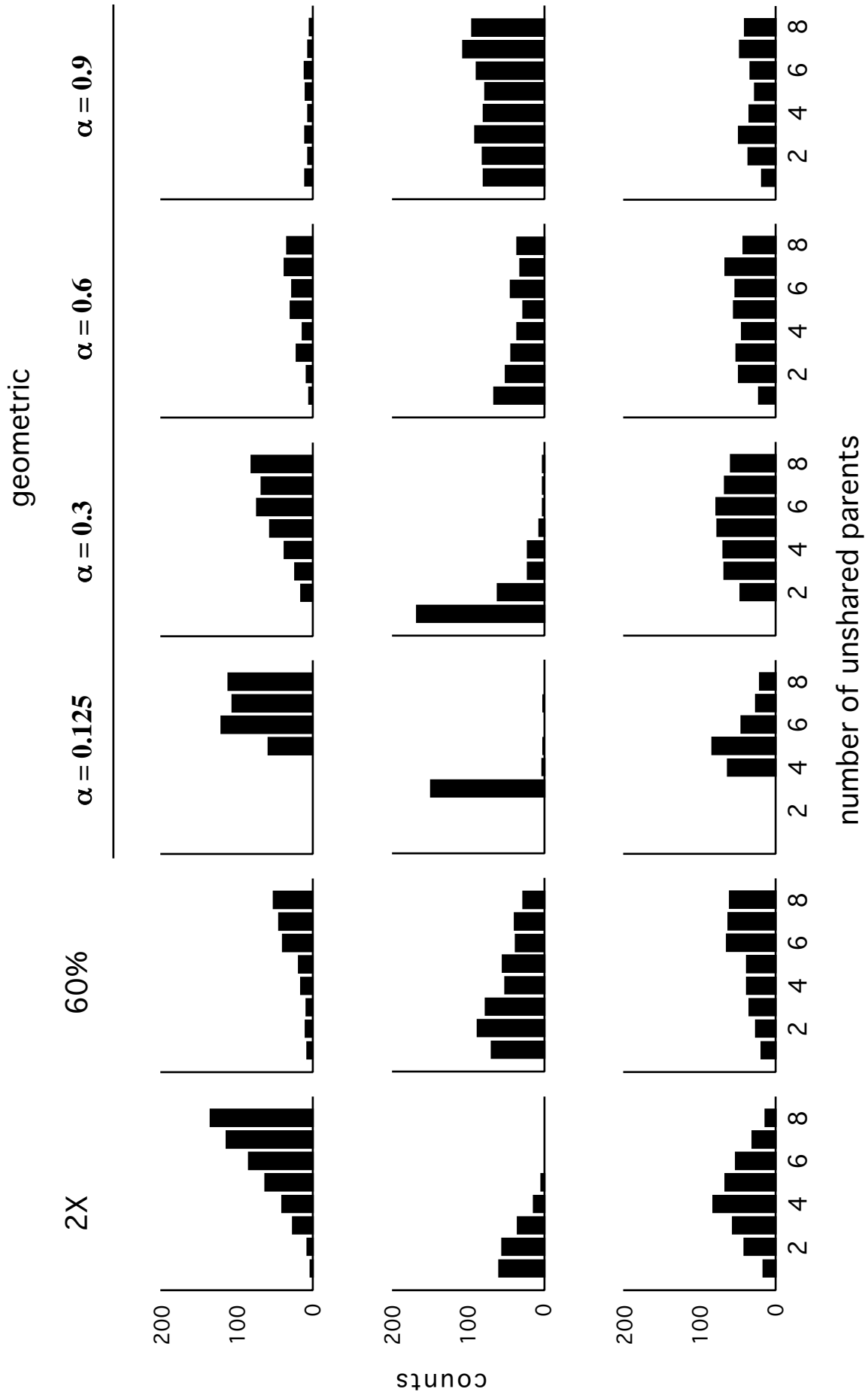


Figure 2

637 Appendix. Allele frequencies for 10 microsatellite loci in a random sample of adult
 638 *Ambystoma maculatum* from Ringwood Pond, Tompkins Co., New York. These
 639 frequencies were used in the HAPLOTYPE simulations to determine the number of
 640 contributing sires to field-collected clutches.
 641

Locus	Allele	Frequency	Locus	Allele	Frequency
Ama61	229	0.085	Ama3-3	169	0.031
	251	0.272		173	0.008
	253	0.638		179	0.004
	255	0.004		180	0.004
Ama5-1	384	0.003		183	0.008
	386	0.234		184	0.004
	388	0.011		186	0.004
	390	0.136		187	0.343
	392	0.250		189	0.038
	394	0.008		193	0.008
	396	0.043		213	0.008
	398	0.313		217	0.004
	400	0.003		219	0.013
Ama9-4	191	0.002	221	0.021	
	205	0.167	229	0.008	
	207	0.070	233	0.025	
	213	0.004	237	0.025	
	215	0.004	241	0.017	
	217	0.044	245	0.004	
	219	0.004	247	0.004	
	221	0.454	249	0.017	
	223	0.118	251	0.008	
	225	0.039	253	0.017	
	227	0.009	255	0.013	
	229	0.013	257	0.013	
	235	0.022	259	0.004	
	237	0.022	261	0.004	
	241	0.004	263	0.025	
243	0.009	351	0.013		
245	0.013	Ama34	86	0.002	
Ama11-2B	237		0.155	90	0.265
	243		0.077	92	0.005
	245		0.027	94	0.005
	247		0.591	98	0.010
	249		0.023	100	0.005
	251		0.005	106	0.010
	255		0.009	108	0.158
	257		0.091	110	0.040
	269		0.005	112	0.015
	271	0.005	116	0.228	
273	0.009	118	0.252		
275	0.005	120	0.005		

642

Locus	Allele	Frequency
Ama4-10	235	0.052
	237	0.157
	241	0.052
	243	0.313
	263	0.004
	265	0.009
	267	0.348
	269	0.065
AmaA	133	0.002
	151	0.025
	157	0.112
	159	0.017
	163	0.013
	165	0.008
	167	0.033
	169	0.548
	171	0.200
	173	0.021
	175	0.004
193	0.004	
197	0.013	

Locus	Allele	Frequency
Ama2C2	203	0.018
	207	0.215
	209	0.039
	211	0.031
	213	0.382
	215	0.035
	217	0.018
	221	0.224
	223	0.031
	229	0.009
Ama12-7	236	0.004
	242	0.004
	290	0.004
	294	0.091
	300	0.161
	302	0.022
	304	0.624
	308	0.013
310	0.074	
330	0.002	

643