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SEXUAL SELECTION IN NORTHERN WATER SNAKES, *NERODIA SIPEDON SIPEDON*: EXAMINATION OF THE MATING SYSTEM AND CORRELATES OF MALE REPRODUCTIVE SUCCESS USING MICROSATELLITE DNA MARKERS

By

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Abstract

Determining how sexual selection operates on males and females can lead to a better understanding of the evolution of specific traits that influence fitness. Of the few studies that have investigated sexual selection in reptiles, most have relied on estimates of mating success from field observations, without knowing whether these behavioural data reliably reflect true reproductive success. However, recently developed molecular tools allow researchers to more accurately assign parentage and thus, measure reproductive success. In this study, I isolated ten northern water snake (*Nerodia sipedon*) microsatellite loci which were then used to conduct genetic studies on wild populations of this species. My major findings include that: 1) There is fine-scale (< 2 km) genetic structure among three water snake populations. 2) Based on paternity assignments involving approximately 200 males and 800 offspring, more than half of the litters were multiply sired, which is lower than previous estimates for this species, and the incidence of multiple paternity was greater where females were more clumped and male competition for mates more intense. 3) Estimates of mating success based on field observations do not reliably predict genetically-determined measures of reproductive success. 4) Securing additional mates resulted in a marginal increase in reproductive success for females, but a large increase for males: the opportunity for sexual selection was more than 70 times greater for males than females. 5) Small male body size was not correlated with reproductive success and therefore, the evolution of female-biased sexual size dimorphism cannot be explained by a reproductive advantage of small males. However, males who were more "active" (*i.e.* more field observations) had higher reproductive success. The results of this study support the view that, to effectively study sexual selection in many reptile species, it is necessary to measure reproductive success using genetically-based techniques.

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Introduction

Sexual selection arises from differences in reproductive success caused by competition over mates (Darwin, 1871). Determining patterns of sexual selection operating on males and females can provide valuable insight into key evolutionary features of animal mating systems. This, in turn, can lead to a better understanding of the factors that influence the evolution of specific behaviours, strategies and / or morphological traits that affect reproductive success.

Females of most species invest more energy in offspring than do males. Therefore, female reproductive success is limited by the number of eggs that females can produce, whereas male reproductive success is restricted by the number of females that males can inseminate (Trivers, 1972). For this reason, male reproductive success is expected to increase upon fertilizing additional females (Bateman, 1948; Clutton-Brock, 1988). In contrast, if one copulation is sufficient to fertilize an entire batch of eggs, female reproductive success should not increase by mating with multiple males. Mating utilizes time and energy and may impose a high risk of predation in some species (Ross and Crews, 1977). Despite these apparent costs to mating, females of many species mate with successive males (*e.g.* Gladstone, 1979; Hanken and Sherman, 1981; Davies and Boersma, 1984; Gibbs *et al.*, 1990; Barry *et al.*, 1992; Murie, 1995; Ochando *et al.*, 1996; Lamunyon, 1997; Trexler *et al.*, 1997). Thus, researchers are interested in what benefits females may derive from multiple paternity that outweigh the costs (Schwagmeyer, 1984; Halliday and Arnold, 1987). For example, one possibility is that if females do not receive enough sperm from one mating to fertilize all of their eggs, then females who are inseminated by multiple males may benefit by producing larger litters (Gromko *et al.*, 1984).

Alternatively, multiple paternity may reduce the potentially deleterious effects of inbreeding. If females cannot discriminate between related and non-related individuals, they may occasionally mate with relatives, which may result in inbreeding depression (Partridge, 1983; Charlesworth and Charlesworth, 1987). However, by mating with multiple males, females may dilute the negative effects of mating with genetically similar partners. Madsen *et al.* (1992) found that multiple mating by females in an inbred population of the European adder, *Vipera berus*, resulted in the production of more viable offspring (*i.e.* fewer stillbirths).

Because male fitness increases by mating with more females, this should result in strong intersexual competition for access to mates (reviewed in Clutton-Brock, 1988). If certain males are at a competitive advantage over rivals, these males might father a large proportion of offspring born in a particular year, while many others may achieve no reproductive success. This will result in strong sexual selection favouring male traits that contribute to male success. In contrast, if female reproductive success does not increase with successive matings, intersexual competition for female access to mates will be less intense. For this reason, reproductive success is typically more uniform among females

(Bateman. 1948; Clutton-Brock. 1988). This results in a greater opportunity for sexual selection (Arnold and Wade. 1984) to act on males than females. However. to demonstrate that variance in male reproductive success exists is not sufficient to imply that sexual selection is operating on male traits. Rather. it must also be demonstrated that specific traits are correlated with this variation (Sutherland. 1985; Fincke. 1988; McVey. 1988).

While patterns of sexual selection have been well studied in a variety of taxa (reviewed in Clutton-Brock. 1988). this field has received little attention for reptiles (but see Madsen *et al.* 1993; Madsen and Shine. 1994). This is no doubt due to the difficulty in studying these animals in the field, since many behave cryptically and are often widely dispersed in their habitats. Furthermore. the few studies that have investigated sexual selection in reptiles have relied on measures of mating success based on field observations of mating attempts (*e.g.* Brown and Weatherhead. 1999; Madsen *et al.*. 1992. 1993; Olsson *et al.* 1994; Weatherhead *et al.*, 1995). However. little is known regarding whether these mating attempts ultimately result in the production of offspring. There are potential limitations to basing estimates of mating success on field observations of mating attempts including: 1) many matings may not be observed. 2) mating attempts do not necessarily result in the production of offspring and 3) patterns of sperm competition and precedence are unknown for many species (Westneat *et al.*. 1987).

Empirical evidence suggests that there may be a correlation between mating success and reproductive success in reptiles. For example. Abell (1997) assigned paternity to striped plateau lizard offspring (*Sceloporus virgatus*) to the male who 1) was seen closest to the mother in the field, and 2) had the greatest home range overlap with the mother. DNA fingerprinting revealed that both of these measures of mating success are reliable predictors of reproductive success. Similarly, Gullberg *et al.* (1997) estimated mating success according to field observations of mating and mate guarding in the sand lizard, *Lacerta agilis*. Where this information was not available, they assigned paternity according to home range overlap between males and females. Both of these estimates of mating success matched their measures of reproductive success based on DNA fingerprinting. However, to determine whether this is a general phenomenon in reptiles, more studies comparing field and molecular estimates of male success are required.

Hypervariable microsatellite DNA loci provide an excellent means of testing the reliability of field estimates of mating success. Because these loci are highly variable, they can detect paternity quite readily from a large panel of potential sires (Morin *et al.*, 1994; Craighead *et al.*, 1995; Primmer *et al.*, 1995). In addition, they are relatively inexpensive and can be used to generate genotypes from degraded or small amounts of DNA (Hughes and Queller, 1993; Dawson *et al.*, 1997). The goal of this thesis was to use recently developed microsatellite DNA markers to investigate various aspects of sexual selection in the northern water snake, *Nerodia sipedon sipedon*.

Northern water snakes are well suited for this type of study because they occur in relatively high densities in isolated populations. Thus, large numbers of individuals can be sampled and marked with relative ease. Since water snakes are fairly conspicuous while basking and mating, the behaviour of individuals during the mating season can be documented easily. In addition, females readily give birth in captivity, which allows a large number of offspring for which maternity is known to be collected (Barry *et al.* 1992: Weatherhead *et al.* 1999). Furthermore, since both male and female water snakes mate with multiple mates (Barry *et al.* 1992; Brown and Weatherhead, 1999), they are useful subjects for investigating whether male or female reproductive success increases upon mating with additional mates.

The first goal of Chapter One is to introduce and characterize ten pairs of microsatellite markers that I developed and used in parentage analyses (see Chapter Two) to estimate male and female reproductive success (Chapter Three). Since these markers are so variable, they are also useful for detecting small-scale microgeographic genetic structure among close populations. Other studies have revealed genetic differences among snake populations less than two kilometers apart in the eastern massasauga rattlesnake, *Sistrurus c. catenatus* (Gibbs *et al.*, 1997) and the black rat snake, *Elaphe o. obsoleta* (Prior *et al.*, 1997). Thus, the second goal of the first chapter is to determine whether three northern water snake populations within two kilometers of each other are genetically differentiated.

In Chapter Two, I present data which bear on methodological issues relevant to using microsatellite loci in parentage analyses. For example, I present the degree of genetic variability among male water snakes and calculate the power of the loci in excluding all males but the true sire. I also present evidence for a null allele at two loci. Null alleles contain mutations at the primer binding site which prevents the primer from binding. This results in no amplification of the product. Therefore, individuals possessing one null and one visible allele are often mis-scored as homozygotes. I discuss the potential danger of using loci with null alleles in paternity analysis by exclusion.

The main goal of the third chapter is to use the parentage assignments from Chapter Two to estimate male and female reproductive success. I classify the mating system in the traditional sense by estimating the mean number of mates of each sex (Emlen and Oring, 1977). In addition, I determine whether reproductive success increases with successive matings for either sex (Duvall *et al.*,1993), and the possible female benefits of multiple paternity are investigated. Barry *et al.*, (1992) found that 86% of all water snake litters were multiply sired. However, in their study, allozyme loci were used, which are typically less variable than DNA loci (*e.g.* microsatellites). As a result, for nearly one-third of their litters, they only had indirect evidence for multiple paternity based on examining for deviations in the offspring genotype frequencies from those expected by chance. I also consider the effect of ecological parameters (*i.e.* OSR, density, length of mating season) on annual variation in the incidence of multiple paternity. Finally, I estimate the degree of variation in male and female reproductive success and determine whether the opportunity for sexual selection (Arnold and Wade, 1984) differs between the sexes.

In the final chapter, I investigate whether any male morphological or behavioural traits are correlated with variation in male reproductive success. In particular, determining how sexual selection acts on male body size may provide insight into the evolution of female-biased sexual size dimorphism exhibited in nearly two-thirds of all snake species (Shine, 1978; 1993; Weatherhead *et al.*, 1995; Brown and Weatherhead, 1999). Although it is generally accepted that in many viviparous species, larger females possess a fecundity advantage (Brown and Weatherhead, 1997; but see Shine, 1988), this does not explain why males do not reach a similar size. In the absence of countervailing selection limiting male body size, genetic correlation among the sexes should result in males growing equally large (Lande, 1980). Shine (1978) proposed that smaller males may attain a greater reproductive advantage because they are more mobile. However, Weatherhead and Robertson (1991) demonstrated that swimming speed is positively correlated with body size in water snakes. In addition, male home range size is not influenced by body size in northern water snakes (Brown and Weatherhead, 1999) or European adders (Madsen *et al.*, 1993). Alternatively, since energy is limited in snakes, males who invest energy into sperm production rather than growth may be physiologically more "ready" for reproduction (Weatherhead *et al.*,1995). Thus, sexual selection may favour small male body size since smaller males are in better reproductive condition.

Because the tail appears to play an important role in stimulating the female to mate and displacing rival males (King, 1989, pers. obs.), I also determine whether tail length is correlated with male reproductive success. Next, I examine whether males in better condition (*i.e.* higher fat content) are more successful. Finally, I investigate whether female encounter rate affects sexual selection in water snakes both at the level of the population and the individual (see Emlen and Oring, 1977). I estimate male activity by 1) home range size and 2) number of times that males were seen in the field. In addition, I take advantage of natural variation in female distribution between my two study sites. Where females are more uniformly distributed, males with larger home ranges and those who are generally more active should encounter more females and thus attain higher reproductive success. In contrast, where females are more clumped, home range size is smaller (Brown and Weatherhead, 1999) because males can access several females without moving far. As a result, I predict that home range size should not influence male reproductive success where females are clumped.

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CHAPTER ONE

Microgeographic population genetic structure in the northern water snake, *Nerodia sipedon sipedon*, detected using microsatellite DNA loci

Introduction

Determining the population structure of a species can lead to a better understanding of important evolutionary aspects its biology, such as levels of inbreeding and the degree of dispersal. The recent advent of molecular tools has revolutionized this field, since population differentiation can more readily and accurately be measured at the genetic level. Allozyme loci were first employed to assess genetic structure in snake populations, but most of these studies found little or no differentiation among populations on a variety of scales (reviewed in Prior *et al.*, 1997). However, allozyme loci are typically less variable than the more recently developed nuclear DNA markers (*e.g.* microsatellites; Hughes and Queller, 1993), and the failure to detect variation among populations may be an artefact of the marker employed.

Indeed, there is increasing evidence from studies using DNA-based genetic markers that snakes show genetic structure on a microgeographic scale. For example, using seven randomly amplified polymorphic DNA (RAPD) loci, Prior *et al.* (1997) found some evidence of genetic structure between black rat snake (*Elaphe o. obsoleta*) populations less than 2 km apart. Similarly, based on six microsatellite loci, Gibbs *et al.* (1997) found evidence of fine-scale (<2 km apart) subpopulation genetic structure in eastern massasauga rattlesnakes (*Sistrurus c. catenatus*). However, more studies are required to assess whether this pattern of structure is a general phenomenon in snakes. With this goal in mind, in this chapter, I: 1) characterize eight microsatellite DNA loci cloned from a northern water snake (*Nerodia s. sipedon*) genomic library, and 2) use these loci to investigate fine-scale population genetic structure among populations within 2 km of each other. This species may be prone to local differentiation because populations often occur in isolated water bodies (*e.g.* beaver ponds), separated by extensive areas of terrestrial habitat. Water snakes spend nearly all of their time in or near water (Scribner and Weatherhead, 1995), so extensive terrestrial habitat might inhibit movement among populations, leading to local differentiation.

Methods

Population samples

In 1994, 1995 and 1996, I captured snakes by hand in three marshes within 2 km of each other at the Queen's University Biology Station, Chaffey's Lock, Ontario (45°37'N, 76°13'W). In terms of the spatial relationship between the populations, the straight-line distance between Barb's and Beaver Marsh is 1.0 km, Barb's and Lindsay Marsh are 1.1 km apart and Beaver and Lindsay Marsh are separated by 1.5 km. However, assuming that water snakes may not traverse extensive amounts of terrestrial habitat, it may be more appropriate to look at the shortest distance that follows as much water as possible. Considering this, Barb's and Beaver Marsh are 3.9 km apart, Beaver and Lindsay Marsh are 1.9 km apart and Barb's and Lindsay Marsh are 1.1 km apart (Fig. 1.1).

Brown and Weatherhead (1999) used Jolly-Seber analysis of mark-recapture data with each of eight years representing one capture period to estimate that Barb's Marsh supports approximately 55 adult males and 50 adult females, while the Beaver Marsh population consists of approximately 50 adult males and 35 adult females (Seber, 1982). I have insufficient mark-recapture data to estimate the population size of Lindsay Marsh. However, based on visual observations, I estimate that this population is approximately 3-4 times larger than the other two. Upon capture, snakes were permanently marked using an electronic passive integrated transponder (PIT tag) and sexed by probing the cloaca for the presence of hemipenes. A 100 µl blood sample was obtained from the caudal vein of each snake using a 0.5 ml insulin syringe with a 28 gauge needle and was stored in 800-1000 µl Queen's lysis buffer (Seutin *et al.*, 1991) at 4°C. DNA was extracted using either a standard phenol-chloroform extraction procedure (as described in Sambrook *et al.*, 1989) or by using DNAzol (GIBCO BRL) and following the manufacturer's protocol. DNA concentrations were determined using a Hoefer TKO 100 DNA Fluorometer.

Isolation and resolution of microsatellite loci

To isolate nuclear microsatellite DNA markers, I followed the protocols outlined by Dawson *et al.* (1997). Briefly, a library consisting of 300-400 bp fragments of genomic DNA from two females and three males from Barb's Marsh was screened simultaneously using three dinucleotide polymers (CA_n, GC_n, and CT_n [Pharmacia]). The positive clones were then sequenced and ten pairs of primers were made from the sequence region that flanked the microsatellite repeat. Approximately one year later, after the completion of the population genetics analysis (see below), a second library was screened and two more primer pairs (Nsμ110 and Nsμ119) were constructed to use in parentage analysis (see Chapter Two). To assay variation in these loci, PCR amplifications were conducted and PCR products were resolved on 6% polyacrylamide gels as per Dawson *et al.* (1997), except that I used 0.2 pmole of the forward end-labeled (^{33}P-ATP[Dupont]) primer, 0.3 pmole of unlabeled forward primer, 0.5 pmole of unlabeled reverse primer, 300uM dNTPs, 0.25U AmpliTaq, and 2.5mM $MgCl_2$ because these modifications reduced the cost of the amplification while still resulting in clean bands. Gels were then dried and exposed to Biomax (Kodak) X-ray film overnight. Products were sized by comparison to the amplification product of the known-size clone which was run on each gel adjacent to the sample products.

Analysis of variation

To determine the extent of microgeographic genetic structure among populations, I assayed variation in eight microsatellite loci in 30 males and 20 females from each of the three marshes. The difference in sample sizes for the two sexes is a result of my need for genotyping more males than females for parentage analyses (Chapter Two). I then estimated the levels of non-random association of alleles within each population by testing for locus-specific heterozygote deficiencies using the exact test (U) of Rousset and Raymond (1995) as implemented in GENEPOP (Raymond and Rousset. 1995). I determined whether the overall heterozygote deficiency for each population was significantly different from zero by pooling all locus-specific p-values (Sokal and Rohlf. 1981). I obtained an estimate of the magnitude of the heterozygote deficiencies by calculating an F_{IS}-estimator, f (Weir and Cockerham. 1984) separately for each locus using GENEPOP (Raymond and Rousset. 1995). and over all loci using FSTAT (Goudet. 1995). I used the sequential Bonferoni correction to adjust the p-value for all multiple tests (Rice, 1989). I also estimated the degree of population differentiation by using the Fisher's exact test for differences in allele frequencies across populations (Raymond and Rousset. 1995). The magnitude of differentiation was determined by calculating an F_{ST}-estimator, θ, (Weir and Cockerham. 1984) overall and for each pair of populations (Goudet. 1995) and overall and pairwise ρ. an R_{ST}-estimator, using R_{ST} CALC (Goodman. 1997). These values were tested for significance of departure from zero using bootstrap and permutation procedures described in Goudet (1995) and Goodman (1997).

Cross amplification with other snake species

To test the effectiveness of my loci in amplifying DNA from other snake species.

and thus, determine whether they might be useful for studies on other species involving population genetics or paternity assignment, low stringency (50°C annealing temperature) PCR reactions were conducted on one male and female from each of the following species: *Thamnophis s. sirtalis, T. s. sauritis, Storeria d. dekayi, S. o. occipitomaculata, Lampropeltis t. triangulus, Crotalis v. viridis, Coluber constrictor flaviventris, Elaphe vulpina gloydi* and *E. o. obsoleta.* Products were run on agarose gels and scored for the presence of a visible band of the expected size.

Results

Isolation and characterization of loci

The first screening involved approximately 6000 colonies and I sequenced all of the 48 positive clones obtained which contained single inserts. I designed primers for 10 of 26 clones that contained repeat sequences; however, only eight of these primer pairs successfully amplified scoreable products. Nearly a year later, approximately 9700 more colonies were screened. Twelve of the 59 positive clones were sequenced and primers were made for two of them (Nsμ110 and Nsμ119). Table 1.1 shows the primer sequences, repeat sequences, optimal annealing temperatures and the range of allele sizes detected in these populations for each locus.

Cross-amplification with other snake species

All eight (from the first screening) *N. s. sipedon* loci successfully amplified *T. s.*

sirtalis DNA. All but two loci (Nsμ6 and Nsμ10) resulted in a product using *T. s. sauritus* DNA. Three loci (Nsμ3, Nsμ7 and Nsμ8) successfully amplified DNA from *S. d. dekayi*. Although *C. v. viridis* is taxonomically distant from water snakes, two loci (Nsμ2 and Nsμ3) produced a band of the expected size. *Storeria o. occipitomaculata* and *C. constrictor flaviventris* DNA each exhibited amplification products at only one locus (Nsμ2 and Nsμ9, respectively). For all other species, no amplification product was seen. This suggests that at least some of these loci might be useful in population or parentage studies with other snake species.

Non-random association of alleles

I found a significant overall heterozygote deficiency in the Barb's Marsh population ($f = 0.053$; $p < 0.005$), which was due to a significant deficiency at Nsμ4 ($f = 0.273$, $p = 0.0003$) since no other loci showed evidence of such a deficiency. Neither the Beaver nor the Lindsay population deviated significantly from overall expected heterozygosity levels ($f = -0.009$, $f = 0.003$, respectively; both $p > 0.25$), although the Beaver population did show a deficiency at Nsμ4 ($f = 0.093$, $p = 0.003$) (Table 1.2).

Since the heterozygote deficiency was detected at only one locus, it is unlikely that it was caused by extensive inbreeding. Instead, null alleles could be present at this locus. Null alleles are caused by a mutation in the primer binding site of certain individuals (Callen *et al.*, 1993), which results in the absence of a visible product for the homologue harboring the mutation. Individuals with one null and one visible allele will be misscored as homozygotes, leading to an underestimation of heterozygosity levels. I have direct evidence for a single or multiple null alleles at two (Nsμ4 and Nsμ9) of my eight loci from a concurrent parentage study (see Chapter Two for details). For one of these loci (Nsμ9), I created new primers (Nsμ9b) that did not overlap with the original primer regions, and samples were re-genotyped using the new primer set. The new primer set constructed for Nsμ4 was not useful (see Chapter Two for reason).

Population differentiation

Since the null allele(s) at Nsμ4 is differentially distributed among the three populations (approximately 12% for Barb's Marsh, 4.2% for Beaver Marsh and 3.7% for Lindsay Marsh; estimates based on formula #4 in Brookfield, 1996), using this locus to measure population differentiation could bias my results. Therefore, I excluded this locus from all subsequent analyses. In addition, I chose to exclude Nsμ7 and Nsμ8 from further analyses since they may be insufficiently variable to detect small population differences due to random genetic drift.

There was no significant difference in allele frequency distribution between Barb's and Beaver populations overall ($p > 0.05$), or at any of the loci (all $p > 0.05$). However, there was an overall significant difference ($p < 0.001$) in allele distributions between Barb's and Lindsay populations and this difference was primarily attributable to the significant differences at two of the eight loci (Nsμ3, $p = 0.0005$; and Nsμ6, $p = 0.002$). There also was an overall significant difference between Beaver and Lindsay populations ($p < 0.001$) due primarily to significant allele frequency differences at Nsμ9 ($p = 0.001$).

Overall, θ was significantly different from zero ($\theta = 0.006$; $p < 0.009$). Significant θ values were also found between all pairs of populations (all $\theta = 0.006$: all $p = 0.02$). These results supported those of the allele frequency distribution analysis in suggesting weak differentiation among the three populations. In contrast, the overall ρ value was -0.003, revealing no differentiation among these populations ($p = 0.76$). Similarly, the pairwise population comparisons revealed no population differences (all $\rho < 0.006$, all $p > 0.33$) (Table 1.3). Because different measures of population differentiation gave varying results, this suggests that these populations are weakly differentiated. Water snake populations that are farther apart may show genetic structure consistently with any measure employed.

Discussion

I have isolated and genetically characterized ten microsatellite loci and have shown that they can be used to detect genetic differentiation among local populations of the northern water snake, *Nerodia s. sipedon*. The level of variation and genetic characteristics of the loci are similar to those of microsatellite loci isolated from other vertebrates (Paetkau and Strobeck, 1994; Primmer *et al.*, 1995; Villareal *et al.*, 1996; Dawson *et al.*, 1997; Gibbs *et al.*, 1998). These loci are a powerful tool for providing valuable insight into population genetics and sexual selection in water snakes, and possibly other snake species.

I found an overall significant deviation from expected levels of heterozygosity for the Barb's Marsh population, due to a significant heterozygote deficiency at one locus. Since the deviation from expected values was detected only at one locus, inbreeding is not likely the cause of the overall heterozygote deficiency.

The fact that different measures of differentiation (*e.g.* θ vs. ρ) gave variable results suggests that at this particular distance, population differentiation is weak. Nonetheless, these results add to the growing literature (Gibbs *et al.*, 1997; Prior *et al.*, 1997) which shows evidence for genetic differentiation on microgeographic scales among a taxonomically and ecologically diverse group of snake species.

In addition, ρ is based on stepwise models of microsatellite evolution (Goldstein *et al.*, 1995; Slatkin, 1995), which suggests that when mutations occur, they result in the gain or loss of one repeat unit (Valdes *et al.*, 1993). Thus, one would expect a continuum of allele sizes, one repeat unit in length apart. For three loci that consist of a "pure" repeat (*i.e.* not interrupted by single base pairs), two have large gaps (4-14 base pairs) that would be inconsistent with stepwise mutation (see Gibbs *et al.*, in press for similar findings). Thus, the more conventional infinite alleles model (*i.e.* every allele size has equal probability of being generated) better describes exceptions to stepwise evolution of microsatellite loci.

My results suggest that limited dispersal has contributed at least partially to the genetic structure of these populations. Therefore, I propose that dispersal may be costly to water snakes. Although it has been suggested that dispersal is beneficial to individuals in a population through reduction in competition for resources and inbreeding (Greenwood and Harvey, 1977; Pusey and Wolf, 1987), dispersal will be selected against if the cost outweighs the benefit of dispersing (Dingle, 1980). Dispersal may be limited in animals if suitable habitat is patchily distributed (Lidicker, 1962). If it is quite difficult for the species to find new suitable habitat, dispersal may be costly (Lidicker, 1962). Water snakes, who spend most of their time in or near the water (Scribner and Weatherhead, 1995) appear to live in a patchy environment, and may be less camouflaged, and therefore more at risk of predation, when away from the water. Additional information is required to test hypotheses for why limited dispersal may occur in these snakes.

Molecular markers, such as microsatellite loci, are becoming more widely employed for population and parentage studies since previously unanswered questions can be addressed more readily with genetics in addition to field work rather than by relying strictly on observational data alone. My use of microsatellite loci has revealed fine-scale population structure in water snake populations consistent with recent findings using other DNA-based markers in other snake species. More genetic studies on closely situated snake populations are required to determine whether such fine-scale population structure is a general phenomenon in snakes.

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Table 1.1- Primer sequences and genetic characteristics of ten *Nerodia sipedon sipedon* microsatellite DNA loci. F and R are the forward and reverse primer sequences, respectively, used to selectively amplify a particular locus. Tm is the annealing temperature used to assay variation in the locus. Size refers to the range of allele sizes detected in 150 individuals.

Locus #	Repeat motif	Primer sequences (5'-3')	Tm (°C)	Size
Nsμ2	$(AC)_{18}$	F- TCCTCTTTGGCAGAGTAATAGT	58	148-176
		R- AGCCGAGAACACACTAGTAAGT		
Nsμ3	$(ATCT)_{14}$ $AT(CA)_{20}$	F- CTGACTCACTTCTGACCCTAAT	58	158-206
		R- AATATTTGCTTGGCTCAAAC		
Nsμ4	$(GT)_{24}$	F- CATGTCTTTAATCCCTTTGACT	58	121-157
		R- TTATGAAGCAAACAGTGCTG		
Nsμ6	$(AC)_{21}(AT)_4(AC)_2GTAC(AT)_4$	F- GCTTCCATTGGTTAACACAT	55	104-204
		R- TTATATTTGTGCTTTTGCCTTA		

Table 1.1 - cont'd

Nsμ7	$(TG)_8TA(TG)_2$ $TA(TG)_3TC$	F- CTTCTCAAACATGACAGGAC	55	151-157
	$(TG)_2TA(TG)_3$ $TA(TG)_3$	R- AGTGAGGACTATCCATCTCC		
Nsμ8	$(GT)_6CT(GT)_3CTG$	F- TGGATTCAAGTTTTGGATTC	55	130-132
	$ATGCT(GT)_5CT(GT)_3$	R- ATAGGAAAAGGAATGAATTGC		
Nsμ9	$(AC)_3CC(AC)_{15}(AG)_4(AGAA)_2$	F- ATGCTACAAAGGGAACTTATTAG	58	143-181
	$(AG)_6AA(AG)_7A$	R- TGTGGGACTAGTAAGATGTATCA		
Nsμ10	$(TG)_{22}$	F- AGAATACAATCATGTGACCATG	50	124-152
		R- GACCGATCATTTAGAAATTGTT		
Nsμ9b	(replaces Nsμ 9)	F- AATGTGATTACATTTGTTTGG	55	200-248
		R- GATGAAGAATGTTCAAAACC		
Nsμ110	$(TC)_{28}CC(TC)_5$	F - GCA GAA GAT CTA ACC ATC AAC A	53	141-207
		R - CCC AAA ATC AAG TGA ATC AAG		
Nsμ119	$(TC)_{17}(TG)_{10}TT(TG)_6$	F - GCT AAA AGA GAT TGG GCA GA	54	140-266
		R - AGT GAA AAA AGT TTC ATG GTT TC		

Table 1.2 - Variation at eight *Nerodia sipedon sipedon* loci. The first column represents the number of alleles detected in 50 individuals from each of the three populations. Observed heterozygosities (H_{obs}) for each locus (proportion of heterozygotes), and expected heterozygosities (Nei and Roychoudbury, 1974) for each population are shown in the second and third columns. Results of exact tests of heterozygote deficiencies (Rousset and Raymond, 1995) are represented by the p values in the fourth column. The F_{IS}-estimator, f, (Weir and Cockerham, 1984) is in the final column.

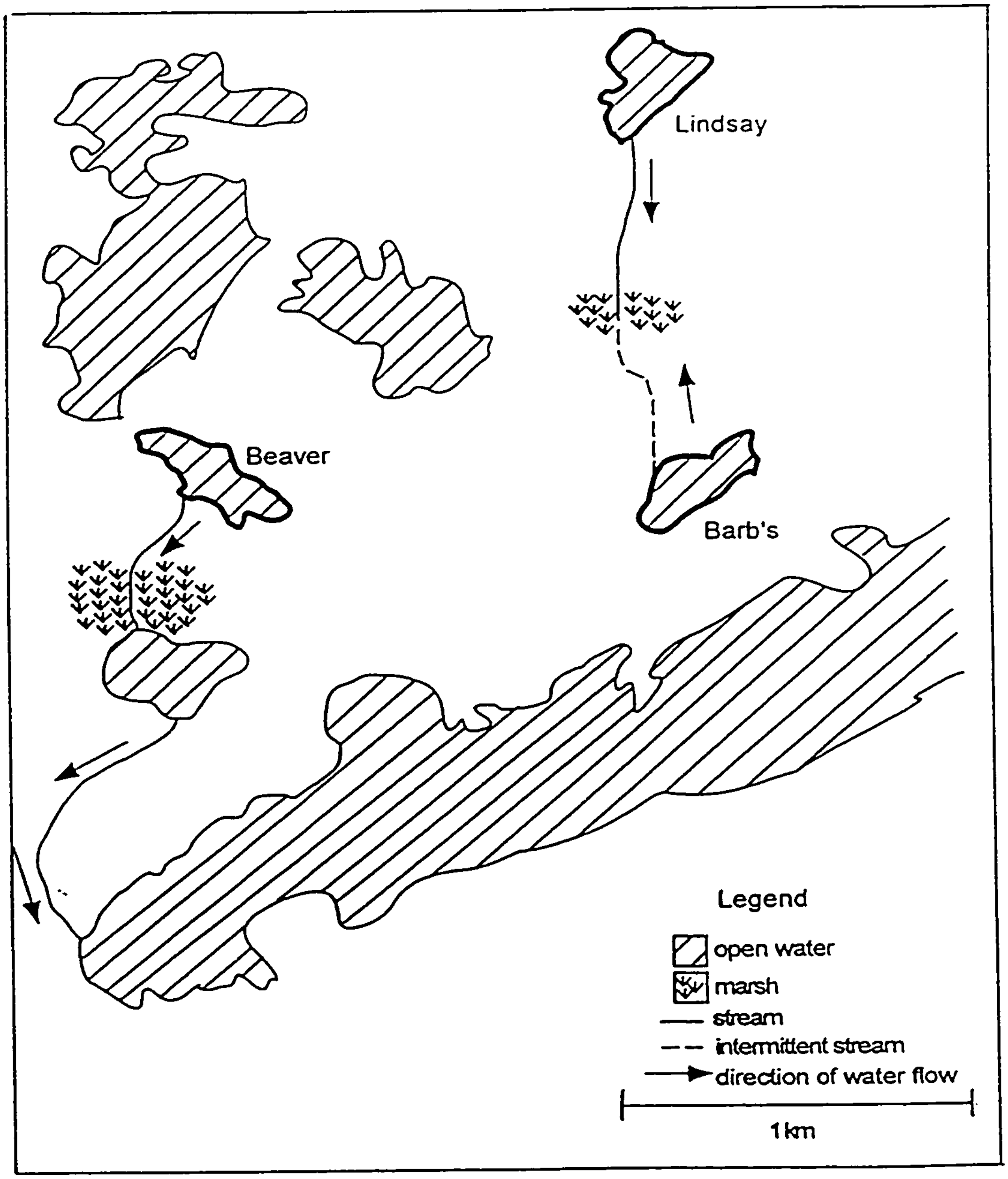
Locus	# alleles	H_{obs}	H_{exp}	p	f
Barbs					
Nsμ2	9	0.720	0.790	0.34	0.090
Nsμ3	18	0.880	0.907	0.52	0.030
Nsμ4	11	0.580	0.796	0.0003*	0.273
Nsμ6	9	0.780	0.738	0.74	-0.057
Nsμ7	2	0.580	0.471	0.98	-0.235
Nsμ8	2	0.160	0.270	0.01	0.413
Nsμ9	12	0.880	0.898	0.40	0.020
Nsμ10	9	0.840	0.849	0.03	0.011
overall					<0.005

Table 1.2 - cont'd

Beaver Marsh					
Nsμ2	13	0.840	0.802	0.94	-0.048
Nsμ3	19	0.880	0.911	0.40	0.034
Nsμ4	9	0.780	0.859	0.003*	0.093
Nsμ6	9	0.860	0.782	0.95	-0.101
Nsμ7	2	0.500	0.471	0.77	-0.062
Nsμ8	2	0.280	0.272	0.76	-0.032
Nsμ9	14	0.900	0.867	0.78	-0.038
Nsμ10	9	0.760	0.784	0.63	0.031
overall					<0.500
Lindsay Marsh					
Nsμ2	12	0.780	0.800	0.54	0.026
Nsμ3	14	0.960	0.908	0.88	-0.058
Nsμ4	10	0.720	0.786	0.14	0.085
Nsμ6	10	0.740	0.807	0.10	0.083
Nsμ7	2	0.440	0.453	0.54	0.030
Nsμ8	2	0.220	0.285	0.13	0.230
Nsμ9	13	0.960	0.913	0.93	-0.052
Nsμ10	10	0.940	0.825	0.95	-0.141
overall					<0.500

Table 1.3 - Overall and pairwise measures of water snake population differentiation (N=50 for each population) estimated by θ (Weir and Cockerham. 1984) and ρ (Goodman. 1997).

Populations Compared	θ (p)	ρ (p)
All	0.006 (0.009)	- 0.003 (0.76)
Barb's and Beaver Marsh	0.06 (0.02)	0.002 (0.33)
Barb's and Lindsay Marsh	0.06 (0.02)	- 0.007 (0.93)
Beaver and Lindsay Marsh	0.06 (0.02)	- 0.003 (0.64)


Lindsay
Beaver
Barb's
Legend
open water
marsh
stream
intermittent stream
direction of water flow
1 km

CHAPTER TWO

Genetic analysis of paternity in northern water snakes

Introduction

Accurately determining parentage is critical to acquiring a thorough understanding of patterns of sexual selection that drive the evolution of mating systems. Until the recent advent of molecular markers, parentage was largely inferred through observations of mating attempts (*e.g.* Birkhead, 1987; Madsen *et al.*, 1993; Brown and Weatherhead, 1999). There are numerous problems with basing estimates of reproductive success on observational data including: 1) many matings may not be observed, 2) mating attempts do not necessarily result in successful fertilization and 3) the importance of sperm competition and sperm precedence in fertilization is unknown for most species (reviewed in Westneat *et al*, 1987; also see Chapter Three). Newly developed, highly variable DNA-based genetic markers (reviewed in Avise, 1994) now allow accurate determinations of parentage which can be used to complement field observations (*e.g.* Quinn *et al.*, 1987; Gibbs *et al.*, 1990; Abell, 1997, Gullberg *et al.*, 1997) in studies attempting to quantify reproductive success.

The remainder of this thesis focuses on using DNA markers to estimate male and female reproductive success in the northern water snake, *Nerodia s. sipedon*, to gain insight into the evolution of the mating system of this species and patterns of selection acting on male and female phenotypic traits. Female water snakes are viviparous and therefore maternity is unambiguously known. To assign paternity, I assayed variation at eight microsatellite loci (Chapter One) among adults and offspring and then used these data for parental exclusion analyses. In this chapter, I report on a number of methodological issues which relate to parentage analyses, namely: 1) the genetic variability among the males sampled, 2) the power of the loci in excluding all males but the true father and 3) the presence of a null allele.

Methods

Field work

Snakes were captured opportunistically by hand from two marshes at the Queen's University Biological Station in Eastern Ontario. Upon capture, snakes were permanently marked using an electronic passive integrated transponder (PIT tag, Anitech). Approximately 100 μl of blood was taken from the caudal vein in the tail of all individuals using a 0.5 ml insulin syringe fitted with a 28 gauge needle. I sexed all individuals by probing the cloaca for the presence of hemipenes.

In late July 1994-1996, 80 (79 after one died in captivity) females from our study populations located in Barb's and Beaver Marsh were taken into the laboratory (Table 2.1) and housed individually or in pairs in fibreglass tanks in which the bottoms were lined with plastic outdoor carpet. The ambient temperature of the room was maintained lined with plastic outdoor carpet. The ambient temperature of the room was maintained at 25-33 °C using electric heaters, and reptile heating rocks were rotated among females to use as basking substrate. Females were fed minnows once or twice a week and were given water *ad libitum* and clean carpets.

Collection of offspring samples

I collected all live and stillborn offspring produced by females in captivity. Approximately 50 µl of blood were collected from live neonates (using the procedure outlined in Chapter One) and approximately a 1 cm length was cut from the tail of stillborns. All offspring blood and tissue samples were stored in 500 µl Queen's lysis buffer (Seutin *et al.*, 1991).

Genotyping Individuals

I isolated and amplified ten pairs of microsatellite DNA markers (see Chapter One for methodology and characterization of primers). Eight of these pairs of markers were sufficiently variable to use for genotyping all males, reproductive females and offspring to identify paternity (see below).

Genetic Variation among males

Over three years, I sampled 94 males from Barb's Marsh and 109 males from

Beaver Marsh. All males were initially genotyped with four loci (Nsμ2, Nsμ3, Nsμ9b, and Nsμ10). For Barb's Marsh, there were two instances where the genotypes of two males were identical at these four loci, and three instances where three males were identical. Similarly, for Beaver Marsh, there were five instances where the genotypes of two males matched perfectly across all four loci. These males were then genotyped at the remaining four variable loci (Nsμ4, Nsμ6, Nsμ110 and Nsμ119) and were found to be genetically identical at these loci as well. I calculated the probability [p(id)] that two randomly chosen unrelated individuals would have the same genotype at one locus by multiplying the population frequencies of each allele present in the individual and then multiplying the product by two (see p. 145 in Avise, 1994). To determine the combined p(id) over all eight loci, I multiplied all locus-specific p(id) values (Avise, 1994). Combined p(id) values ranged from 1.73×10^{-19} to 5.62×10^{-7} (mean = 8.44×10^{-9}) for Barb's Marsh and from 3.80×10^{-16} to 3.48×10^{-5} (mean = 9.33×10^{-7}) for Beaver Marsh Males (overall mean = 5.14×10^{-7}). Since these p(id) values are so small, I assumed that these samples with identical genotypes actually came from the same male. To determine how this happened, I checked the field records of these individuals. Seven (70%) records all revealed that the snake may have lost its original PIT tag because of the presence of a scar where the PIT tag was usually inserted. Since I was unable to determine the identity of any individuals with lost PIT tags, whenever this occurred I inserted a new PIT tag and created a new record for that individual. To confirm further that these and the other three duplicate records belonged to only one snake. I compared the growth rates in the two independent records, and when available, I looked at photocopies taken of the individuals in a concurrent study (G.P. Brown, unpublished data) to compare the ventral scale pattern of the two allegedly separate snakes. In all cases, growth rates and/or ventral scale patterns were consistent with my assumption that these records documented the same individual. Therefore, these duplications are likely to be due to the replacement of a lost PIT tag, rather than the individuals being monozygotic twins. With these errors accounted for, the actual number of captured males from Barb's Marsh was 86, and from Beaver Marsh was 104.

Parentage

Probability of exclusion

I calculated the probability of excluding a male who is actually not the father at each locus using the formula:

$$P(E)_i = 1 - 2a_2 + a_3 + 3(a_2a_3 - a_5) - 2(a_2^2 - a_4)$$ (Westneat *et al.* 1987)

where $P(E)_i$ is the probability of exclusion at the *i*th locus, and:

$$a_n = \sum_{i=1}^{k} p_i^n$$

where p_i is the population frequency of the *i*th allele. This calculation assumes that the allelic frequencies are in Hardy-Weinberg equilibrium, that dominance of one allele is negligible, and that all possible parents are sampled randomly from the population. The combined probability of exclusion PE(C) over all loci was calculated using the formula:

$$PE(C) = 1 - \prod_{i=1}^{n} (1 - P(E)_i) \quad \text{(Westneat } et\ al.\text{, 1987)}$$

This gives an estimate of the overall probability of excluding a male who is actually not the father. However, this value represents a population-wide exclusion probability which assumes that the allele frequencies are in Hardy-Weinberg equilibrium, alleles are codominant and mating occurs randomly with respect to genotype (Westneat *et al.*, 1987). I also calculated individual locus specific P(E) and combined PE(C) values for each offspring using the formulae:

$P(E)_i = 2p_i - p_i^2$ where p_i is the population frequency of the paternal allele at the *i*th locus; and

$$PE(C) = \prod_{i=1}^{n} P(E)_i \quad \text{(Jeffreys } et\ al.\text{, 1992)}$$

Exclusion Analysis

Since the average overall PE(C) value across all eight loci was so high (see results), I predicted that I would not have to genotype all of the neonates at all eight variable loci to accurately assign paternity. Therefore, to minimize the resources and time required for the analyses. I followed a two-step process. First, I assayed all neonates, males and maternal females with Nsμ2, Nsμ3, Nsμ9b, and Nsμ10. These loci were those with the highest P(E) values, based on thirty males from Barb's Marsh (Chapter One). Although Nsμ110 and Nsμ119 are quite variable, they were not used in this initial step since they were developed after much of the genotyping had been begun. The genotypes determined at these four loci were used in a preliminary exclusion analysis using a computer algorithm. This program compares the genotypes of mother and offspring to determine which are the maternal alleles in the offspring. Then, it generates a list of putative sires, based on the paternal alleles of the offspring. Males who do not match the paternal alleles of the offspring are excluded.

I then genotyped the remaining offspring and putative sires at the other four loci until either all males were excluded or a single male remained. If the males had been genotyped previously and were different at any locus, the offspring and female were then also genotyped at this locus to determine the paternal allele contributed. Otherwise, the loci were used as needed in the order Nsμ4, Nsμ6, Nsμ110 and Nsμ119. The loci Nsμ7 and Nsμ8 were not used at all in these analyses since they show such low variability. Although Nsμ4 has at least one null allele (see results) which could cause incorrect paternal exclusions due to inaccurate scoring of individuals, I had genotyped many of the males prior to discovering the null allele. Since this locus is quite variable and has a high P(E), I only used it to perform exclusions with heterozygous individuals that could not have had the null allele. The loci Nsμ110 and Nsμ119 were used last because few males had previously been genotyped at these loci. Individual P(E) values, which are calculated on the basis of allele frequencies, might be biased if loci with few genotyped individuals are used.

I also compared the paternity assignment of each neonate with the putative sires of all other offspring in the litter. If one or two males appeared to be responsible for fertilizing the entire litter, then these males were accepted as the most probable sires. On the other hand, if several males were each responsible for the paternity of only one or a few offspring, then the entire litter was analyzed using the remaining loci. Using this approach, I always found either a scoring error, or that all marked males could eventually be eliminated.

Results

Litter Sizes

Over the three years of the study, forty-six females gave birth to a total of 793 live (mean = 17.2, sd = 5.4, range 0-28) and 39 incomplete (*i.e.* stillborn or partially developed follicles) offspring (Table 2.1) (mean = 0.84, s = 3.3, range = 0-22). The overall mean litter size (*i.e.* live and incomplete combined) was 18.1 (s = 4.7, range = 5-28) offspring. One gravid female was found dead in the field just after the mating season. I was able to extract DNA from 19 of her 22 follicles and used these samples in my determination of multiple paternity. Similarly, one female died in captivity from undetermined causes prior to giving birth, and I dissected out sixteen partially developed offspring from which I was able to extract DNA and also included these offspring in the multiple paternity analyses.

There were no significant year effects at Barb's Marsh (all $U < 42.00$, all $p > 0.94$, $df = 1, 21$) or Beaver Marsh (all $H < 2.80$, all $p > 0.242$, $df = 1, 21$) with respect to numbers of live or incomplete offspring, or in overall litter sizes (Table 2.1). I should note here that only one female gave birth at Barb's Marsh in 1995, and thus the comparison of litter sizes across years for Barb's Marsh only involved two groups (*i.e.* 1994 and 1996). When the data for each marsh were pooled across years, I found no significant differences in live, incomplete or overall litter sizes between the two sites (all $U > 222.0$, all $p > 0.35$). Therefore, all litter sizes were pooled across years and sites.

Probability of exclusion

Locus-specific P(E) values ranged from 0.21 for Nsμ2 at Beaver Marsh to 0.82 for Nsμ3 at Barb's Marsh (Table 2.2). The overall PE(C) was >0.9999 for both sites, but this is an average population estimate based on allele frequencies in the population. If a particular offspring only has alleles that are quite common in the population, the probability of exclusion for that individual will be much higher. Individual PE(C) values for offspring ranged from 1.24×10^{-7} to 0.019 for Barb's Marsh (mean = 0.001, s =

0.002, N = 418) and from 3.70 X 10^{-8} to 0.026 (mean = 0.002, s = 0.003, N = 435) for Beaver Marsh. This suggests that, on average, the probability that another male in the population has the same genotype across all eight loci as the sire is approximately 0.0015. Therefore, in my panel of 100 males, all males should have unique genotypes. Nineteen of the individuals had estimates of PE(C) that were higher than 0.01, which suggests that one in 100 males may by chance perfectly match the true sire and thus, fail to be excluded. However, in all of these cases, the sire had been unambiguously assigned using only 4 loci. Therefore, if there were any discrepancy with the assignment of paternity of the other offspring in the litter, I could have used more loci to determine paternity of the offspring in question.

Exclusion

Using only Nsμ2, Nsμ3, Nsμ9b and Nsμ10, I was able to exclude unambiguously all but one male for 290/415 (69.9%) of the neonates from Barb's Marsh and 207/432 (47.9%) of the offspring from Beaver Marsh. On average, all but 1.21 ± 0.62 Barb's Marsh males were excluded, and at Beaver Marsh, all but 1.19 ± 1.50 were excluded. To gain some insight into the number of loci required for each exclusions, I examined the number of loci that excluded each male in five randomly chosen broods in which only one male was not excluded based on four loci. For both populations, more than 92% of the male exclusions occurred at two or more loci (Fig. 2.1 and 2.2). This suggests that most exclusions are unlikely to have occurred as a result of either a mutation at a single locus or errors in scoring.

The four additional loci were successful in determining paternity for many of the remaining neonates (Fig. 2.3 and 2.4). For the remaining offspring, all marked males were excluded. The large proportion of unassigned offspring is due to a small number of litters in which paternity could not be assigned to any or some of the offspring. For example, paternity was assigned to all offspring in all but three and ten litters at Barb's and Beaver Marsh, respectively. However, in less than two percent of the offspring, two males matched at all of the paternal alleles and thus could not be distinguished. To assign paternity to those offspring, I assumed that if only one of the males had fathered other neonates in the brood, then he likely had also sired the offspring in question.

Null Allele

In Chapter One, I reported a significant heterozygote deficiency at Nsμ4, which is probably due to a null allele at this locus. Null alleles are caused by a mutation in the primer binding site of certain individuals (Callen *et al.*, 1993, which results in the absence of a visible product for the homologue harboring the mutation. Individuals with one null and one visible allele will be mis-scored as homozygotes, which may result in the exclusion of the actual sire in parentage analyses. I have direct evidence for a null allele(s) at two loci. My preliminary parentage analysis detected three families in which the alleles possessed by approximately half of the offspring could not have been inherited from the documented genotype of the mother. These genetic mismatches are best explained by the presence of a null allele at either of two loci. In an attempt to overcome this problem, I constructed new primer pairs using sequences from regions flanking the repeat sequence that did not overlap with the previous primer regions (See Chapter One). The new set of primers flanking Nsμ9 (now called Nsμ9b) were successful in amplifying two bands in many samples that had previously only exhibited one. In contrast Nsμ4b failed to detect a product for the null allele at this locus and there was not enough sequence on one side of the repeat to construct yet a third distinct primer set.

Unsampled Males

For 30 (7.2%) and 135 (31.2%) neonates from Barb's and Beaver Marsh, respectively, I excluded all putative sires, which implies that the true father was not sampled. However, I was still able to obtain information on the minimum number of males siring the litter, which can be used in Chapter Three. To do this, I determined the number of paternal alleles present at each locus for each litter, and then used the locus with the greatest number of paternal alleles to estimate the minimum number of fathers. For example, if the most paternal alleles at any locus was five, I would conclude that there must have been a minimum of three males that fathered this litter.

To determine the minimum number of males that were unsampled in each population, I determined all paternal genotype(s), based on the paternal alleles contributed to the offspring in the brood. Based on this analysis, I determined that at least five males from Barb's Marsh and 17 males from Beaver Marsh were not captured.

Discussion

I have demonstrated that these loci are quite powerful in excluding all but one putative father for a large proportion of water snake offspring. My extremely high probabilities of exclusion imply that, typically, all but one male will be excluded for a population as large as 10,000 males. However, in this study, there were a few instances in which I was unable to exclude all but one male, despite the fact that I was dealing with a population of approximately one hundred males. This discrepancy may be due to the fact that when calculating P(E) values, it is assumed that all males in the population are unrelated (Morin *et al.*, 1994). Since the patterns of dispersal are not known for this species, it is possible that related individuals sharing many similar alleles may reside in the same marsh.

Null alleles can drastically affect paternity analyses, especially if paternity is assigned by excluding all non-sires. If the non-amplifying allele is passed from father to offspring, the genotypes of father and offspring might appear quite different, and thus, the actual sire would be excluded. However, despite the fact that one locus (Nsμ4) in this study had a null allele, it was still successful in excluding some males. If the mother, the offspring, and all putative males are heterozygotes at this locus, it can still be used to attempt to eliminate some males.

I had greater success at determining paternity for Barb's Marsh than for Beaver Marsh because at Barb's Marsh, there were fewer unsampled males who had sired offspring. This discrepancy between the populations is probably due to the nature of the marshes. For instance, Barb's Marsh is approximately 400 meters from the nearest permanent body of water, a distance that may be sufficient to limit much migration (Chapter One). In addition, I was able to walk transects through the entire site. In contrast, Beaver Marsh is directly adjacent to the nearest water body during wetter times of the year, so snakes may be able to move among these sites with relative ease. Furthermore, due to the depth of water and lack of surface vegetation (*i.e.* basking sites) in the middle of the marsh, I concentrated my efforts on two large beaver dams and the marsh periphery. Thus, snakes that spent a lot of time in other areas of the marsh may have been missed by my sampling methods.

Once paternity is assigned, male reproductive success can be estimated, which can give valuable insight into patterns of sexual selection, and the evolution of the mating system. In the past, studies of sexual selection in reptiles have estimated mating success using field observations of mating attempts (*e.g.* Madsen *et al.*, 1993; Brown and Weatherhead, 1999; see other references in Chapter Three). However, few studies have investigated whether such mating observations are reliable indices of true reproductive success (but see Abell, 1997; Gullberg *et al.*, 1997). In Chapter Three I compare water snake data from field observations with actual estimates of reproductive success determined by the parentage analysis described in this chapter. In the remainder of Chapter Three, I investigate aspects of the mating system of the northern water snake. In particular, I determine whether multiple paternity of litters exists, and whether there are any ecological factors that affect the degree of multiple paternity. In Chapter Four, I determine whether a variety of male morphological and behavioural traits are correlated with male reproductive success. I also investigate whether male body size affects male reproductive success in such a way to explain the occurrence of female-biased sexual size dimorphism despite the apparent mating advantage attributed to large males in this species.

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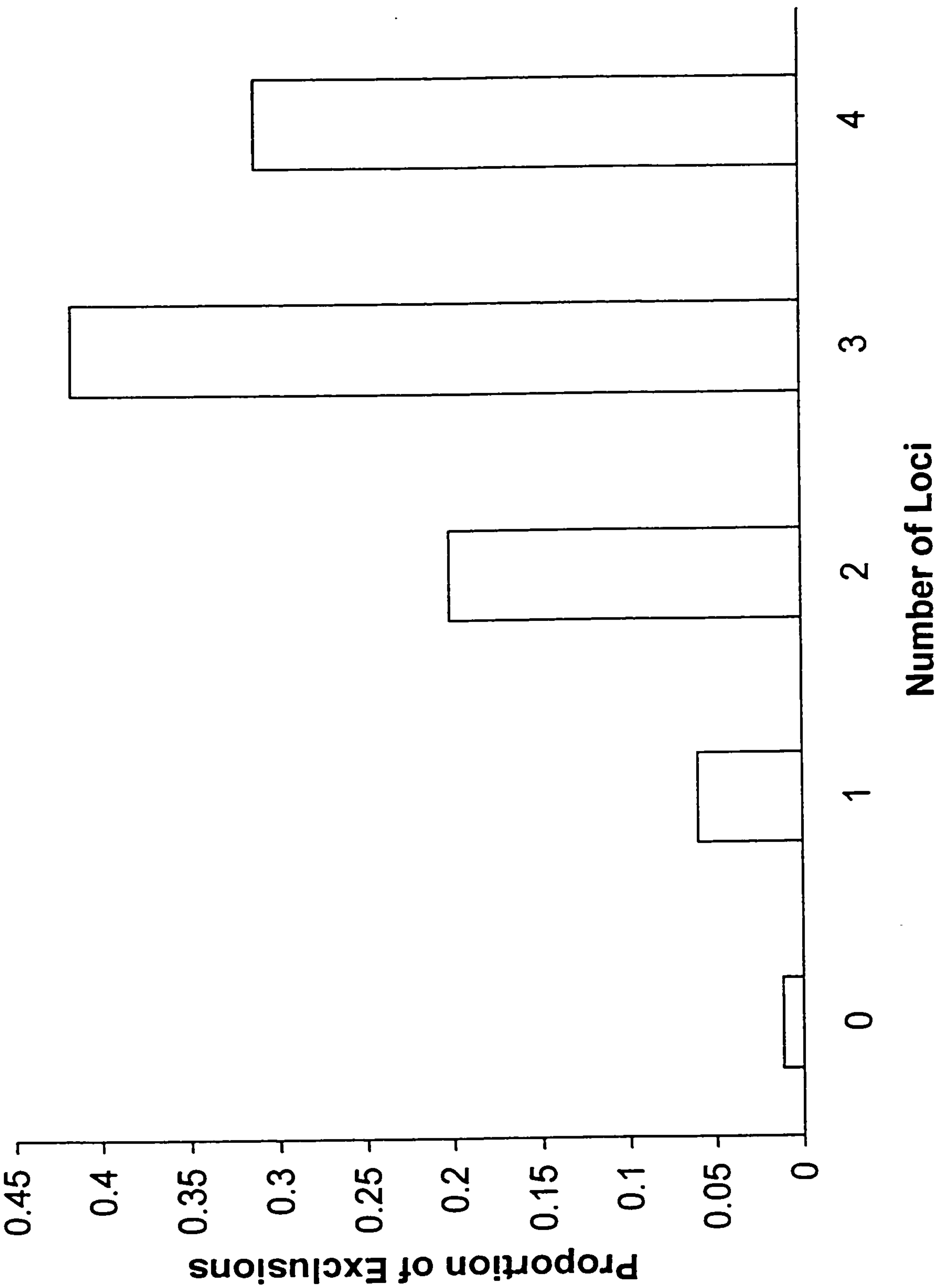
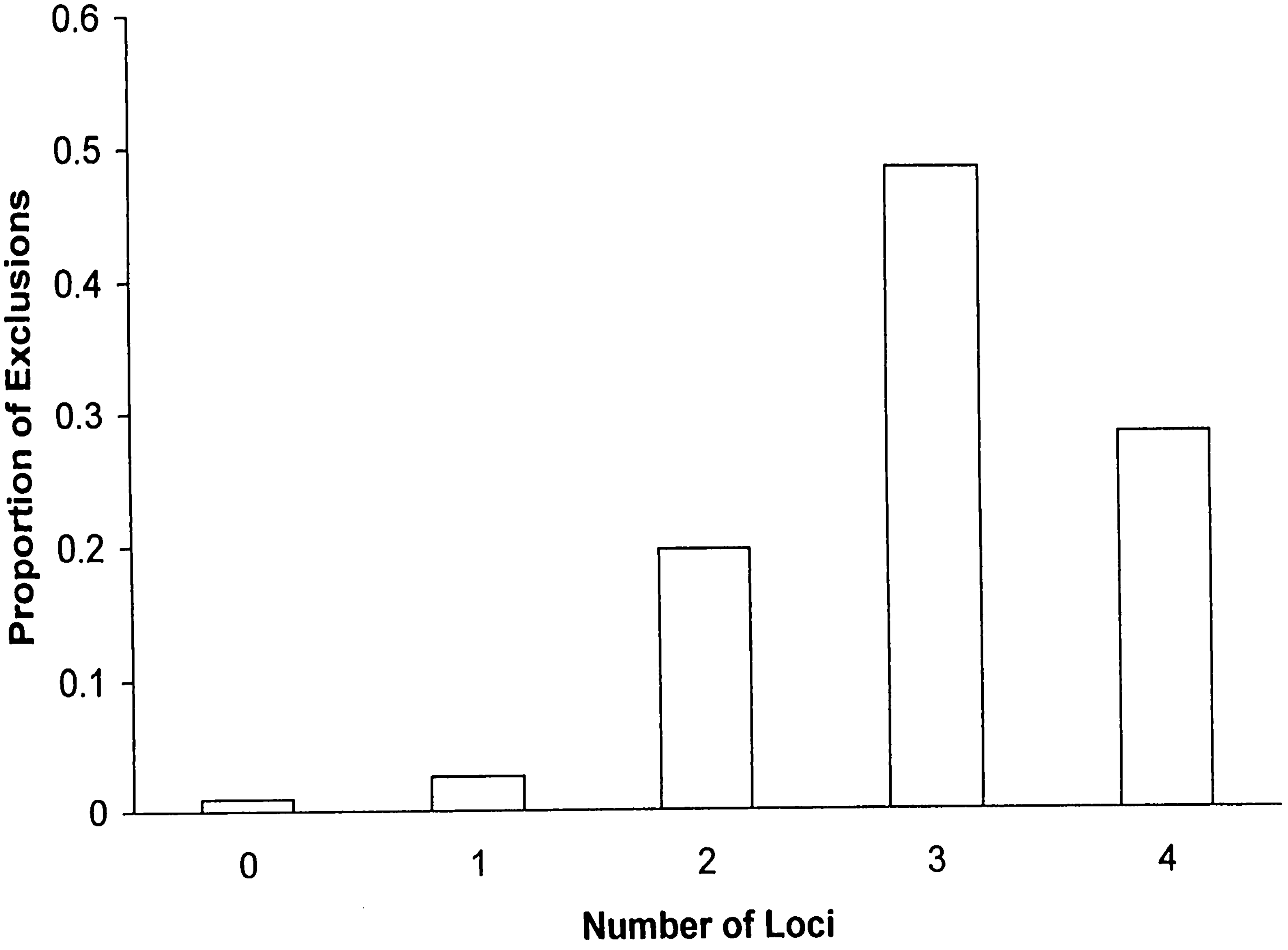
Table 2.1 - Summary statistics of the number of females collected, the number who gave birth and litter sizes per year for each population. Litter sizes are represented by the number of live-born neonates ("live"), the number of stillborn offspring or partially developed follicles ("incomplete") and the overall litter size (live and incomplete combined).

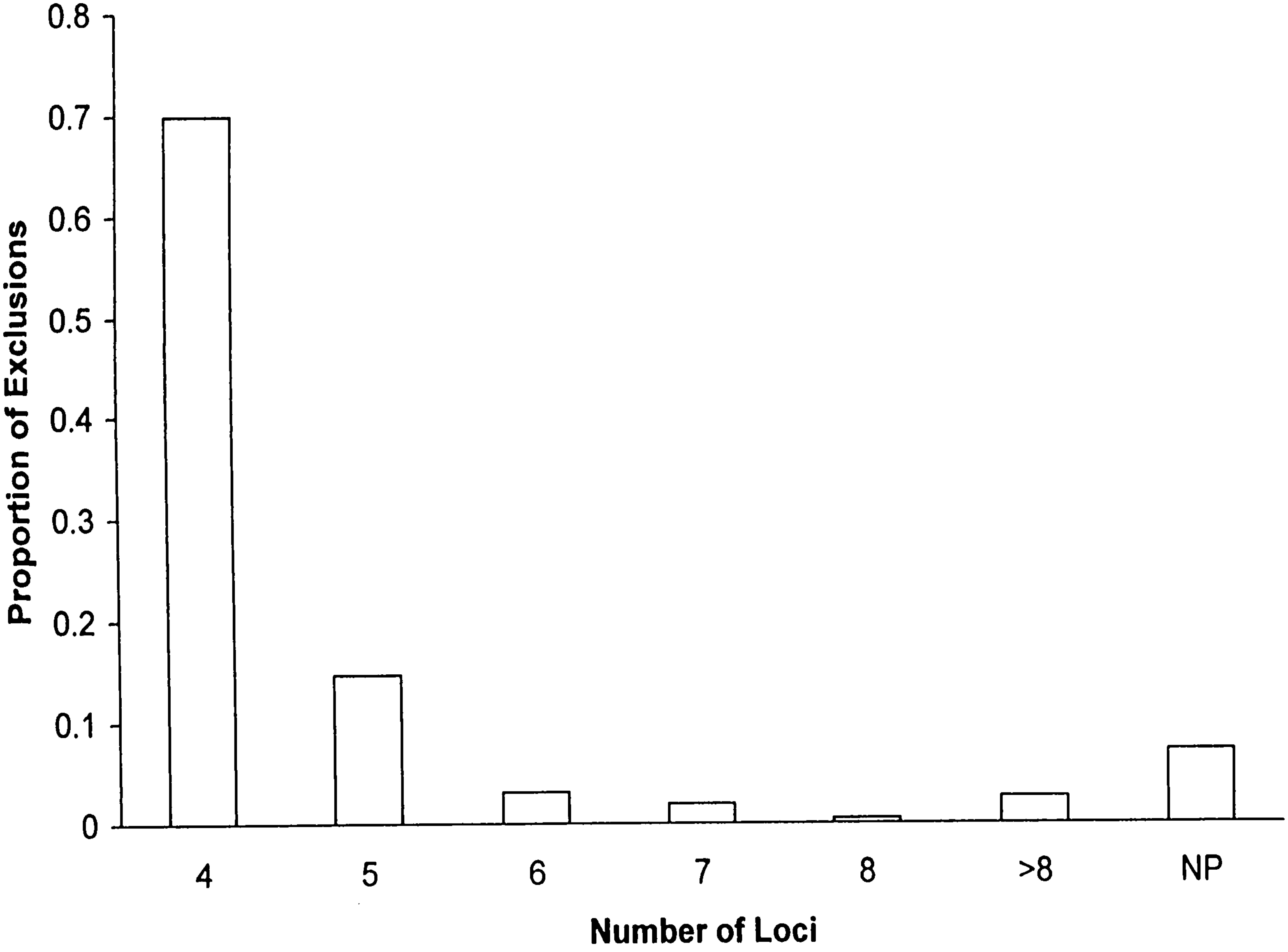
				litter size		
		# collected	# gave birth	overall	live	incomplete
Barb's Marsh	1994	12	6	108	105	3
	1995	7	1	17	17	0
	1996	19	16	284	257	27
Beaver Marsh	1994	12	7	138	135	3
	1995	12	8	159	156	3
	1996	17	8	126	123	3
	Total	79	46	832	793	39

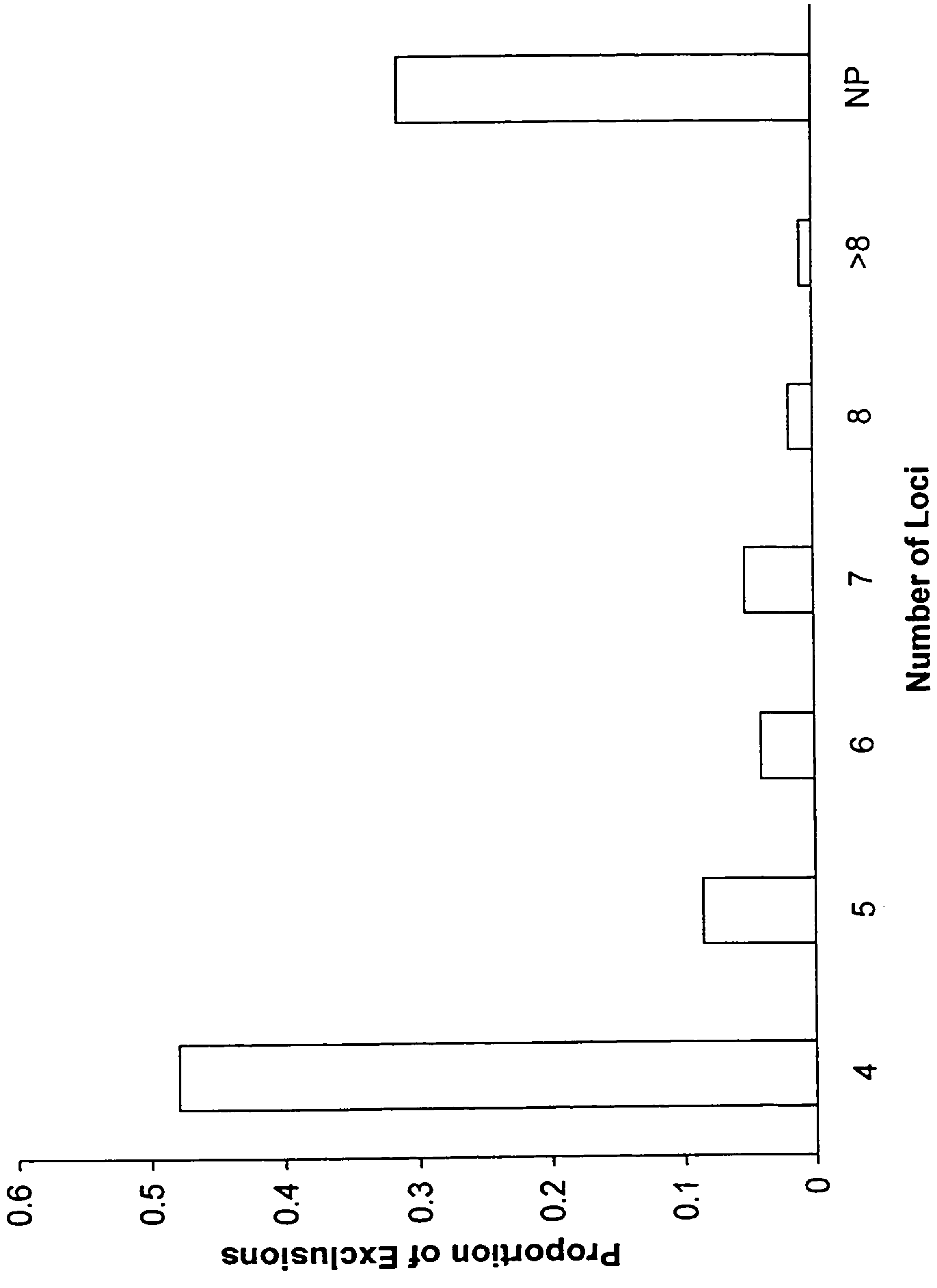
Table 2.2 - Exclusion probabilities for each locus P(E) and for all loci combined PE(C) for males in Barb's and Beaver Marsh (calculated as in Westneat *et al.*, 1987)

	P(E)	
Locus	**Barb's Marsh**	**Beaver Marsh**
Nsμ2	0.65	0.21
Nsμ3	0.82	0.81
Nsμ4	0.64	0.70
Nsμ6	0.55	0.55
Nsμ9	0.79	0.75
Nsμ10	0.66	0.61
Nsμ110	0.78	0.80
Nsμ119	0.86	0.86
Overall	0.999977	0.999946


Proportion of Exclusions
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
1
2
3
4
Number of Loci


0.6
0.5
0.4
0.3
0.2
0.1
0
Proportion of Exclusions
0
1
2
3
4
Number of Loci


Proportion of Exclusions
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
4
5
6
7
8
>8
NP
Number of Loci


Proportion of Exclusions
0.6
0.5
0.4
0.3
0.2
0.1
0
Number of Loci
4
5
6
7
8
>8
NP

CHAPTER THREE

Mating system of northern water snakes: multiple paternity and variation in male and female reproductive success

Introduction

Accurate estimates of individual reproductive success are essential to the description and understanding of reproductive behaviour in natural populations (Clutton-Brock, 1988). Such data can yield information on key evolutionary features of animal reproductive behaviour, such as the mating system, and the variation in male and female reproductive success and thus the potential for sexual selection. Despite the importance of this information, few estimates of individual reproductive success have been obtained for reptiles such as snakes (but see Duvall *et al.*, 1993 and references therein). This dearth of information is no doubt due to the difficulty in observing reptiles in the wild. Because reptiles are often widely dispersed and behave cryptically (Reinert, 1993), it is difficult to obtain sufficient observations to study patterns of mating.

A potentially important limitation of the studies that have been done on sexual selection in reptiles is that most have relied on the untested assumption that observed male mating success accurately predicts true success (Brown and Weatherhead, 1999a; Madsen *et al*, 1992, 1993; Madsen and Shine, 1993, 1994; Olsson 1993; Olsson and

Shine, 1996; Weatherhead *et al.*, 1995). DNA-based techniques have been used to test this assumption in ornithological research and have revealed that true patterns of success can sometimes be very different from those estimated from behaviour (*e.g.*, Gibbs *et al.* 1990; Westneat, 1987). There are various reasons that estimates of reproductive success based on behaviour may be unreliable (Westneat *et al.*, 1987), and some of these may be particularly relevant to snakes. For instance, it is highly unlikely that all matings will be observed. Even when mating attempts are observed, they may not result in copulation and insemination because a female can resist mating by not exposing her cloaca (Devine, 1984). Even if intromission is observed, the high incidence of multiple paternity reported for some species of snakes (Gibson and Falls, 1975; Schwartz *et al.*, 1989; Barry *et al.*, 1992) means that one would need to observe all copulations by a female to estimate male success. Even if one could observe all copulations, little is known about sperm competition and patterns of sperm precedence in snakes (Devine, 1984).

Some recent herpetological studies have used genetic approaches to analyze parentage, and in some cases, a close match between behavioural and genetic estimates of success have been found. For example, Gullberg *et al.* (1997) used field observations of mating and mate guarding combined with multi-and single-locus DNA fingerprinting to compare observed versus successful copulations in the sand lizard, *Lacerta agilis*. When mating or guarding had not been observed, mating success was estimated through the overlap of male and female home ranges. Gullberg *et al.* (1997) found that their assigments of paternity based on field observations corresponded to the genetically-determined paternity for 77% of the offspring. Similarly, Abell (1997) estimated mating success in the striped plateau lizard, *Sceloporus virgatus*, in two independent ways. She assigned paternity of all offspring produced by a female to the male who 1) was seen closest to the female in the field and 2) had the greatest proportion of home range overlap with the female. DNA fingerprinting indicated that both estimates of mating success were reliable indices of relative reproductive success among males, and that spatial proximity was an excellent measure of absolute reproductive success. However, in species in which intromission is more difficult to observe, male competition is more intense, and larger litters provide more potential for multiple paternity, estimating paternity through mating observations may be less reliable. My goal in this chapter is to use genetic analysis of parentage in northern water snakes to determine how well observed mating behaviour predicts true reproductive success, and in doing so, to determine both the mating system and the potential for sexual selection in this species.

There are a number of aspects of the biology of northern water snakes that make them well suited to this type of study. Because this species is aquatic, it is possible to find marsh populations that are sufficiently small and isolated (see Chapter One) that most of the adult population can be sampled. This, in turn, makes it possible to identify the true parents of a substantial proportion of the young produced (see Chapter Two), and thus to estimate the reproductive success of most individuals in the population. In addition, past work has indicated that multiple paternity occurs frequently (86%) in this species (Barry *et al.*, 1992), so *N. sipedon* is an appropriate species for addressing questions regarding the basis for multiple mating by females.

I have five specific goals in this chapter. My first goal is to assess how well behavioural measures of mating success predict true reproductive success. Females observed mating should give birth, but it is possible that some females not observed mating may also give birth. For males, behavioural estimates of mating success should be less reliable predictors of reproductive success.

My second goal is to determine the frequency of multiple paternity in northern water snakes. The estimate of 86% for this species by Barry *et al.* (1992) was based on a single season and used females collected from a number of different ponds. I assess multiple paternity within pond populations for several consecutive years. Barry *et al* (1992) also used allozyme electrophoresis in their study, and thus, they could only estimate the minimum incidence of multiple paternity. I use microsatellite DNA markers (Chapter Two) to determine the precise number of sires per litter and the exact number of young fathered by each of those males.

My third goal is to determine the factors that contribute to both individual and population variation in multiple paternity. At the individual level, one might expect no difference between females that produce singly-sired litters and those that produce multiply-sired litters because there is no apparent reason that the proclivity to mate with multiple males should vary with female size, for example. Alternatively, it is possible that males might pursue larger females more vigorously because larger females are more fecund, and thus provide males greater potential for reproductive success (Brown and Weatherhead, 1997; Duvall *et al.* 1993). If the number of males with which a female mates is related to the number of males that court her, then larger females should more often give birth to multiply-sired litters.

At the population level, the occurrence of multiple mating, and thus multiple paternity, might depend on the number of males that a female encounters while receptive. The operational sex ratio (OSR), which is the ratio of fertilizable females to reproductively active males in the population at any given time (Emlen and Oring 1977), is male biased in northern water snakes but can vary substantially between years (Weatherhead *et al.* 1995). I test the prediction that, as the OSR becomes more male-biased, the incidence of multiple paternity will increase. Similarly, longer mating seasons will allow females more opportunity to solicit multiple matings, assuming that each female is receptive longer when the mating season is longer. Thus, I predict that the incidence of multiple paternity varies with the length of the mating season (*i.e.* number of days between the first and last observation of mating in the field).

Water snakes bask (and mate) on solid substrate on the surface of the water (*e.g.* muskrat lodges, beaver dams). The distribution of suitable basking substrate varies substantially between my two study populations. Whereas solid substrate occurs throughout Barb's Marsh, suitable basking substrate is restricted to the periphery of Beaver Marsh. In fact, at Beaver marsh, the majority of basking (or mating) snakes were sighted along two beaver dams. Thus, receptive females were four times more clumped

(Brown and Weatherhead, 1999a) and were more predictably located at Beaver Marsh. At Beaver Marsh, males probably do not need to undergo extensive searching to locate receptive females. Indeed, Brown and Weatherhead (1999a) found that male home ranges were larger at Barb's Marsh than at Beaver Marsh. Thus, males may have more opportunity to encounter and mate with receptive females at Beaver Marsh. I test the prediction that the more clumped distribution and predictable locations of females in Beaver Marsh should result in greater incidence of multiple paternity relative to Barbs Marsh.

My fourth goal is to determine, at least proximately, whether multiple mating has any adaptive consequence for females. Many hypotheses have been proposed to explain why females of many species mate with multiple males, even though a single male should be capable of fertilizing all of a female's eggs (Schwagmeyer, 1984; Halliday and Arnold, 1987). I consider just two of those hypotheses here. First, if a single mating is not sufficient to fertilize an entire batch of eggs, females that mate with more than one male should have fewer unfertilised ovules, and thus larger litters, than females who mate once (Gromko *et al.*, 1984). Second, multiple mating may be a bet-hedging strategy, whereby the chance exists that the second mate may be superior in quality (or less related) to the first. This, in turn, may reduce the number of stillborn offspring. However, there is an equal likelihood that the second male may be inferior, which may increase the risk of producing stillborn young (Pusey and Wolf, 1996). However, Madsen *et al.* (1992) found no difference in the variance in the proportions of inviable offspring. In contrast, they found that multiple mating reduced the mean proportion of stillbirths in an inbred population of European adders (*Vipera berus*). They interpreted this by suggesting that multiple copulations may promote intrauterine sperm competition, whereby sperm of higher quality males are more likely to fertilize eggs. I determine whether multiple paternity reduces the mean or variance in proportion of inviable offspring in northern water snakes, which although not inbred, do appear to have restricted gene flow between pond populations (Chapter One).

My final goal is to use the data on male and female reproductive success and mating patterns to determine the mating system of northern water snakes, and hence the extent to which sexual selection is likely to be operating in this species. Behavioural observations suggest that competition to mate among male water snakes can sometimes be quite intense (Barry *et al.* 1992, Weatherhead *et al.* 1995), but one needs to know the true reproductive success for all individuals in the population to determine the intensity of sexual selection (Duvall *et al.* 1993). First, I document the mean number of mates for males and females. Then, I determine whether sexual selection is greater in males than females by comparing their respective slopes of the regressions of reproductive success on the number of mates (*i.e.* sexual selection gradients; Arnold and Duvall, 1994). If sexual selection acts more strongly on males than females (*i.e.* the slope is greater for males), this indicates a polygynous mating system. Given the observations that suggest a male-biased operational sex ratio (OSR), and the potential for males to mate with multiple females, the mating system should be polygynous. Thus, the variance in reproductive success should be much higher for males than for females, and therefore, sexual selection should act more strongly on males (Arnold and Wade, 1984a). The results of these analyses will set the stage for Chapter Four, in which I determine the factors that contribute to variance in male reproductive success, and thus the factors likely to be influenced by sexual selection.

Methods

Capturing and "processing" individuals

Snakes were opportunistically captured by hand from two marshes (Barb's Marsh and Beaver Marsh) at the Queen's University Biology Station, Chaffey's Lock, Ontario. Upon each capture, I measured the mass, total length and tail length, and determined the snout-vent length (SVL) by subtracting tail length from total length. Snakes captured for the first time each season were painted with a unique three-colour paint code so that individuals could be identified in the field with minimal disturbance. This paint was reapplied throughout the season as required. Any snakes captured for the first time ever were also marked by inserting an electronic passive integrated transponder (PIT tag, Anitech) just under the skin. In addition, I sexed the individual by probing the cloaca for the presence of hemipenes and drew approximately 100 µl of blood from the caudal vein in the tail which then was stored 800-1000 µl of Queen's Lysis Buffer (Seutin *et al.*, 1991).

Behavioural observations

After processing, snakes were returned to their respective marshes. Each day when snakes were active, each marsh was visited at least twice to record basking or mating behaviours during the hours corresponding to peak snake activity (*i.e.* approximately 10:00 to 17:00, pers. obs.). Barb's Marsh has various mats of vegetation, muskrat and beaver lodges and dead willows that the snakes use for basking, throughout the marsh. Therefore, I walked transects through the entire site, targeting popular basking spots. In contrast, at Beaver Marsh, there is no material adequate for basking in the center of the marsh; rather snakes were active primarily on two large beaver dams and a beaver lodge, and in the grass along the perimeter. Therefore, at Beaver Marsh, I restricted my observations to the perimeter of the marsh.

Estimation of individual reproductive success

In late July and early August 1994-1996, 80 females were captured from Barb's and Beaver Marshes and were retained in the laboratory until parturition. Blood or tissue samples were collected from live and stillborn offspring, respectively (see Chapter Two). Offspring and potential parents were genotyped at a minimum of four microsatellite loci until paternity could be assigned unambiguously (see Chapter Two for details).

The comparison between behavioural and genetically determined estimates of reproductive success was performed in two ways. First, the raw reproductive success values were pooled across years and used in all analyses. Second, reproductive success values were standardized by adjusting the mean for each year such that it equaled the overall mean. Since I obtained similar results for both sets of analyses, I only report the results of the raw data.

In addition, since some snakes were present in multiple years, I was concerned about non-independence of data if I pooled results from all three years. To avoid this bias, each snake can be represented by one random score, or a mean score of all measures of that individual. However, the latter approach would substantially reduce the sample size, especially since a very small proportion of individuals actually produced offspring (see results). In addition, Leger and Didrichsons (1994) demonstrated that for data in which the variance is larger within than between individuals, a more accurate estimate will be obtained if all records of individuals are pooled rather than if a single random or mean value for each individual is included. Since very few of the snakes in this study reproduced in multiple years, I did find more variance within than between individuals in both populations, and for both sexes, (all sum of squares at least 1.31 times greater within than between; ANOVA with individual as factor). Thus the inclusion of all data points for individuals should have introduced very little bias to the analyses. However, to avoid any potential for bias, I calculated individual reproductive success by pooling all observations for a given individual and then repeated the analysis using only a mean score. Since the magnitude of the estimate of variation in male and female reproductive success was similar for both methods, I present the results of the analyses using the pooled data only.

Mating system

To classify the mating system (Arnold and Duvall, 1994), I first documented the mean number of mates for both males and females. I then investigated whether female reproductive success increases with multiple inseminations in two ways. First, I tested for a significant difference in litter sizes between singly and multiply sired litters, and between litters sired by a different number of males. Second, I compared the average slope of the partial regression of fecundity on number of mates (sexual selection gradients) of all adult males and females. Under this model, a polygynous mating system occurs in the situations in which the slope of the male selection gradient is steep, and that of females is flat, whereas if both slopes are not significantly different from zero, a monogamous mating system is implied (see Arnold and Duvall, 1994 for a more detailed discussion). I then modified this approach slightly. In contrast to the method suggested by Arnold and Duvall (1994), I used the results from sexual selection gradients only from those individuals who reproduced. For animals with fairly large litter sizes, such as snakes, including individuals who obtained no matings may introduce bias into the results of sexual selection gradients. The portion of the line from zero mates to one mate will show the greatest slope. In turn, this may influence the overall slope of the line. Besides, I am not interested in how much better it is to mate than not to mate; rather I wish to determine how much fecundity increases with every mating after the first.

Results

Field observations

Overall, I had 120 observations of mating attempts. An observation was classified as a mating attempt if the male was performing a courtship behaviour such as chin-tapping, body-jerking or dorsal advance movements (Perry-Richardson *et al.*, 1990; Mushinsky, 1979). Forty-four of these mating groups (36.7%) involved more than one male. Based on my genetic data, 41 males reproduced, ranging in snout-vent length (SVL) from 43.25 to 69.05 cm. All males longer than the smallest male to reproduce (43.25 cm SVL) were considered sexually active, since I have no *a priori* reason to believe that males are unable to reproduce in successive years. This number ranged from 29 to 46 in any given year and population (mean = 36.5, sd = 6.1) (Table 3.1). The 46 females who reproduced (see above) ranged in SVL from 63.3 to 83.1 cm. In contrast to males, female water snakes may not reproduce in consecutive years (Feaver, 1977; Brown and Weatherhead, 1999b; this study). For this reason, I estimated the number of sexually active females by the number of females seen mating in the field plus the number of females who gave birth. This gave me an estimate of from four to 17 reproductive females per marsh in any given year and population (mean = 13.8, sd = 5.2) (Table 3.1).

Reproductive frequency

Fifty-eight percent of the females that I brought into captivity gave birth. This is similar to the value (70%) obtained by Brown and Weatherhead (1999b) based on captive females, which they used to imply that the probability that a water snake female will reproduce in a given year may be as high as 70%. The reproductive frequency of other water snake populations also appears to be greater than 70% (Feaver, 1977; King, 1986) This suggests that many females would be capable of reproducing in successive years. If this were true, I should have found that, in this study, of the 14 females who gave birth and were held in captivity the following year, approximately 10 should have also given birth the second year. However, none of my females gave birth in successive years. The error may be in assuming that reproductive and non-reproductive females are equally catchable after the mating season. Reproductive females bask to maintain high body temperatures to accelerate the rate of follicular development (Brown and Weatherhead, 1999c). Therefore, my sampling at this time of year may have been biased toward reproductive females who were much more conspicuous due to their basking behaviour. Indeed my three years of field observations indicated that females who reproduced comprised 172 of the 685 sightings (25.1%) of females without transmitters during the mating season, whereas these females comprised 47 of the 56 sightings (83.9%) of females during the two week period at the end of July when I typically collected gravid females in the field (*i.e.* July 20 - Aug 3). Females with radiotransmitters as part of another study (Brown and Weatherhead 1999 a-c) were excluded from this comparison because Brown and Weatherhead (1999a) determined that snakes with transmitters were sighted significantly more often than other snakes. This evidence supports the hypothesis that reproductive females are disproportionately more active during the latter half of the summer.

Behavioural versus genetic estimates of reproductive success

Males

When I pooled the data from the two populations, I estimated that 85, 73 and 62 males were large enough to be considered sexually active in 1994, 1995 and 1996, respectively. Of these 220 males, 94 (43%) were observed courting or mating with a female in the wild. However, of these 94 males who were judged sexually active based on behaviour, only 31 (33%) fathered offspring born in captivity. In total 52 (24%) mature males achieved some reproductive success. However, 21 (40%) males that had successfully reproduced had never been observed mating in the field. Nearly half of the mature males (47.8%) were neither seen mating nor reproduced (Table 3.2). There was a significant correlation between whether males were seen mating and whether they produced offspring based on the genetic data ($\chi^2 = 7.94$, $p < 0.005$). This suggests that field observations of mating attempts may be reliable predictors of which males are likely to reproduce. However, this relationship is largely influenced by the disproportionately large number of males who were neither seen mating nor reproduced, and may be influenced very little by the males who were seen mating and reproduced. In addition, the large proportion of males who: 1) were not observed mating in the field but sired offspring (10%) and 2) were observed mating in the field yet failed to reproduce (29%) indicates that estimates of mating success based on field observations are not reliable indices of true reproductive success.

Females

Over the three years, I brought 81 females into captivity. One female died in captivity and one female gave birth to a whole litter of stillborn offspring (see Chapter Two) and both were excluded from subsequent analyses. Of these remaining 79 females. 46 (58%) gave birth (see Chapter Two for litter sizes). However. of these 46 reproductive females, only 22 (48%) had previously been seen mating in the field. Twenty-five females for whom no mating observations were recorded produced offspring. Similarly. 15 of the females who were held in captivity did not give birth. despite having been courted in the field (Table 3.2). In contrast to the males. there was no significant correlation between whether individuals were seen mating and whether they produced offspring ($\chi^2 = 0.00$, $p > 0.99$). which suggests that field observations are not reliable predictors of which females are likely to reproduce in a given year.

Do mating observations reliably predict reproductive success?

There was no significant difference in the number of offspring produced (reproductive success) by females (see Chapter Two) or males among years (Barb's Marsh: $U = 428.5$, $p = 0.06$; Beaver Marsh: $H = 0.76$, $p = 0.47$) or between populations ($U = 3528.5$, $p = 0.12$). Only three males and two females produced offspring in multiple years. I then pooled the results of those individuals who were seen mating and produced offspring to determine whether there was a relationship between the number of times seen mating and the number of offspring produced.

The number of offspring produced by males (*i.e.* male reproductive success) was not significantly correlated with the number of times seen mating (r_s = -0.03, p = 0.86) or the number of different females that males were seen mating with (r_s = -0.026, p = 0.89). The number of females that a male was seen mating with and the number of females actually fertilized were not significantly correlated (r_s = 0.196, p = 0.28). However, the number of females fertilized was highly significantly correlated with male reproductive success (r_s = 0.528, p = 0.002) (Fig. 3.1), suggesting that males can increase their fitness by fertilizing multiple females.

There was no significant correlation between the number of times a female was seen mating and the number of offspring that she produced (r_s = 0.12, p = 0.45). Nor was there a significant correlation between the number of different males females were seen mating with and female reproductive success (r_s = 0.18, p = 0.27). The number of males that fathered a female's offspring was not correlated with the number of times that she was seen mating (r_s = 0.02, p = 0.90) or the number of different males that she was seen mating with (r_s = 0.06, p = 0.72). In contrast to the male data, there was no significant correlation between the number of males that fathered the litter and the number of offspring produced (r_s = 0.25, p = 0.08) (Fig. 3.2), which indicates that females may not significantly increase their reproductive success via multiple paternity of their litters.

Overall, these results imply that while field observations of mating may be useful in predicting which males are likely to reproduce based on whether they were seen mating, they do not provide the same information for females. Furthermore, the number of observed matings in the field does not reliably predict individual reproductive success for either sex. In addition, since males can increase their fitness by fertilizing multiple females, but females do not increase their reproductive success by accepting the sperm of multiple males, this suggests that the mating system is polygynous, according to the model of Arnold and Duvall (1994).

Incidence of multiple paternity

Barb's Marsh litters were sired by a mean of 1.71 (sd = 1.20) males, whereas 2.00 (sd = 0.88) males fathered Beaver Marsh litters, on average. There is a marginally non-significant difference in the number of sires between the two populations (U=175.5, p=0.06). Although one litter had as many as six different fathers, nearly 80% of all litters were fathered by one or two different males (Fig. 3.3). The proportion of offspring fathered by each single male in multiply sired litters ranged from 0.05 to 0.95 (Fig. 3.4). In 8 of 20 (40%) litters, males fathered significantly different proportions of neonates.

The proportion of multiply sired litters in a given year from Barb's Marsh was 0.33 (N=6), 1.00 (N=1) and 0.41 (N=17) for 1994, 1995 and 1996, respectively (Table 3.3). These proportions are not significantly different (χ^2=1.576, p=0.90, df=5). At Beaver Marsh, the proportion of multiply sired litters was 0.43 (N=7), 0.75 (N=8) and 0.77 (N=9), in 1994, 1995 and 1996, respectively (Table 3.3). These proportions are not significantly different (χ^2=2.536. p=0.77). In total. 10 of the 24 (41.7%) litters from Barb's Marsh females and 16 of the 24 (0.67) litters from Beaver Marsh females were fathered by more than one male.

Factors affecting the incidence of multiple paternity

The length of the mating season is defined as the interval between the first and last day in which mating was observed in the wild and ranged from 11 to 29 days (Table 3.3). The low value of 11 days was the estimate for Barb's Marsh in 1995. in which only one female gave birth in captivity. Thus. this estimate may inaccurately reflect the actual length of the mating season for this year. Initially. I predicted that longer mating seasons would result in more opportunity for males to inseminate females. and consequently. a higher incidence of multiple paternity. However. my raw data do not support this prediction. The longest mating season was observed in Beaver Marsh in 1994. and this group of females had one of the lowest incidences of multiple paternity. In addition. the two groups with the highest incidence of multiple paternity (aside from Barb's Marsh 1995. in which N=1) were Beaver Marsh 1995 and 1996. and these groups had the shortest mating season.

The incidence of multiple paternity is expected to be higher if males encounter receptive females more frequently (*i.e.* higher density or more male-biased operational sex ratio). To determine the OSR. I divided the number of sexually active females by the number of sexually active males in the population. The operational sex ratio ranged from

0.59 to 1.09, and did not differ significantly among years or between marshes (χ^2=0.16, p=0.99). Similarly the density was determined by the total number of adult snakes divided by the area of the site in hectares. It ranged from 13.5 to 26.3 adult snakes per hectare, and did not differ significantly among years or between sites (χ^2=7.83, p=0.16).

There was no correlation between the proportion of multiply sired litters and OSR (r_s = 0.49, p = 0.32) or density (r_s = 0.08, p = 0.87). In contrast, the proportion of multiply sired litters was significantly negatively correlated with the length of the mating season (r_s = - 0.81, p = 0.05). However, this relationship was greatly affected by the very low estimate of the length of the mating season for Barb's Marsh in 1995 (11 days), when only one female gave birth (to a multiply sired litter). When this entry was excluded, there was no correlation between the proportion of multiply sired litters and OSR (r_s = 0.70, p = 0.19) or density (r_s = 0.50, p = 0.39) or the length of the mating season (r_s = - 0.67, p = 0.22). These results suggest a non-significant trend whereby multiple paternity is more likely to occur in shorter mating seasons because females are predictably located for a shorter period of time and thus, male competition is intense during this time frame.

Although it is evident that there is a substantial amount of variation in the incidence of multiple paternity among years and between sites (Table 3.3), there is no significant difference in the incidence of multiple paternity between these two populations in any one year (Fisher exact test, all p > 0.11). However, when the data are pooled across years, it is evident that multiple paternity occurred more frequently at Beaver Marsh (16 of 24 litters) than at Barb's Marsh (8 of 24 litters), which is in the direction of my prediction. The difference in the incidence of multiple paternity between marshes is marginally non-significant (χ^2=3.02, p=0.08), which provides weak evidence to support my prediction that multiple paternity would be more prevalent at Beaver Marsh where females were more predictably located by males. However, small sample size might reduce the power of my statistical tests.

Within each marsh, I found that female SVL did not vary across years (U = 26.0, p = 0.09, Barb's Marsh; H = 2.45, p = 0.294, Beaver Marsh), or between the two sites (years pooled) (U = 255.5, p = 0.50), so the data for all females were pooled. I found no significant effect of SVL on the occurrence of multiple paternity (U = 232.5, p = 0.268). This suggests that variation in the incidence of multiple paternity cannot be explained by variation in female SVL.

Adaptive significance of multiple paternity

Since there were no significant differences in litter sizes or the proportion of multiply sired litters across years or sites (Chapter Two), I pooled these data to investigate whether singly and multiply sired litters differed in litter size. Similarly, there was no significant difference in the proportion (or absolute number) of unfertilized ovules between singly and multiply sired litters (both U = 73.00, p = 0.37). Nor was there any significant difference in live, or overall litter size between singly and multiply sired litters (both U > 64.5, both p > 0.35). These results suggest that one mating is sufficient to fertilize a batch of eggs. The variance in number of stillborn offspring was significantly greater in litters with multiple than single paternity ($F_{25,19} = 20.85$, $p < 0.001$), which is in contrast with my predictions. However, this result was largely affected by an entire litter of stillborn offspring (N=22). With this outlier removed, there was no significant difference in the variance of the number of stillborn offspring in singly or multiply sired litters ($F_{24,19} = 0.188$, $p > 0.50$). Finally, there was no significant difference in the mean ($U = 66.5$, $p = 0.31$) in the proportion of stillborn (including partially developed follicles) between singly and multiply sired litters. These results suggests that mating with multiple males does not reduce any negative consequences of inbreeding in water snakes.

Mating system

The traditional method of classifying mating systems is to determine the mean number of mates obtained by each sex. I only included females who reproduced because failure to mate is more likely a life history "decision" for females rather than a sexual selection issue. In contrast, I have no reason to believe that males should forego reproduction in any given year. Indeed, males who fathered offspring in a given year were no less likely to be successful or attempt mating the following year ($\chi^2 = 0.889$, $p = 0.34$). Males fertilized a mean of 0.33 and 0.26 females at Barb's Marsh and Beaver Marsh, respectively. However, many males were unsuccessful. Considering only successful males, Barb's Marsh males fertilized 1.46 females, on average, while Beaver Marsh males fertilized a mean of 1.15 females. This suggests that many males are unable to secure a mating, that most males fertilize only one female, and that few males mate multiply. Because I demonstrated earlier that many females do not mate, but of those that do, over half mate with multiple males, the mating system is best classified as polygynandrous.

A second method of defining the mating system involves comparing sexual selection gradients for males and females. If sexual selection is greater for males than females, the system can be defined as polygynous (Arnold and Duvall, 1994). In this study, I found that female reproductive success increased by 5.64 offspring for each additional mating secured ($p < 0.05$), whereas male reproductive success increased by 10.11 offspring for each additional mate ($p < 0.001$). After the first mating, female reproductive success increased by 1.75 offspring for each additional mating secured ($p < 0.05$), whereas male reproductive success increased by 11.36 offspring for each additional mate ($p < 0.001$). This demonstrates that male reproductive success increases uniformly from zero to three mates, but for females, the number of mates has little effect on reproductive success for females mating with at least one male. However, my finding that reproductive success may increase by nearly two offspring for each additional mate contrasts with my other results which detected no difference in litter size between singly and multiply sired litters. This discrepancy may be due to the nature of the parametric test conducted here, which may be more sensitive to detecting significant trends than Spearman rank correlation tests used previously. In addition, these results suggest that the mating system is polygynous, which is similar to the conclusion based on the traditional method of classification (*i.e.* number of mates).

Individual reproductive success

Male reproductive success in any given year was highly skewed (Fig. 3.5). with the proportion of males achieving no reproductive success ranging from 59 to 94%. Mean male reproductive success per year in each marsh ranged from 0.51 (sd = 2.24) to 8.10 (sd = 12.43) offspring. Since the variance in male reproductive success was quite high. the opportunity for sexual selection on males, I_m. (the standardized variance of the mean) was fairly high, ranging from 2.35 to 19.29 (Table 3.4). In contrast to males. female reproductive success was not quite as skewed (Fig. 3.6). The proportion of females brought into captivity that did not reproduce ranged from 18 to 86%. Mean female reproductive success ranged from 2.43 (sd = 6.43) to 13.94 (sd = 8.07) offspring and the opportunity for sexual selection, I_f, ranged from 0.34 to 7.00. By dividing I_m by I_f. I determined that the opportunity for sexual selection is from 2.76 to 10.58 times greater for males than females (mean 5.97, sd = 3.24).

However, female water snakes appear not to reproduce in successive years and this is more likely to be a life history decision than a sexual selection issue (*i.e.* outcompeted by other females). In contrast. males who failed to reproduce probably were outcompeted by rival males. Therefore, it may be more appropriate to calculate I_f based only on females who reproduced. Mean female reproductive success of reproductive females per year in each marsh ranged from 8.50 (sd = 4.95) to 19.75 (sd = 4.80) offspring and the opportunity for sexual selection, I_f, ranged from 0.05 to 0.39. By dividing I_m by I_f, I determined that the opportunity for sexual selection is from 6.02 to

180.00 times greater for males than for females (mean 73.48, sd = 60.25) (Table 3.4). It is evident that the magnitude of these two values are quite different depending on which values of female reproductive success are used. However, both analyses revealed that the opportunity for sexual selection is greater in male than female water snakes.

Discussion

The small and isolated nature of my populations along with conspicuous behaviours (*i.e.* basking and mating) occurring in predictable locations, allowed rare insight into the mating system of a snake species. I found that traditional measures of mating success (*e.g.* field observations of mating) are not reliable indices of true reproductive success. My genetic results revealed that over half of the litters were fathered by multiple males. However, multiple paternity does not appear to greatly benefit females in terms of number of unfertilized follicles, stillborn offspring or total litter size. However, I found weak evidence that female reproductive success may be slightly increased by mating with more than one male. Since male reproductive success greatly increases by fertilizing more than one partner, and many males and females fail to reproduce in a given year, the mating system of this species can be classified as polygynandrous.

Are mating observations reliable indices of reproductive success?

Males become sexually mature at a snout-vent length of approximately 43.5 cm.

which corresponds to four years of age based on the age equation of Brown and Weatherhead (1999b). Although mature females are much larger (at least 60.85 cm SVL), their growth rate is faster, and they also mature at approximately four years of age (Brown and Weatherhead, 1999b). These values are roughly similar to those estimated by Weatherhead *et al.* (1995) based on the smallest males and females seen mating in the field. In contrast, it has been estimated that males and female water snakes in other populations mature at three years of age (Feaver, 1977; King, 1986). This discrepancy is probably a result of the reduced growing season due to higher latitude. This result suggests that water snakes do not engage in any sexual activity until they are capable of producing offspring. I found a significant correlation between whether a male was seen mating and whether he reproduced. This may suggest that primarily, only males who are physiologically capable of fertilizing females engage in mating activities. An unexpected result was the large number of males neither seen mating nor reproducing, since once males reach a large enough size to reproduce, they should be sexually active each year. However, perhaps the energetic costs of searching for and mating with females are too great for annual reproduction in males. Certainly, sperm production has recently been shown to be more costly than once believed. Olsson *et al.*, (1997) found that male adders lose mass while producing sperm, indicating a substantial investment of energy for this process. However, reproductive males do not lose more weight than non-reproductive males in *Nerodia sipedon* (Brown and Weatherhead, 1999a). A more intensive study needs to be conducted to determine whether reproduction in individual male water snakes occurs less frequently than once per year.

It is possible that I did not detect some reproductive success for some males due to not capturing all reproductive females in a marsh. However, upon careful evaluation of the history of the potentially reproductive females in the population who were not brought into captivity, I felt quite confident that I captured most who were capable of reproducing that year. For instance, in Beaver Marsh in 1996, there were 36 females of reproductive size. Of these, 17 were brought into captivity, and one was found dead with follicles (see methods). Of the 18 that were not taken into the laboratory, two were found dead during or after the mating season. Three others were only captured once ever (after the mating season) and therefore, may have been transient residents. Two females gave birth the year before, and since I suspect that females of this species do not reproduce in successive years, I assume that they were not reproductive. Of the remaining eleven females who were not taken into captivity, only three were observed to have been courted by males in the field. Furthermore, since reproductive females need to maintain high body temperatures to develop their follicles (Brown and Weatherhead, 1999c), my sampling at this time of year was likely to be biased toward reproductive females. Therefore, I am quite confident that many of the females that were not maintained in the laboratory were non-reproductive. Although I found a correlation between whether males were seen mating and whether they reproduced, I found that of 156 dyads of males and females seen together in the field, only seven produced offspring together (4.5%). This further confirms that mating observations not reliable predictors of reproductive success.

Of males seen mating and reproducing, there was no significant correlation between the number of times seen mating and the number of offspring fathered. This is in contrast to theory which predicts that males who mate more should father more offspring (Trivers, 1972). This inconsistency between behavioural and genetic data could arise for various reasons. For instance, it is very difficult to observe intromission in this species in the wild since often portions of the individuals in mating pairs or aggregations are obscured by vegetation or logs. In addition, females must accept courtship by the male by opening their cloacae (Devine, 1984). For these reasons many of the observations for which I assumed that mating had occurred may simply have been courtship attempts by males which may not have resulted in successful matings. In addition, since we have very little idea of how sperm competition operates in most reptile species, I cannot predict how it might have affected paternity in this study. Overall, these results imply that although mating observations may predict which males are likely to reproduce, they are not reliable in estimating reproductive success.

There was no correlation between whether a female was mating and whether she reproduced. The fact that some females who were never seen mating reproduced demonstrates that there were some matings that I did not observe. In addition, there were several females who were seen being courted yet failed to produce a litter. This is unexpected since mating is costly in terms of time and energy spent, and conspicuousness to predators. However, there are three reasons why non-reproductive females may have remained near courting males. First, although mating may be costly, it may actually be less costly to mate than to be harassed by males (Rowe, 1992). This is unlikely in this species because on several occasions I witnessed females easily escape from courting males. Second, females may mate in a year in which they are physiologically incapable of reproducing, and then store the sperm for the following year. Although between-year sperm storage has been documented in many species of snakes (*e.g.* Fox, 1956; Halpert *et al.*, 1982; Schuett, 1982; Gist and Jones, 1987), there is no evidence that female water snakes are capable of long-term sperm storage. Direct evidence of sperm storage could have been obtained from our data if males who were recorded as dead in one year fathered offspring in the next. However, in none of my 52 cases of paternity did this occur. Third, the females may have actually rejected the male. This alternative is plausible since the initiation of mating necessarily is controlled by the female (Devine, 1984). Perhaps males are unable to discriminate between reproductive and non-reproductive females and may waste time and energy courting some non-reproductive females who ultimately reject them.

Incidence of multiple paternity

My findings that multiple paternity is a common natural occurrence confirm previous work using phenotypic, allozyme and DNA markers in many snakes (Gibson and Falls, 1975; Zweifel and Dessauer, 1983; Schuett and Gillingham, 1986; Stille *et al.*, 1986; Schwartz *et al.*, 1989; Barry *et al.*,1992; Höggren and Tegelström, 1995). I found evidence for multiple paternity in more than half (54%) of all northern water snake litters born in two populations over three years. This accumulating evidence for multiple paternity suggests that copulatory plugs are unlikely to play a role in sperm precedence. Even in garter snakes, in which copulatory plugs have been documented (Ross and Crews, 1977), multiple paternity occurs (Gibson and Falls, 1975; Schwartz *et al.*, 1989). Therefore, the function of copulatory plugs may be other than preventing additional male sperm from fertilizing the eggs. However, female snakes of many species are capable of between-season sperm storage (Fox, 1956; Gist and Jones, 1987). Therefore, multiple paternity may be the result of mating with two males in the same, or two consecutive, mating seasons. To distinguish between these two alternatives, females could be captured just prior to hibernation in the fall and mated in captivity the following spring. Any males not identified must have mated with the female prior to her capture.

My estimate for the incidence of multiple paternity is substantially lower than that of 86% obtained by Barry *et al.* (1992) using allozyme data from water snakes from the same location in 1990. This demonstrates the potential for annual variation in multiple paternity. In addition, my estimates varied substantially from year to year, but this variation was not found to be significant, likely due to small sample sizes.

Factors such as the length of the mating season, the density of snakes in the population, the operational sex ratio (Emlen and Oring, 1977) and/or the distribution of receptive females could affect the total number or rate of encounters between sexually active males and females (Barry *et al.*, 1992) and thus affect the opportunity for females to be fertilized by multiple males. However, for the duration of my study, neither the operational sex ratio, the density, nor the length of the mating season was correlated with the incidence of multiple paternity. I was unable to directly compare my results to those of Barry *et al.* (1992) in terms of these three variables, since they collected their behavioural observations and sex ratio data in a separate location from where they collected gravid females. Finally, the overall (*i.e.* all years pooled) incidence of multiple paternity was greater at Beaver Marsh, although this difference was marginally non-significant. This provided weak support of my prediction that there would be a higher incidence of multiply-sired litters at Beaver Marsh, since here, females were more clumped and predictably located and thus, easier for males to locate.

Adaptive significance of multiple paternity

One hypothesis regarding a potential benefit to females in mating with multiple males is that they do not receive enough sperm from one mating to fertilize all of their eggs (Gromko *et al.*, 1984). If this were true for this species, I would have expected to find that singly sired litters would have a smaller total litter size, and a larger proportion of unfertilized ovules than multiply sired litters. However, comparisons between singly and multiply sired litters revealed no difference in either of these measures. In contrast, female sexual selection gradients revealed a slight increase (*i.e.* nearly two offspring) with each additional mate. Together, these results provide weak evidence that females may not receive enough sperm from one mating to fertilize all of their eggs.

A second hypothesis is that mating with multiple males reduces the variance in the proportion of stillborn offspring. If females cannot discriminate close relatives from other males, mating with multiple males might reduce the risk that all of a female's litter would be fathered by a close relative. If inbreeding has a deleterious effect on the offspring, this is crucial. In a highly inbred population of the European adder, *Vipera berus*, Madsen *et al.* (1992) found that multiple mating reduced the mean proportion of stillbirths, rather than the variance. They interpreted this by suggesting that multiple mating promotes intrauterine sperm competition such that higher quality sperm are more likely to fertilize ova. However, I was not able to test this hypothesis because in the northern water snake, there are very few stillborn offspring (see Table 2.1). Furthermore, the few stillbirths that did occur were just as likely to have come from multiply as singly sired litters (data not shown).

Definition of mating system

Both female and male water snakes mate with more than one partner, on average. Despite the fact that female water snakes mate with multiple males (this study; Barry *et al.*, 1992), female reproductive success increases very little by doing so. In contrast, male reproductive success increases greatly by inseminating multiple females. Thus, I broadly define the water snake mating system as polygynandrous.

Reproductive success

Sexual selection theory predicts that in polygynous species, males should compete for access to females. For this reason, reproductive success should be more variable and more skewed than that of females (Trivers, 1972). In support of theory, I found male reproductive success to be quite skewed. At least 59% of males each year achieved no reproductive success while the rest fathered all of the offspring. In contrast to theory, many females (47%) also achieved no reproductive success. However, in this species, females do not seem to reproduce in successive years. Therefore, a large number of females that fell in this "zero" category may simply have been between reproductive years.

Measures of lifetime reproductive success are preferred to measures of seasonal reproductive success (Lande and Arnold, 1983; Arnold and Wade, 1984 a,b) for examining how sexual selection acts on specific traits. However, I felt that the data collected in this study were more conducive for estimating seasonal reproductive success. For example, the data were collected during only three field seasons. Not only is this a small proportion of the breeding lifespan of water snakes, but only two males reproduced in multiple years during this study. Because of this, calculating a mean reproductive success for males over the three years would be misleading. Hypothetically, a male may have been outcompeted in the first two years when he was small, but may have been highly successful when he was larger. This information would have been lost if I had combined all three years to estimate lifetime reproductive success. In addition, there was high overwinter mortality in males (50 % never seen again). For males present for all three years, a mean annual reproductive success can be calculated. However, the same cannot be done for males present for only one year because they were not present for the other two years. On the other hand, nor can their full value reproductive success be compared to the mean annual reproductive success of males present in multiple years. Therefore, I felt that the best approach for these data was to estimate seasonal, rather than lifetime, reproductive success.

My estimates of the opportunity for sexual selection for males and females fall within the range of those determined for a variety of taxa (Clutton-Brock, 1988). The opportunity for sexual selection has been calculated in only one other species of snake. Madsen and Shine (1994) calculated an I_m of 0.96 and I_f of 0.17 for the European adder, *Vipera berus*. Thus the opportunity for sexual selection is 5.64 greater in males than females, which is very similar to my mean value of 5.97. However, if only reproductive females are considered, the opportunity for sexual selection is over 70 times greater in males than females. To demonstrate variance in reproductive success is not adequate to imply that selection occurs on phenotypic traits (Sutherland, 1985; Fincke, 1988; McVey, 1988). Rather, it must be demonstrated that a trait(s) contributes to this variance. In the next chapter, I conduct such analyses using male morphological and behavioural traits and relate my findings to the evolution of the mating system, and the evolution of female-biased sexual size dimorphism.

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Table 3.1 - Numbers of adult and reproductive males and females for each year and population. Adult males are those who were larger than the smallest male to reproduce. Adult females are those who were seen mating in the field or reproduced in that year. Reproductive individuals were those who reproduced in that year.

			Adult	Reproductive
Barb's Marsh	1994	Males	39	9
		Females	12	6
	1995	Males	33	2
		Females	4	1
	1996	Males	29	13
		Females	17	17
Beaver Marsh	1994	Males	46	8
		Females	17	7
	1995	Males	40	9
		Females	17	8
	1996	Males	33	11
		Females	17	9

Table 3.2 - Numbers of water snake males and females with (+) or without (-) mating success and reproductive success. Mating success refers to whether they were seen attempting to mate in the field. Reproductive success refers to whether they produced offspring in the laboratory (females) or whether genetic work found them to have sired young (males).

Males

		Mating Success		
		+	-	Total
Reproductive	+	31	21	52
Success	-	63	105	168
	Total	94	126	220

Females

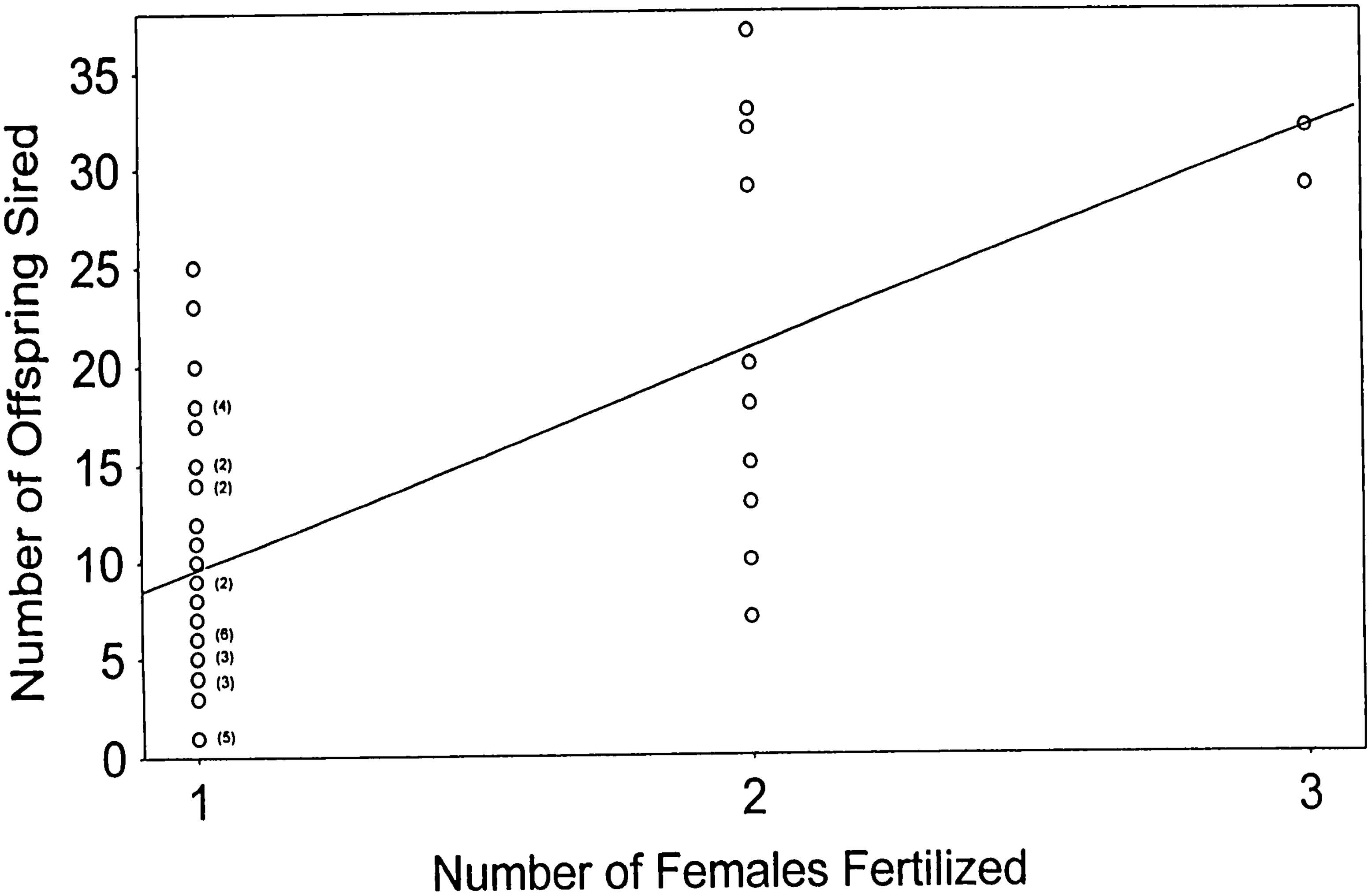
		Mating Success		
		+	-	Total
Reproductive	+	22	24	46
Success	-	15	18	33
	Total	37	42	79

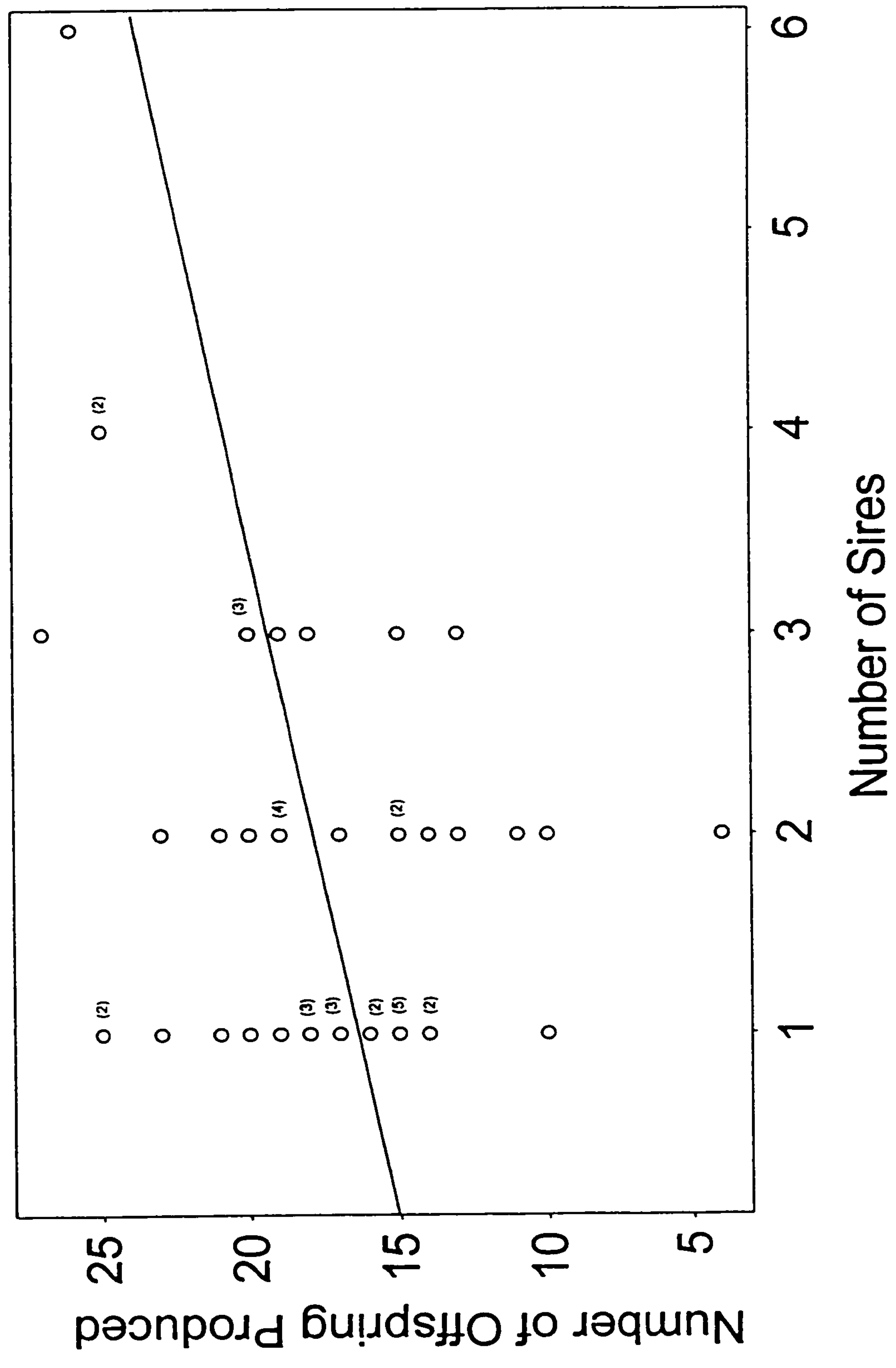
Table 3.3 - Incidence of multiple paternity of water snake litters and potential factors for each year and population. The incidence of multiple paternity (Inc. mp) refers to the proportion of multiply sired litters. The length of the mating season (LOMS) is represented by the interval between the first and last observed mating in the field. The operational sex ratio (OSR) is the ratio of sexually mature females to males (Emlen and Oring, 1977). Density is measured as number of adult snakes per hectare.

Site	Year	N	Inc. mp	LOMS (days)	OSR	density (#/ha.)
Barb's Marsh	1994	6	0.33	25	0.59	15.5
	1995	1	1.00	11	0.85	15.3
	1996	17	0.41	23	0.86	13.5
Beaver Marsh	1994	7	0.43	29	0.72	26.3
	1995	8	0.75	19	0.85	24.6
	1996	9	0.77	19	1.09	23.0
mean			0.54	21	0.83	19.7

Table 3.4 - Reproductive success and the opportunity for sexual selection of male and female water snakes. Zero RS refers to the proportion of males producing no offspring. RS refers to the number of offspring produced and is presented as mean ± SD. I_m and I_f is the opportunity for sexual selection for males and females, respectively (Arnold and Wade, 1984a).

		Males			Females			
Site	Year	zero RS	RS	I_m	zero RS	RS	I_f	I_m/I_f
Barb's	1994	0.77	2.45±5.26	4.60	0.50	17.33±4.32	0.06	76.67
	1995	0.94	0.51±2.24	19.29	0.86	8.50±4.95	0.34	56.74
	1996	0.59	8.10±12.43	2.35	0.18	19.58±12.17	0.39	6.02
Beaver	1994	0.83	2.20±6.60	9.00	0.42	19.29±4.27	0.05	180.00
	1995	0.75	1.90±4.40	5.36	0.33	19.75±4.80	0.06	89.33
	1996	0.73	2.91±6.20	4.50	0.53	15.38±5.70	0.14	32.14
mean		0.77±0.12	3.0±2.6	7.52±6.16	0.47±0.23	16.64±4.33	0.17±0.15	73.48±60.25


Number of Offspring Sired
0
5
10
15
20
25
30
35
(4)
(2)
(2)
(2)
(6)
(3)
(3)
(5)
1
2
3
Number of Females Fertilized


Number of Offspring Produced
Number of Sires
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10
15
20
25
1
2
3
4
5
6
(2)
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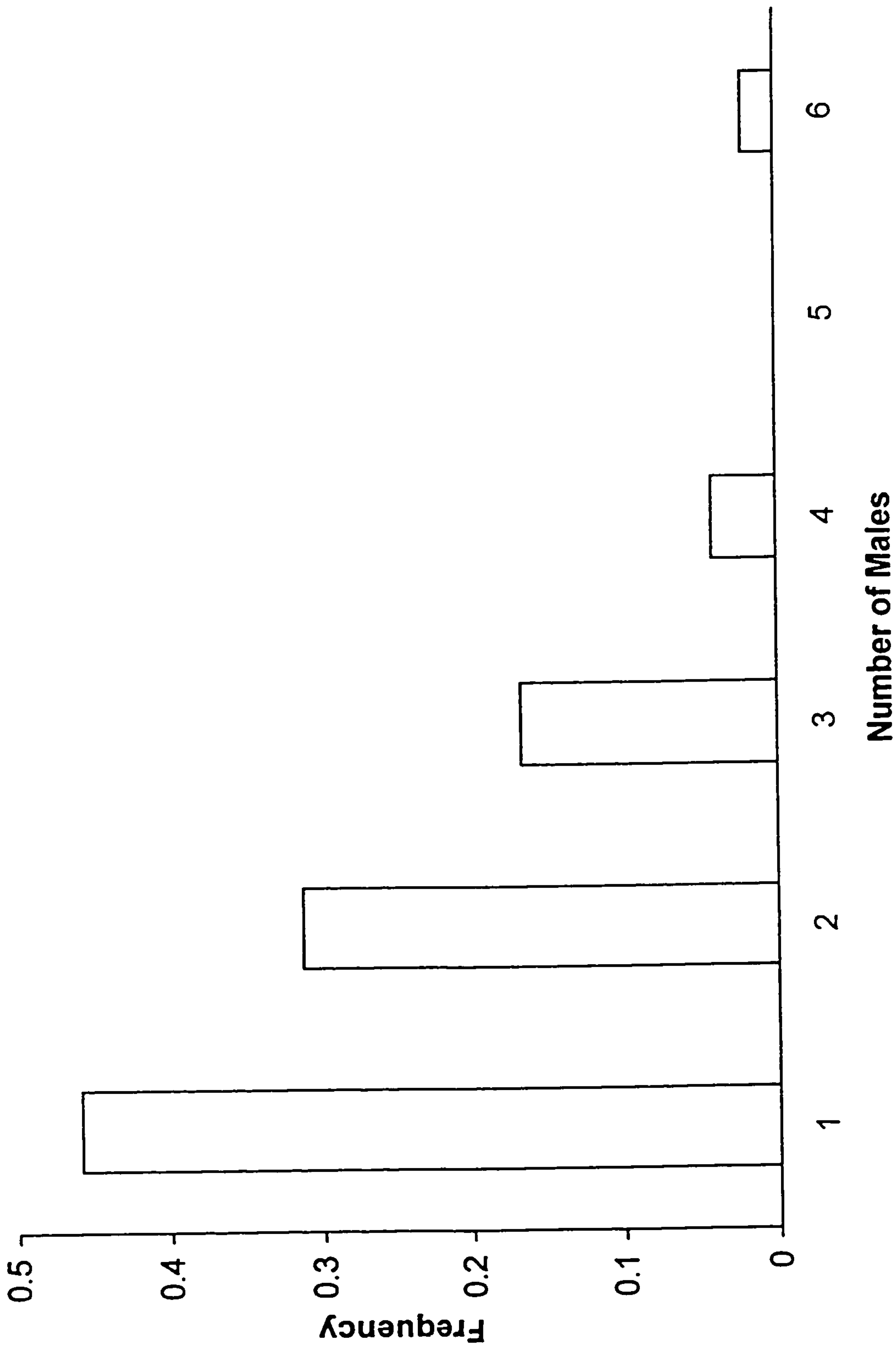
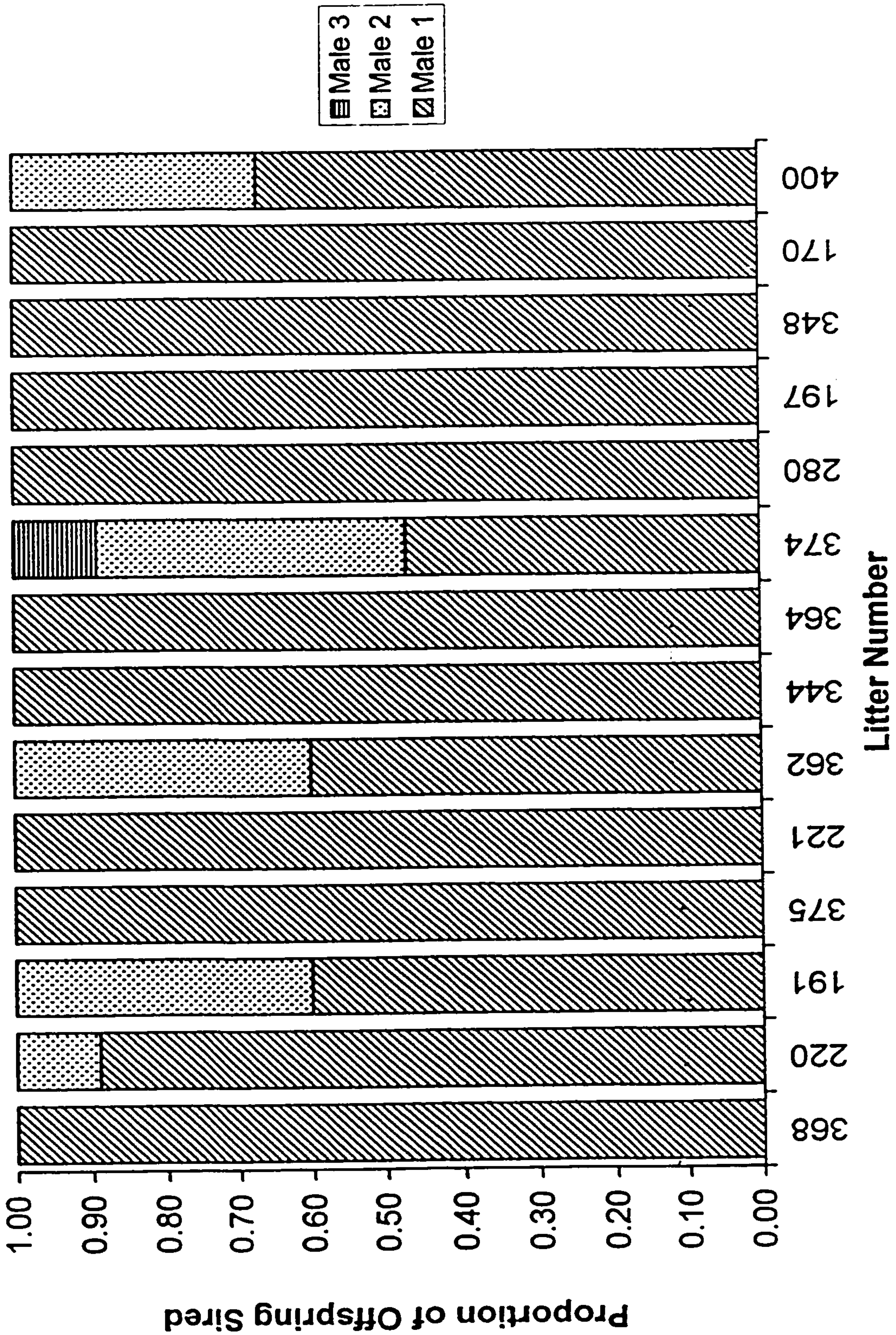
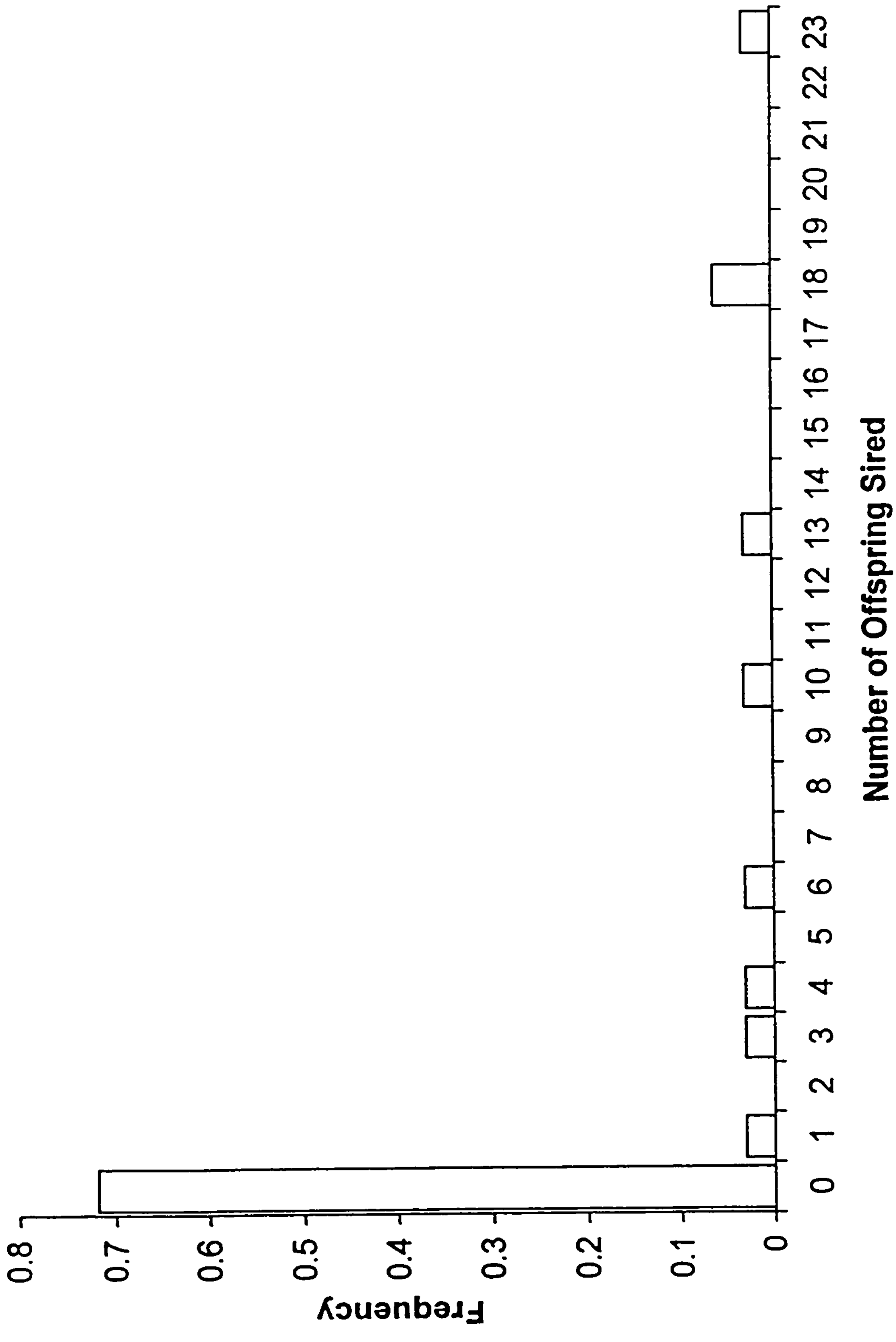
(2)


Frequency
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
Number of Males


▤ Male 3
▨ Male 2
▧ Male 1
Proportion of Offspring Sired
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Litter Number
400
170
348
197
280
374
364
344
362
221
375
191
220
368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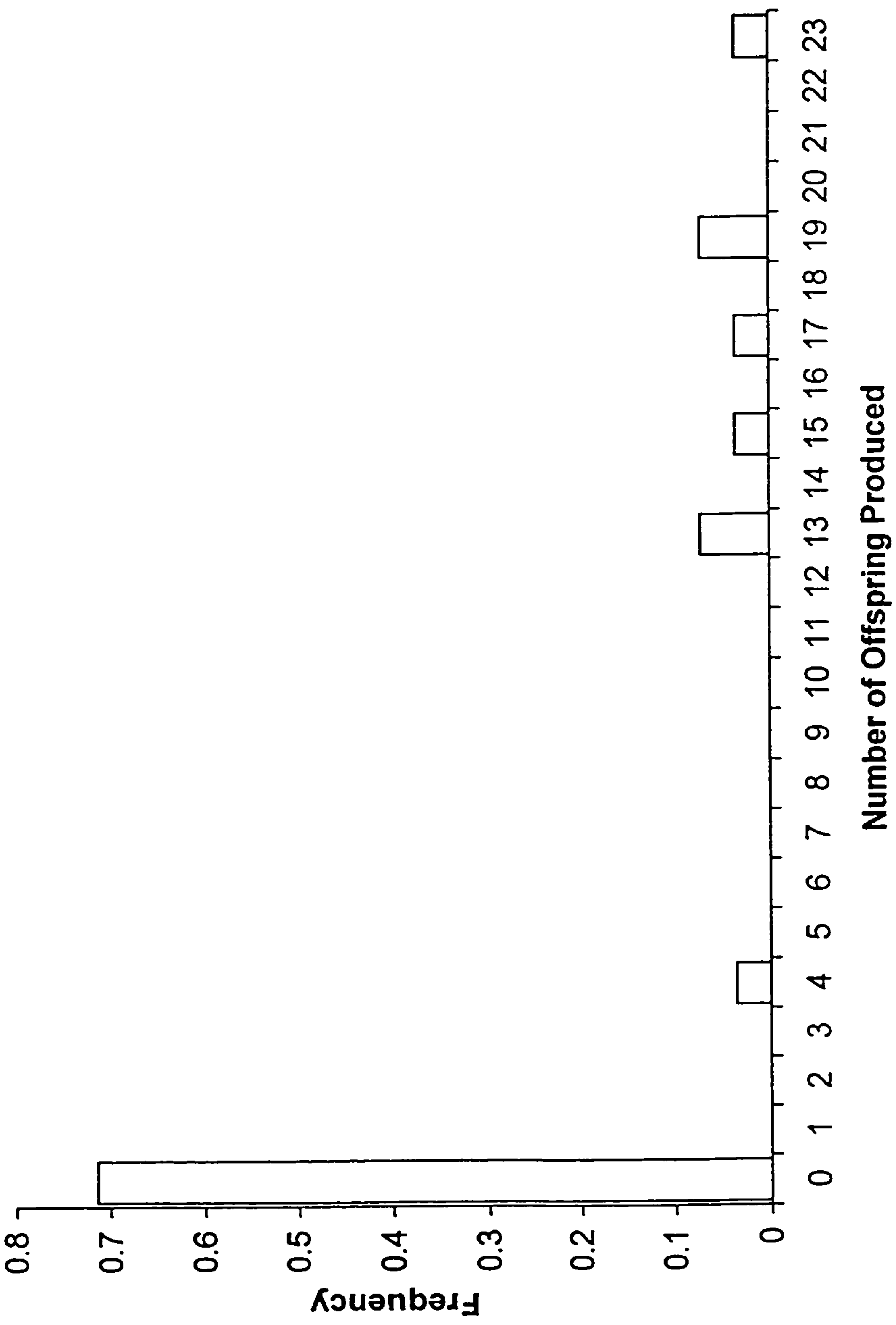
19
20
21
22
23
Number of Offspring Sired


Frequency
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0.1
0.2
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Number of Offspring Produced
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CHAPTER FOUR

Sexual selection on morphological and behavioural traits in male water snakes

Introduction

According to Darwin (1871), sexual selection arises from variance in reproductive success due to competition over mates. In populations of many species, relatively few males sire most of the offspring while many males achieve little or no reproductive success; thus the potential for sexual selection to operate on male traits can be relatively high (reviewed in Clutton-Brock, 1988). However, merely determining that variance in reproductive success exists is not sufficient evidence to infer that selection is acting on phenotypic traits. Rather, it must be demonstrated that specific traits contribute to this variation (Sutherland, 1985; Fincke, 1988; McVey, 1988).

Snakes lack conspicuous secondary sexual characters. However, body size varies among males and thus may be under sexual selection. As a consequence, body size has most commonly been investigated as a possible correlate of male reproductive success in snakes. In snakes in which males compete via direct combat (ritualized fights), large male body size is advantageous (Madsen *et al.*,1993; Schuett, 1997) and males tend to be larger than females in those species (Shine, 1978). Even in snake species in which males do not fight, but in which mating occurs in aggregations involving one female and several males, one would still expect a reproductive advantage associated with large male body size, because larger males should be more successful at displacing rivals from the aggregation.

Despite the apparent advantage for large male body size in many snakes, in two-thirds of all snake species females are larger than males (Shine 1978; 1993). Sexual size dimorphism (SSD) is thought to arise due to differences between males and females in the costs and benefits of various body sizes. As a result, natural and/or sexual selection may act to set optimal body sizes that are different between the sexes. Therefore, to understand SSD, one must not only explain why one sex gets large, but also determine the selective pressures that keep the other sex small (Montgomerie and Lundberg, 1989; Weatherhead *et al.*, 1995). Large size in female snakes is advantageous because female fecundity increases with body size (Brown and Weatherhead, 1997; but see Shine, 1988). Some researchers have suggested that genetic correlation between the sexes means that selection for increased female size could result in males increasing in size as much as females, in the absence of counterbalancing selection on males (Lande, 1980).

In northern water snakes, females are substantially larger than males despite strong competition among males for access to females (Weatherhead *et al.*, 1995). This size differential between males and females could be a consequence of natural selection, sexual selection, or both. Brown and Weatherhead (1999a) investigated how body size affects survival (*i.e.* natural selection) in northern water snakes. They found that males appear to survive slightly better than females, as indicated by population sex ratios becoming more male-biased among older snakes. They also found that mating appears to impose strong survival costs on males that give a slight advantage to intermediate-sized males. However, they concluded that this survival advantage was not sufficient to explain why males are so much smaller than females in northern water snakes, and that the explanation must lie with sexual selection. In this chapter, I determine whether sexual selection limits male body size by investigating how variation in body size affects male reproductive success in northern water snakes.

There are at least two potential ways in which sexual selection might favour small male body size in snakes. First, smaller males may be more mobile (Shine, 1978). Because male reproductive success in many snakes relies on the ability of males to locate receptive females (Madsen *et al.*, 1993), enhanced mobility should increase reproductive success. Second, in snakes, energy is limited and is required for growth, maintenance, movement and reproduction. Therefore, energy used for one function is not available for other purposes. Males who do not invest much energy in growth may be able to devote that energy to sperm production (Weatherhead *et al.*, 1995). Olsson *et al.*, (1997) demonstrated that male adders (*Vipera berus*) lose mass during sperm production. In contrast, Brown and Weatherhead (1999b) found no difference in mass loss between water snake males who were engaged in mating and those who were not. If males tradeoff growth for reproduction, smaller males may produce sperm of higher quality and thus may be more competitive in sperm competition. This could be particularly relevant to a species such as the northern water snake, in which multiple paternity, and thus multiple insemination, is common (Barry *et al.*, 1992; Chapter Three).

Few studies have investigated sexual selection in snakes, presumably due to the difficulty in obtaining adequate data on variation in male reproductive success to do so. Water snakes are well suited to such studies because they exist in isolated marshes (Chapter One) in fairly high densities (Feaver, 1977; Barry *et al.*, 1992) and most adults can be marked and sampled with relative ease (Chapter Two). In addition, females readily give birth in laboratory conditions (Barry *et al.*, 1992), so offspring also can be sampled easily. Brown and Weatherhead (1999b) took advantage of these features to investigate how sexual selection acts on male water snakes up to the point where males find receptive females. At the study site where females were relatively dispersed, both male condition and body size were positively correlated with male success. Males in this marsh also had relatively large home ranges during the mating season and home range size was correlated with male mating success. However home range size was not related to male size or condition. In the marsh in which females were relatively clumped, no male attributes explained significant variation in male success. Thus, the evidence up to the point of mating suggests that where sexual selection acts on body size in male water snakes, it favours larger males. Perhaps larger males are better able to outcompete smaller rivals to gain access to females. Therefore, if sexual selection limits male size in water snakes, it must do so after males have located females.

Brown and Weatherhead's (1999b) evidence that sexual selection favours larger males is consistent with data from other snake species (Madsen and Shine, 1993; Madsen *et al.*, 1993; Schuett, 1997; but see Joy and Crews, 1988). However, all of these studies have used estimates of mating success based on observations of mating attempts in the field, without any knowledge of whether these mating attempts result in the production of offspring. In Chapter Three, I found that the relationship between the number of times male water snakes were seen attempting to mate in the field and the number of offspring that they sired was at best slight. In this chapter, I use estimates of individual male reproductive success obtained by parentage analyses using microsatellite genetic markers to retest several of these hypotheses regarding how sexual selection acts on male water snakes.

In Chapter Three, I demonstrated that the opportunity for sexual selection (Arnold and Wade, 1984) was over seventy times greater for male than for female water snakes. Thus, my first objective in this chapter use multiple regressions of reproductive success on various male morphological characters to determine whether directional sexual selection acts on any of the traits that I measured. If smaller males are more competitive, I should find directional selection favouring smaller males. Alternatively, there may be an optimum size for males where males are large enough to dislodge rival males from aggregations, but small enough to be competitive in sperm competition. By including a quadratic term for SVL in my regression models, I can test whether stabilizing selection favours intermediate-sized males.

My second objective is to determine whether male attributes other than body size affect male reproductive success. Male water snakes appear to use their tails to stimulate females to mate and to displace rival males from aggregations (King, 1989; pers. obs.). Furthermore, males have proportionately longer tails than females. Thus, I test the prediction that males with longer tails are more successful. In addition, I test the prediction that males in better condition are more successful, either because they have more energy to spend finding mates, or because they can invest that energy in sperm production and thus succeed through sperm competition.

My third objective is to determine whether male activity during the mating season predicts male reproductive success. Male activity is represented by 1) home range size corrected for the number of observations (see methods this chapter; Brown and Weatherhead 1999b) and 2) the number of times the male was seen during the mating season. I test the prediction that both measures of male activity will be positively correlated with male reproductive success.

Very little is known regarding the role of mate choice in snake mating systems. Females may choose their mates according to one or several traits that I consider here. Alternatively, females may prefer male attributes that are unrelated to their morphology or behaviour. For example, if mating with genetically similar individuals results in inbreeding depression (Partridge, 1983; Charlesworth and Charlesworth, 1987), females may choose to mate with unrelated males. Olsson *et al.*, (1996) found that although female sand lizards, *Lacerta agilis*, appeared to mate at random, unrelated males sired more offspring. Therefore, the fourth objective is to investigate whether males and females that produce offspring together are less closely related than expected by chance.

I conducted my study in the same two marshes used by Brown and Weatherhead (1999b). Thus, I was able to take advantage of the fact that females were relatively clumped in Beaver Marsh and relatively dispersed in Barb's Marsh. Brown and Weatherhead (1999b) found that female dispersion affected male mate searching behaviour and the factors that affected males' ability to locate females. In Chapter Three I showed that female distribution in these marshes affected the frequency with which multiple paternity occurred. Thus, I expected that female dispersion might also affect other paternity patterns. Specifically, I predict that where females are more clumped (Beaver Marsh), and thus competition among males is more intense, any reproductive advantage to a male attribute (*i.e.* morphology, behaviour, genetics) should be more pronounced than where females are more dispersed (Barb's Marsh). I also consider the possibility that the intensity of male competition could have other effects on mating. Given that larger females are more fecund (Brown and Weatherhead, 1997; Semlitsch and Gibbons, 1982; Shine, 1988), males should preferentially mate with larger females if they have the opportunity. It is possible that when females are clumped, male choice is greater, so the most competitive males should disproportionately sire the offspring of the largest females.

Methods

Estimating Reproductive Success

Snakes were captured by hand from two marshes (Barb's Marsh and Beaver

Marsh) approximately 10 km from the Queen's University Biological Station in eastern Ontario in 1994-1996. For all individuals captured, I measured the total length, the tail length (the distance from the cloaca to the tail tip), and calculated the snout-to-vent length (SVL) by subtracting these two measures. In addition, I measured the mass and took a blood sample from each snake captured. To estimate male condition, I first used the formula of Weatherhead and Brown (1996) to estimate the fat-free wet mass of each male from its SVL. Then I determined the fat mass by subtracting the fat-free wet mass from the wet mass. Finally, I divided fat mass by the total mass to determine the relative fat content (percent fat). This method is preferred to conventional estimates of condition which are based on the residuals from a regression of mass on SVL, because it allows condition to vary non-randomly with body length (Weatherhead and Brown, 1996). Each adult was permanently marked with an electronic PIT tag (Anitech). In late July, females presumed to be gravid based on size and shape were taken into the lab until parturition. All neonates were collected, counted, measured and had a blood sample taken (see Chapters One to Three for more detail). My measure of reproductive success was the number of offspring sired by each male, as determined through paternity analysis using microsatellite loci (see Chapter Two). Other studies (*e.g.* Brown and Weatherhead, 1999b) have also used the number of mates as a measure of male mating success. However, since the number of females fertilized is highly correlated with male reproductive success (Chapter Three), I did not use it as an independent measure. Because only one female from Barb's Marsh gave birth in 1995, I excluded data from

Barb's Marsh in 1995 from the analyses.

Male characteristics

Because water snakes can grow in length substantially over the active season (Brown, 1997), my measurements of SVL for males captured after the mating season may not be indicative of their relative size during the mating season when they were competing for access to females. To compensate for this, I used the formula in Brown and Weatherhead (1999c) to adjust the SVL to the median capture date from measures taken earlier or later. I was unable to adjust tail length in this fashion, since there is no formula available. However, this character is so small that what little change may occur over the season would probably be less than the measurement error, and thus is not worth considering. In contrast, mass (and thus condition) is quite dynamic over the season. Because very little is known about the patterns of fluctuation in mass, I was unable to estimate mass early in the season from measures taken later. Therefore, for analyses involving condition, I only considered males captured prior to and during the first two weeks of the mating season. During this period of time, water snake males feed very little and therefore, mass (and condition) is unlikely to change substantially (Feaver, 1977; Brown, 1997). Seventy-two and 50% of all males were captured during this time for Barb's and Beaver Marsh, respectively.

Measuring selection

To measure the intensity of sexual selection on various male characters, selection differentials and selection gradients were calculated. All male characters were standardized to have means of zero and standard deviations of one. Relative reproductive success was estimated by dividing the individual's score by the mean reproductive success of the sample. There were several males whose tails were more than 3 cm shorter than all other males and did not taper to a point, which I judged to be due to loss of their tail tips. All analyses were conducted including and excluding these individuals. Since the results did not differ qualitatively between these two sets of analyses, I report only the results of the analyses with these individuals included. I conducted analyses for each year separately, and pooled across years, for each marsh. Few (38%) males were present in multiple years (only 1% was shown to have reproduced in multiple years), and those that were present grew between years. Therefore, each year that a male was present, the group of males with which he competed was different. For this reason, I felt justified in including each year as a separate event. All regression analyses were repeated using mass instead of condition. However, these results did not differ qualitatively from those involving condition. In addition, mass is highly correlated with SVL and tail length, but condition is not (see results). Therefore, I report the results of the regression analyses involving condition only.

Univariate Analyses

To determine whether SVL, tail length or condition influenced male reproductive success, I estimated selection differentials (which represent the mean value of a trait before and after selection) by determining the covariance of each trait and the number of offspring sired (reproductive success).

Multivariate Analyses

I compared traits between successful and unsuccessful males by performing logistic multiple regression analyses where success was the dependent variable (*i.e.* whether or not a male sired any offspring), and SVL, tail length and condition were the independent variables. Second, selection gradients were estimated as the standardized partial regression coefficient of each character from a multiple regression of relative reproductive success on each trait. To estimate directional selection, linear regressions were performed using relative reproductive success as the dependent factor and standardized values of SVL, tail and condition as the independent factors. The model was:

$$\text{relative reproductive success} = \text{intercept} + \beta_1 \text{ SVL} + \beta_2 \text{ tail length} + \beta_3 \text{ condition}$$

The coefficients (β_1, β_2, β_3) represent the intensity of directional selection on each trait, after the effects of correlated traits have been removed. A positive coefficient indicates that directional selection is acting to increase that trait. To measure the effect of stabilizing selection on body size, non-linear regressions were performed by introducing a squared term for SVL. The model for this regression was:

$$\text{relative reproductive success} = \text{intercept} + \beta_1\ \text{SVL} + \gamma_1\ \text{SVL}^2 + \beta_3\ \text{tail length} + \beta_4\ \text{condition}$$

The coefficient of the quadratic term (γ_1) represents stabilizing or disruptive selection acting on the traits. Stabilizing selection is inferred to be acting on traits if the sign (*i.e.* positive or negative) of the quadratic term is opposite to that of the corresponding linear term. For example, in the equation above, if β_1 is positive and γ_1 is negative, this indicates that stabilizing selection favours intermediate-sized males. Alternatively, if β_1 and γ_1 are both positive or negative, this indicates that disruptive selection is occurring. Quadratic terms were not included for tail length or condition because there was no a priori reason to expect that stabilizing selection might operate on these traits.

Variations of the analyses:

All analyses were repeated using three different variations of how the data were compiled. In the first variation of the analysis, relative tail length (tail corrected for SVL) was used, resulting in a reduced sample because some males had damaged tails and were excluded (see above). In the second variation, only males that sired offspring were included. in case unsuccessful males included individuals that had not attempted to mate for some reason, as opposed to having been unsuccessful. Third, for the analyses involving data pooled across years, a mean trait and reproductive success value were used for each male present in multiple years. Because none of these analyses produced results qualitatively different from the original analyses (*i.e.* those involving 1) absolute tail length, 2) all males larger than the smallest sexually mature male, and 3) males represented in multiple years), I do not present them here.

Male activity

I recorded the location of all snakes seen in the field during daily observations according to a grid system which divided the marshes into 20 m X 20 m quadrats (see Brown, 1999b). Any time a painted snake was sighted, I recorded its grid location. Male activity was measured in two ways. First, the home range size was calculated using the concave polygon method (see White and Garrot, 1990; Brown and Weatherhead, 1999b) for all males seen at least five times in at least three different quadrats that were not in a straight line. The center of each grid in which snakes were sighted was used as location reference. A microcomputer software package was used to measure the areas of home ranges (see Brown and Weatherhead, 1999b for more details). Because home range size is related to the number of times that a male was observed (Brown and Weatherhead, 1999b), I used the residuals from a regression of home range size on number of observations to estimate home range size. Second, the number of times that the male was observed in the field was also used as an estimate of male activity. However, some of the males were equipped with radio-transmitters as part of a telemetry study (Brown, 1997). Since these males were observed a disproportionate number of times, they were excluded from analyses involving number of observations. In addition, seven males in total were known to have died prior to the completion of the mating season and were also excluded, since the number of observations would be underestimated for these males. Both estimates of male activity level were investigated for their effect on reproductive success using logistic multiple regressions and multiple regressions, where reproductive success was the dependent variable, and SVL, tail length, condition and either home range size or number of observations were the independent variables. Home range size and number of observations could not be included in the same regressions because I had estimates of both of these parameters for too few males. However, because home range size had no evident effect on reproductive success (see results), I felt justified in removing this variable from subsequent analyses.

Mate choice

To determine whether water snakes avoid mating with relatives, I first generated relatedness scores (*i.e.* r values; (Queller and Goodnight, 1989)) as estimated using Kinship 1.2 (Goodnight *et al.*, 1997) for each pairwise combination a male and a female in each population, pooled across years. Therefore, individuals were included each year that they were present. I then compared the distributions of r-values of reproducing pairs of snakes and pairs of snakes who did not reproduce using a contingency table analysis in which the significance of the differences in the distribution of r values for reproductive and non-reproductive pairs was assessed using a randomization technique (STRUC subroutine of GENEPOP; Raymond and Rousset, 1995).

To determine whether water snakes mate assortatively with respect to body size, I calculated correlations (Spearman rank) between the SVL of each female and her mates for each year and marsh combination. I did not include data from Barb's Marsh in 1995, because the sample size was only one litter. To pool the data across years within a marsh, I transformed the SVL data such that the mean SVL was zero and the standard deviation was one. Thus each snake was given a SVL score that was relative to all others of the same sex and site and in the same year and then all years were combined for a given site.

Results

Variation in male characters

The median capture date within a year ranged from May 5 to May 13 at Barb's Marsh and May 19 to May 22 at Beaver Marsh. The means and standard deviations of all morphological characters are presented in Table 4.1. After the SVL of each male had been adjusted to represent the size of the individual on the median capture date for the year, the adjusted SVL ranged from 43.59 to 68.02 cm and 43.98 to 62.82 cm in 1994 and 1996, respectively, at Barb's Marsh. At Beaver Marsh, adjusted SVL ranged from 43.64 to 69.05 cm, 41.85 to 64.90 cm and 44.99 to 60.40 cm in 1994, 1995 and 1996, respectively.

Correlation among male morphological characters

All male morphological characters were normally distributed in all years and both populations (all $p > 0.0033$; true $p = 0.05$ adjusted to $p = 0.0033$ by sequential Bonferroni procedure; Rice, 1989). Thus, I looked for correlations among male characters within each year and population using parametric tests (Pearson correlation) and the sequential Bonferroni procedure (initial adjusted $p = 0.0017$). In both populations, adjusted SVL, tail length and mass were significantly correlated in all years (all $r > 0.499$; all $p < 0.001$), except that tail length was not significantly correlated with adjusted SVL ($r = 0.500$, $p = 0.006$) or mass ($r = 0.536$, $p = 0.03$) in Barb's Marsh in 1996, and adjusted SVL and tail were not significantly correlated in Beaver Marsh in 1994 ($r = 0.447$, $p = 0.002$). In both marshes, adjusted SVL, tail length and mass were significantly correlated using data pooled across years (all $r > 0.531$; all $p < 0.001$) (Table 4.2). Condition varied independently of all measures of body size (all $r < |0.423|$ all $p > 0.02$), except that it was negatively correlated with adjusted SVL in Beaver Marsh males in 1994 ($r = -0.615$, $p < 0.001$) and pooled across years ($r_s = -0.314$; $p = 0.004$). Since there is a high degree of correlation among these traits, it is necessary to measure selection using selection gradients which identify the effects of direct selection on a character independently of indirect selection due to correlated characters.

Sexual selection and morphological characters

To estimate the univariate effect of each morphological trait on reproductive success, I calculated the covariance between each male morphological character and reproductive success for each marsh and year, and for each marsh pooled across years (Table 4.3). Measures of condition were used only for males caught prior to or during the first two weeks of the breeding season. All p-values were adjusted using the Bonferroni procedure for multiple tests (Rice, 1989).

I found no significant covariation between male body size, tail length and condition and the number of offspring sired in 1996 at Barb's Marsh (all $s < |0.15|$, all $p > 0.40$), although there was a marginal non-significant covariance between reproductive success and SVL ($s = 0.495$, $p = 0.051$) and tail length ($s = 0.949$, $p = 0.04$) (adjusted level of significance $p = 0.0033$) at Barb's Marsh in 1994. However, since none of the characters covaried with reproductive success in Barb's Marsh pooled across years (all $s < |0.277|$, all $p > 0.09$), the marginally non-significant results in 1994 may have been largely influenced by a small number of males. At Beaver Marsh, none of the characters covaried with reproductive success in any year (all $s < |- 0.281|$, all $p > 0.21$), or pooled across years (all $s < |- 0.475|$, all $p > 0.28$). This suggests that male morphological characters had little effect on male reproductive success. To investigate this further, I examined all traits simultaneously, using multivariate regressions to estimate selection gradients, which account for correlations among traits and thus estimate only the direct selection on a trait (Lande and Arnold, 1983).

Multivariate logistic regression analysis revealed no relationship between whether males reproduced and any morphological character at Barb's Marsh for either year (SVL both $\beta < |0.066|$, $p > 0.73$; tail length both $\beta < |0.303|$, $p > 0.46$; condition both $\beta < |10.012|$, $p > 0.30$), or both years pooled (SVL $\beta = - 0.134$, $p = 0.18$; tail length $\beta = 0.256$, $p = 0.34$; condition $\beta = - 0.948$, $p = 0.84$). Similarly, at Beaver Marsh there was no relationship between whether males reproduced and SVL (all $\beta < |0.454|$, $p > 0.10$) or tail length (all $\beta < |1.794|$, $p > 0.05$) condition (all $\beta < |14.485|$, $p > 0.14$) for any year or all years pooled (SVL $\beta = 0.0008$, $p = 0.99$; tail length $\beta = - 0.117$, $p = 0.48$, condition $\beta = 2.537$, $p = 0.56$; Bonferroni adjusted level of significance $p = 0.0033$).

Similarly, using linear multiple regression analyses, I found no evidence for significant directional selection on SVL, tail length, or condition in either population in any year or pooled across years (all $\beta<|1.189|$, all $p>0.21$; Bonferroni adjusted level of significance $p = 0.01$) (Table 4.4). To test for stabilizing or disruptive selection acting on SVL, I performed non-linear regressions by introducing a squared term for SVL. Again, there was no evidence for directional selection acting on SVL, tail length or condition in either population, in any year or all years pooled (all $\beta < |0.740|$, $p > 0.14$). In addition, there was no evidence for stabilizing or disruptive selection acting on SVL in either population, in any year or all years pooled (all $\beta < |0.375|$, $p > 0.13$).

Male activity

I investigated the influence of each measure of activity (*i.e.* home range size and number of observations) on reproductive success independently by estimating the covariance between reproductive success and each trait. Due to the small number of males for which I had home range estimates, I could estimate covariance of home range size and reproductive success for each population pooled across years only. Home range size did not covary significantly with reproductive success at Barb's Marsh ($s = 0.344$, $p = 0.28$) or Beaver Marsh ($s = -0.558$, $p = 0.20$) (Table 4.3). However, the number of times males were seen in the field did covary with reproductive success at Barb's Marsh ($s = 0.523$, $p = 0.007$) and Beaver Marsh ($s = 0.475$, $p = 0.04$) (Table 4.3) (Fig 4.1 and 4.2). To investigate this relationship further, I included these measures of activity in multiple regressions where reproductive success was the dependent variable and the measures of activity and morphological characters were the independent variables. Since there were few (<10) males for which I had estimates of home range size and number of observations, I was unable to include both measures of activity in the same regressions. However, since the univariate results indicated no effect of home range size, I felt justified in removing this variable from the regressions involving the number of observations.

Logistic regressions and selection gradients

At Barb's Marsh, there was no relationship between whether or not males sired offspring and SVL, tail length, condition (all $\beta < |-0.928|$, all $p > 0.20$), or home range size ($\beta = 660.583$, $p = 0.12$). Similarly, at Beaver Marsh, no morphological character affected whether or not males were successful (all $\beta < |- 19.584|$, all $p = 0.16$); nor did home range size ($\beta = - 798.578$, $p = 0.14$). There was no relationship between the number of offspring produced and any morphological character (all $\beta < |1.34|$, all $p > 0.18$), or home range size ($\beta = 0.541$, $p = 0.18$) at Barb's Marsh or Beaver Marsh (morphological characters all $\beta < |1.404|$, all $p > 0.32$; home range size $\beta = - 0.460$, $p = 0.44$).

In contrast, the positive relationship between the number of observations and whether males produced offspring at Barb's Marsh approached significance ($\beta = 0.173$, $p = 0.06$) and Beaver Marsh ($\beta = 0.185$, $p = 0.056$). However, none of the morphological traits tested in this model influenced male success at Barb's Marsh (all $\beta < |4.831|$, $p > 0.18$) or Beaver Marsh (all $\beta < |8.991|$, $p > 0.32$) (all years pooled). Similarly, multiple regression analyses revealed that the relationship between the number of offspring produced and the number of field observations just failed to attain statistical significance for males in Barb's Marsh ($\beta = 0.038$, $p = 0.058$) and Beaver Marsh ($\beta = 0.036$, $p = 0.098$). Again, no morphological traits tested in this model affected reproductive success at Barb's Marsh (all $\beta < |1.074|$, $p > 0.17$), or Beaver Marsh (all $\beta < |0.957|$, $p > 0.47$).

I then assessed whether being active imposed a cost on males. To determine whether more active males lost more weight during the mating season, I used all males for whom I had two measures of mass within the mating season. Because mass in snakes can increase rapidly due to a recent large meal (pers. obs.), I only included males whose measurements were at least two weeks apart. I calculated the mean rate of change of mass by subtracting the two measures of mass and dividing by the number of days between samples. Analyses were performed for data pooled across years for each population due to the small number of males for which I could calculate change in mass (N = 14 for Barb's Marsh, N = 24 for Beaver Marsh). There was no correlation between the number of times males were observed and the rate of change of mass for Barb's Marsh ($r_s = 0.0033$, $p = 0.99$) or Beaver Marsh ($r_s = - 0.231$, $p = 0.28$). Similarly, there was no correlation between the number of observations and the condition of the male at the beginning of the season for Barb's Marsh ($r_s = 0.156$, $p = 0.59$) or Beaver Marsh ($r_s = - 0.025$, $p = 0.91$).

To determine whether there may be a survival cost for reproductive males, I performed a 2 x 2 contingency analysis using whether or not males reproduced as one variable and whether or not they were ever seen again after the mating season as the second factor. Because of the apparent rarity of dispersal (Chapter One), I assumed that if males were never seen again that they had died. Because I have no information regarding male survival for after my study period, I was not able to make this comparison for males who reproduced in 1996. I found no relationship between whether males reproduced and male survival in Barb's Marsh in 1994 ($\chi^2 = 0.17$, $p = 0.67$), or in Beaver Marsh in 1994 ($\chi^2 = 0.47$, $p = 0.25$) or in 1995 ($\chi^2 = 0.09$, $p = 0.38$). Similarly, a logistic regression with whether or not males were seen after the mating season as the dependent variable and the number of times males were observed during the mating season as the independent variable revealed that males who were seen most often were not less likely to survive until the following year (all $\beta < 0.167$, $p > 0.19$). These results suggest that there may not be a survival cost for "active" males.

Mate choice

Figure 4.3 and 4.4 show the frequency distribution of r-values for reproductive and non-reproductive pairs in Barb's and Beaver Marsh, respectively. Mean values for reproductive and non-reproductive pairs were similar for both marshes (- 0.039 and 0.014, and 0.073 and 0.005, for Barb's and Beaver Marsh, respectively). There was no significant difference between the distributions of relatedness scores of reproducing pairs and the remainder of the population at Barb's Marsh ($p = 0.64$), or Beaver Marsh ($p = 0.16$), which suggests that water snakes mate at random with respect to degree of relatedness.

At Barb's Marsh, the SVL of females was significantly correlated with that of their mates only in 1994 ($r_s = 0.75$, $p = 0.02$), although in 1996, the correlation approached significance ($r_s = 0.72$, $p = 0.055$). In addition, the pooled data for Barb's Marsh also revealed a significant correlation between female and male SVL ($r_s = 0.58$, $p = 0.0006$) (Fig. 4-5). In contrast, at Beaver Marsh, I found no such correlation in any given year (all $r_s < 0.33$, $p>0.08$). In fact, in two of the three years, the correlation was negative. Similarly, there was a non-significant negative correlation between male and female SVL in the pooled data ($r_s = -0.23$, $p = 0.23$) (Fig. 4-6). This suggests that assortative mating with respect to size may occur at Barb's Marsh but not at Beaver Marsh.

Discussion

In Chapter Three, I demonstrated that the opportunity for sexual selection was substantially greater for male than female water snakes. Therefore, the main objective of this chapter was to determine whether any male traits contributed to the variance in male reproductive success. The main contributions of this chapter are: 1) there was no evidence for selection on the morphological traits measured in this study; 2) there was evidence for positive size assortative mating at one marsh and; 3) male activity may influence male reproductive success. I discuss each of these results below.

I found no correlation between male reproductive success and male body size, tail length or condition. These results are consistent with those of Joy and Crews (1988) who found that neither body size nor weight influenced mating success in red-sided garter snakes, *Thamnophis sirtalis parietalis,* whose mating system is similar to that of water snakes (*i.e.* also mate in aggregations). In contrast, larger male grass snakes, *Natrix natrix*, were more successful in mating aggregations in captivity (Madsen and Shine, 1993), while male water snakes, *Nerodia sipedon,* with longer SVL and in better condition were more likely to be observed in mating aggregations in the field (Weatherhead *et al.*, 1995; Brown and Weatherhead, 1999b).

The results of this study suggest that, in contrast with my predictions, the morphological traits measured do not affect a male's competitive ability in mating aggregations. In addition, females do not appear to discriminate among potential males based on these characters. In the absence of parental care, the only benefit that female water snakes may be able to derive from males is "good genes". Therefore, if female water snakes are able to discriminate among potential mates, this suggests that none of the morphological traits measured in this study are reliable indicators of male quality.

Alternatively, female mate choice may not be possible in water snakes. Although female distribution, and thus, male competition differs between the two study sites, the mating system at both sites appears to be dominated by scramble competition. In this form of competition, mate searching is typically more important than direct interaction among males. Spatial scattering of females (*e.g.* Barb's Marsh), a key feature of scramble competition, is thought to promote non-aggressive mate searching by males (Alcock, 1980; Schwagmeyer, 1988). In contrast, where females are clumped (*e.g.* Beaver Marsh), males congregate where females are located. Therefore, once these "hotspots" are found, male mate searching is likely to play little or no role in acquiring mates. Although the mating system here can be quite different from that at Barb's Marsh, scramble competition also describes mating systems in which females are concentrated and male density is high (*e.g.* "explosive breeding assemblage"; Thornhill and Alcock, 1983; Schwagmeyer, 1988). In this situation, males and females are likely to mate whenever possible and may have little or no opportunity to exercise mate choice. Therefore, selection may not act on particular morphological traits at either site because these traits do not give males a reproductive advantage through male competition or female choice in either form of scramble competition.

However, there may be several reasons for lack of detection of selection when it actually exists (Endler, 1986). For instance, the power of regression analyses in detecting statistical significance is largely dependent on sample size. In this study, there were 67 Barb's Marsh males included in the analyses but only 21 of these males achieved reproductive success. Similarly, 118 Beaver Marsh males were included but only 27 sired offspring. Some of my selection coefficients were similar in magnitude to those found to be significant by Gibbs and Grant (1987) and Brown and Weatherhead (1999b). The difference may be that these other studies boasted substantially higher sample sizes (> 300 finches; Gibbs and Grant, 1987; 225 water snake mating aggregations, Brown and Weatherhead, 1999b). Therefore, if I had doubled or tripled my sample size, I may have been able to detect statistically significant sexual selection acting on body size or tail length.

The selection gradient for SVL was consistently positive at Barb's Marsh (*i.e.* each year and both years pooled), but negative at Beaver Marsh (each year and all years pooled), which also supports the hypothesis that selection is acting on body size but my statistical analyses failed to detect it. This also suggests that selection may slightly favour large male body size where competition is less intense, but small male body size where competition is stronger. This is consistent with my findings of positive size-assortative mating at Barb's Marsh (see below). Perhaps larger males sire proportionately more offspring because they selectively pair with larger, more fecund, females. However, I cannot explain why small male size may be favoured at Beaver Marsh. Small male size does not enhance mobility, nor are small males more likely to court females (Brown and

Weatherhead, 1999b).

In addition, the selection coefficient for tail length was positive in each year and all years pooled at both sites, with the exception of males in 1996. which suggests that sexual selection may favour males with longer tails. Tail length appears to play an important role in courtship (King, 1989; pers. obs.). Thus. perhaps males with longer tails are better able to align their cloaca with that of the female, or stimulate the female to mate. In contrast, the direction of the selection coefficient for condition varied across years. Therefore, it is unlikely that selection acts on condition. To determine whether these patterns of selection on male body size and tail length are real, a larger sample size is required.

Finally, selection may act on these traits intermittently due to variation in the physical and social environment, but was not operating in the years of this study due to benign conditions (Endler, 1986). Long-term studies which monitor patterns of selection over a broad range of conditions are necessary to assess this possibility.

Assortative mating

Despite the lack of significant correlation between male body size and male reproductive success, water snakes appeared to exhibit positive size assortative mating at one marsh (Barb's Marsh). In some species, positive size assortative mating may occur because partners of similar size may be more physically compatible during copulation. Brown (1993) demonstrated that the degree of size-match between pairs of leaf beetles, *Trirhabda canadensis*, was correlated with the probability of successful intromission. Although water snakes are capable of mating with individuals that are not necessarily similar in body size, as evidenced by the apparently random (with respect to body size) mating exhibited at Beaver Marsh, perhaps mating between size-matched pairs is more efficient. Because mating is conspicuous and therefore likely to be costly in this species due to increased risk of predation (Ross and Crews, 1977), short durations of courtship and mating should be favoured. Male water snakes are unlikely to decline any mating opportunities. However, females may choose to mate with similar-sized males, when possible. Therefore the positive size-assortative mating exhibited at Barb's Marsh, where male competition was relatively low, may have been due to overt mate choice by females which may have resulted in enhancing the efficiency, and thus reducing the duration, of mating. However, at Beaver Marsh, females are more frequently courted simultaneously by multiple males (Brown, 1997; pers. obs.) than are Barb's Marsh females. Thus, Beaver Marsh females may not be able to accurately assess the body size of all suitors. In fact, although one male may be successful in stimulating a female to mate, she may ultimately mate with another male in the aggregation. For this reason, females at Beaver Marsh may not be able to exert preference for similar-sized males. Tests of the idea that there is an advantage to size-assortative mating would require: 1) assessing the duration of copulations between males and females that vary in the degree to which they are matched in body size, and 2) determining if there is a cost, in terms of increased predation, for example, for pairs with longer mating durations.

Encounter rate with females

Males with larger home ranges were not more successful than males with smaller home ranges. However, there was weak evidence that a male's general "activity" level may influence his reproductive success. Males who were seen basking, swimming or mating in the field more often were more likely to reproduce. Furthermore, there was a positive correlation between the number of times observed and the number of offspring sired. Together, these results suggests that ranging *per se* is not as important as general activity level. Females are relatively stationary (Barry *et al.*, 1992; pers. obs.) and therefore, once males have discovered where females tend to bask, they may not need to move much, particularly in populations in which several females bask in close proximity (*e.g.* Beaver Marsh). However, it appears that reproductive males are more likely to be involved in conspicuous activities (*e.g.* swimming, basking, or attempting to mate). Despite this tendency, there was no evidence of a survival or energetic cost associated with being more active. In addition, large body size or condition is not correlated with the proclivity to be more active. Perhaps "active" males are more successful simply because they are more likely to encounter receptive females. Alternatively, "activity" level may operate as an indicator of male quality or vigour which females can use to discriminate among potential mates.

Brown and Weatherhead (1999b) demonstrated that male body size in water snakes is related to the number of times that males were observed attempting to mate in the field. Thus, sexual selection appears to favour larger male water snakes up to the point of mating with females. However, according to my results, this advantage does not appear to persist after males have located females. This suggests that although few smaller males are seen courting females, some do successfully mate. Therefore, small males must secure matings less conspicuously. Even though larger males may be more successful in displacing rival males from aggregations, small males are often seen within two meters of a mating pair or aggregation (Brown, 1997; pers. obs.). Because female water snakes often produce multiply sired litters (Barry *et al.*, 1992; Chapter Three), these smaller males may attempt to mate with females after larger males have left the aggregation.

Finally, one objective in this chapter was to determine whether sexual selection acting on male body size could explain the evolution or maintenance of female-biased SSD in northern water snakes. I found no evidence that sexual selection was operating on body size or any other male morphological characters measured, suggesting that selection may not act to limit male size in water snakes through a reproductive advantage of smaller males. Given the lack of a relationship between male body size and male reproductive success, I suggest that female-biased SSD may simply be a consequence of the fecundity advantage enjoyed by larger females outweighing the cost of growing larger than the optimum size for both sexes which is set by natural selection (Price, 1984).

In relation to this idea, Brown and Weatherhead (1999a) investigated size-specific survival in male and female water snakes. They found that beyond 53 cm SVL, females incurred greater mortality, but found no such size effect on male survival. However, in keeping with my hypothesis above, few males exceed this optimum body size and thus, selection against large size in males would be difficult to detect because of small sample sizes. My idea also requires that the genetic correlation between the sexes be less than one, which remains untested in snakes.

This study was the first to use molecular techniques to estimate sexual selection gradients on male characteristics and I found little evidence of selection acting on males. However, some of my results require additional testing. For instance, the coefficients for selection gradients on SVL and tail length were large, yet my analyses did not detect statistically significant selection acting on these traits. In addition, the selection gradient for the number of field observations per male was positive, but marginally non-significant, while univariate analysis revealed a significant covariance between the number of observations and reproductive success. To determine whether any of these traits do, in fact, affect reproductive success, I suggest that: 1) a larger sample size is required and 2) the study should be conducted over several years so that the effect of selection on these traits can be tested over a broader range of ecological conditions. The next step might be to study selection in a semi-natural environment (in large enclosures), where ecological factors such as density and sex ratio can be controlled, and thus, the effect of these factors determined. In addition, the researcher can control the attributes of males competing against each other by matching them in all but one trait, and/or by ensuring that the traits of the males competing in the semi-natural environment fall along the entire range of those of males in nature.

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Table 4.1 - Summary Statistics for a) male morphological and b) behavioural characteristics for each population and year.

Values presented are means with standard deviations in parentheses.

	Barb's Marsh		Beaver Marsh		
	1994	*1996*	*1994*	*1995*	*1996*
median capture date	May 5	May 13	May 22	May 19	May 21
N	38	29	46	40	32
a) Morphology					
SVL(adjusted, cm)	51.4 (5.6)	51.1 (4.9)	53.9 (6.1)	52.0 (5.4)	49.9 (5.2)
tail length (cm)	18.3 (2.0)	17.0 (2.7)	18.0 (2.6)	17.0 (2.8)	16.9 (2.4)
condition	0.18 (0.08)	0.20 (0.07)	0.19 (0.09)	0.18 (0.08)	0.20 (0.07)
N (condition)	29	19	14	17	25
b) Behaviour					
Number of Observations	5.38 (3.61)	5.52 (4.71)	4.15 (3.06)	3.34 (3.63)	4.21 (3.66)
Home Range Size	0.0006 (0.0020)	0.0020 (0.0027)	- 0.0007 (0.0016)	- 0.0016 (0.0013)	- 0.0022 (0.0014)
N (home range size)	8	11	8	8	5

Table 4.2 - r values (Pearson correlations) among morphological characters in male water snakes for a) Barb's Marsh and b) Beaver Marsh populations, pooled across years. Significance of correlations is represented by p values in parentheses. Values in bold font represent those correlations that were significant at the 0.008 level (Bonferroni adjustment to 0.05 level; Rice, 1989).

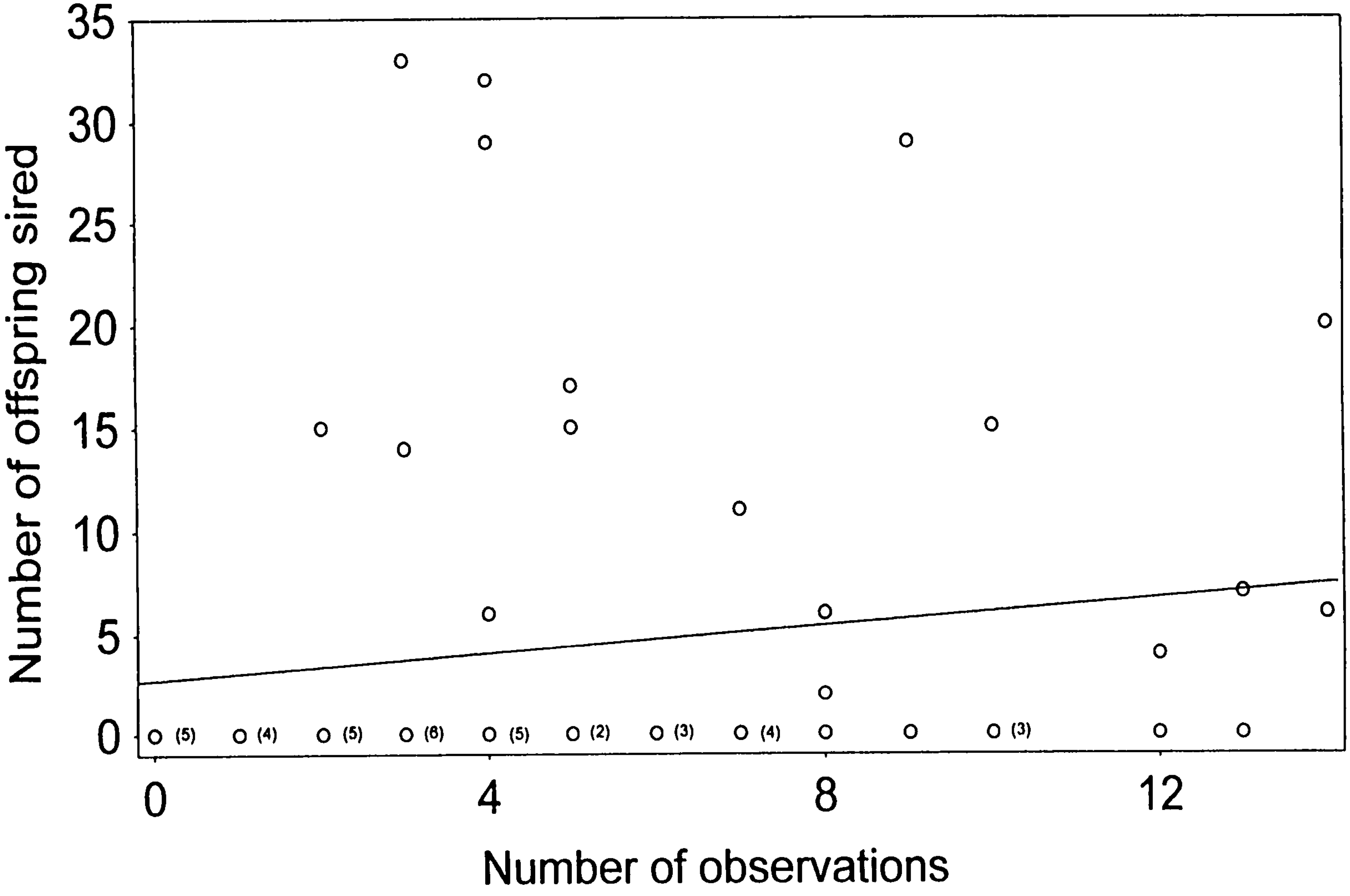
	AdjSVL	tail length	mass
a) Barb's Marsh			
tail length	**0.703**	-----	
	(<0.001)		
mass	**0.930**	**0.678**	-----
	(<0.001)	**(<0.001)**	
condition	- 0.142	- 0.042	0.159
	(0.28)	(0.75)	(0.23)
b) Beaver Marsh			
tail length	**0.533**	-----	
	(<0.001)		
mass	**0.944**	**0.531**	-----
	(<0.001)	**(<0.001)**	
condition	**- 0.314**	- 0.092	- 0.063
	(0.004)	(0.41)	(0.57)

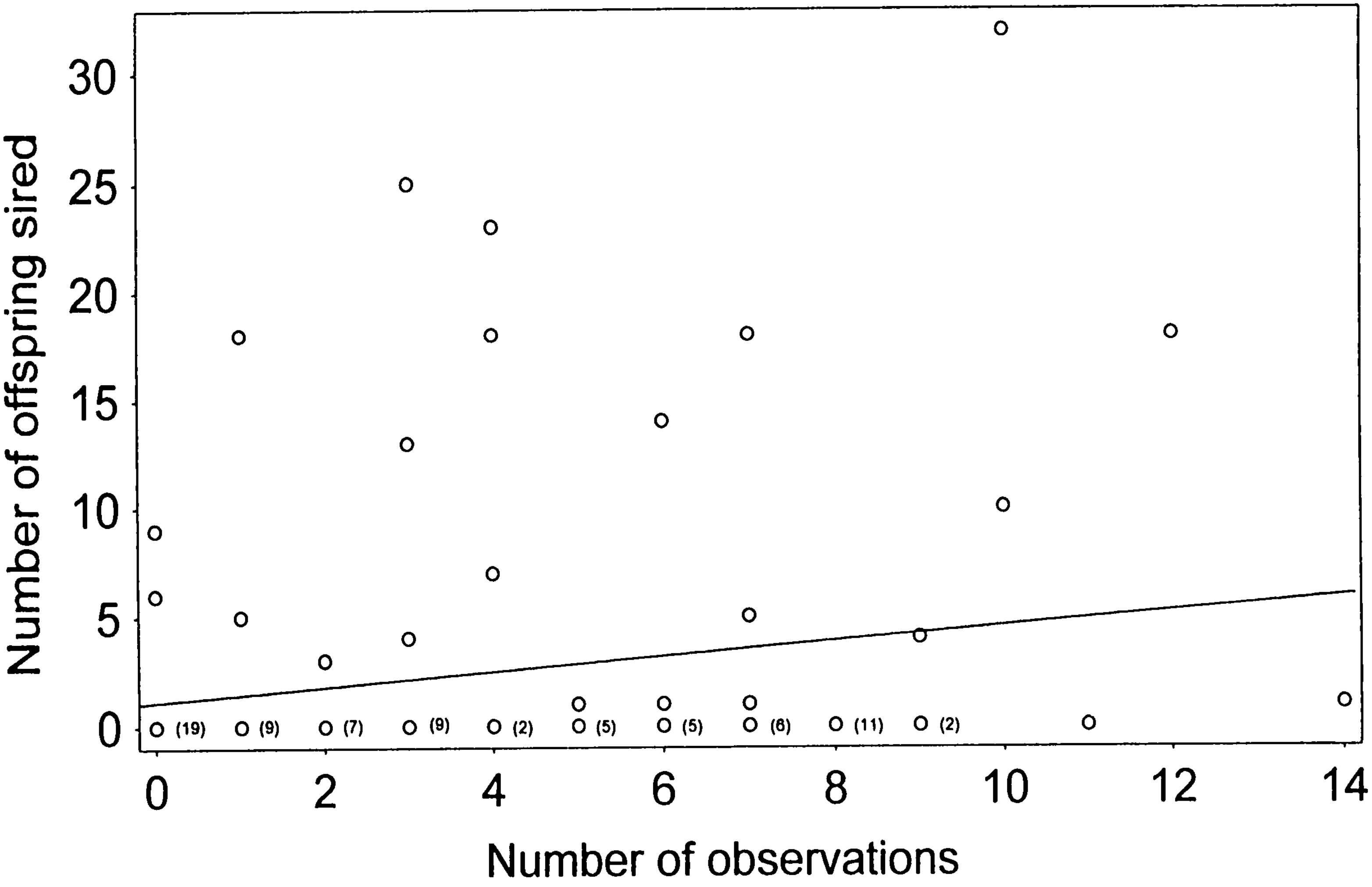
Table 4.3 - Selection differentials of morphological and behavioural traits of male water snakes from Barb's and Beaver marsh.

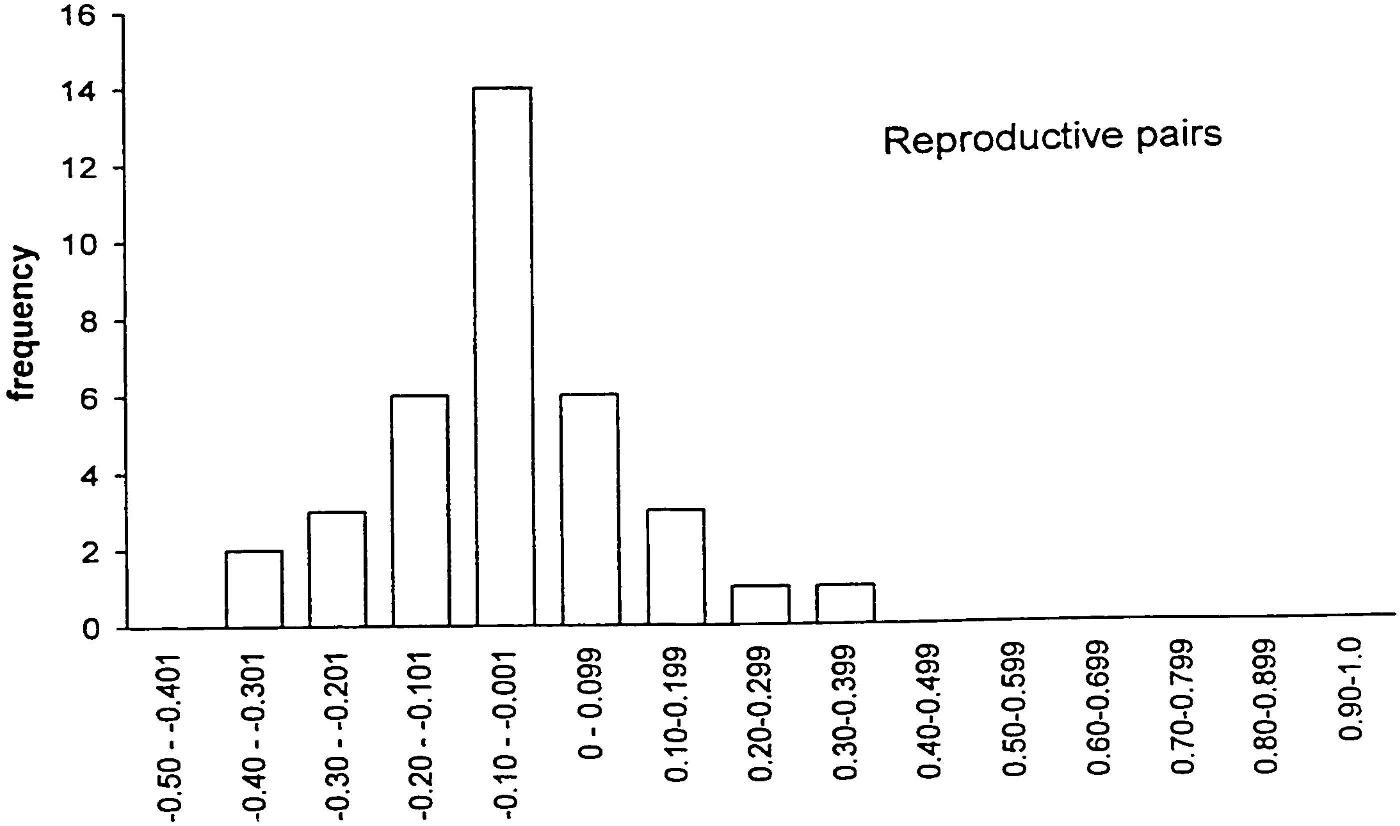
	Barb's			Beaver			
	1994	1996	pooled	1994	1995	1996	pooled
SVL	0.495	0.018	0.277	0.108	- 0.018	- 0.254	- 0.101
tail length	0.494	- 0.084	0.189	0.142	- 0.225	0.210	0.104
condition	- 0.266	0.150	- 0.099	- 1.061	- 0.200	- 0.281	- 0.475
home range size			0.280				- 0.558
# observations	- 0.008	0.597	0.523	0.812	0.820	- 0.081	0.475

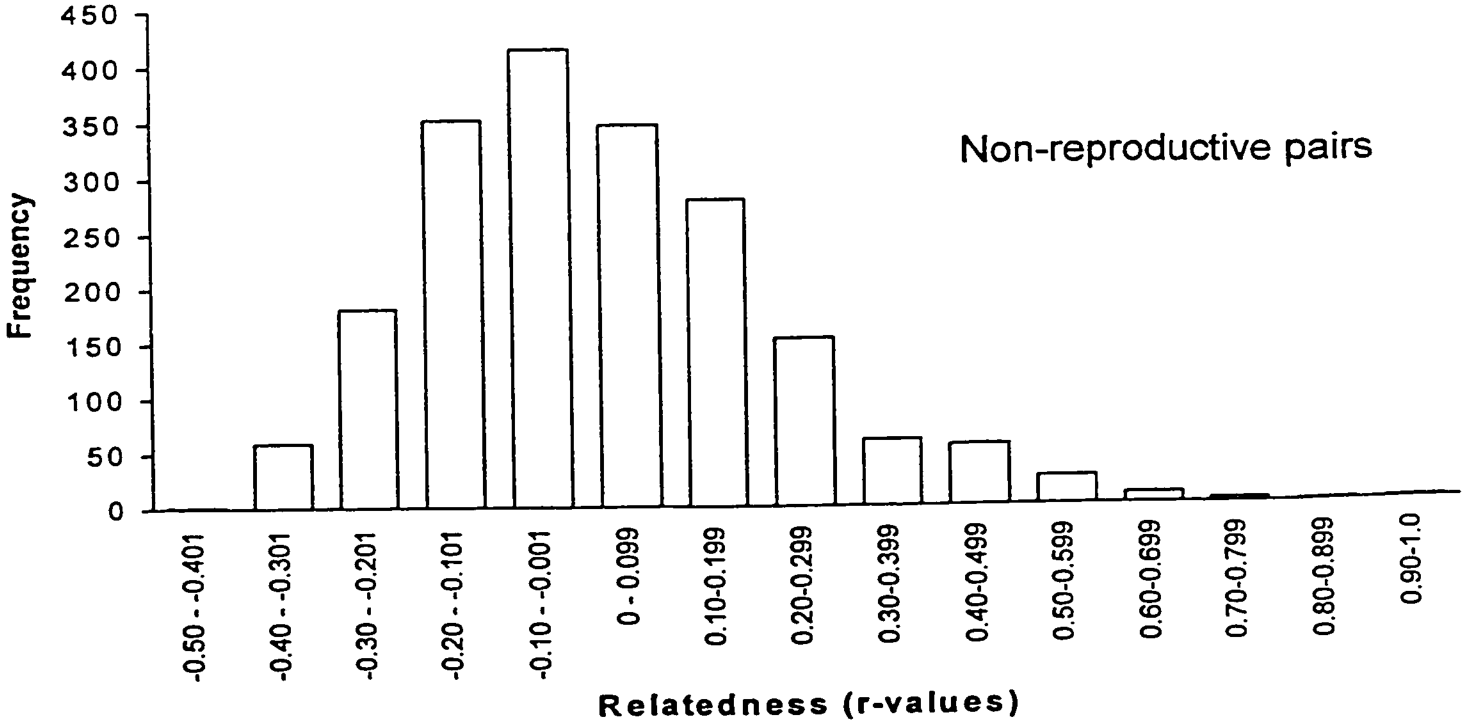
Table 4.4 - Selection gradients (Arnold and Wade, 1984) derived from regressions of number of offspring produced and morphological characters in male water snakes from Barb's and Beaver marshes. Values in the cells represent β scores from the regression analyses. Significance of the overall model and of each β score is represented by p values in parentheses.

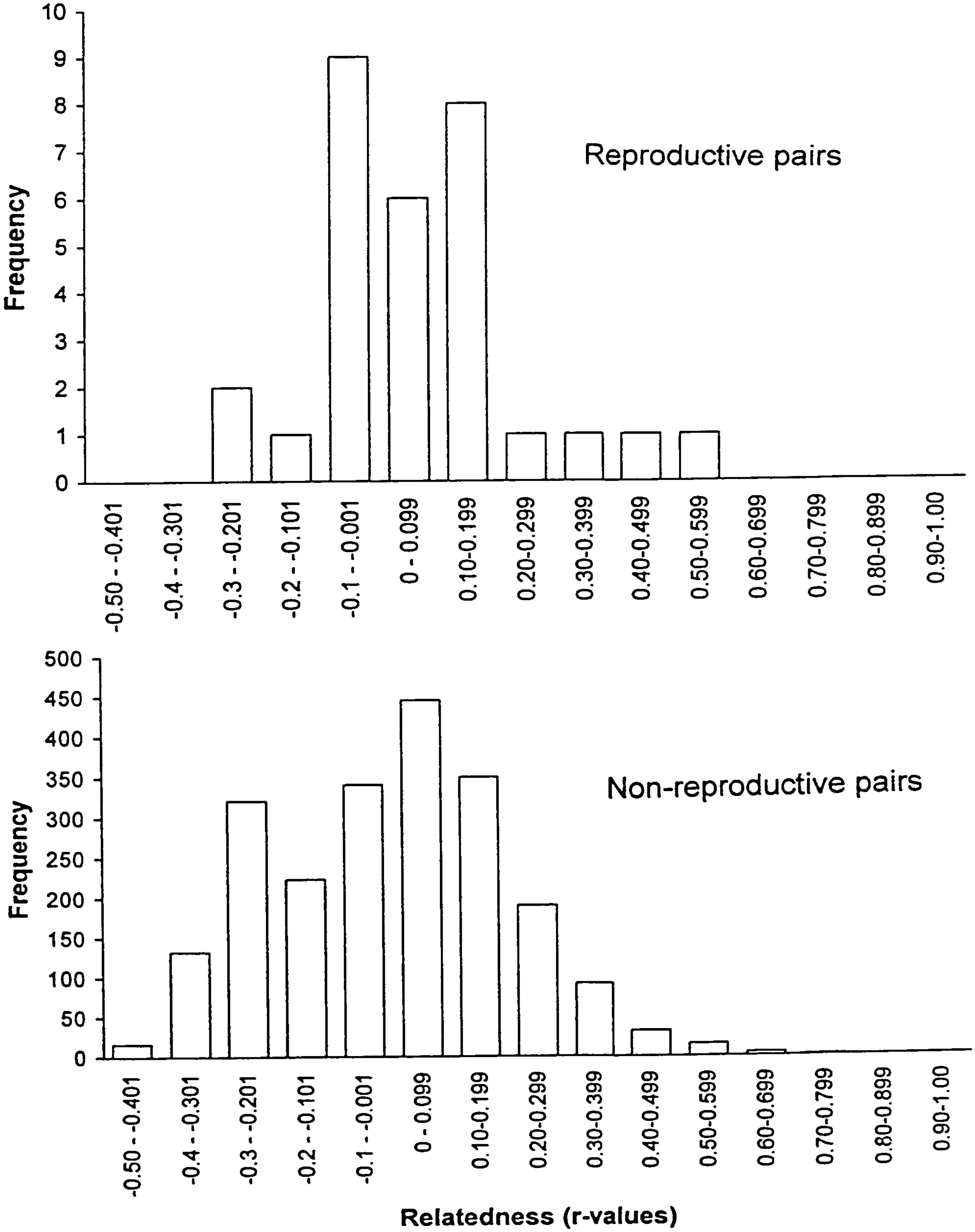
	Barb's			Beaver			
	1994	1996	pooled	1994	1995	1996	pooled
Linear Model	(0.49)	(0.83)	(0.70)	(0.28)	(0.73)	(0.50)	(0.79)
SVL	0.072 (0.93)	0.224 (0.57)	0.251 (0.47)	- 0.100 (0.86)	- 0.338 (0.48)	- 0.545 (0.26)	- 0.245 (0.38)
tail	0.529 (0.58)	- 0.227 (0.54)	0.043 (0.91)	0.161 (0.75)	0.245 (0.62)	0.592 (0.22)	0.233 (0.40)
condition	- 0.233 (0.51)	0.290 (0.39)	- 0.008 (0.97)	- 0.261(0.59)	0.376 (0.37)	- 0.279 (0.46)	- 0.106 (0.65)
Non-linear Model	(0.65)	(0.51)	(0.26)	(0.01)	(0.05)	(0.38)	(0.51)
SVL	0.184 (0.83)	0.372 (0.35)	0.399 (0.27)	- 0.059 (0.92)	- 0.340 (0.49)	- 0.740 (0.14)	- 0.260 (0.36)
SVL^2	- 0.078 (0.77)	- 0.375 (0.13)	- 0.229 (0.19)	- 0.194 (0.62)	- 0.049 (0.88)	- 0.246 (0.18)	- 0.112 (0.45)
tail	0.445 (0.66)	- 0.102 (0.78)	0.043 (0.91)	0.187 (0.71)	0.263 (0.60)	0.594 (0.22)	0.244 (0.38)
condition	- 0.195 (0.61)	- 0.302 (0.36)	0.056 (0.82)	- 0.324 (0.52)	0.389 (0.37)	- 0.405 (0.29)	- 0.143 (0.55)

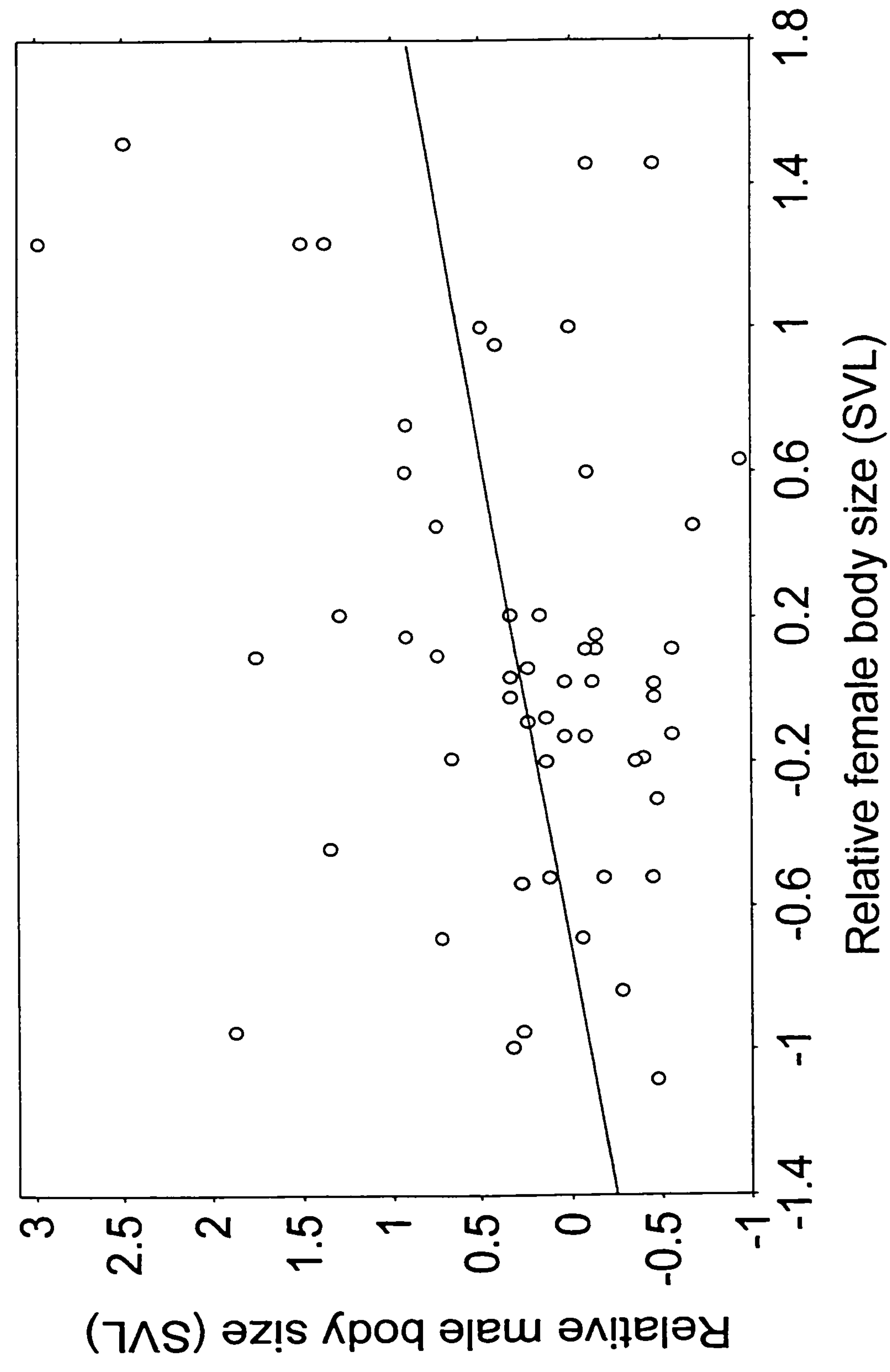

35
30
25
20
15
10
5
0
Number of offspring sired
0
4
8
12
Number of observations
(5)
(4)
(5)
(6)
(5)
(2)
(3)
(4)
(3)

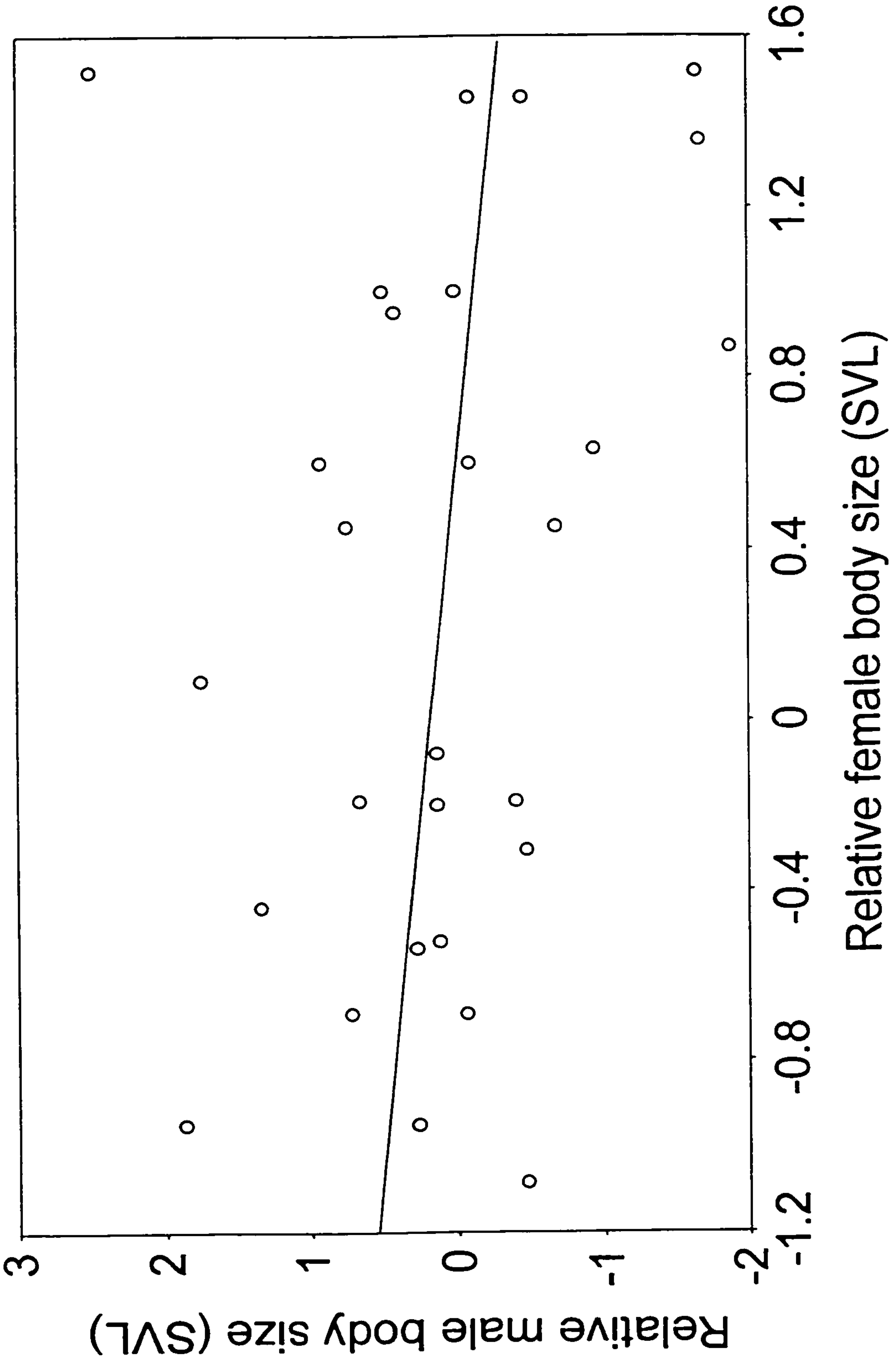

Number of offspring sired
0
5
10
15
20
25
30
(19)
(9)
(7)
(9)
(2)
(5)
(5)
(6)
(11)
(2)
0
2
4
6
8
10
12
14
Number of observations


Reproductive pairs
frequency
0
2
4
6
8
10
12
14
16
-0.50 - -0.401
-0.40 - -0.301
-0.30 - -0.201
-0.20 - -0.101
-0.10 - -0.001
0 - 0.099
0.10-0.199
0.20-0.299
0.30-0.399
0.40-0.499
0.50-0.599
0.60-0.699
0.70-0.799
0.80-0.899
0.90-1.0


Non-reproductive pairs
Frequency
0
50
100
150
200
250
300
350
400
450
-0.50 - -0.401
-0.40 - -0.301
-0.30 - -0.201
-0.20 - -0.101
-0.10 - -0.001
0 - 0.099
0.10-0.199
0.20-0.299
0.30-0.399
0.40-0.499
0.50-0.599
0.60-0.699
0.70-0.799
0.80-0.899
0.90-1.0
Relatedness (r-values)


Reproductive pairs
Frequency
10
9
8
7
6
5
4
3
2
1
0
-0.50 - -0.401
-0.4 - -0.301
-0.3 - -0.201
-0.2 - -0.101
-0.1 - -0.001
0 - 0.099
0.10-0.199
0.20-0.299
0.30-0.399
0.40-0.499
0.50-0.599
0.60-0.699
0.70-0.799
0.80-0.899
0.90-1.00
Non-reproductive pairs
Frequency
500
450
400
350
300
250
200
150
100
50
0
-0.50 - -0.401
-0.4 - -0.301
-0.3 - -0.201
-0.2 - -0.101
-0.1 - -0.001
0 - 0.099
0.10-0.199
0.20-0.299
0.30-0.399
0.40-0.499
0.50-0.599
0.60-0.699
0.70-0.799
0.80-0.899
0.90-1.00
Relatedness (r-values)


Relative male body size (SVL)
3
2.5
2
1.5
1
0.5
0
-0.5
-1
Relative female body size (SVL)
-1.4
-1
-0.6
-0.2
0.2
0.6
1
1.4
1.8


Relative male body size (SVL)
3
2
1
0
-1
-2
Relative female body size (SVL)
-1.2
-0.8
-0.4
0
0.4
0.8
1.2
1.6

Conclusions

The primary goal of this study was to use microsatellite DNA markers to investigate various aspects of sexual selection in northern water snakes, *Nerodia s. sipedon*. In Chapter One, I presented the primer sequences and characteristics of the microsatellite loci developed for this study. I also used these loci to determine that three water snake populations less than two kilometers apart exhibited microgeographic genetic differentiation.

In Chapter Two, I described a variety of methodological issues that may arise when using microsatellite markers to analyze parentage. First, I revealed that there were some offspring that possessed alleles that could not have been inherited from the known mother. These genetic mismatches between offspring and mother were best explained by the presence of a null allele at either of two loci. I discussed the potential consequences of using loci with null alleles for paternal exclusions. Second, I highlighted the great degree of variation of these loci in the males of each population. Finally, I calculated that the overall probability of exclusion (of the eight most variable loci) is 0.9999, which suggests that theoretically, one in 10 000 males should have the same genotype as the actual sire.

In Chapter Three, I used the parentage assignments from Chapter Two to estimate male and female reproductive success for all individuals in the two study populations.

Reproductive success was more variable for males than females, which is consistent with sexual selection theory. Few males sired most of the offspring, while many males achieved no reproductive success. Although many females did not reproduce, this is likely a life history "decision", due to insufficient energy to reproduce each year, rather than a result of being outcompeted. Despite the large variance in female reproductive success, the opportunity for sexual selection was still over 70 times greater for males than females.

I also determined that just over half of my litters were sired by multiple males. The incidence of multiple paternity was greater at the location in which females were more clumped. Female reproductive success increases slightly but significantly (<2 offspring with each successive mate) with multiple paternity. In contrast, male reproductive success greatly increases with additional matings. Therefore, the water snake mating system was classified as polygynandrous. In addition, this supports the hypothesis that females may benefit from multiple paternity because one mating is not sufficient to fertilize an entire batch of eggs. A second potential benefit of multiple paternity is that it reduces the variance (or mean) in proportion of stillborn offspring. However, in this study I found no difference in the mean or variance in stillbirths between singly and multiply sired litters.

Finally, since previous estimates of mating success in reptiles were based on observations of mating attempts in the field, I compared results from field data to actual parentage, as indicated by parentage analyses (Chapter Two). I found no correlation between the number of male mating attempts and true reproductive success. Similarly, there was no correlation between the number of times females were courted and true female reproductive success. This demonstrates that field observations may not be reliable indices of reproductive success, at least in some reptile species.

In Chapter Four, I found no correlation between male reproductive success and male body size, tail length and condition. Therefore, I rejected the hypothesis that sexual selection favours small body size because small males have devoted their energy to reproduction rather than to growth. Instead, I proposed that female-biased SSD in water snakes is the result of the fecundity advantage of larger females outweighing the cost of growing larger than the optimum body size that is set by natural selection. However, I also remark that 1) because the coefficients from the selection gradients on SVL and tail length were quite large, that my samples sizes were not sufficiently large to detect selection on these traits 2) selection may act intermittently in these populations due to benign conditions during the three years of the study, it was not detected. Therefore, I suggested that selection should be studied for a longer period of time and with a larger sample size, if feasible.

I rejected the hypothesis that males with large home ranges are more successful due to higher female encounter rate. However, I found that males who were seen most frequently during the mating season had higher measures of reproductive success. "Activity" was not correlated with any male morphological trait measured. I proposed that "activity" level may be an indicator of vigour, and thus, male quality.

Finally, I found that the SVL of males and females that produced offspring together was correlated at the marsh in which females were spatially distributed. I suggested that a close size-match between partners may result in more efficient mating. Because mating activity is conspicuous, mating snakes may be more vulnerable to predation. Therefore, selection should act to reduce mating duration and if mating with similar-sized partners is more efficient, females should choose to similar-sized males when possible. However, I also proposed that where females are more clumped, male competition is so intense, that females are unable to exercise mate choice.

Microsatellite loci are valuable tools for investigating patterns of genetic differentiation and sexual selection in reptiles. The markers used in this study were sufficiently variable to detect genetic differences among water snake populations less than 2 km apart, and perform paternal exclusions from a panel of approximately 100 prospective males. It is evident that it is essential to use molecular tools to accurately measuring reproductive success because I found no correlation between traditional measures of mating success and the number of offspring produced. Thus, to more fully understand how sexual selection operates in snakes and other taxa, researchers need to recognize the limitations of solely using behavioural data, and must consider using genetic-based techniques for assigning parentage. In addition, researchers may require rather large sample sizes to detect significant selection acting on particular traits in water snakes. I suggest that the next step might be to study sexual selection in a semi-natural environment (*e.g.* outdoor enclosures) in which various factors can more easily be controlled, and studies replicated. For instance, the OSR and density of the snakes can be controlled to determine whether these factors influence how selection acts on male and female traits. In addition, individuals can be selected for study based on their morphological characteristics; competitors can be well-matched in all but one trait, or can differ greatly in size. Also, if snakes are more predictably located, it might be possible to observe a larger proportion of the mating attempts and determine whether these observations more accurately predict reproductive success. Finally, the survival of the offspring can be monitored to determine whether females benefit by multiple paternity through increased survival of their young.