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The majority of female animals are polyandrous: offspring within a single reproductive event are sired by more than one male. However, we lack a clear understanding of the ultimate causes of polyandry, and of the male reproductive strategies which have evolved in response to selection occurring after insemination. I addressed these issues by conducting three studies of the Ocoee salamander, *Desmognathus ocoee* (Family Plethodontidae).

In the first study, I examined the nature of polyandry. I collected *D. ocoee* clutches from the field and assessed the number of males siring offspring in each clutch. The data suggest that polyandry is common in *D. ocoee,* but females do not appear to mate multiply in order to increase clutch size (via female benefits) or from pressure by males (sexual conflict).

In the second study, I staged a series of laboratory encounters between a female and two males to investigate the effects of male body size and sperm competition on male reproductive strategies. Paternity data, combined with data from sperm counts (Ch. 3), revealed that large males allocate more sperm per spermatophore, and experience greater insemination and fertilization success, as

compared to smaller males. Thus, selection for greater insemination and fertilization success favors the evolution of large male body size in *D. ocoee.*

In the third study, I examined one aspect of male reproductive strategy, sperm allocation. I counted the number of sperm per spermatophore provided to mated and unmated females by males of differing body sizes. I found that males can assess female mating status and vary sperm number to provide more sperm to unmated females. On average, larger males produced spermatophores with more sperm than did smaller males. Thus, variable sperm allocation is an important male strategy resulting from competition for the fertilization of ova (polyandry).

Overall, factors other than increased or decreased clutch size have resulted in the evolution of polyandry in *D. ocoee.* In addition, polyandry clearly has shaped the evolution of both male traits and reproductive strategies in *D. ocoee.*

Reproductive Strategies of the Ocoee Salamander, *Desmognathus ocoee.*

by

Erika M. Adams

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Erika M Adams, Author

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CONTRIBUTION OF AUTHORS

Dr. Adam Jones and Dr. Steve Arnold were directly involved in the design and data analysis of Chapter 2. Therefore, they are co-authors on the manuscript submitted for publication resulting from that study.

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CHAPTER 1

GENERAL INTRODUCTION

The use of molecular DNA markers to determine paternity in natural populations of animals has revealed unexpected results. In many species originally assumed to be monogamous, females are now known to produce offspring sired by multiple males. Increasingly, it appears that multiple mating by females is no longer the exception, but the rule (birds, Birkhead 1998; mammals, Gomendio et al. 1998; insects, Simmons and Siva-Jothy 1998). These findings contradict the general assumption that, because a single mating usually provides sperm in excess of what is needed to fertilize all of a female's ova, female monogamy should prevail. Also, multiple mating may be costly to a female (Blanckenhorn 2002). Potential costs include reduced foraging opportunities (Rowe 1994), reduced paternal care (Emlen 1978, Burke et al. 1989), increased risk of predation (Eberhard 1996), and increased exposure to sexually transmitted diseases (Birkhead and Møller 1992, Hunter et al. 1993). All of these reasons strongly suggest that most females should mate only once, and yet evidence shows that mating occurs more frequently. Given this apparent

paradox in female mating behavior, how might widespread female multiple mating have evolved?

The use of laboratory-based model systems (Chapman et al. 1995, Rice 1996, Tregenza and Wedell 1998, Evans and Magurran 2000), particularly in insects, has lead to remarkable advances in our understanding of the ultimate causes of polyandry, in which a female mates with several males. These studies suggest that female benefits and sexual conflict may be the primary forces affecting the evolution of polyandry. Generally speaking, the female benefit model assumes that female fitness is increased by intersexual interactions associated with mating with several males. Direct benefits that increase the fitness of the female may include nuptial gifts (insects, Thornhill and Alcock 1983), ejaculate nutrients (insects, Friedel and Gillot 1977), and acquisition of parental care from additional males (primates, Hrdy 1977). Alternatively, polyandry may not provide tangible benefits but instead may increase female fitness by increasing the survival and reproductive success of her offspring (e.g., "good genes" model, Fisher 1915, 1930; Williams 1966).

The sexual conflict model, on the other hand, suggests that there is an inherent imbalance in the roles of males and females with respect to optimal mating frequency Parker 1979, Rice 1992, Rice 1996). Males often seek to increase mating frequency and females seek to resist male mating attempts (Holland and Rice 1998). Traits affecting mating propensity have evolved in each sex even though these traits may reduce fitness in the other sex (Chapman et al. 1995, Rice 1996). In this manner, the sexes evolve antagonistically. The spread in the population of a male trait that increases insemination frequency can be accompanied by the concordant spread of a

female trait for resistance to increased insemination frequency (Holland and Rice 1998). As a result, male and female traits evolve in response to each other in a never-ending cycle of persuasion and resistance (Holland and Rice 1998; Gavrilets 2000, 2001). Thus, in order to understand how selection pressures from female benefits and sexual conflict may interact to shape polyandry, both models must be taken into account when studying polyandry.

Polyandry has important consequences not only for female reproductive success, but for the evolution of male reproductive strategies as well. When a female is inseminated by more than one male within a single reproductive event or season, sperm from each male compete for access to ova within the female reproductive tract (sperm competition, Parker 1970). Sexual selection on males is not only relegated to events leading up to insemination, but also affects fertilization success. As a result, in order to reproduce successfully, a male must not only to be able to inseminate females, but his sperm must out-compete sperm from rival males in the female reproductive tract. Some of the most spectacular phenotypic traits important to sperm competition have evolved in insects. Male damselflies, for example, have a modified intromittent organ that allows them to remove previously inseminated sperm from the female's reproductive tract, thereby reducing sperm competition (Waage 1979). In *Drosophila*, sperm and semen have been shown to displace and incapacitate rival male sperm (Price et al. 1999). These traits have in common an effect on the number of sperm retained in the female reproductive tract. The more sperm from a particular male that is available to compete for fertilization of ova, the higher the probability that offspring will be sired by that male.

The reproductive biology of the plethodontid salamander, *Desmognathus ocoee*, is well-suited to research examining polyandry. Found in the Appalachian Mountains of North Carolina, *D. ocoee* inhabits terrestrial sites, usually near running water (Tilley 1977). The relative simplicity of behavioral interactions during reproduction in *D. ocoee* has great advantages over other systems in which polyandry has been investigated (e.g., insects and birds). This simplicity is primarily because male and female mating strategies affecting mating success in *D. ocoee* are limited to a discrete window of time between insemination and fertilization leading to a single reproductive event (in this case, a single clutch of eggs). Males are not known to provide resources to either the female or her offspring. Females may become inseminated throughout the fall and spring (Organ 1961, Tilley 1977), and individuals usually only seek cover below ground during periods of freezing temperatures in the winter (Organ 1961). Sperm are stored in the female's spermatheca for up to nine months prior to ovulation, fertilization, and oviposition, which occur in rapid sequence. The long period of time separating insemination and ovulation may provide an opportunity for large post-insemination effects during sperm storage. Stored sperm require hospitable physiological conditions and may need female resources to survive until ovulation (Sever and Kloepfer 1993). The temporal separation between insemination and fertilization also allows for the clear separation of pre- and post-insemination effects of polyandry on offspring paternity. Oviposition takes place on land in late summer (Organ 1961), after which the female broods a single clutch of 9-32 eggs (see Ch. 2) for 6-8 weeks. After hatching, the young disperse and there is no further maternal care of offspring.

Although a great deal of research on polyandry and sperm competition has been conducted in insect and avian systems since Parker's (1970) landmark paper on sperm competition, relatively few investigations have been conducted on amphibians, and on salamanders in particular. Basic information on insemination frequency and other topics in *D. ocoee* is limited primarily to experimental results from matings staged in the laboratory. In these laboratory encounters, females may be inseminated up to 26 times by different males (Houck et al. 1985b), but this may not be a biologically relevant upper bound. Tilley and Hausman (1976) used allozymes and were only able to detect multiple paternity in 7% of field collected eggs clutches. Labanick (1983) used color polymorphism to estimate that 25% of females mated multiple times. Although it is usually assumed that female *D. ocoee* mate with multiple males in the field, results presented in the second chapter of this thesis are the first direct estimates of the number of males siring offspring in each clutch in *D. ocoee*.

The research described in this dissertation addresses: (1) the role of female benefits and sexual conflict in the ultimate causes of polyandry in *D. ocoee* (Ch. 2); (2) the association between those male traits important to competition for insemination and those important for fertilization (Ch. 3); (3) the sperm allocation strategies males employ based on a female's mating status (Ch. 4); and (4) fundamental information concerning the mating system and reproductive biology of *D. ocoee* (Chs 2, 3, and 4).

CHAPTER 2

MICROSATELLITE ANALYSIS OF PATERNITY IN THE OCOEE SALAMANDER (*DESMOGNATHUS OCOEE)*

Erika M. Adams, Adam G. Jones, and Stevan J. Arnold

ABSTRACT

Despite important advances using model systems, the ultimate causes of polyandry remain largely unresolved. Recently, the theory of antagonistic coevolution between the sexes has received a great deal of attention as being a major factor affecting the evolution of polyandry. However, little is understood about how selection on male and female traits important during antagonistic coevolution may interact with selection on male traits that provide benefits to females from mating multiply. An understanding of these interactions is crucial to understanding the evolution of female multiple mating. Studies of polyandry in salamanders are largely non-existent, despite the fact that both antagonistic coevolution and female benefits may be operating in their mating systems. We developed microsatellite DNA markers to assess offspring paternity in field-collected clutches of the salamander *Desmognathus ocoee*. We determined that slightly over half of the clutches that we

analyzed (n=28) were sired by two males (54%; n=15). Thirty-two (n=9) and 11 (n=3) percent of the clutches were sired by 3 and 4 males, respectively. Only one clutch was sired by a single male (3%). Thus, female multiple mating is an important aspect of the mating system in *D. ocoee.* Within clutches sired by 2 males, one male consistently sired a majority of the offspring in the clutch (avg=80%). These last data suggest that sperm precedence could be one mechanism affecting offspring paternity in this system. Finally, we found no evidence that females experience either direct benefits (increased clutch size) or sexual conflict (decreased clutch size when not mating at the optimal frequency) from mating multiply. This result was surprising in light of the reproductive biology of *D. ocoee.* However, given the low level of statistical power to detect either female benefits or sexual conflict (41-46%), additional studies with larger sample sizes may be needed to conclude definitively whether female benefits or sexual conflict are operating in this sytem.

INTRODUCTION

Mating strategies have important consequences for reproductive success and differ between the sexes. A male typically can produce more offspring by mating with many females. We lack an equally simple explanation for why females mate with multiple males. The use of laboratory-based model systems has lead to remarkable advances in our understanding of the evolution of polyandry. These studies suggest that female benefits and sexual conflict may be the primary forces affecting the

evolution of polyandry (Chapman et al. 1995, Rice 1996, Tregenza and Wedell 1998, Evans and Magurran 2000). Females may increase their reproductive success by mating with males that provide direct benefits (e.g., nuptial feeding, fertility assurance, additional paternal care) or indirect benefits (e.g., good genes) (see Birkhead and Møller 1998 for review). These benefits outweigh any costs incurred from the additional mating. Thus, the sexes might evolve mutualistically, such that both males and females increase their reproductive success by mating multiply. Sexual conflict theory, on the other hand, suggests that there is an inherent imbalance in the roles of males and females with respect to optimal mating frequency (Parker 1979, Rice1992, Rice 1996). Males seek to increase mating frequency and females generally seek to resist male mating attempts (Holland and Rice 1998). Traits affecting mating propensity have evolved in each sex even though these traits may reduce fitness in the other sex (Chapman et al. 1995, Rice 1996). In other words, the sexes evolve antagonistically. The spread in the population of a male trait that increases insemination frequency is accompanied by the concordant spread of a female trait for resistance to increased insemination frequency (Holland and Rice 1998). As a result, male and female traits evolve in response to each other in a never-ending cycle of persuasion and resistance (Holland and Rice 1998; Gavrilets 2000, 2001). Thus, in order to understand how selection pressures from female benefits and sexual conflict may interact to shape polyandry, both models must be taken into account when studying polyandry and the evolution of male and female reproductive strategies.

We have focused on the plethodontid salamander *Desmognathus ocoee* as a useful system in which to study polyandry. Females can be inseminated throughout

the fall and spring courtship seasons (Organ 1961, Tilley 1977) and produce clutches sired by more than one male (Tilley and Hausman 1976, Labanick 1983). Pairs are not known to interact outside of courtship nor are males known to provide tangible resources to the female or the offspring. After mating, the female stores sperm in her spermatheca. Several studies have been conducted on the histology of the *D. ocoee* spermatheca (Sever and Hamlett 1998). However, nothing is known about the physiology of sperm storage, including possible short- and long-term effects of cloacal gland (seminal) proteins, throughout the nine month mating season. In July or August, a female oviposits a single clutch of between 9 to 32 eggs, which she broods for 4 to 6 weeks (Organ 1961). Females are not known to store sperm from one oviposition event to the next (Sever and Hamlett 1998, but see Houck and Schwenk 1984). Soon after hatching, the young disperse and there is no known further maternal care (Organ 1961). There is currently little agreement as to whether females produce clutches on an annual or biennial cycle (Organ 1961, Bruce 1993, Tilley and Bernardo 1993, Bruce 1996). Nevertheless, since females produce a maximum of one clutch per year, male-female interactions during the breeding season affect only a single clutch. Unlike studies of many species of birds and insects, interactions between male and female *D. ocoee* are relegated to a discrete window of time prior to a single oviposition event, facilitating investigation of these interactions.

Although female Ocoee salamanders in the field have been shown to produce offspring sired by more than one male (Tilley and Hausman 1976, Labanick 1983), the proportion of females in the field that mate multiply and the number of males siring offspring in each clutch is unknown. In laboratory studies, females can mate with up

to 26 different males within a single mating season (Houck et al. 1985b). While such high insemination frequency may be indicative of the normal mating propensity, it may likely be a laboratory artifact. Females in the field simply may not encounter and become inseminated by as many males in a single mating season. However, from these studies we know that females are capable of being inseminated many times throughout the mating season, indicating that high rates of multiple mating could occur in the field (Houck et al. 1985a). An important question that results from the observations that female Ocoee salamanders mate multiply, and potentially with many different males, is to ask why females engage in this behavior. Do females benefit from mating with several males? What roles, if any, do male persuasion and sexual conflict play in this system?

Female *D. ocoee* appear to have a high level of control over all aspects of insemination and fertilization, suggesting that female benefits may explain polyandry. Females are not restrained physically in any way by the male and can (and often do) leave the male at various points throughout courtship (Organ 1961, Houck et al. 1988, Verrell and Arnold 1989). Toward the completion of courtship, the male deposits a spermatophore on the ground in front of the female. The female then moves over the spermatophore and picks up only the sperm mass in her cloaca (the gelatinous base supporting the sperm mass is left behind). Frequently, the female will fail to retrieve the sperm mass, although whether this is on purpose or whether it is simply an error is unclear (pers. obs.). In addition, a female potentially has a great deal of influence over the transfer of sperm from the male to her spermatheca. Males do not have an intromittent organ and, other than the movement of sperm under their own power, the

transport of sperm from the sperm mass to the spermathecal storage site may be controlled largely by the female. Furthermore, the female may be able to influence sperm during storage, potentially through resource allocation in the form of secreted proteins or, conversely, through spermiophagy (Sever and Hamlett 1998). Altogether, these observations strongly suggest that female *D. ocoee* salamanders have the potential to exert a high level of control over insemination and fertilization. This level of control, combined with potentially high rates of insemination, suggests that females may benefit from inseminations by multiple males. However, these benefits remain to be established empirically. Thus, despite this apparently high level of female control over reproduction, we cannot discount the possibility that males may exert a large, potentially chemical, effect on female insemination rate through the use of courtship pheromones (Arnold and Houck 1982, Houck 1986, Verrell 1988, Houck and Reagan 1990).

During courtship, male Ocoee salamanders use their premaxillary teeth to "inject" pheromones into the female (Arnold 1977, Houck 1986). These pheromones have been shown to reduce courtship time (Houck and Reagan 1990) and may result in higher insemination rates. The full extent of the effects of these pheromones on female physiology is still unknown. However, the existence of male-delivered pheromones and their known action on female receptivity suggests that males may be able to persuade females to mate beyond their optimum frequency. Thus, sexual conflict may also shape polyandry in this system, although it should be noted that evidence of female harm from male pheromones has not been demonstrated and must

be established before concluding that sexual conflict is operating in *D. ocoee* (Pizzari and Snook 2003).

To address the issues of female benefits and sexual conflict in the Ocoee salamander, we first need to consider three basic questions: (1) What proportion of females in the field produce offspring sired by more than one male? (2) How many males sire offspring within a clutch, and (3) in a multi-sired clutch, does one male sire the majority of the offspring? The first question addresses whether or not polyandry is a major component of the mating system of *D. ocoee.* If most females produce offspring sired by more than one male, female benefits and male persuasion may be important. Conversely, if few females mate multiply, female choice and male-male competition prior to insemination potentially are the primary determinants of reproductive success. With respect to the second question, we will test (a) whether female fitness (clutch size) increases with the number of sires in a clutch (i.e., female benefits), and (b) whether clutch size as a function of the number of sires is a quadratic function. This last test indicates the presence or absence of an intermediate optimal female mating frequency and hence tests for sexual conflict (Gavrilets 2000, 2001; Mead and Arnold *In Press*). Clutch size is an ideal trait on which to begin investigations of female benefits and sexual conflict because of its clear connection to fitness. In *D. ocoee,* females mate throughout the year-long period of time in which they are yolking eggs (Organ 1961). Potentially, the act of mating, or substances transferred to the female during mating, may affect the size and composition of a females clutch. Although, to date, there is no evidence that this is the case in *D. ocoee* salamanders, as has been found in other organisms (e.g., *Drosophila,* Manning 1967).

Finally, the third question addresses whether sperm precedence, which can have a large effect on patterns of offspring paternity, may be operating in *D. ocoee*.

In order to assess offspring paternity, we developed novel markers for tetranucleotide microsatellite loci in *D. ocoee*. We used these markers to determine the number of males siring offspring in clutches of field-collected *D. ocoee*, as well as to determine the distribution of offspring paternity within each clutch. Although laboratory-based research is ideal for studying responses to selection, it is crucial to conduct studies in natural or natural-like systems in which animals presumably are currently adapted to natural selection pressures (Pizzari and Birkhead 2002). Without field studies, the biological relevance of selection pressures affecting polyandry may not be addressed.

METHODS

Microsatellite Development

Genomic DNA from a single *D. ocoee* individual collected at Deep Gap (Macon Co.), NC, USA (35 02'42"N, 083 33'19"W) was used to clone sequences containing microsatellite repeats ($GATA_n$), as described by Jones et al. 2001. Twenty-nine positive recombinants were sequenced by automated sequencing (Applied Biosystems, ABI 3100). From these sequences, 9 primer pairs were designed to amplify the microsatellite-containing sequence. Of these, only 3 primer

pairs produced polymorphic results that could be scored. Following primer optimization, each 20µl PCR reaction included a final concentration of 10X taq buffer, 1.5mM $MgCl_2$, 10pmol of each forward and reverse primer, 0.5 units of taq polymerase, and approximately 50ng/µl template DNA. PCR conditions for each of the 3 loci differed from the others only in annealing temperature (52°C -56°C) and consisted of: 5 min at 94°C followed by 2 min at 92°C, 1 min at the annealing temperature, and 1 min at 70°C (Table 2.1). These last three steps were repeated 36 times, followed by a final 10 min lengthening step at 70°C.

We used template DNA from 42 unrelated males and females collected at Deep Gap in a separate parentage study (See Ch. 3) to calculate the size range of alleles, allele frequencies, observed and expected heterozygosities, and to test for Hardy-Weinberg equilibrium, heterozygote deficit, and linkage disequilibrium at each of the 3 loci (GENEPOP 3.1b, Raymond and Rousset 1995). We also calculated the frequency of null alleles at each locus and noted any *de novo* mutations from the offspring arrays produced in the same study.

Table 2.1. Variation in three novel *Desmognathus ocoee* microsatellite DNA loci was studied in a sample of 42 individuals. Shown for each locus are the number and nature of tetranucleotide repeats in the original microsatellite clone, primer sequences, size range of alleles, number of unique alleles, observed heterozygosity (H_o), expected heterozygosity (H_e), and optimal annealing temperatures for each locus.
* denotes significant heterozygote deficit due to null alleles ($p<0.01$).

Locus	Repeat motif	Primer sequences (5'-3')	Size range (bp)	No. of unique alleles	H_o	H_E	Optimal annealing temperature (C)
Doc01	$(GATA)_8$... $(GATA)_3$	F: (6-FAM) TGTGAAGGGTGTTCTCTTACTG R: GCTGTTTGTGCTTTGACTTTAC	115-211	22	0.786*	0.905	52
Doc02	$(GATA)_{10}$	F: (HEX) TCAATCCAAGCACCATCAAAAG R: ACCCAAAACAGCACCAGCA	202-576	47	0.905*	0.976	56
Doc03	$(GATA)_{10}$	F: (NED) CTCTCCCACTCTTCCTCAAGTA R: CTTCACCTTCGCTATGACTGT	105-183	17	0.952	0.929	54

Clutch collection and genotyping

We collected 30 females and their clutches between 11 - 15 August 2001 from two sites in the Nantahala Mountains of North Carolina, USA: Deep Gap and Standing Indian. Eggs and maternal tail-tip tissue samples were frozen upon collection (-80°C) and transported to Oregon State University. Using calipers, we measured the snout-to-vent length of each female (from the tip of the head to the posterior edge of the vent). Prior to molecular analysis, we dissected each egg to remove all surrounding membranes and yolk from the embryos. Whole embryos were then ground using an epi-pestle in a standard solution of 10X STE buffer, 10% SDS and proteinase-K (Sambrook et al. 1989). Tissues were incubated at 56°C for 4 hrs, followed by phenol-chloroform extraction and ethanol precipitation. For the extractions, we used 2ml phase-lock gel tubes (light, ISC BioExpress$_{tm}$) with 500µl of aqueous digested tissue. We performed 1 extraction with 500µl phenol, followed by 1 extraction with 250µl phenol, 240µl chloroform, and 10µl isoamyl alcohol. The aqueous layer was then transferred to a clean 1.5µl eppendorf tube and extracted one final time with 490µl of chloroform and 10µl of isoamyl alcohol. Alcohol precipitation was conducted according to standard protocols using 95% and 70% ethyl alcohol (ETOH) (Sambrook et al. 1989). After PCR using the same conditions developed during primer optimization discussed above, amplified fragments were run on an ABI 3100 automated sequencer and scored using Genescan$_{tm}$ fragment analysis software.

Of the 30 clutches collected in the field, 2 clutches (clutches #7 and #8) had to be excluded from the analysis because they failed reliably to amplify discrete

genotypes at any of the three loci. Re-extraction of template DNA and optimization of PCR conditions did not correct the problem. Also, for one of the clutches (#13 having 2 sires), the female escaped collection in the field. Prior to parentage analysis with GERUD1.0 (Jones 2001), the maternal genotype from clutch 13 was reconstructed by hand, based on offspring genotypes.

Parentage analysis

The analysis of maternal and offspring genotypes from field-collected samples was accomplished using the computer software GERUD 1.0 (cf Jones 2001). We determined the minimum number of males siring offspring in each clutch and reconstructed the paternal genotypes. In cases where more than one genotype reconstruction was possible for the sires in a clutch, the program listed all the possible paternal genotype combinations and ranked the families based on probability (see Jones 2001 for details on ranking paternal genotypes). However, we chose conservatively to accept the program-generated paternal genotype reconstructions only when one unique solution was possible.

We found low frequencies of null alleles at all three loci (Doc01: 3.6%, Doc02: 4.7%, and Doc03: 1.2%). The only cases in which the possibility of paternal null alleles resulted in uncertainty regarding the number of sires per clutch was in clutches in which offspring appeared to be homozygous for one or the other of the heterozygous maternal alleles. In these cases, it appeared as though 1 or 2 sires might share maternal alleles. When only one of the maternal alleles appeared in multiple homozygous offspring, the estimate of the total number of sires per clutch was not

affected, even if the allele was actually a null and not a shared allele. However, when both maternal alleles appeared in homozygous offspring, we needed to address the possibility that there really were not two unique paternal alleles, but a single null allele. This situation occurred in 5 of the 28 clutches analyzed; three clutches at the Doc02 locus and two clutches at the Doc01 locus. To resolve this uncertainty, the 5 clutches were analyzed two ways: first, as if the offspring really were homozygotes; and, second, as if the offspring shared a paternal null allele. Assuming the offspring were homozygotes resulted in the conclusion that there was one additional sire, when compared with the null allele model (see GERUD manual for a discussion of this). In the end, for all 5 clutches, we concluded that the offspring were not homozygotes (i.e., their parents shared alleles), but instead that the offspring all shared a single paternal null allele. The homozygote model always resulted in paternal genotype reconstructions at the other two loci (without null alleles) that were more complicated and less probable than those generated under the null allele model. Furthermore, none of the offspring in the 5 clutches were heterozygous for both maternal alleles, which would have provided support for the homozygote model by revealing the presence of alleles shared by the mother and sire(s). In all 5 cases, analysis with only the two loci not having null alleles suggested that the third locus had a paternal null, not that alleles were shared among parents. Thus, although null alleles slightly reduced our power to detect males siring offspring in a clutch, the probability of correctly determining the number of males siring offspring in each clutch was still high.

Reliability of parentage analysis

We used GERUDSIM 1.0 simulation software and the allele frequency data (from 42 individuals, see Ch. 3) at each of the three loci to assess the probability of accurately detecting all sires in each clutch and to determine the probability of correctly reconstructing paternal genotypes from known maternal and offspring genotypes (Jones 2001). For each iteration of a given simulation, GERUDSIM 1.0 draws alleles at random from known allele frequency data at each of the three loci, and then uses those alleles to construct a single maternal genotype and several paternal genotypes. (We used the same allele frequencies here as were used in the GENEPOP analysis.) Up to 5 sires can be included in the program parameters. From the parental genotypes, GERUDSIM 1.0 constructs an offspring array based on user-specified paternity distribution and clutch size. GERUDSIM1.0 then removes the paternal genotypes from the analysis and, using the same analysis algorithm as in GERUD1.0, attempts to determine the number of males siring offspring per clutch, as well as to reconstruct all paternal genotypes (cf Jones 2001). Finally, these results are compared to the originally constructed known paternal genotypes. We ran 1000 iterations (i.e., constructed 1000 families) to determine the probability of correctly determining the number of males siring offspring per clutch and of correctly reconstructing all paternal genotypes based on the allele frequencies at the three loci.

Statistical analysis

We used GENEPOP software (Raymond and Rousset 1995) to test for Hardy-Weinberg Equilibrium and linkage disequilibrium among three microsatellite loci in a

population of 42 unrelated individuals (Ch. 3). We computed linear and curvilinear regressions using the procedure RSREG of SAS v. 8.1 (SAS Institute Inc. 2000) and tested the null hypothesis that the number of sires in a clutch and female body size do not affect the number of embryos in a clutch. The procedure RSREG employs the least squares method to fit quadratic regression models. The RSREG procedure also fits a linear regression model to the data for use as a basis for comparison with the quadratic model. In the MODEL statement we specified the LACKFIT option to measure the adequacy of the quadratic model. The statistical software nQuery Advisor® (Statistical Solutions 2003) was used to calculate the power (1-β) of the linear and quadratic regression analyses (testing female benefits and sexual conflict, respectively) at the alpha (α) = 0.05 level. Chi-square analysis was conducted using the SAS procedure FREQ. The procedure GENMOD, which performs a logistic regression analysis, was used to assess whether the proportion of offspring sired in two-sire clutches differed from 50%.

RESULTS

Microsatellite development.

Analyses of allele frequencies for 42 unrelated individuals using GENEPOP 3.1b (Raymond and Rousset 1995) showed that, while Doc01 and Doc03 were in Hardy-Weinberg equilibrium (H_0 = random union of gametes; p=0.23, p=0.26, respectively), Doc02 was not (p=0.017) (Table 2.1). There was also a significant

heterozygote deficit in both Doc01 and Doc02 ($p<0.0001$ and $p<0.001$, respectively). No pairs of loci were found to be in linkage disequilibrium (H_0 = genotypes at one locus are independent of genotype at the other loci; $p=1.0$). Using offspring array data from a separate parentage study in which all parental genotypes were known (see Ch. 3), we determined that the lack of Hardy-Weinberg equilibrium and the presence of heterozygote deficiencies were due to null alleles. Although null alleles did occur at each locus, the loci were also very polymorphic and hence ideal for parentage analysis. Also, one embryo was found to have a *de novo* mutation at the maternal allele (from 244bp to 248bp) at the Doc02 locus, indicating that there had been a single-repeat mutation of the maternally-inherited allele.

Parentage analysis - Number of sires in each clutch

Analysis of the field-collected clutches revealed that only 1 clutch had been sired by a single male, 15 clutches had been sired by 2 males, 9 clutches had been sired by 3 males, and 3 clutches had been sired by 4 males (Figure 2.1). Thus, most females (96%) produced clutches sired by more than one male. Of the multiply sired clutches, over four-fifths (86%) were sired by either 2 or 3 males.

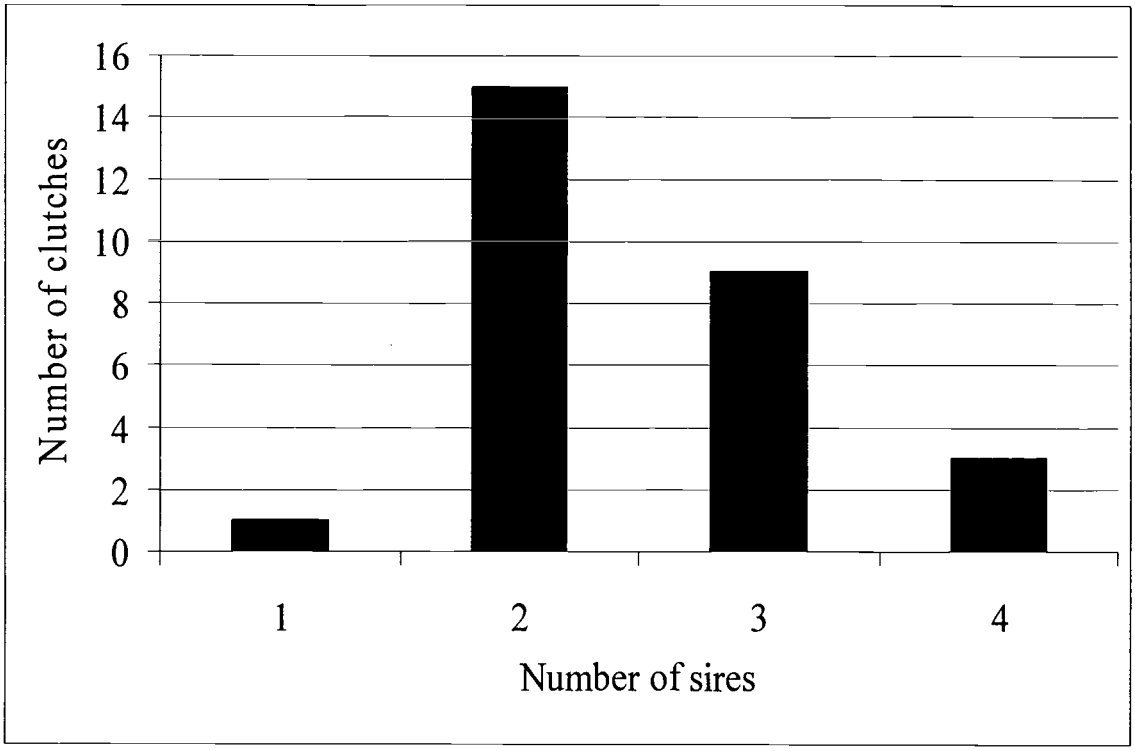

Figure 2.1. Microsatellite parentage analysis of 28 field-collected clutches of *D. ocoee*. Ninety-six percent of the clutches had more than one sire, and over 86% of clutches were sired by either 2 or 3 males.

Parentage analysis - Distribution of offspring paternity

We were able to reconstruct paternal genotypes for all but two of the 15 clutches sired by 2 males (Figure 2.2). In one of the excluded clutches, at all three loci too many alleles were shared among the mother and both sires to be able to decide between two possible paternal genotype reconstructions. In the second of the excluded clutches, we were unable to decide between two equally probably genotype reconstructions. For each of the remaining 13 clutches sired by two males, one male sired the majority (between 58%-86%) of the offspring. Using logistic regression analysis, we determined that these values deviated significantly from 50% (H_0= equal paternity among males; $p < 0.001$). For those clutches having either 3 or 4 sires, analyses with

GERUD 1.0 resulted in a single unequivocal paternal genotype reconstruction for 6 of the 12 clutches (Figure 2.3). In these 6 clutches, one male sired most of the offspring, but paternity was more evenly distributed among sires than in clutches sired by only two males (Table 2.2).

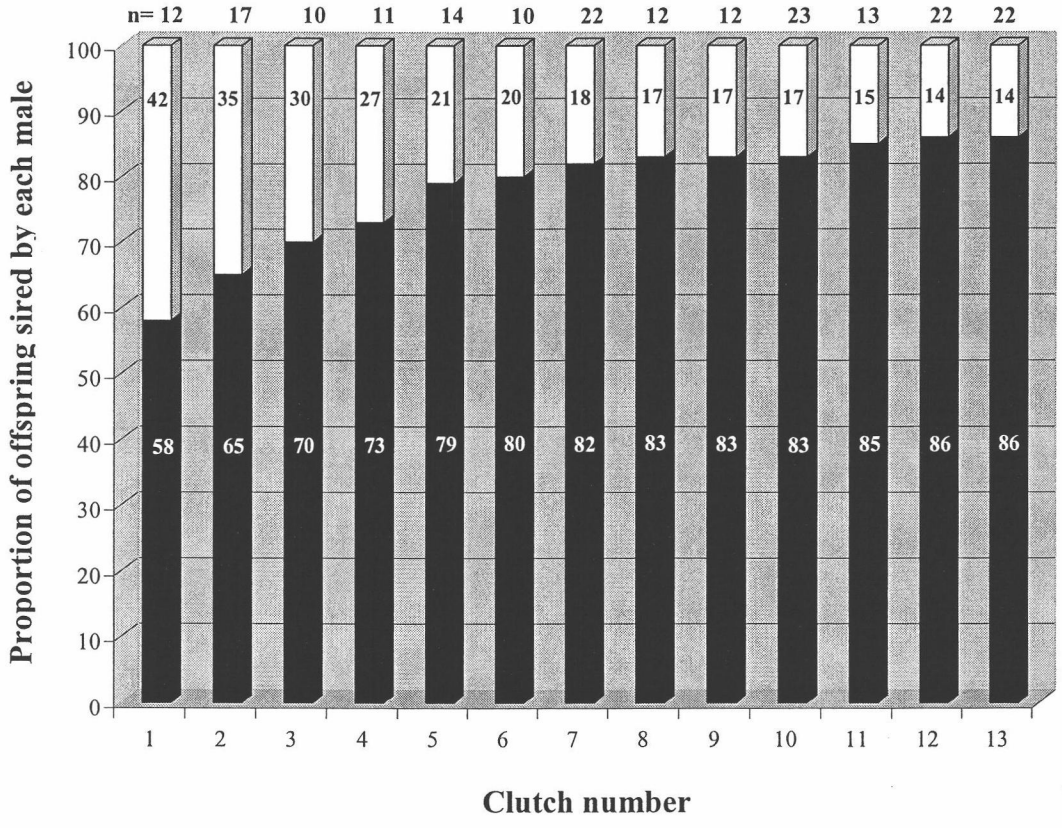

Figure 2.2. Distribution of paternity within clutches sired by two males. Numbers within the light and dark regions of each bar reflect the respective proportion of offspring sired by each of the two males. Numbers above each bar indicate the number of eggs in each clutch. One male tended to sire the majority of offspring in each clutch. (Note: Bar color does not imply a particular insemination order)

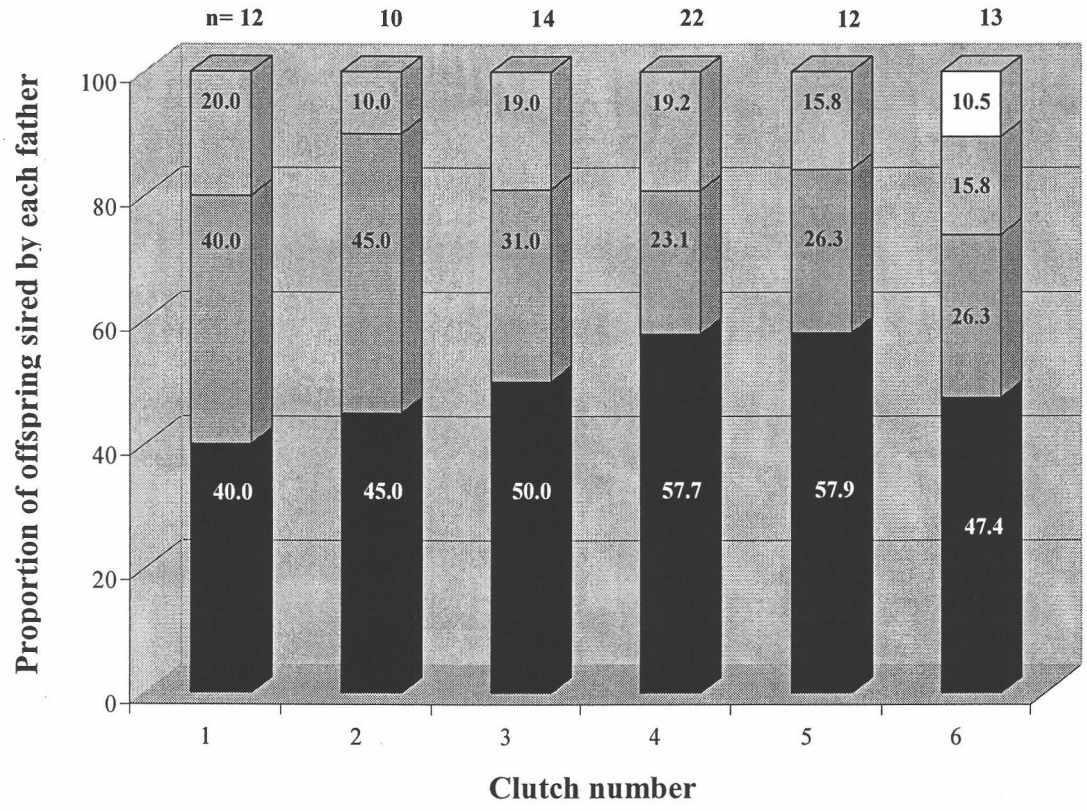

Figure 2.3 Distribution of paternity within clutches sired by three and four males. Genotype reconstruction was possible for five of the clutches sired by three males, but only one of the clutches sired by four males. Numbers within the light and dark regions of each bar reflect the respective proportion of offspring sired by each male. Numbers above each bar indicate clutch size. (Note: Bar color is not meant to imply a particular insemination order.)

Table 2.2 Average allocation of parentage as assessed by GERUDSIM 1.0 simulations.

Number of Sires	Average percent offspring sired by each male	No. of clutches
2	80:20	13
3	50:33:17	5
4	47:26:16:11	1

Reliability of parentage analysis

We used the program GERUDSIM 1.0 to assess the reliability of GERUD 1.0 in determining the number of sires having offspring in a clutch, and to evaluate how well this program reconstructed paternal genotypes. Since our results from the GERUD1.0 analysis determined that one sire had the majority share of offspring, we based our parameters in GERUDsim1.0 on the skewed distribution of paternity seen in the field data (see Table 2.2 for ratios). Clutch size was also parameterized in GERUDsim1.0 based on average values obtained from the field data. Average size for clutches with two sires was 15 eggs, for 3 sires was 19 eggs, and for 4 sires was 18 eggs. The results of the simulation examining the probability of correctly determining the number of males siring offspring per clutch and of correctly reconstructing paternal genotypes are presented in Figure 2.4. Since the total probability of correctly reconstructing paternal genotypes (as shown in Figure 2.4) relies heavily on the number of offspring each male sired, we further separated probabilities into two categories: one category for those males that sired the majority of offspring and a second category for those males that sired fewer offspring (Figure 2.5).

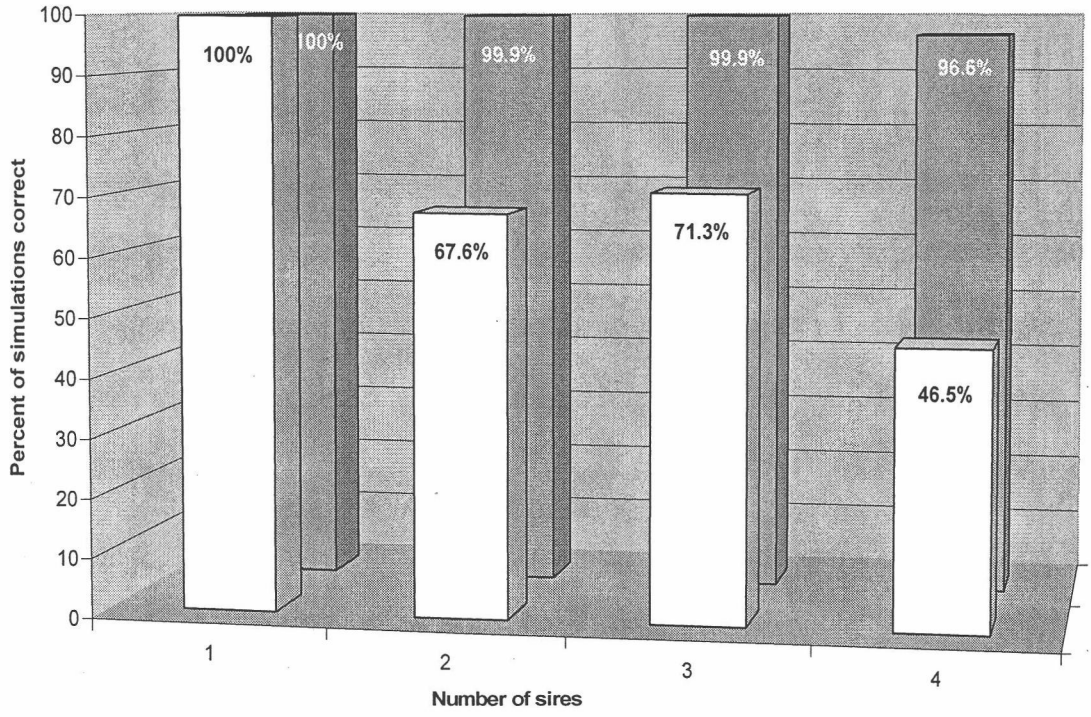

Figure 2.4. Results of GERUDsim1.0 simulations. Dark bars represent the probability of correctly determining the number of sires per clutch for clutches with 1 to 4 sires. Light bars represent the probability of correctly reconstructing all paternal genotypes. Simulations were based on paternity data from the field in which average clutch sizes were: 1 sire = 12 offspring, 2 sires = 15 offspring, 3 sires = 19 offspring, 4 sires = 18 offspring. We had a very high probability of correctly determining the number of sires per clutch, but a low overall probability of correctly reconstructing paternal genotypes.

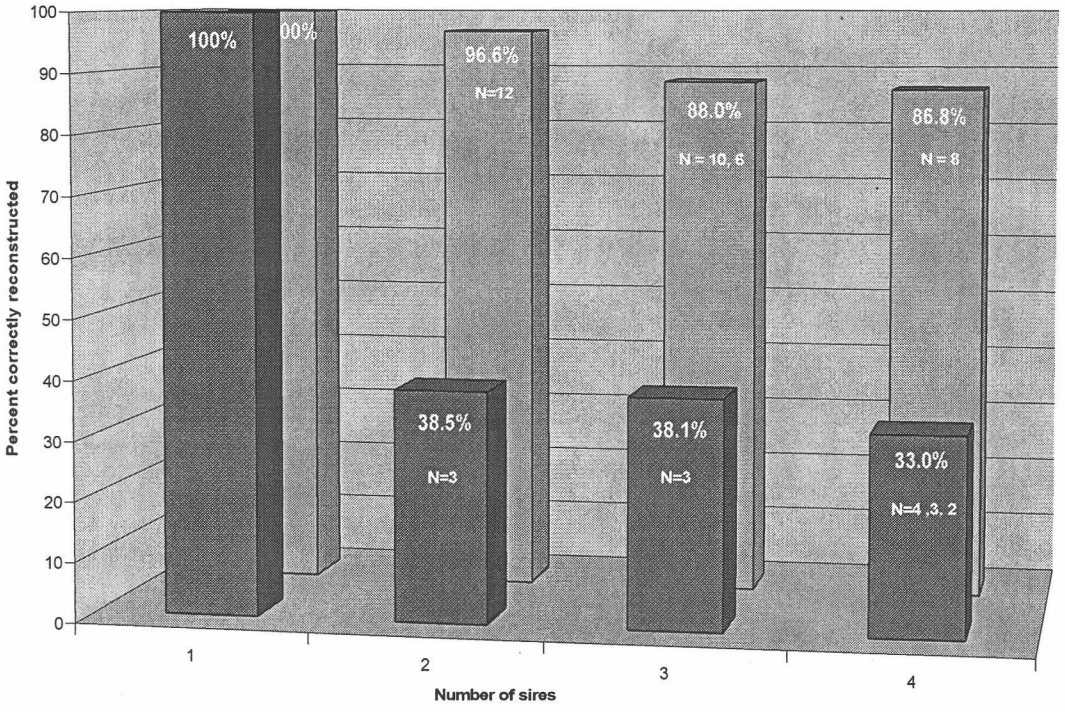

Figure 2.5. Results of GERUDsim1.0 simulations. There was a high probability of correctly reconstructing paternal genotypes when males sired more than six offspring in a clutch. However, this probability declined dramatically for 5 or fewer offspring. Dark bars represent the probability of correctly reconstructing the paternal genotypes for males siring 5 or fewer offspring in a clutch. Light bars represent the probability of correctly reconstructing the paternal genotypes for males siring 6 or more offspring per clutch. The number of offspring sired by each male is noted in the corresponding bar (e.g., n=3 means that one of the sires in a clutch had three offspring). The number of offspring sired by each male in a clutch used in this analysis was based on the field data (see Table 2.2 for ratios).

Female benefits and sexual conflict

We tested the statistical hypothesis that the number of sires in a clutch and female size (SVL) both affect clutch size (as evaluated at stage 26, Marks 1995) in order to determine whether females may benefit from polyandry. Previous studies have shown a correlation between female size and clutch size in many plethodontid

salamanders (including *Desmognathus ocoee,* although the authors point out that many factors may contribute to variation in clutch size; Tilley 1968, 1977; Houck 1977a, 1977b) such that it was necessary to control for variation in clutch size attributable to female size in our analyses. Using simple linear regression, we found that there was a significant effect of female size on clutch size (n=27, p= 0.001). However, the number of sires in a clutch did not have a significant effect on clutch size when both number of sires and female size were included in the model (number of sires: p= 0.653, and female size: p= 0.004, respectively; Figure 2.6), nor was the interaction term between female size and number of sires significant (p= 0.139).

Addition of a squared term to the regression (procedure RSREG) did not result in a better fit of the model to the data, and the relationship between the number of sires per clutch and clutch size was not statistically significant (n=27, p= 0.733; Figure 2.6). A good fit of the data to a quadratic model would be expected under the sexual conflict model. R-square values for the linear and quadratic regression models were 0.361 and 0.402, respectively, indicating that the quadratic model only accounted an additional 4% of the variance as compared with the linear model. The Lack of Fit test was significant, indicating that the quadratic model did not account for a significant increment in variance in clutch size (p= 0.042).

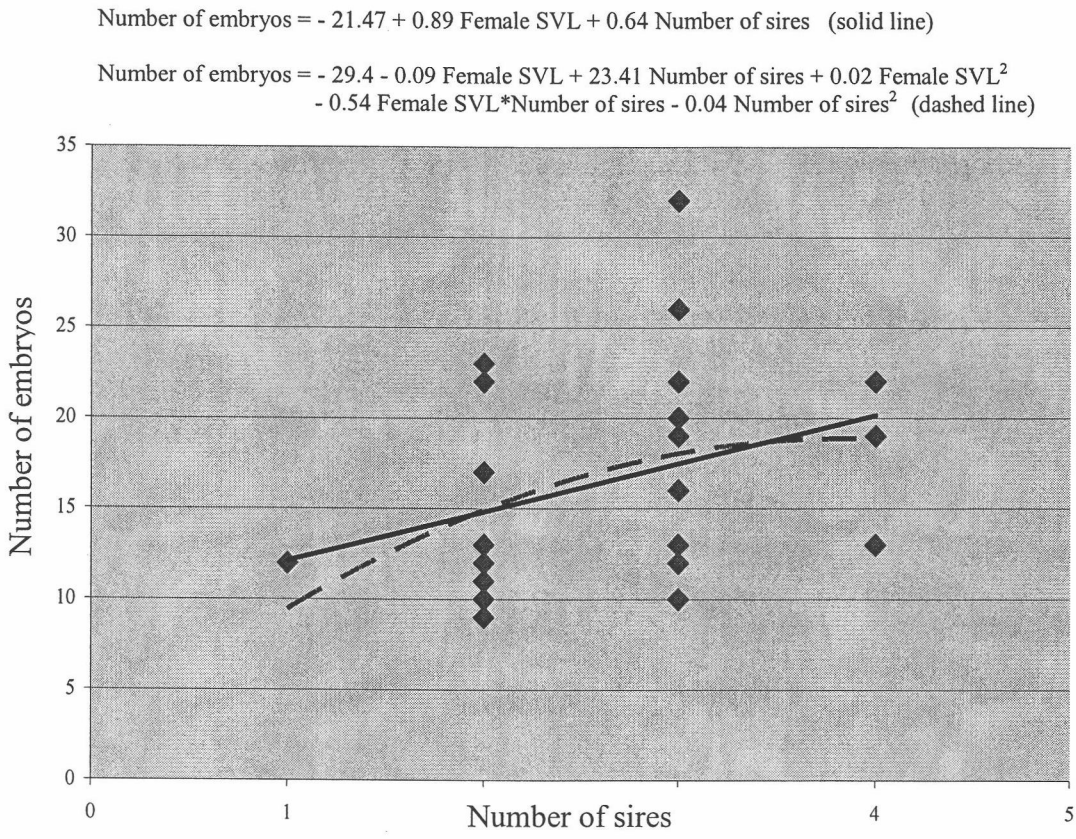

Figure 2.6. Number of embryos per clutch as a function of the number of sires. Shown are the trendlines for both the linear (solid line) and quadratic (dashed line) regression models, neither of which was statistically significant ($p= 0.653$ and $p= 0.733$, respectively). As a result, our data do not provide evidence that female *D. ocoee* are mating multiply either to gain female benefits or as a result of sexual conflict.

Since we were unable to reject the null hypotheses for the multiple linear and quadratic regression models, our data do not provide support for either the female benefits or sexual conflict models acting in *D. ocoee*. In order to test whether rejection of these models was warranted or was potentially due to a lack a statistical power ($1-\beta$), we conducted power analyses using the software nQuery (Statistical

Solutions 2003). For the simple linear regression of clutch size on the number of sires (α= 0.05), we had a 41% chance of rejecting the null hypothesis if it was actually false. Including female size in the model increased power by 5%, for a combined power of 46%. The addition of a squared term to the model did not increase the power of our analysis.

DISCUSSION

Our data indicate that polyandry is an important aspect of the mating system in *D. ocoee*. Of the 28 clutches analyzed, 96% were sired by more than one male. In contrast, Tilley and Hausman (1976) used allozymes to assess paternity in over 150 field-collected clutches of *D. ochrophaeus* (=*D. ocoee*) and concluded that only approximately 7% had been sired by more than one male. In a separate study, Labanick (1983) used color polymorphisms and assumptions of Mendelian inheritance to assess paternity in field-collected clutches. He concluded that only two of eight clutches had been sired by multiple males. Both studies suffered from severe limitations imposed by low levels of polymorphism in the markers used to assess paternity. With the use of highly polymorphic microsatellites, we were able to assess paternity with a higher level of accuracy. Our results indicate that multiple mating in the field is far more common in natural populations of *D. ocoee* than was previously thought. While sexual selection acting prior to insemination has been studied

extensively in *D. ocoee* (Houck et al. 1985b, Houck et al. 1988, Verrell and Arnold 1989), our study is the first to show that polyandry and the resulting post-insemination sexual selection (e.g., sperm competition and cryptic female choice) is a large component of the mating system in the field. However, results similar to ours in *D. ocoee* have been found in a field study of the rough-skinned newt (*Taricha granulosa,* Jones et al. 2002). Over two-thirds of females in the field produced clutches were sired by more than one male. Females of the alpine newt (*Triturus alpestris)* also appear to be polyandrous (Rafinski and Osikowski 2002). Thus, polyandry may be a common occurrence among caudate amphibians.

Although polyandry is common in *D. ocoee*, the number of sires per clutch seems to be limited. Of the 28 *D. ocoee* clutches we analyzed, the majority (86%) were sired by either 2 or 3 males. The remaining clutches were sired by either one (3%) or four (11%) males. Houck et al. (1985b) showed that female *D. ocoee* have a propensity for high insemination rates in the laboratory. However, we found that relatively few males sire offspring in field-collected clutches. One explanation for this discrepancy is that the high insemination rates observed by Houck et al. (1985b) are a result of unnatural encounter rates and conditions found in the laboratory. Alternatively, it is possible that only a fraction of the males that inseminate a female also sire offspring. Whether or not a male that inseminates a female sires offspring has important implications, not only for male reproductive strategies, but for female reproductive strategies as well. If not all males that inseminate a female sire offspring, a female may be able to increase her offspring quality by mating with many, increasingly higher quality males as she encounters them, if only high-quality males

fertilize offspring. On the other hand, if all males that inseminate a female also sire offspring, we expect selection on females to reject low-quality or incompatible males prior to insemination, and mate only with a few males. Rejection of low-quality males is plausible given observations that female *D. ocoee* can and do reject males during courtship encounters staged in the laboratory (Houck et al. 1988, Verrell and Arnold 1989). However, additional study is needed to determine whether all males that inseminate a female always sire offspring in the clutch, and how this affects female mating strategies.

In their preliminary study of sperm competition in *D. ocoee*, Houck et al. (1985a) showed that the last male to inseminate a female always sired at least a small proportion of the offspring. This effect was observed even if the female previously had been inseminated by up to 11 different males. In the present study, we found that one male in each clutch sired the majority of offspring in the clutch, the proportion of which was relatively consistent among clutches sired by the same number of sires (Figures 2.2 and 2.3). This finding, together with evidence from Houck et al. (1985a), suggests that sperm precedence, favoring the first male to inseminate a female, may be acting in *D. ocoee* (although we note that one of seven clutches in their study showed last-male sperm precedence). Another ramification of the observation that the last male to inseminate a female always sires offspring (Houck et al. 1985a) is that males always benefit from inseminations just prior to oviposition. As a result, we expect strong directional selection on males to achieve inseminations late in the breeding season, independent of whether the female previously has been inseminated. This selection, together with the fact that males deliver courtship pheromones known to

affect female receptivity, suggests that sexual conflict could be present in *D. ocoee*. Nevertheless, we did not detect sexual conflict in our study.

There was no support for a model of stabilizing selection on mating frequency in females (Figure 2.6) An intermediate optimum mating frequency would have indicated sexual conflict was operating in *D. ocoee* (Gavrilets 2000, 2001; Mead and Arnold *In Press*). One explanation is that mating may not be costly to females, which removes the conflict between males and females with regards to mating frequency. The high mating frequency observed in the laboratory (Houck et al. 1985b) makes sense in light of this assumption. Cost-free mating is unlikely, however, given that females may experience increased risk of predation and reduced foraging time while courting with additional males. Furthermore, sexual conflict may affect aspects of female fitness other than clutch size. Given the low power (46%) of the test for sexual conflict, however, additional studies with larger sample sizes are needed. This presents a significant challenge as clutches in the field are hard to find, and large samples sizes may be difficult to achieve. Nevertheless, larger sample sizes will be needed in order to resolve whether sexual conflict affects clutch size in *D. ocoee*.

Although a female may mate with several males, there is no significant effect of the number of sires per clutch on clutch size, even after accounting for female size. This finding is similar to that shown by Bateman (1948) in *Drosophila* and suggests that females cannot increase their clutch size with increased number of inseminations. Thus, our results indicate that female *D. ocoee* may not gain direct benefits in the form of increased clutch size from mating multiply. In contrast to other organisms in which females continuously produce mature ova throughout the breeding season (e.g., many

insect species), salamander clutch size is determined in large part by resource availability throughout the one or two years required to yolk the ova (Tilley 1977). It appears that our original hypothesis, that males may be able to influence clutch size by inseminating females throughout the period when females are yolking ova, may not be occurring. Again, however, the lack of a sufficiently large sample size decreased our power to detect a significant effect of number of sires on clutch size. Thus, while our results suggest that females may not benefit from increased clutch size due to multiple mating, further study is required clearly to establish this.

The simultaneous use of multiple, variable loci to analyze paternity data was a crucial aspect of our study. The three microsatellite loci used in this study were highly polymorphic. However, the high mutation rate that produced this high level of allelic diversity also resulted in null alleles. Although not desirable, the null alleles did not affect the reliability of our results. For the five clutches in which several offspring appeared to be homozygous for both heterozygous maternal alleles, we were able to determine that these individuals actually had null alleles. In each case, if we assumed the offspring in question were homozygous, paternal genotype reconstructions were more complicated and required more paternally and maternally shared alleles than assuming that offspring simply had a single paternal null allele. Furthermore, given the high levels of polymorphism at all three loci, reliance on the two loci not expressing null alleles was sufficient to detect all males siring offspring in a clutch.

We used computer simulations to show that, with three microsatellite loci we had a very high probability of correctly determining the number of sires per clutch, as well as correctly reconstructing paternal genotypes. In addition, GERUDsim1.0

clearly demonstrated the inherent problems associated with reconstructing paternal genotypes when males sire only a few offspring in a clutch. The primary reason for the precipitous decline in ability to correctly reconstruct paternal genotypes when more than 2 males sire offspring in a clutch is the small clutch sizes found in *D. ocoee*. The average size of *D. ocoee* clutches collected from the field was just 16 eggs (range of 9 to 32 eggs). Assuming equal paternity, this means that, if a clutch had two sires, each male sires 8 offspring. With 3 males, each male sires either 5 or 6 offspring, and with 4 males, each only sires 4 offspring. If several males sire fewer than 5 offspring each within a clutch, there frequently are not sufficient unique paternal allele combinations represented among the offspring to determine which offspring belong to which male (i.e., to reconstruct the paternal genotypes). Reconstructing paternal genotypes is further confounded if any alleles are shared among males. Such shared alleles did occur, despite the fact that the loci we used were highly polymorphic. However, if one male sires the majority of offspring in a clutch (or if a clutch is particularly large), the probability of correctly reconstructing that male's genotype is high, no matter how many males sire offspring in a clutch. Furthermore, the distribution of paternity as presented in our analyses are very likely correct (although the specific alleles assigned to each male may be interchanged) since, if the true number of offspring sired by each male were to differ by more than one or two offspring, our power to reconstruct a male's genotype is greatly increased.

In summary, our results indicate that polyandry is an important component of the mating system in the salamander *Desmognathus ocoee*. We found that: (1) Females produce clutches with 1-4 sires, although the great majority of clutches had

either two or three sires; and (2) the distribution of paternity in clutches sired by multiple males confirms previous indications that sperm precedence may be acting in this system (Houck et al. 1985a). We also tested the hypotheses that female benefits and sexual conflict over mating frequency were operating in this system. We found that female *D.ocoee* do not appear to benefit from increased clutch size when increasing numbers of males sire offspring in each clutch. In addition, we found no evidence that females produced fewer offspring when clutches were sired by either 1 or 4 males than when they were sired by 2 to 3 males. These findings suggest that either sexual conflict does not occur in *D. ocoee* or conflict affects aspects of female fitness other than clutch size. Further study is needed to identify additional traits, and the type of selection on these traits, which may explain the ultimate causes of female multiple mating in *D. ocoee.*

CHAPTER 3

SPERM PRECEDENCE AND THE EFFECTS OF MALE SIZE ON COMPONENTS OF MATING SUCCESS IN THE OCOEE SALAMANDER (*DESMOGNATHUS OCOEE)*

Erika M. Adams

ABSTRACT

I investigated the effects of male body size on insemination and fertilization success in the Ocoee salamander (*Desmognathus ocoee)*, a species that shows extensive natural variation in male size. Larger males (>41mm SVL) had increased insemination success and sired a greater proportion of offspring than did smaller males. Sperm number per spermatophore also increased with male size. In all but 2 of 14 clutches, the first male to inseminate a female sired the majority (90%) of offspring in the clutch. Male and female body sizes and insemination frequency were unrelated to a female's probability of ovipositing. Overall, large male body size increased reproductive success for males, demonstrating strong directional selection on male size in *D. ocoee*. These results contribute to a greater understanding of the

effects of interactions among traits important to insemination success and fertilization success on the evolution of male reproductive strategies.

INTRODUCTION

Body size has long been recognized as an important male trait affecting insemination success in many animal mating systems (Andersson 1994). Large males are often more successful than are smaller males during male-male competition and female choice. However, in promiscuous mating systems in which females mate with several males, sexual selection occurring after insemination also can have a large effect on male reproductive success. Thus, a male must not only be able to successfully inseminate females, but must be able to compete successfully in post-insemination processes (occurring within the female) that affect fertilization success.

Recently, a great deal of research has focused on male traits important to fertilization success. Male damselflies, for example, have a modified intromittent organ that allows them to remove previously deposited sperm from the female's reproductive tract, thereby reducing sperm competition (Waage 1979). In *Drosophila*, seminal proteins delivered to the female (along with sperm) reduce female receptivity and stimulate ovulation, increasing the probability that sperm in that ejaculate will fertilize ova (Harshman and Prout 1994, Rice 1996, Wolfner 1997). Also in *Drosophila*, sperm and semen have been shown to displace and incapacitate rival male sperm (Price et al. 1999). Clearly, selection to increase fertilization success plays an

important role in the evolution of male reproductive traits (Parker 1979). Therefore, male reproductive success is a function of both insemination and fertilization success.

Selection on male traits important prior to and after insemination (e.g., plumage coloration to achieve inseminations and sperm motility to gain access to the ova) are expected jointly to shape a male's reproductive strategy and may be positively or negatively correlated. In the simplest case, body size allometry alone may explain the positive correlation between a physical trait such as male body size (often important in male-male competition; Andersson 1994) and testes size (which has been shown to affect sperm number and fertilization success; Møller 1988, 1989). However, selection on males to achieve inseminations and to fertilize ova may also result in a positive correlation among those traits important to each stage (Birkhead and Pizzari 2002). For example, certain traits important to insemination success, such as male coloration and levels of sigmoid displays in guppies (Evans and Magurran 2000, Evans et al. 2003), and body size in butterflies (Wedell and Cook 1998) are correlated with other traits that produce higher fertilization success and, ultimately, a higher percentage of offspring paternity. In these examples, variation in sperm number resulting in increased paternity was not attributable to increased success at transferring sperm to the female (insemination frequency), but to traits not directly selected by female choice (e.g., testes size and ejaculate size). The positive correlation between pre- and post-insemination traits is particularly clear in the study by Evans et al. (2003). The authors showed that increased coloration in male guppies was correlated with post-insemination fertilization success, even though the females were artificially inseminated with equal numbers of sperm from two differently colored males. Thus,

in the above systems, males experienced selection pressures to increase post- as well as pre-insemination success. Traits that increase insemination success, however, are not always positively correlated with traits that affect fertilization success.

In systems in which pre- and post-insemination male traits are condition dependent, or are costly to maintain, selection favors males that are particularly successful either prior to insemination or after insemination (Birkhead and Pizzari 2002). In these cases, a negative correlation is expected between pre- and post-insemination traits. For example, in marine isopods, different-sized isopods were able to achieve similar reproductive success through different traits and reproductive strategies (Shuster and Wade 1991). Thus, males having different reproductive strategies are expected to co-exist in a population and have similar overall reproductive success. Furthermore, selection maintains variation in male reproductive strategies within a population. In contrast, when pre- and post- insemination traits are positively correlated, males are expected to evolve toward a single optimum. Thus, by examining the nature of correlations between male traits important to pre- and post-insemination success, one can determine male reproductive strategies and predict how varying reproductive scenarios during inter- or intrasexual selection may affect male fitness.

Adult male body size varies extensively within and among populations of Ocoee salamanders (*Desmognathus ocoee;* Tilley 1973, Bruce 1993). In the experiment described below, the snout-to-vent length (SVL) of males that successfully inseminated a female varied between 31.0mm-56.9mm, almost a two-fold difference in male size. Male size in *D. ocoee* has previously been shown to be important in

male-male competition for mates, with larger males successfully chasing away smaller males and inseminating the female (Houck 1988). To date, there is no evidence of female choice based on male size (Houck 1988), however, males vary in their insemination success in this system, and this variation potentially is due to female choice (Houck et al. 1985b). An additional source of variance in male insemination success could also be due to interactions between the sexes during courtship. Courtship in *D. ocoee* requires the completion of a series of coordinated behaviors (Arnold and Houck 1982), any of which might be unsuccessful if the male is too small (Bruce 2000). Thus, male size is, potentially, an important factor affecting male insemination success in Ocoee salamanders. However, little is known about male traits important to post-insemination success.

A recent study of paternity in field-collected clutches of *D. ocoee* revealed that the majority of females mate multiply and produce clutches sired by between 1 and 4 males (see Ch. 2). Most clutches were sired by either 2 or 3 males. Given that polyandry is common in *D. ocoee*, post-insemination processes could have a major effect on male reproductive success. Insemination in *D. ocoee* occurs indirectly via a spermatophore deposited on the substrate by the male (fertilization is internal). In order to become inseminated, the female must walk over the spermatophore and lodge the sperm mass in her cloaca (Arnold and Houck 1982). Personal observation suggests that the size of the sperm mass varies among males, with small males producing smaller spermatophores than do larger males. However, variation in sperm number among different-sized males has never been measured experimentally. In addition to behavioral observations, *D. ocoee* physiology suggests that large males

may have greater sperm producing capacities than smaller males. Amphibians experience indeterminate growth and the size of a male Ocoee salamander's testes (number of testes lobes) increases throughout its lifetime (Humphrey 1922, Tilley 1977, Houck and Francillon-Vieillot 1988). Larger and older males have larger testes and may be able to produce more sperm. Thus, larger males may be able either to provide more sperm per spermatophore, or they may be able to produce more spermatophores than can a smaller male. As a result, size may be correlated with sperm production and, ultimately, with male fertilization success in Ocoee salamanders.

In order to determine whether male *D. ocoee* body size is a factor affecting insemination success and paternity, I staged replicated encounters in the laboratory between a female and two males. In a prior study, I determined that over half of the clutches sampled from the field were sired by two males (see Ch. 2), suggesting that two males represents a biologically relevant number of mates for the current experiment. In this study, I determine whether larger males have greater insemination success and sire more offspring than do smaller males. I then compare these results with data from a separate study on the effects of body size and female mating status on sperm number per spermatophore to determine whether there is a correlation between insemination success (pre-insemination selection) and both fertilization success (paternity) and sperm number (post-insemination selection) as a function of male size. From these data I identify the reproductive strategies of *D. ocoee* males and predict how these reproductive strategies may function in natural populations of *D. ocoee.*

METHODS

Animal collection - females

Fifty female Ocoee salamanders were chosen (based on their similar reproductive status) from a group of females collected during August 2000 and 2001 (25 females from each year; collected from Deep Gap, NC). Only females having large, yolked ova ready for oviposition were used in the experiment. Mature ova were clearly visible and easily counted within the ovaries by holding each female up to a fiber-optic light and gently compressing her abdomen. All animals were housed individually, and none of the females were inseminated in the laboratory prior to the experiment. Females were not collected from the field just prior to start of the experiment as there is a high probability that field-collected females may have already mated and thus are storing fresh sperm from that year's breeding season (Sever and Hamlett 1998). Microsatellite analysis (described below) verified that none of the offspring were sired by males other than those used in our experiment. In addition to counting the number of ova in each ovary, I measured each female's snout-to-vent length (SVL, from the tip of the snout to the posterior edge of the cloaca) using digital calipers.

Animal collection - males

One hundred males were randomly chosen from males collected at Deep Gap, NC during May 2002, just prior to the start of the experiment. The reproductive maturity

of each male was confirmed by transilluminating the animal using a fiber-optic light and noting the presence of darkly pigmented testes and vasa (Houck and Francillon-Vieillot 1988). I measured each male's SVL using digital calipers.

Animal Care and Maintenance

All animals were housed individually in plastic containers lined with moist paper towels and crumpled wet paper towels as refugia. Light and temperature conditions in the animal room were adjusted to match the natural photoperiod and temperature regimes experienced at Highlands Biological Station (HBS), Macon Co., NC (HBS; 38° 45'N 79° 30' 00W). HBS experiences similar seasonal conditions as the site from which the animals were collected (Deep Gap). Individuals were fed weekly with 2-week old crickets dusted with vitamins.

Experimental protocol

I controlled for possible differences associated with year of collection (females) and with body size (males). I ranked males from smallest to largest and assigned every other male to a female collected in 2000, with the remaining males paired with females collected in 2001. Thus, one group of 50 males was assigned to females collected in 2000, and another group of 50 males to female collected in 2001. The range of body sizes was similar for both male groups. Each of the 50 females was randomly assigned two males from her assigned male group. Each female was paired

sequentially with each of two males during separate courtship encounters. I did not attempt to classify males into small and large categories as I wanted to capture as much of the variation in male reproductive success based on male size as possible. Each male was used to inseminate only one female, and a male was allowed two courtship opportunities in which to inseminate her. Once inseminated, the sperm mass remained visible in the female's cloaca for up to 24hr, allowing for unambiguous scoring of insemination success (Verrell 1991b). If a female was inseminated during the first encounter with a male, she was not paired with that male again. A total of four courtship encounters were conducted between 2000 hr to 0100 hr each night from 20 June to 17 July 2002. Individuals were allowed a minimum of 6 days between courtship encounters. Males require only 4 days off to sustain maximal reproductive output (Verrell 1991b). Thus, 6 days allowed for complete recovery between courtship encounters if a male had deposited a spermatophore (but failed to inseminate the female).

On a given trial night, a male and female were put together in a clear plastic shoebox (31 x 17 x 9cm) lined with moist paper towel. Pairs were observed throughout the 5 hr courtship period to determine whether a female was inseminated and to control for multiple spermatophore deposition events within a single courtship encounter. After the male deposited a spermatophore, the female occasionally deserted the male without picking up the sperm mass in her cloaca and becoming inseminated. Spermatophore size appears to decrease with sequential failed insemination attempts within a single night (pers. obs). Thus, it was important to control for the number of spermatophores produced by a male prior to inseminating

the female, as reduced spermatophore size may affect post-insemination fertilization success. Only 9 of 154 courtship encounters (5.8%) resulted in the deposition of an additional spermatophore prior to insemination of the female. None of the females that were inseminated by the second spermatophore deposited within a single night went on to mate twice and successfully oviposit a full clutch of eggs. Thus, these females did not meet our protocol requirement for two inseminations and thus were excluded, so there was no chance that repeated spermatophore production affected the results of the paternity data.

Hormone injections

Although females maintained in the laboratory will occasionally oviposit spontaneously without the use of hormones, few actually do so. Therefore, I injected females to increase the probability that the experimental females would oviposit. Ten days after the last insemination date (16 July 2002), all females were injected intraperitoneally with 10μl of 10ng/μl of leutinizing hormone releasing hormone (LHRH, Arnold et al. 1993). All of the clutches included in the analysis for this experiment appeared to have been oviposited normally: eggs within each clutch were clustered together (as is observed in the field, pers. obs.) and the female attended the eggs until they were collected. Other laboratory experiments in which the female *D. ocoee* were injected with LHRH to produce oviposition revealed that, occasionally, females will oviposit haphazardly in multiple locations throughout the maintenance box (Arnold et al. 1993). In these cases, the females did not attend the clutch and the

eggs failed to develop, suggesting that they had not been fertilized. I injected each female once every 2 weeks until she oviposited, for up to 6 weeks (3 injections). Once a female was observed guarding a clutch of eggs, she was left undisturbed for approximately 4 weeks. After ensuring that the eggs contained darkly pigmented embryos (approximately stage 26, Marks 1995), the entire clutch, as well as a small sample of tail-tip from each parent, was frozen to -80°C.

Parentage analysis using microsatellite markers

Prior to molecular analysis, I dissected each egg to remove all surrounding membranes and yolk from the embryo. Whole embryos were then ground using an epi-pestle in a solution of 10X STE buffer, 10% SDS and proteinase-K. Tissues were incubated at 56°C for 4 hr, followed by standard phenol-chloroform extraction and ethanol precipitation (Sambrook et al. 1989). Following PCR at three unlinked microsatellite loci (see Ch. 2), amplified fragments were run on an ABI 3100 automated sequencer (Applied Biosystems) and scored using Genescan$_{tm}$ fragment analysis software. Using the simulation program GERUDsim1.0 (Jones 2001), I determined that the exclusion probabilities for the three loci were above 98%. Thus, there was never any ambiguity as to the parentage of offspring. Furthermore, I did not encounter novel alleles not found in the sires used in the experiment.

Sperm counts

In a separate laboratory study (Ch. 4), 24 males were allowed to court with a mated female and with an unmated female. Just after spermatophore deposition

during each courtship trial, I collected the sperm mass before the female could lodge it in her cloaca. Sperm were dyed with ethidium bromide (EtBr binds to nucleic acids) and, using a fluorescence microscope, I counted the number of sperm in each sperm mass (see Ch. 4 for details).

Statistical analyses

I used the procedure LOGISTIC in SAS v. 8.1 (SAS Institute Inc. 2000), which performs logistic regression analysis, to test for associations between male body size, insemination success, and paternity (P_2 or the number of offspring sired by the second male). I used a Chi-square test of independence to determine whether insemination frequency (once or twice) was statistically independent of oviposition success (whether a female oviposited or not). In the study of sperm counts, I used a paired *t*-test to test for differences between mean sperm number per sperm mass deposited for mated versus unmated females. Linear regression was used to determine whether there was a significant effect of male size on sperm number per sperm mass.

RESULTS

Body size and insemination success

At the end of the courtship trials, 27 females were inseminated twice, 20 females were inseminated 1 time, and 3 females failed to become inseminated by either male.

Using logistic regression, I determined that, during both the first and second courtship encounters, large males had a significantly higher probability of inseminating a female than did small males (first encounter: $p= 0.0008$, second encounter: $p=0.0002$). During the first encounter, males that successfully inseminated the female had on average a 43.6 ± 0.94 mm SVL (mean ± SE). Males that did not successfully inseminate the female had a mean of 34.9 ± 0.81 mm SVL. In the second encounter, males that successfully inseminated the female had on average a 47.7 ± 0.97 mm SVL, and males that did not had a mean of 38.8 ± 0.99 mm SVL. Thus, for every millimeter increase in male SVL, the odds of inseminating a female during the first encounter increased 1.56 times. In the second encounter, a 1mm increase in SVL increased the odds of insemination 1.39 times. To ensure that small males that failed to inseminate females did not unduly influence the results, I removed from the data set all males smaller than the smallest male to achieve an insemination and re-ran the analysis. I chose conservatively to remove 8 males <34.9mm from the first encounter, despite the fact that, of the 8 males excluded, 1 male of 31.0mm had successfully inseminated a female during the first encounter (9 males <36.9mm were removed from the second encounter). The results were still highly significant ($p=0.0106$ for the first encounter and $p=0.0036$ for the second encounter).

Effects of insemination frequency and male body size on oviposition success

Of the 27 females that were inseminated twice, 14 (52%) oviposited a full clutch of eggs and raised the eggs until the embryos were large and nearly ready to

hatch (approximately stage 26, Marks 1995). In contrast, only 5 of the 20 females (25%) that were inseminated a single time oviposited a full clutch of eggs and raised them until just prior to hatching. Despite this apparently large difference in oviposition success (52% vs 25%), insemination frequency was statistically independent of the probability of ovipositing (χ^2= 3.44, n= 47, p= 0.064).

Effects of insemination order and body size on offspring paternity

The first male to inseminate a female sired the majority of the offspring in 12 of the 14 clutches (Figure 3.1). Using logistic regression analysis, I determined that the P_2 values deviated significantly from 50% (H_0= equal paternity among males; $p < 0.001$). Among the 12 clutches exhibiting first-male sperm precedence, the second male to inseminate a female (P_2) sired an average of 11.9% ± 4.1 (mean ± SE) of the offspring. In the two clutches (Numbers 13 and 14) having second-male sperm precedence, the P_2 average was 67.1% ± 2.9 (mean ± SE).

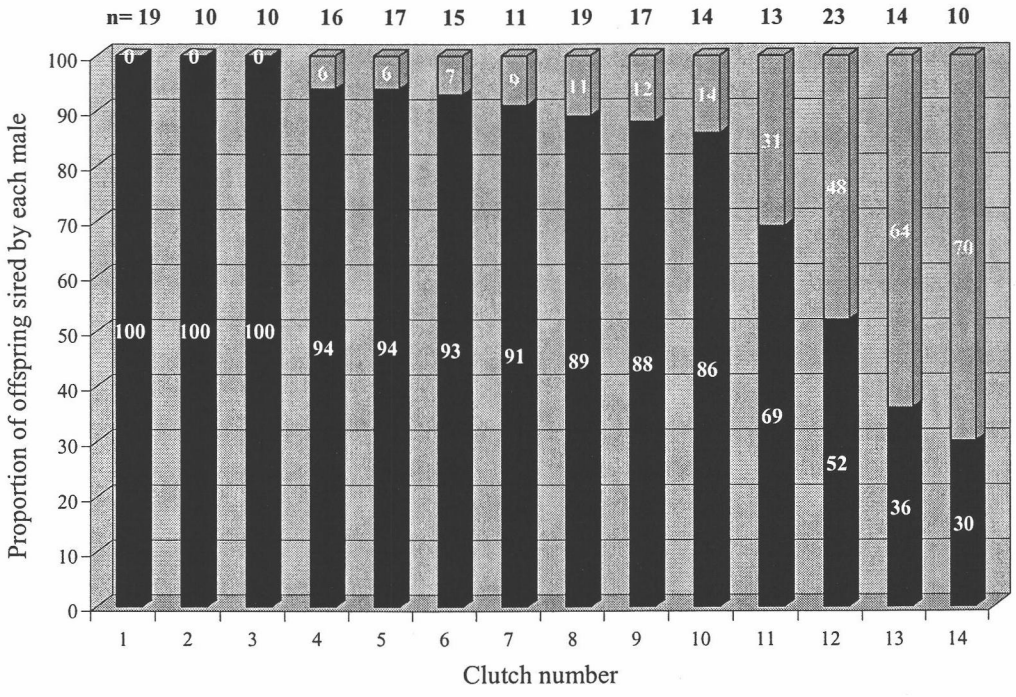

Figure 3.1 Proportion of offspring sired by each of two males to inseminate a female. Dark bars indicate the proportion of offspring sired by the first male to inseminate a female and light bars indicate the proportion of offspring sired by the second male to inseminate a female. Numbers above each bar indicate the number of eggs in each clutch. In all but two clutches (13 and 14), the first male to inseminate a female sired the majority of the offspring.

Offspring paternity varied with male size. There was a highly significant ($p < 0.001$) negative relationship between size of the first male to inseminate a female and P_2. Thus, the larger the first male to inseminate a female, the fewer offspring were sired by the second male. The size of the second male had a marginally insignificant effect ($p=0.056$) on P_2. As the size of the second male increased, there was a trend for the number of offspring he sired to increase as well (Figure 3.2). There was no effect of female size ($p= 0.8$), the number of days between the first and second insemination (13 to 27 days; $p= 0.21$), and the number of courtship encounters prior to insemination ($p= 0.24$).

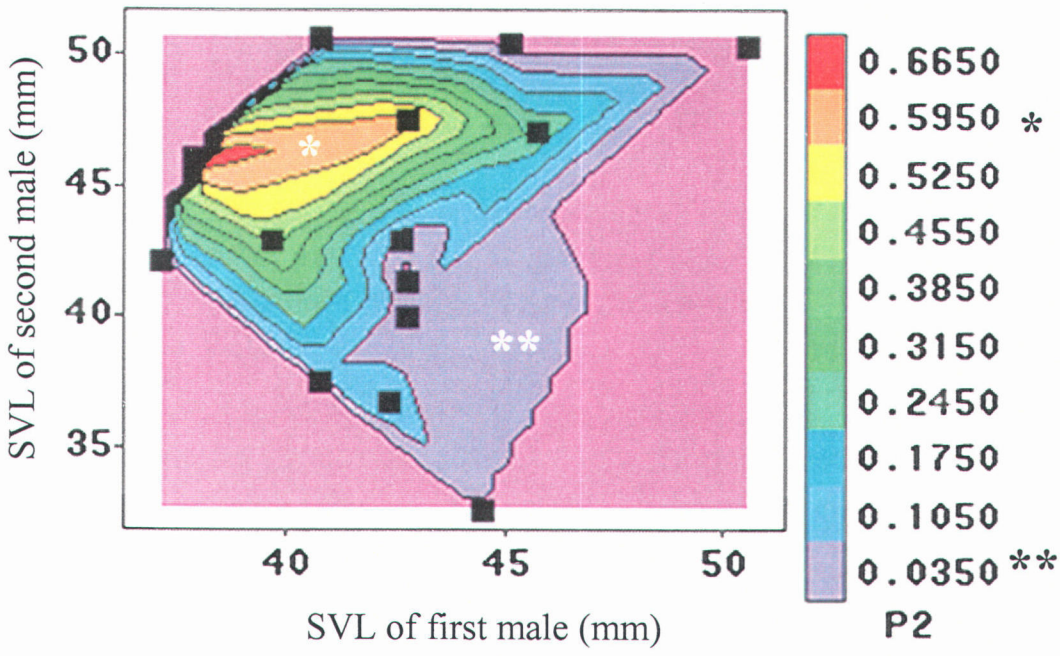

Figure 3.2 Proportion of offspring sired by the 2nd male (P_2) as a function of the snout-to-vent length (SVL) of the first and second males to inseminate a female. Colors in the contour plot denote different proportions of P_2, which are defined in the legend to the right of the plot (red represents the highest point in the plot and purple the lowest). Stars indicate regions with the same shading in both the plot and the legend. The black squares represent each of the 14 clutches included in the analysis. P_2 decreased significantly as the SVL of the first male to inseminate the female increased ($p< 0.001$). However, there was a marginally insignificant effect of the SVL of the second male on P_2 ($p= 0.056$).

Effects of male body size on sperm number

There was a significant effect of male body size on sperm number, but only when males were paired with unmated females (separate linear regression analyses of unmated females and mated females had p-values of 0.02 and 0.6, respectively). The effect of male size on sperm number for unmated females was significant even when the smallest male was removed from the analysis ($p= 0.04$). The paired *t*-test comparing the number of sperm in the sperm masses provided by the same male to

mated and unmated females was highly significant (t_{crit}= 2.78, df= 4, p= 0.007), with unmated females on average receiving 256,697 sperm per sperm mass and mated females receiving only 171,249 sperm. However, these results should be interpreted with caution because the majority of the paired counts (4 of 5) were from large males (two counts from unmated females had to be excluded because they were not paired). Visual inspection of the data suggests that, for small males, the opposite trend could be occurring, such that small males may provide less sperm to unmated females than to mated females.

DISCUSSION

Our results clearly show that large male *D. ocoee* experience greater reproductive success than do small males at almost every stage of reproduction, from insemination to siring offspring. There were positive correlations between male size and: (a) insemination success, (b) sperm number per spermatophore, and (c) fertilization success. Thus, it appears that selection in *D. ocoee* favors the evolution of large male body size as a result of increased reproductive success both prior to insemination and after. Although body size allometry is probably a factor contributing to the observed correlation between male body size and sperm number (assuming larger testes produce more sperm), it is likely that selection helps maintain the correlation. In *D. ocoee*, individuals continue to grow throughout their lives, and older/larger males are known have more testes lobes than younger/smaller males. However, selection on males to

increase their reproductive success clearly favors large males throughout reproduction. I found no evidence that small males were able to compensate for decreased insemination success with increased fertilization success (or vice-versa), thereby achieving equivalent reproductive success as larger males. Thus, our data suggest that selection favors large body size in *D. ocoee*. Large males have greater success inseminating females and producing spermatophores with more sperm, and also are more successful in siring offspring than are smaller males.

The effect of male size on insemination success did not arise because smaller males were not reproductively mature or sexually experienced. Even when the smallest males that did not successfully inseminate females were removed from the analysis, the results were still highly significant. Only 10 of the 100 males were smaller than 34mm. Of these, one male (31.0mm) successfully inseminated a female. Thus, the size range of males used in this experiment represented a realistic range of reproductively active males in the field and the observed effect of male size on insemination success is, in fact, real.

The observed effect of male size on insemination success is in contrast to the findings of other studies of courtship in *D. ocoee* (Houck 1988, Houck and Francillon-Vieillot 1988, Verrell 1991a) which revealed no association between male body size and insemination success. All three studies included a size range of males (36.9-58.6mm, 46.2-57.7mm and 31-52mm SVL, respectively) similar to that in the present study, but used different experimental protocols. In those studies, male-female pairs were allowed to court overnight (for over 12 hours) and the authors did not observe the courtship interactions. In contrast, I observed pairs throughout the 5 hr period of

time in which the animals were together (2000-0100hr). The observation period included the period of time when Ocoee salamanders in the field have been observed to be most active (10pm-12am, Houck pers. com.). Furthermore, during the courtship trials, I found that most courtship interactions took place within approximately 2 hr of putting the animals together. For all trials, 5 hr of observation exceeded the period of time in which pairs were active and attempting courtship (initiated by either the male or female). Thus, the period of time in which I conducted the courtship trials may reflect a more realistic outcome of courtship than the studies by Houck 1988, Houck and Francillon-Vieillot 1988, and Verrell 1991a. By virtue of being kept together overnight in these studies, pairs may have re-initiated courtship in the early morning hours and females may have been inseminated by small males that were initially rejected. Also, this extended courtship period is highly unlikely in the field, given that pairs are free to move apart after a courtship interaction; this separation may reduce their risk of predation while exposed during courtship, and maximize the amount of time each forages for food.

The observation that first-male sperm precedence operates in *D. ocoee* is in accordance with a study by Houck et al. (1985a). They used allozymes to determine the number of offspring sired by the last male to inseminate a female, and concluded that either first-male sperm precedence or mixed sperm precedence was operating in *D. ocoee*. However, my results conflict with predictions made from histological studies of sperm storage (Sever and Hamlett 1998) in which the authors observed that sperm found in the distal portions of the spermathecal tubules (female sperm-storage organ) were embedded in the epithelium and appeared to be undergoing

spermiophagy. From this observation, they predicted that last-male sperm precedence was likely in *D. ocoee*. Clearly, this is not the case. Given first-male sperm precedence and the fact that sperm may be stored for up to nine months during the breeding season, an interesting line of future study may be to determine what factors keep sperm viable and in sufficient quantity to fertilize the majority of ova in a female's clutch.

In addition to the effects of sperm precedence, variation in sperm number per spermatophore may explain a great deal of variation in male fertilization success. As the size of the first male to inseminate a female increased, the number of offspring sired by the second male decreased. However, there was only a marginal effect of size of the second male on P_2. I found that the number of sperm per spermatophore provided to unmated females (i.e., by the first male to inseminate a female) increased as a function of male size, but that sperm number did not vary significantly among differently sized males paired with mated females (i.e., the group of second males to inseminate a female). Thus, patterns of sperm allocation (by differently sized males) to mated and unmated females explain the observed patterns of offspring paternity. Potentially, small males may hold back when allocating sperm to unmated females, particularly as the effects of first-male sperm precedence already result in high fertilization success. On the other extreme, a large male may be able to allocate extra sperm to an unmated female, potentially as a defense against sperm competition from males that later may inseminate the same females. The lack of an effect of male size seen both in sperm number and P_2, suggests that males may simply allocate a set amount of sperm (approximately 200,000 sperm) to mated females, so that at least a

few ova may be fertilized. Given first male sperm precedence, a greater increase in sperm number may be too costly for the second male, as relatively few ova are available for fertilization.

The fact that small males provide less sperm per spermatophore than do large males could have consequences for female reproductive success if large numbers of sperm are required for optimal fertilization success. Although *D. ocoee* females that were inseminated by two males appeared to have a greater probability of ovipositing than did females that were only inseminated by one male (52% vs. 25%), this result was not statistically significant. There was a trend ($p=0.064$) toward increased oviposition success for females that had been inseminated twice. Also, neither male size nor female size had a significant effect on oviposition. These results, however, should be interpreted with caution. Our experimental design did not include insemination frequency (once or twice) as an *a priori* treatment. In essence, females chose whether to become inseminated once or twice. Our results relating the effects of male size on insemination success suggest that females that mated only once may have done so because they were offered only one male of acceptable size (>41mm). Nevertheless, oviposition success is confounded with female mating propensity and female condition. Females with a low probability of ovipositing may have chosen to become inseminated by only one male. Therefore, our results suggest only the possibility that interactions occurring during courtship, (e.g., pheromone delivery or number of sperm transferred to the female) may affect a female's decision to oviposit.

Summary

In conclusion, large male body size apparently is correlated with overall increased reproductive success for males. Larger males had increased insemination success and had a greater probability of siring offspring. Furthermore, large males allocated more sperm to each spermatophore, particularly when paired with an unmated female. The positive correlation among male traits important both prior to and after insemination suggest that sexual selection is acting on *D. ocoee* males in a manner that promotes large body size. It appears from patterns of sperm precedence, sperm allocation, and offspring paternity, that small males may be able to take advantage of first-male sperm precedence to allocate fewer sperm to individual females (ones not previously inseminated), and still sire the majority of offspring in a clutch. Larger males that potentially have larger sperm reserves may be able to increase the number of offspring sired and defend against sperm competition by allocating large amounts of sperm to unmated females.

CHAPTER 4

EFFECTS OF MALE BODY SIZE ON SPERM NUMBER IN THE OCOEE SALAMANDER (*DESMOGNATHUS OCOEE*)

Erika M. Adams and Lynne D. Houck

ABSTRACT

As in many species, female plethodontid salamanders (*Desmognathus ocoee*) mate multiple times and can store sperm. Male *D. ocoee* are likely to experience sperm competition if a female mate is inseminated a second time. If so, a male presumably would benefit by adjusting the amount of sperm that is delivered to a female, depending on whether that female had been previously inseminated. We hypothesize that another factor affecting the amount of sperm delivered to a female is the potentially greater sperm production capacity of large males. We tested whether a male could vary the amount of sperm transferred to a female by staging and observing courtship encounters between male-female pairs. Males ranged in size from 34.9 to 56.0 mm in snout-vent length (SVL). Females either had been inseminated once, immediately prior to our experiment (termed mated females), or had not been inseminated (unmated females). When a male deposited a spermatophore during a staged courtship encounter, the sperm mass was collected (before the female could

become inseminated) and sperm were counted. Three results from these courtship trials are: (1) Male body size was positively correlated with sperm number per sperm mass, but only when a male was paired with an unmated female; (2) For a male of any size, sperm number was greater when that male was paired with an unmated female, rather than a mated female; and (3) The duration of courtship was increased by 50% when a male was paired with a mated female vs. an unmated female. We conclude that male *D. ocoee* can assess whether a female has been inseminated previously, and can adjust the delivery of sperm accordingly.

INTRODUCTION

In many animals, the number of sperm a male provides to a female upon mating has a direct effect on the number of offspring that male will sire (insects: Simmons and Achmann 2000, Gage and Morrow 2003; birds: Birkhead 1998; mammals: Gomendio et al. 1998). Male competition for fertilization success can be particularly intense if females mate with multiple males and then store sperm prior to fertilization and ovulation (Parker 1970). In these cases, the differential use of sperm to fertilize ova, termed sperm competition, may have a significant effect on the reproductive success of males that have inseminated the female. Among the variety of animals in which sperm competition can occur, there is a wide range of sperm competition outcomes: only the first mate sires offspring, all or most mates sire at least some offspring, or only the last mate sires offspring (Simmons and Siva-Jothy 1998).

The nature of sperm competition for a particular species presumably may affect a male's strategy of allocating sperm to different mates. One theory of sperm allocation predicts that males should provide more sperm to females when the risk of sperm competition is high (Parker 1998). The rationale here is that male fertilization success will be raised by contributing more sperm to the female, thus increasing the male's own proportion of sperm within the female's sperm-storage organ (spermatheca). This rationale assumes that sperm are mixed freely within the female's spermatheca, and that fertilization success for a particular sire is directly proportional to the relative amount of his sperm. Another assumption implicit in this theory is that males are capable of discerning their risks, particularly in terms of whether a female has been previously inseminated. The reproductive consequences of contributing large amounts of sperm simply to "top off" the female (cf Jones et al. 2002) may provide strong selection on males to be able to assess female insemination status. Sperm allocation by males also may be influenced by male body size, which often has been found to be correlated with sperm number per ejaculate (Møller 1988, 1989). Male body size thus may be a major co-factor affecting sperm number per insemination.

In terms of sperm competition, certain plethodontid salamanders are opportune study organisms because the females mate multiple times (see Ch. 1) and then store sperm for weeks or even months prior to fertilization and oviposition (Houck 1986). The lengthy period of courtship and mating that occurs after the first insemination presents an opportunity for intense sperm competition. In particular, the Ocoee salamander (*Desmognathus ocoee*) has a prolonged period of courtship and mating

(Tilley 1977): fall mating typically is interrupted by retreating underground during the winter, but mating is resumed in spring and early summer (Organ 1961). Females oviposit in late July or early August, and use stored sperm to fertilize the ova just prior to oviposition. The male spermatogenic cycle is typical of that known for other North Temperate amphibians (Houck and Woodley 1995): the process of spermatogenesis is initiated in early spring, culminating in the production of mature spermatozoa in late summer or early fall. For salamanders, the end of the spermatogenic cycle is characterized by spermiation, the process of transferring mature sperm from the testes to the vasa (where sperm are stored until mating). The significance of the Ocoee reproductive cycle is that a male produces his entire yearly complement of mature spermatozoa at the beginning of the fall breeding season. The availability of sperm, therefore, potentially is a limited resource. A male must carefully allocate sperm to each sperm mass in order to be able to take advantage of mating opportunities that may arise during the fall mating period, in addition to those that occur in spring or early summer. We also note that, once a sperm mass is formed, additional sperm cannot be added. This prevents the duration of courtship from being coupled with the amount of sperm transferred to the female (as is found in many insect species, Simmons and Siva-Jothy 1998).

Sperm allocation in Ocoee salamanders occurs when sperm are released from the vasa and join with other glandular secretions to form a sperm mass. The sperm mass is part of the male's spermatophore, which also includes of a gelatinous base (which elevates the apical sperm mass) that is formed by secretions from additional cloacal glands. The sperm transfer phase of courtship in Ocoee salamanders is

characterized by the following events (Arnold 1972, Houck and Arnold 2003): (a) the female closely follows after the male, (b) the male deposits a spermatophore on the substrate in front of the female, (c) the female then moves forward to position her cloaca over the spermatophore, and (d) the female lowers her body and lodges the sperm mass in her cloaca. When the sperm mass is in the female's cloaca, sperm emerge from the mass and migrate dorsally into the opening of the female's spermatheca.

The nature of sperm allocation (number of sperm per sperm mass) in Ocoee males may be strongly influenced by male size. Smaller males appear to produce proportionately smaller spermatophores, and thus smaller sperm masses. Spermatophore size may simply be a function of the fact glands within a small male's cloaca are proportionally smaller than are comparable secretory glands within the cloaca of a larger male. In addition, testes size increases proportionally with male age and size (Houck and Francillon-Vieillot 1988), suggesting that absolutely more sperm can be produced by larger males. Thus, as small males simply may not be able to produce as many sperm per year as do larger males, there may be increased pressure on small males to allocate sperm efficiently during the prolonged mating season.

Patterns of sperm allocation also may reflect the recent finding that paternity in the majority of Ocoee clutches reveals strong first-male sperm precedence (Ch. 3). Given this finding, males might be able to detect whether a female has mated previously. If so, sperm allocation patterns should vary depending on a female's recent insemination status and the corresponding risk of sperm competition

In the experiments described below, we address the following questions:

(1) Does sperm number (per sperm mass) covary positively with male size? We predict that these variables will have a positive covariance, based on the observation that the physical size of a sperm mass appears to be correlated with male size.
(2) Does sperm number vary if a male courts a mated (inseminated) female vs a previously unmated female? That is, does a male vary the number of sperm in his sperm mass if he will be the second (or third) male to inseminate a female? Our null hypothesis is that the number of sperm is unrelated to female insemination status, and that an individual male produces a similar number of sperm per spermatophore (although sperm number may vary among males).

In order to address these questions, we designed an experiment in which we determined the effects of female reproductive status (mated vs. unmated) and male size on the number of sperm contained in a sperm mass.

METHODS

Selection of females

Forty-eight gravid female *D. ocoee* were collected from Deep Gap, NC between 1999 and 2001 for experimental use in 2003. We selected the females based on the fact that each had oviposited a full clutch of eggs during the summer of 2001 and none had been allowed access to males for two years after ovipositing. Thus, our experimental group of unmated females did not have viable stored sperm. In contrast, for our experimental group of mated females, each had stored sperm from only a

single insemination (which occurred as a result of a courtship encounter that was staged in our laboratory just prior to this experiment). In addition to controlling for prior insemination, we also controlled for prior occurrence of oviposition. Although it is possible for a female to produce a clutch of eggs every year, females in the field probably are food limited and oviposit on a biennial cycle (Organ 1961, Tilley 1977). Consistent with oviposition on a biennial cycle, examination of our experimental females in October 2002 revealed that none had large, yolked ova ready for oviposition that year. Thus, the females used in this experiment were not artificially forced to forgo oviposition in 2002.

In mid-June 2003, two weeks prior to beginning the experiment, we weighed each female using a digital balance, measured the distance from the tip of the snout to the posterior edge of the vent (snout-to-vent length, SVL) using digital calipers, and counted the number of large, yolked ova in each female's paired ovaries. Ova were easily visible through the ventral body wall and could be counted by holding the female up to a fiber-optic light and gently compressing the female's abdomen. Females were ranked in ascending order according to body mass and assigned in alternating order to either the mated or unmated experimental treatments. In this way, we minimized the possibility of confounding female mass (which is correlated with fecundity) with treatment effects.

Mated and unmated female treatments

Each of the 24 females assigned to the mated treatment was paired with one male (total number of males = 24) during a staged courtship encounter. Each of the

inseminating males was used only once and was not involved in later portions of the experiment. In order to minimize possible variance in treatment effects based on the size of the inseminating male (and, potentially, the number of sperm delivered to the female), we restricted the size of the inseminating males to 39-47mm SVL. This size range reflects the center third of the distribution in SVL of reproductively mature males (see Ch. 3). Each female assigned to the mated treatment was placed together with a male for 7 hr, after which time the female's cloaca was examined for the presence of a sperm mass. Sperm masses are clearly visible in the female's cloaca for up to 24 hr after insemination (Houck 1986). At the end of 7 hr, all but three of the 24 females had been inseminated. Each of these three females was left with the male overnight; by 0945hr the next morning, each of these females also had been inseminated a single time. Females in the unmated treatment were not allowed to interact with males at any time prior to the experiment.

Selection of experimental males

All males used in this experiment were collected from the same locality as were the females (Deep Gap, NC) during May 2002. We used digital calipers to measure the SVL of approximately 50 males. The reproductive maturity of each male was confirmed by transilluminating the animal using a fiber-optic light and noting the presence of darkly pigmented testes and vasa (cf Houck 1977a). Twenty-four males, ranging in size from 37.3mm to 56.0 mm SVL, were selected to reflect the natural range of adult male size for this experiment.

Assignment of animals to experimental treatments

Each of the 24 experimental males was paired with a mated female and with an unmated female during two courtship trials staged 10 days apart. Our study allowed for 10 days between courtship trials to ensure that we did not observe an effect of prior spermatophore deposition on the subsequent production of a typically sized spermatophore (cf Verrell 1991b). We collected two sperm masses from each male: one mass was obtained after spermatophore deposition when the male was paired with a mated (previously inseminated) female, and another mass when paired with an unmated female. For the courtship trials, each male-female pair was put together in a large, clear plastic box lined with moist paper towel and allowed to court. However, due to logistical constraints, only half (12) of the pairs could be observed reliably during a given courtship trial. As a result, we ranked males from smallest to largest SVL and then assigned the males in alternating order to two separate courtship trial dates. Thus, courtship trials were staged on four dates in 2003: 4 July, 6 July, 14 July and 16 July. The first encounter with a female for all the experimental males was on either 4 July or 6 July, and the second encounter was on either 14 July or 16 July. For each courtship trial (e.g., 4 July), males were ranked according to SVL and alternately assigned either a mated or unmated female. A female within the mated or unmated treatment was randomly selected and assigned to a male. If a male encountered a mated female on the first trial, he was randomly assigned a female from the unmated group for his second encounter (and vice versa). Each female (regardless of her treatment group) was used in only one experimental courtship encounter.

Protocol for courtship trials

All animals were maintained on a 14/10hr light/dark cycle. Several weeks prior to the start of the experiment, the daily photoperiod was shifted such that the lights were turned off at 1200hr PST (Pacific Standard Time) each day. Courtship trial observations began at approximately 1445 hr, when all pairs had been put together. The specific time when each male-female pair was put together was recorded. We observed the pairs throughout the courtship trial, which terminated only after the male had deposited a spermatophore. The time at which each male deposited a spermatophore was recorded. Therefore, the total time that a pair spent together prior to spermatophore deposition was known. In only one instance (on 16 July) did we take a pair apart without spermatophore deposition: after 2.5 hr of courtship, the male had been unable to pause long enough to deposit a spermatophore without the female moving away from him.

When a spermatophore was deposited during the staged encounters, we used forceps to gently push the female aside and then retrieved the sperm mass with the forceps. Each sperm mass was immediately submerged in 200μl of sterile water in a 1.5ml Eppendorf tube. The male and female were then returned to their respective maintenance boxes to ensure that no further spermatophores were deposited. After all sperm masses has been collected on a given trial night, we confirmed by visual inspection that none of the females has been inseminated without our knowledge, and that only one gelatinous base was present on the substrate of the courtship arena.

Eppendorf tubes containing sperm masses were placed at 4° C until processing occurred the following morning.

Preparation for counts of sperm number per spermatophore

At 1000 hr, we vortexed each tube containing a sperm mass for 60s. We then removed the sperm mass from the tube and placed it on a slide. Under a dissecting scope, we used forceps and a glass pipette containing a known volume of water to gently dissociate sperm from the central sperm mass. The dissociated sperm and water were transferred back to the original Eppendorf tube. This portion was reserved and stored separately at 4° C (= aliquot #1). The remaining undissociated sperm mass was transferred to a new Eppendorf tube containing a final concentration of 3.6mg/ml collagenase Type IV (Worthington Chemicals) and Ringer's solution to a final volume of 700µl. Tubes containing collagenase were incubated at 37° C for 1hr. Following incubation, each sample was vortexed for 60s until the sperm mass was completely dissociated (= aliquot #2). If any clumps remained after incubation with collagenase, these samples were briefly ground using an epi-pestle. We added 1.5µl of ethidium bromide (EtBr) to every 10µl of sperm solution for both aliquots #1 and #2. After 15 min, a 1µl sample from each aliquot was viewed under a fluorescence microscope and diluted with sterile water to a concentration that could be counted reliably. Using a calibrated P10 micropipette, we placed ten 1µl drops of each aliquot onto a single glass slide and allowed the drops to dry at room temperature (one slide per sperm mass). A random number was assigned to each slide that was not related in any way to either male size or female status to assure that the observer counting the sperm was blind to treatment category.

Protocol for sperm counts

We used a fluorescence microscope at a total magnification of 12.5X to take digital images of 10 drops per slide (5 drops from each aliquot). As drops were larger than the microscope's field of view, approximately 15-25 images were required to capture each drop. Adobe Photoshop 7.0 was used to join images into a composite image of the drop. Each drop was assigned a unique random number, such that all sperm counts were made completely blind (i.e., we did not know if subsequent drops came from the same slide or not, nor did we know what treatment the drop had come from). We averaged the number of sperm counted in each of the 5 drops obtained from a single sperm mass. We then determined the total number of sperm in the total volume of each aliquot and added aliquots #1 and #2 together to get an estimate of the number of sperm contained in each sperm mass. While counting the sperm in each drop, we occasionally encountered small clumps of sperm. We recorded their presence and estimated the number of sperm in each clump. The estimated values for these clumps were not added into our totals of the number of sperm in each sperm mass, but were noted in case we encountered any drops with counts that varied drastically from other drops on the slide.

The tendency for *D. ocoee* sperm to clump was the main challenge we faced when developing a protocol to count Ocoee sperm. Clumping prevented the use of other techniques for counting sperm, including the use of flow cytometry. Clumping, in combination with relatively long sperm tails (approx. 2 mm in length), also prevented the use of a Coulter counter, which operates on the interruption of electrical current as a cell passes through the chamber. There was no way to ensure that two or

more sperm wouldn't enter at the same time, and no way to correct for this error. Also, we could not use a hemocytometer because the Ocoee sperm were too large and got caught at the junction between the cover slide and the counting chamber (i.e., the sperm cells simply would not enter the counting chamber).

Statistical analysis

We used a paired *t*-test to test for differences between mean sperm number per sperm mass produced during encounters with mated versus unmated females. Multiple linear regression was used to determine whether there was a significant effect of male size on courtship time and on sperm number per sperm mass. Using the procedure GLM (general linear model) of SAS v. 8.1 (SAS Institute Inc. 2000), we assessed the repeatability of counts among the 5 drops per aliquot per slide (see Schaus and Sakaluk 2002).

RESULTS

Of the 47 courtship events that resulted in spermatophore deposition, we failed to recover 6 sperm masses: either the male or female's body came into contact with the sperm mass (the sperm mass binds almost instantly to salamander skin and it cannot be removed intact), or the female was inseminated within the 15 min period during which pairs were being put together (all 3 instances occurred on 14 July). Of the remaining 41 samples, only 14 were included in the statistical analyses due to time

constraints in analyzing recently available data. We selected 14 initial samples that represented the extremes in male body size in order to maximize the chance of observing size-related differences in the initial data. Additional samples will be incorporated and analyzed at a later date.

There was no effect of male body size on courtship duration for either mated or unmated females [linear regression of mated females ($p = 0.4$) and unmated females ($p = 0.6$); Figure 4.1]. However, the duration from when a pair was introduced into a courtship box to the deposition of a spermatophore was significantly longer when a male was paired with a previously mated female than with an unmated female ($n = 47$ courtship events; mean of 2 hr 19 min for mated females and 1 hr 9 min for unmated females; Table 4.1).

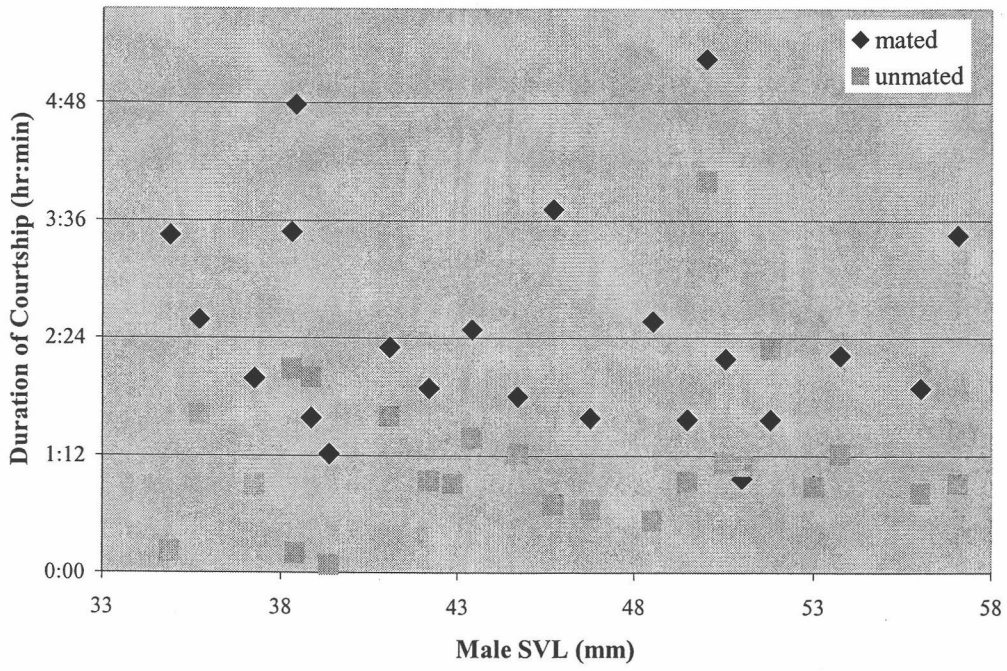

Figure 4.1. Duration of courtship as a function of male body size when paired with mated and unmated females. Neither regression was statistically significant (p-values for mated females and for unmated females were 0.4 and 0.6, respectively).

Table 4.1. Average duration of courtship (hr:min) for all four courtship trials. A male took approximately twice as long to deposit a spermatophore when paired with a mated female than with an unmated female. (*One pair was excluded from the analysis because spermatophore deposition did not occur within the 4hr observation period.)

	Unmated Female	n	Mated Female	n
4 July	1:29	6	3:33	6
6 July	1:07	6	2:06	6
14 July	1:12	6	2:06	6
16 July	0.49	6	1:33*	5

We did find a significant effect of male body size on sperm number, but only when a male was paired with an unmated female (linear regression of unmated females and mated females had p-values of 0.02 and 0.6, respectively; Figure 4.2). The positive correlation between male size and sperm number with unmated females was significant, even when the smallest male was removed from the analysis ($p = 0.04$). In addition, the paired *t*-test comparing the number of sperm in the sperm masses provided by the same male to mated and unmated females was highly significant ($t_{\alpha=0.05} = 2.78$, d.f. = 4, $p = 0.007$): unmated females received an average of 256,697 sperm per sperm mass, and mated females received an average of 171,249 sperm per sperm mass.

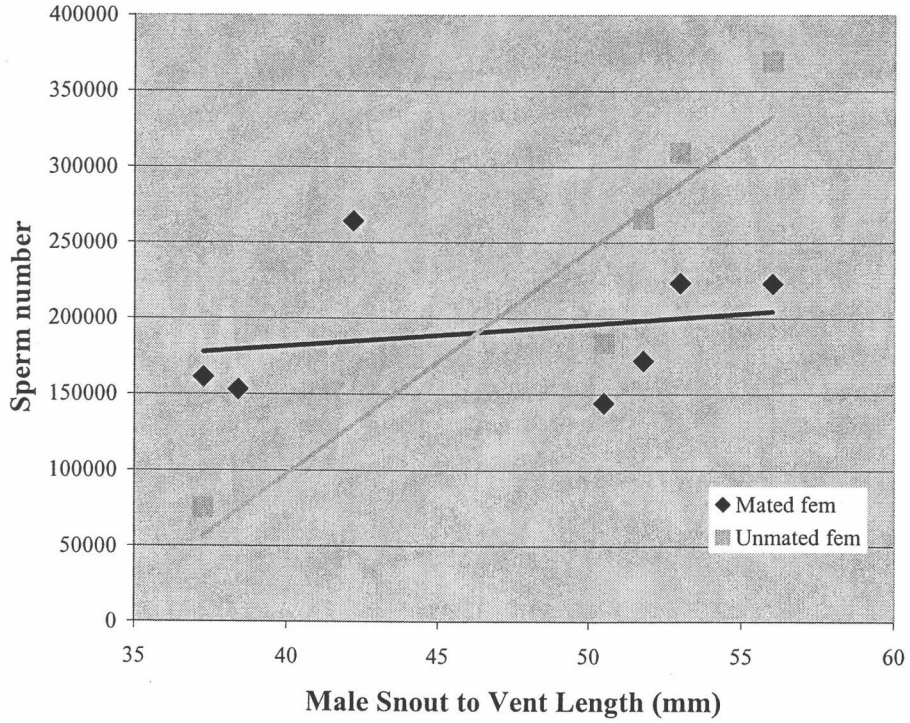

Figure 4.2. Sperm number per sperm mass as a function of male size (SVL). Sperm number increased as a function of male size for males paired with mated females ($p = 0.02$), but not for males paired with unmated females ($p = 0.6$). The effect of male size on sperm number with mated females was significant, even when the smallest male was removed from the analysis ($p = 0.04$).

DISCUSSION

This study revealed a surprising departure from theoretical expectations: male *D. ocoee* provided approximately 50% more sperm to an unmated female than to a female that previously had been inseminated. This result is contrary to competition models that indicate males should provide more sperm to females only when the risk of sperm competition is high (i.e., if the female was previously inseminated and has stored sperm; Parker 1998). This prediction is based on a "fair raffle" model in which each sperm cell has an equal probability of fertilizing ova (Parker 1998). The equal fertilization probability is clearly not the case in Ocoee salamanders as the first male to inseminate a female fertilizes a significantly larger proportion of the ova (first-male sperm precedence, see Ch. 3). For unmated female *D. ocoee*, the percentage of ova that are sired by the first male is substantial: 80% in field-collected clutches and 90% in laboratory clutches (Chs 2 and 3). We speculate that the larger number of sperm (approx. 250,000 sperm) provided to an unmated female may fill the majority of space within her spermatheca. We further speculate that sperm from a subsequent male may displace only a small portion of the first male's sperm. This modest sperm displacement by a subsequent sire would be consistent with other experimental results pertaining to *D. ocoee*: in a study of sperm competition, Houck et al. (1985a) found that the last male to inseminate a female would sire at least one offspring, even if that female already had been inseminated up to 11 times.

Male *D. ocoee* delivered significantly less sperm to females that had been recently inseminated, and therefore acted as though they were capable of discerning

the recent insemination history of a reproductively active female. An additional result bolsters the concept that male *D. ocoee* can discern cues that enable them to vary the number of sperm that are made available in a given sperm mass. Males of large body size produced significantly more sperm per mass than did small males, but only when the large male was paired with an unmated female. The mechanism for regulating sperm allocation to each sperm mass is unknown. Nor do we know the cues that the male uses to assess female insemination status. One possibility is that the male can detect specific female traits (e.g., change in body or cloacal odors) that are correlated with insemination. Another possibility is that female receptivity is greater in females that have not recently been inseminated. This possibility is supported by another major result: in staged courtship encounters, the time until spermatophore deposition was significantly greater if the female has been previously inseminated. The potential connection between female receptivity and insemination history easily could be tested by observing courtships staged between male-female pairs in which the females varied in their insemination status. Regardless of what cue the male is using, however, a male that can act to increase the amount of sperm delivered to an uninseminated female presumably would be more likely to sire the majority of offspring produced by that female (see Ch. 1). The significantly greater percentage of offspring sired by the first male to inseminate a female also debunks the concept of sperm mixing freely within the spermatheca (a theoretical possibility proposed by Parker 1998).

Another major result was that, for males courting an uninseminated female, larger males deposited spermatophores in which the sperm masses contained significantly more sperm than did the sperm masses produced by smaller males. This

is the first documentation for salamanders that male body size confers an advantage in terms of delivering more sperm to the female. That larger males are able to transfer more sperm per spermatophore suggests that, overall, these males have the advantage of greater amounts of sperm stored in their vasa. The large male advantage in sperm number, however, does not negate the advantage obtained should a small male be the first to inseminate a female (Ch. 3). Given the lengthy mating period experienced by *D. ocoee* salamanders, the question is whether small males are more likely to be limited by the amount of sperm that they produced at the start of the reproductive season. The question of sperm limitation could be addressed by examining the vasa of large and small males during mid-summer (when females are ovipositing, and before new spermatozoa have been transferred to a male's vasa). Given the relatively limited rate of encounters with a receptive female in the field (LH, personal observations), it is possible that all males –regardless of size– would have viable sperm remaining in their vasa at the mid-summer period. Whether or not sperm are limiting during a reproductive season, though, males clearly are allocating their sperm differentially in accordance with female insemination history.

A potential issue in our experimental protocol is that the treatment group of "unmated" females did not court prior to our experimentally staged courtship interactions designed to estimate sperm number per spermatophore. These females did not totally lack courtship experience, however, as all were collected after each had previously oviposited a clutch. Moreover, courtship behavior is highly stereotyped in these salamanders (Arnold 1972), and even a small amount of experience is sufficient for an individual to perform normally (Verrell 1991a, 1994). Therefore, we reject the

possibility that lack of recent courtship experience *per se* had any significant effect on the number of sperm that males allocated to these females in our experimentally staged courtships.

An unexamined area that is potentially important is understanding the nature of the male-produced matrix that serves as a vehicle for transferring the sperm from the male to the female. In species of *Drosophila*, males deliver toxins to the female, along with sperm (Wolfner 1997). We do not know of any chemical evaluation of the sperm mass matrix in plethodontid salamanders.

In conclusion, we have answered our initial two questions to show that the number of sperm transferred to a female varies positively with male size, and that sperm number is higher if a male courts a previously unmated female. Addressing these questions helps to begin to determine sources of variance in offspring paternity. Although male size has been suggested to affect male paternity though male-male competition for mates, we now know that male size also affects paternity in part due to variance in sperm number. In addition, we have demonstrated that males apparently can take advantage of knowledge of insemination order to increase the probability of paternity. Overall, determining all sources of variance in offspring paternity will help address the ultimate causes of female multiple mating, and will help determine male strategies during sperm competition that maximize reproductive success.

CHAPTER 5

REPRODUCTIVE STRATEGIES IN THE OCOEE SALAMANDER, *DESMOGNATHUS OCOEE*

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The research presented in this thesis addresses the ultimate causes of polyandry and the effects that female multiple mating has on male reproductive strategies. Initial results (Ch. 2) indicate that selection on clutch size does not explain the evolution of polyandry in *D. ocoee.* However, future studies addressing the effects of insemination frequency on female body condition, reproductive physiology, and offspring survival on reproductive success are warranted. Salamanders such as *D. ocoee* are an ideal system in which to investigate the ultimate causes of female polyandry, in large part because females are inseminated and store sperm throughout a long breeding season which culminates in the oviposition of a single clutch of eggs. Therefore, any effects of mating frequency and traits of the inseminating males can be ascribed to one clutch. This is in contrast to many birds and insects in which females mate multiply and produce several clutches throughout the reproductive season. Often it is difficult to ascribe the effects of particular matings to a single clutch, since sperm from one male may fertilize more than one clutch.

Chapters 3 and 4 address the consequences of polyandry to male reproductive strategies, both on the population level (Ch. 3) as well as to individual males (Chs 3

and 4). Results reveal that large males enjoy higher reproductive success than do smaller males. Large males have greater insemination success, sire more offspring relative to small males, and allocate more sperm to each spermatophore. These results are crucial to determine what reproductive strategies males use to increase their reproductive success. In the future, two additional issues in particular will need to be addressed. First, we must determine the exact relationship between insemination frequency and fertilization frequency. There is potentially conflicting evidence between data presented in this thesis (Ch. 3), which shows that not all males that inseminated a female sired offspring, and research by Houck et al. (1985a) in which the last male to inseminate a female always sired offspring. Studies are needed to determine the number of males having sperm within a female's spermatheca, both in the fall and spring. These data can then be compared with data presented here which show that most clutches are sired by either 2 or 3 males. This analysis will reveal not only the temporal dynamics of insemination in *D. ocoee*, but will address in greater depth how males partition insemination effort and sperm number per spermatophore based on female mating status (which males clearly can assess, Ch. 4).

The second fundamental issue that needs to be addressed in *D. ocoee*, is the nature of the physiological mechanisms of sperm storage and sperm use within the female reproductive tract. In particular, the possible role of epithelial cell secretions within the spermatheca should be addressed. To date, there have been no studies addressing the physiology of long-term sperm storage in *D. ocoee*. Sperm storage on such a long time scale is relatively rare among animals, and presents a compelling opportunity to determine novel cellular and physiological interactions between sperm

and the spermathecal epithelium during sperm storage. Furthermore, determining the mechanisms of sperm use has long been recognized as a fundamental step in determining how male reproductive strategies evolve (Birkhead and Møller 1998).

In conclusion, this thesis provides much needed data on the ultimate causes of polyandry and the effects of multiple mating affects male reproductive strategies in an animal system in which very little previously was known. Additional studies certainly can continue to elucidate the unique aspects of the reproductive biology of *D. ocoee*, thus contributing further to our understanding of female multiple mating.

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