

## Bateman–Trivers in the 21st Century: sexual selection in a North American pitviper

BRENNA A. LEVINE<sup>1\*</sup>, CHARLES F. SMITH<sup>2,3,4</sup>, GORDON W. SCHUETT<sup>3,4,5</sup>,  
MARLIS R. DOUGLAS<sup>1,6</sup>, MARK A. DAVIS<sup>6</sup> and MICHAEL E. DOUGLAS<sup>1,6</sup>

<sup>1</sup>Department of Biological Sciences, University of Arkansas, Fayetteville, AR 72701, USA

<sup>2</sup>Department of Biology, Wofford College, Spartanburg, SC 29303, USA

<sup>3</sup>The Copperhead Institute, PO Box 6755, Spartanburg, SC 29304, USA

<sup>4</sup>Chiricahua Desert Museum, PO Box 376, Rodeo, NM 88056, USA

<sup>5</sup>Department of Biology and Center for Behavioral Neuroscience, Georgia State University, Atlanta, GA 30303, USA

<sup>6</sup>Illinois Natural History Survey, Prairie Research Institute, University of Illinois, Champaign, IL 61820, USA

Received 14 July 2014; revised 4 September 2014; accepted for publication 5 September 2014

Assessment of sexual selection in organisms with cryptic life histories is challenging, although accurate parentage assignments using genotypic markers, combined with behavioural observations and a method to account for open population bias, allow for robust estimation of metrics. In the present study, we employed 22 tetranucleotide microsatellite DNA loci to interpret mating and reproductive success in a population of Copperhead (*Viperidae*, *Agkistrodon contortrix*) in Connecticut, USA. We sampled DNA from 114 adults (56 males, 58 females) and 137 neonates from known mothers to quantify Bateman gradients ( $\beta_{ss}$ ), as well as sex-specific opportunities for selection ( $I$ ) and sexual selection ( $I_s$ ). We also estimated selection on male size [snout-to-vent length (SVL)], a trait important for successful combat and subsequent copulations. Estimates of male  $I$  and  $I_s$  differed significantly from those of females when estimated with four different methods and only males had a significant Bateman gradient. As predicted, male reproductive success was positively correlated with increasing SVL. These results contrast with those derived in another study investigating the same population but based solely on observational data and without correction for open population bias. We thus argue that molecular approaches to quantifying reproductive success and strength of sexual selection provide more accurate results than do behavioural observations alone. © 2014 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, **114**, 436–445.

**ADDITIONAL KEYWORDS:** *Agkistrodon contortrix* – Bateman's principles – copperhead – male body size – mating systems – reproductive success.

### INTRODUCTION

The characterization of mating systems, quantification of reproductive success, and identification of phenotypic characters influenced by sexual selection have a long history of investigation and are clearly important in both micro- and macro-evolutionary processes (Andersson, 1994; Shuster & Wade, 2003). Bateman's (1948) principles, derived from laboratory breeding

experiments using *Drosophila*, are particularly useful in understanding mating system evolution by allowing sexual selection gradients (i.e. Bateman gradients; Arnold & Duvall, 1994) to be estimated via regression of reproductive success onto mating success, and by facilitating estimation of the opportunity for selection ( $I$ ; also referred to as opportunity for fecundity selection; Scharf & Meiri, 2013) and the opportunity for sexual selection ( $I_s$ ) in both males and females (for definitions of terminology, see Appendix; Wade, 1979; Jones, 2009; Mobley & Jones, 2012). Bateman (1948) predicted that variances in mating

\*Corresponding author. E-mail: blevine@email.uark.edu

and reproductive success are greater among the sex with 'cheaper' gametes and in greater abundance (often males) and that this sex will gain in fitness from an increased number of copulations whereas the less abundant sex (often females) will experience no such gain. This consideration prompted Trivers (1972) to extend the argument by postulating that females will not only be a limiting factor in reproductive equations, but also a subject of intense male–male competition. Subsequently, regressions of reproductive success onto male phenotypic characters important in competition for access to females (e.g. horn size, body length) can be used to identify traits hypothesized to be under sexual selection (Shuster & Wade, 2003).

Despite their utility, accurate estimation of  $I$ ,  $I_s$ , Bateman's gradients, and selection on male phenotypic characters (hereafter referred to as Bateman's metrics) is sensitive to a variety of complications associated with the study of natural populations (Parker & Tang-Martinez, 2005). Post-copulatory processes such as multiple paternity, long-term sperm storage, and cryptic female choice can result in offspring paternity that is incongruent with field observations of mating (Prosser *et al.*, 2002). Additionally, incomplete population sampling may bias estimates, particularly when one sex is not sampled with their respective offspring (Mobley & Jones, 2012). Furthermore, copulations that do not result in offspring (e.g. as a result of sperm competition, cryptic female choice, long-term sperm storage) are important in the estimation of Bateman's metrics but can easily be missed in observations of taxa with cryptic life histories (Collet *et al.*, 2014).

Fortunately, recent technological and statistical advances can circumvent these difficulties. The capacity to genotype individuals using microsatellite DNA now permits many taxa with complicating post-copulatory processes to be evaluated with regard to their mating systems by facilitating accurate parentage analysis (Prosser *et al.*, 2002; Dubey *et al.*, 2009). Maximum-likelihood methods have been developed to correct for potential statistical biases associated with sampling open populations (Mobley & Jones, 2012) and the use of behavioural observations of mating in conjunction with genotypic parentage assignments has been shown to increase the accuracy of Bateman's metrics estimates (Collet *et al.*, 2014).

Nonetheless, the evaluation of mating systems in wild vertebrates has been and remains largely dominated by species that are abundant and/or easy to observe and sample (Clutton-Brock, 1988), with other taxa often being neglected or insufficiently sampled as a result of small size, cryptic behaviour, unpredictable sampling, and a range of other complexities, such as long-term sperm storage, cryptic female choice, and

facultative parthenogenesis. Furthermore, although the ability to overcome these complications now exists, studies often still rely only on behavioural observations for estimating Bateman's metrics (Fritzsche & Arnqvist, 2013; Smith & Schuett, 2014) and ignore potential sampling biases, which may result in overestimation (Williams & DeWoody, 2009; Mobley & Jones, 2012; Collet *et al.*, 2014).

Squamate reptiles have been particularly affected by these difficulties. Although multiple paternity, long-term sperm storage, and facultative parthenogenesis have been documented in this taxon, studies still rely solely on behavioural observations to estimate Bateman's metrics (Smith & Schuett, 2014) or neglect the potential for statistical biases in open populations (Weatherhead *et al.*, 2002). To our knowledge, there have been no studies to date of snake mating systems that simultaneously incorporate genotypic parentage analysis, behavioural observations of mating, and open population statistical corrections. This is unfortunate because the convergence of multiple methods on estimates of Bateman's metrics will yield unique and robust insights into snake mating systems.

For those snake taxa thus far investigated with one or more methods, with some exceptions (Lukoschek & Avise, 2011), it appears that both sexes have multiple mates, with mating failure being especially high in males, and with multiple paternity being common (Duvall & Schuett, 1997; Jellen & Aldridge, 2011). Snake mating systems are now considered polyandrous (Shuster & Wade, 2003; Kissner, Weatherhead, & Gibbs, 2005; Rivas & Burghardt, 2005) with considerable female sexual selection (Fritzsche & Bookmythe, 2013). Because male snakes do not supply female mates with direct benefits, such as nuptial gifts or care of offspring (Blouin-Demers, Gibbs & Weatherhead, 2005), estimations of sexual selection and fitness are simplified (Shuster & Wade, 2003). Furthermore, phenotypic characters such as snout-to-vent length (SVL) contribute to a priority-of-access by males to females (Madsen & Shine, 1993, 1994; Duvall & Schuett, 1997) and are therefore predicted to be under sexual selection (Shuster & Wade, 2003).

In the present study, we employed 22 uninterrupted tetranucleotide microsatellite DNA loci (Castoe *et al.*, 2010) to evaluate the mating system of a North American pitviper and test hypotheses driven by sexual selection theory. Specifically, from 2001 to 2003, we sampled adults and neonates (known mothers) in a population of copperhead (Viperidae, *Aghkistrodon contortrix*; Linnaeus, 1766) aiming to estimate  $I$ ,  $I_s$ , and  $\beta_{ss}$  for adults of both sexes using four methods. Litters were analyzed and the relationship between litter size and the number of sires in

multiple paternity litters was assessed. Additionally, we tested for and found selection on male SVL, which is an important phenotypic trait with respect to successful combat and subsequent copulations (Duvall & Schuett, 1997; Schuett, 1997). We also compared our results with those from a previous study in the same population in which estimates of Bateman's metrics were derived solely with behavioural observations and without accounting for open population bias (Smith & Schuett, 2014).

## MATERIAL AND METHODS

### HYPOTHESES

Our hypotheses extend from mating system and sexual selection theory (Wade & Shuster, 2005): (H<sub>1</sub>) males exhibit variance in mating and reproductive success; (H<sub>2</sub>) multiple paternity occurs in litters; (H<sub>3</sub>) estimates of  $I$ ,  $I_s$ , and  $\beta_{ss}$  are significantly greater for males than females; and (H<sub>4</sub>) an estimate of selection on male SVL is statistically significant.

### SAMPLING AND MOLECULAR METHODS

From 2001 to 2003, SVL and blood samples were obtained from 114 wild adult *A. contortrix* (males = 56, females = 58) and shed skins from 137 neonates ( $N = 251$  total) caught at a field site (486 ha) in the Central Connecticut River valley (Connecticut, USA) (Smith, 2007). Offspring were collected from 19 females and maternity was unequivocal in that parturition occurred in a University of Connecticut laboratory (for a complete description of study site and field methods, see Smith, 2007; Smith *et al.*, 2009; Smith & Schuett, 2014).

Genomic DNA was extracted using the PureGene® DNA Isolation Kit, with concentration standardized at 20.0 ng/μL. Genotype profiles were generated across 23 tetra-nucleotide microsatellite loci (Castoe *et al.*, 2010). Polymerase chain reactions (PCRs) consisted of 1X GoTaq® Flexi PCR buffer, 2.0–2.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 15.15 μM bovine serum albumin, 0.2–0.65 mM forward and reverse primer, 0.5 units of GoTaq® Flexi *taq* DNA polymerase, and 20–40 ng DNA. The temperature profile comprised an initial denaturation of 95 °C for 3 m followed by 15 cycles of 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 30 s, followed by 16–19 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 15 s, with a final extension of 72 °C for 5 m, conducted on GeneAmp® 9600 PCR System or Veriti® 96-Well Thermal cyclers. Microsatellite fragments were resolved on an ABI Prism 3730xl automated sequencer and sized after comparison to the LIZ500 size standard. Alleles were scored using PeakScanner™ (Applied Biosystems) software.

### POPULATION GENETIC ANALYSIS

All loci were evaluated for null alleles, large allele dropout, and genotyping errors in MICROCHECKER, version 2.2.3 (Van Oosterhout *et al.*, 2004). Departures from Hardy–Weinberg equilibrium (HWE) were examined in GENEPOP (Raymond & Rousset, 1995) using a Markov chain method with 200 batches, 5000 iterations per batch, and with Bonferroni-adjusted  $\alpha = 0.0002$ . A Markov chain test for linkage disequilibrium (LD) was also conducted in GENEPOP (batches/iterations as above).

### PARENTAGE ANALYSIS AND DETECTION OF MULTIPLE PATERNITY

COLONY, version 2.0 (Jones & Wang, 2010) employs a full-pedigree likelihood method to estimate familial relationships that is robust with respect to genotyping errors (Wang, 2004) and was used to infer parentage for each juvenile. All adult males and females were included in the candidate father and mother pool, respectively, and the mating system for both sexes was classified as polygamous. Additional parameters were a run of medium length, no inbreeding, no sibship size prior, no update of allele frequencies to account for inferred relationships, and a genotyping error rate conservatively set at 0.001 for each locus (Wang, 2004). Parentage inferences were first derived without employing known maternal data or maternal sibships to confirm that known and inferred offspring maternity and maternal sibships were congruent, thereby confirming the ability of our microsatellite loci to resolve parent–offspring relationships. Multiple paternity was noted when more than one male was assigned parentage within a single litter. The mean numbers of offspring in single versus multiple paternity litters were compared with a two-sample *t*-test ( $\alpha = 0.05$ ).

### ESTIMATING SEXUAL SELECTION METRICS

The construction of a parental table (see Supporting information, Appendix S1; Arnold & Duvall, 1994) allowed reproductive success and mating success to be calculated for each individual. We first estimated mating success via genotypic analysis ( $MS_{gen}$ ), then generated a second parental table (see Supporting information, Appendix S2) that included observed copulations in estimates of mating success ( $MS_{tot}$ ) (Collet *et al.*, 2014). Bateman's metrics were gauged via traditional calculations (Arnold & Duvall, 1994), as well as by a maximum likelihood method (Mobley & Jones, 2012). Opportunities for selection ( $I$ ) for both males and females were estimated by calculating variance in reproductive success by sex and dividing each by the squared mean sex reproductive success

**Table 1.** Opportunity for selection ( $I$ ) was calculated for all males ( $\sigma$ ;  $N = 71$ ) including males of unknown identity for which paternity was inferred through genotypic parentage analysis, and all females that produced litters ( $\varphi$ ;  $N = 19$ )

	$\sigma$	$\varphi$
Sample size ( $N$ )	71	19
Mean	1.93	7.21
Variance	11.47	8.95
Relative variance ( $I$ )	3.08	0.17
Bias corrected $I$	0.84	0.17

$I$  is equal to sex-specific relative variance in reproductive success (= number of offspring) and is calculated by dividing sex-specific variance in reproductive success by the square of mean sex-specific reproductive success.  $I$  was also estimated with the maximum-likelihood software BATEMANATER (Mobley & Jones, 2012) to correct for open population bias (bias corrected  $I$ ), with 1000 bootstrap replicates. Male and female  $I$  were significantly different ( $F$ -ratio test;  $P < 0.05$ ) when calculated with traditional methods and when estimated with BATEMANATER.

**Table 2.** Opportunity for sexual selection ( $I_s$ ) was calculated for all males ( $\sigma$ ;  $N = 71$ ) including those of unknown identity for which paternity was inferred through genotypic parentage analysis and females that produced litters ( $\varphi$ ;  $N = 19$ )

	$\sigma$	$\varphi$
Sample size ( $N$ )	71	19
Mean	0.42	1.58
Variance	0.39	0.48
Relative variance ( $I_s$ )	2.19	0.19
Bias corrected $I_s$	0.74	0.19

$I_s$  is equal to sex-specific relative variance in number of mates and is calculated by dividing sex-specific variance in number of mates by the square of mean sex-specific number of mates.  $I_s$  was also estimated with the maximum-likelihood program BATEMANATER (Mobley & Jones, 2012) to correct for open population bias (bias corrected  $I_s$ ), with 1000 bootstrap replicates. Male and female  $I_s$  were significantly different ( $F$ -ratio test;  $P < 0.05$ ) when calculated with traditional methods and when estimated with BATEMANATER.

(Table 1). Similarly, opportunities for sexual selection ( $I_s$ ) were assessed in both sexes by dividing the sex-specific variance in mating success by the sex-specific squared mean mating success (Table 2). Bateman gradients ( $\beta_{ss}$ ) represent the slope of the least-squares regression line of sex-specific reproductive success on that of sex-specific mating success, and were calcu-

lated by dividing the sex-specific covariance of reproductive success and mating success by sex-specific variance in mating success (Arnold & Duvall, 1994; Duvall, Schuett, & Arnold, 1994).

To account for potential statistical biases associated with sampling open populations, we also estimated Bateman's metrics using the maximum-likelihood software BATEMANATER (Mobley & Jones, 2012), with 1000 bootstrap replicates. Traditional calculations for males included those individuals that achieved no reproductive success, whereas calculations for females included only those that produced offspring (for justification, see Prosser *et al.*, 2002). By contrast, BATEMANATER calculations employ only those males that achieved mating or reproductive success (Mobley & Jones, 2012). Differences among male and female estimates of  $I_s$  and  $I$  were assessed with  $F$ -ratio tests ( $\alpha = 0.05$ ; Williams & DeWoody, 2009; Fritzsche & Arnqvist, 2013), whereas differences among male and female  $\beta_{ss}$  were compared statistically with an analysis of covariance (ANCOVA) ( $\alpha = 0.05$ ).

#### ESTIMATING SELECTION ON MALE BODY LENGTH

Relative reproductive success for each known male that produced offspring was calculated by dividing the number of offspring sired by the mean number of offspring sired ( $N = 10$ ; one of the 11 known males that sired offspring was excluded as an outlier). SVL data were standardized to have a mean of zero and SD equal to one. Relative fitness was regressed onto standardized SVL, and the standardized selection gradient estimated from the slope of the regression line. All statistical analyses were conducted with R (R Core Team, 2013). The standardized selection gradient was calculated for all known males that sired offspring ( $N = 11$ ), as well as for a reduced data set ( $N = 10$ ) in which one of the 11 males was excluded as an outlier.

## RESULTS

#### GENOTYPING, PARENTAGE ANALYSES, MULTIPLE PATERNITY, AND PATERNAL SKEW

One microsatellite locus displayed HWE disequilibrium and evidence of a null allele ( $P = 0.12$ ) and was subsequently removed from analyses. GENEPOP also detected LD between three pair of loci, although these comparisons were less than expected by chance alone (Bonferroni-adjusted  $P > 0.0002$ ). Mean observed and expected heterozygosities and allelic richness are provided in Levine (2013).

COLONY inferred offspring maternity at a probability greater than or equal to 0.997, except for five offspring with a probability less than or equal to 0.976. However, known maternity and maternal

sibships matched maternal and sibship assignments for all 137 offspring, confirming the ability of our microsatellites to reliably delineate familial relationships.

Paternity for 66 of 137 offspring (48.2%) was assigned to 11 known males at a probability greater than 0.996 ( $N = 65$ ), and a probability of 0.964 ( $N = 1$ ). Paternity of the remaining 71 offspring was assigned to 15 unknown males, thus eliminating our opportunity to evaluate the probability of correct paternity. Importantly, four males were previously observed mating with females that subsequently had offspring (Smith & Schuett, 2014), although they were not supported by molecular parentage analysis as being fathers of those offspring. Similarly, known males that sired offspring were also not observed mating with the respective female parent (Smith & Schuett, 2014).

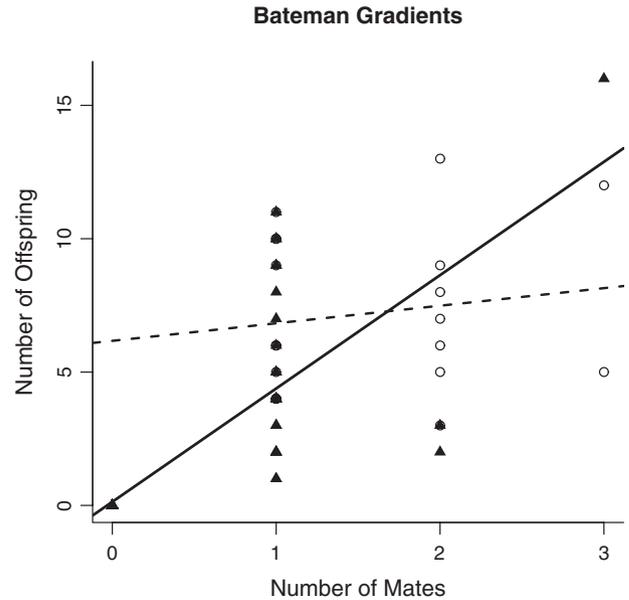
We identified multiple paternity in nine of 20 litters (eight with two sires, one with three sires), and thus rejected the null hypothesis of no multiple paternity. There was no significant difference in litter size (number of offspring) among single paternity (mean offspring =  $6.82 \pm 2.89$ ) and multiple paternity litters (mean offspring =  $6.89 \pm 2.68$ ) (two-sample  $t$ -test,  $t_{18} = -0.06$ ,  $P > 0.05$ ).

#### SEXUAL SELECTION METRICS AND THEIR ESTIMATES

We reject the null hypotheses that both sexes have statistically equivalent estimates of  $I$ ,  $I_s$ , and  $\beta_{ss}$ . The estimate for  $I$  derived with traditional calculations (i.e. no correction for open population bias) was equal to 3.08 for males, and was significantly different from that for females (female  $I = 0.17$ ;  $F$ -ratio test;  $P < 0.05$ ) (Table 1). Values of  $I$  estimated with BATEMANATER were also significantly different between males (0.84) and females (0.17) ( $F$ -ratio test;  $P < 0.05$ ) (Table 1).

Utilizing  $MS_{gen}$ , we found that  $I_s$  for males (2.19) was significantly different from that for females (0.19) ( $F$ -ratio test;  $P < 0.05$ ) (Table 2). When using  $MS_{tot}$ , estimates of  $I_s$  for males and females were equal to 1.02 and 0.26, respectively, and were, again, significantly different ( $F$ -ratio test;  $P < 0.05$ ). Male and female  $I_s$  estimated with BATEMANATER (Mobley & Jones, 2012) were equal to 0.74 and 0.19 (Table 2), respectively, and were again significantly different from each other ( $F$ -ratio test;  $P < 0.05$ ). When  $I_s$  was estimated for males and females with both BATEMANATER and  $MS_{tot}$ , male and female estimates were also statistically different from one another (male  $I_s = 1.01$ ; female  $I_s = 0.26$ ;  $F$ -ratio test;  $P < 0.05$ ).

The Bateman gradient ( $\beta_{ss}$ ) for males was statistically significant when calculated with  $MS_{gen}$  or  $MS_{tot}$



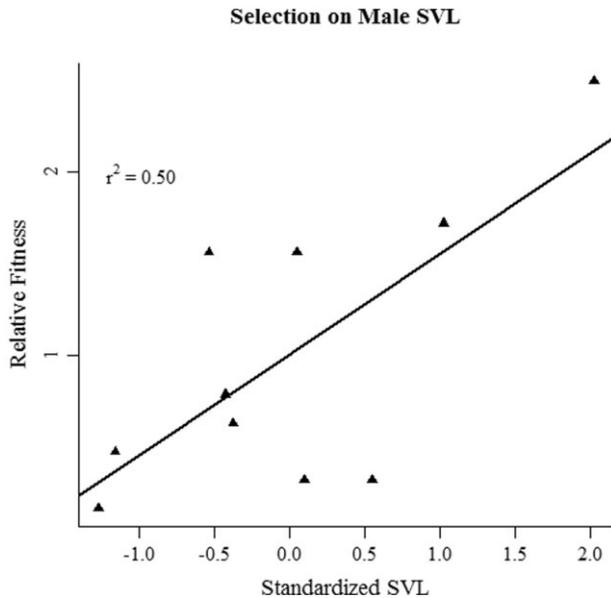
**Figure 1.** Bateman gradients for female (dashed line) and male (solid line) copperhead (*Agkistrodon contortrix*) based on 71 males of both known ( $N = 56$ ) and unknown ( $N = 15$ ) identity, and the 19 females that produced litters sired by both known and unknown males. Open circles and filled triangles denote females and males, respectively. Only males had a statistically significant Bateman gradient (males  $\beta_{ss} = 4.25$ ;  $r^2 = 0.61$ ;  $P < 0.0001$ ; females:  $\beta_{ss} = 0.66$ ;  $r^2 = 0.02$ ;  $P > 0.05$ ).

( $MS_{gen}$ :  $\beta_{ss} = 4.25$ ,  $r^2 = 0.61$ ,  $P < 0.0001$ , Fig. 1;  $MS_{tot}$ :  $\beta_{ss} = 2.15$ ,  $r^2 = 0.28$ ,  $P < 0.0001$ , not shown). By contrast,  $\beta_{ss}$  for females was not significantly different from zero, again regardless of the MS used ( $MS_{gen}$ :  $\beta_{ss} = 0.66$ ,  $r^2 = 0.02$ ,  $P > 0.05$ , Fig. 1;  $MS_{tot}$ :  $\beta_{ss} = 0.07$ ,  $r^2 = 0.000$ ,  $P > 0.05$ , not shown). Male and female Bateman gradients were also significantly different from each other (ANCOVA,  $P < 0.05$ ).

With the complete data set of 11 known males, the null hypothesis of no correlation between male SVL and the number of offspring sired could not be rejected ( $P > 0.05$ ). However, with the removal of one male data point as an outlier, the relationship between male reproductive success and SVL was statistically significant, with larger males siring more offspring than smaller males ( $r^2 = 0.50$ ;  $P = 0.023$ ) (Fig. 2).

## DISCUSSION

We quantified and characterized the mating system of a population of copperhead using an extensive number of robust polymorphic microsatellite loci and by employing four estimators of Bateman's metrics. Our results lend strong empirical support to the



**Figure 2.** The standardized selection gradient (solid line) for snout-to-vent length (SVL) of male copperhead (*Agkistrodon contortrix*,  $N = 10$ ) was estimated by regressing relative fitness data on standardized SVL measurements. Relative fitness was calculated by dividing individual reproductive success by mean male reproductive success. SVL measurements were standardized to have a mean equal to zero and a standard deviation equal to one. Filled triangles denote males. A significant selection gradient on male SVL was detected ( $r^2 = 0.50$ ;  $P = 0.023$ ).

argument that male pitvipers display elevated opportunities for selection and sexual selection compared to females. To our knowledge, this is the first study to employ multiple methods to estimate Bateman's metrics in pitvipers, or any other snake taxa, and to yield consistent and statistically significant differences between males and females.

#### PARENTAGE ANALYSES AND MULTIPLE PATERNITY

Although multiple paternity has been described in a variety of animals (e.g. mammals: Carling, Wiseman & Byers, 2003; birds: Gowaty & Karlin, 1984; fish: Soucy & Travis, 2003); insects (Boomsa, Fjerdingstad & Frydenberg, 1999), and *A. contortrix* under laboratory conditions (Schuett & Gillingham, 1986)], our findings are novel for wild copperhead, and only the second for New World pitvipers (Jellen & Aldridge, 2011; Clark *et al.*, 2014). Multiple paternity may occur under several scenarios: when females mate multiply within a single season or when long-term sperm storage from different males is maintained across breeding seasons, or a combination of the two (Uller & Olsson, 2008; Jellen & Aldridge, 2011). Long-

term sperm storage has been documented in laboratory crosses of *A. contortrix* (Schuett & Gillingham, 1986) and is an obligate component of the mating system in the study population (Smith *et al.*, 2009). With a single mating season in late summer, females then store sperm throughout hibernation, and then ovulate and fertilize ova in spring. Although males in the study population are capable of mating multiple times within a season (Smith, 2007; Smith & Schuett, 2014), precise estimates of the number are lacking. Sperm competition or cryptic female choice may obscure the number of mates for a female, particularly if ova have not been fertilized (Uller & Olsson, 2008). Furthermore, the effects of sperm competition on multiple paternity are difficult to separate from cryptic female choice (Thornhill, 1983), which may also influence patterns of paternity.

Variance in male reproductive success will be reduced by multiple paternity and thus diminish sexual selection (Duvall, Schuett & Arnold, 1993) unless a single male sires all (or almost all) offspring in a litter (Shuster, Briggs & Dennis, 2013). As in other snakes, gamete production is finite in male copperhead and this aspect combined with multiple paternity yields fewer offspring for each male in a multiply-sired litter, particularly compared to males as sole sires. In the present study, the majority of litters (55%) were singly-sired, supporting the hypothesis of strong male sexual selection, particularly given the statistical equivalence between the numbers of offspring produced in single versus multiple paternity litters. Our present analysis, however, does not clarify whether a first-male advantage occurs in *A. contortrix* (Höggren & Tegelström, 2002), and this point will be addressed in future studies on this population.

#### OPPORTUNITIES FOR SELECTION AND SEXUAL SELECTION

The influence of the operational sex ratio (OSR) on sexual selection metrics was first described based on studies of mammals and birds (Emlen & Oring, 1977) and has subsequently been extended to other mating systems (Douglas, 1979; Wade & Shuster, 2005). In the present study, the adult sex ratio is almost unity ( $N_m = 56$ ,  $N_f = 58$ ), although the OSR is male-biased (between 1 : 2 and 1 : 3; Smith, 2007; Smith *et al.*, 2009; Smith & Schuett, 2014) in any given year as a result of low frequency (biennial or triennial) female reproductive patterns, a situation common in temperate viperid snakes (Brown, 1993; Smith & Schuett, 2014). Consequently, the elevated abundance of available males contributes to variance in male reproductive success. Therefore, it was not unexpected that our estimates of  $I$  and  $I_s$  were greater in males than in females.

$I_s$  in the study population was estimated from behavioural observations (i.e. number of mates and copulations) in a previous study (Smith & Schuett, 2014) with no sex-specific differences found. However, in the present study,  $I_s$  differed significantly between males and females, highlighting the widely-varying results that can emerge when different methods are used to estimate sexual selection (Shuster & Wade, 2003). We argue that molecular analyses provide greater resolution when sex-specific estimates are derived. For example, none of the four males previously observed mating with three females (Smith & Schuett, 2014) was assigned paternity when the offspring were evaluated using molecular parentage analyses. Furthermore, known males that did sire offspring were never observed to mate with those females in the field (Smith & Schuett, 2014). Cryptic copulation may be commonplace in snakes because 35% of males in a population of northern water snake (*Nerodia sipedon*) sired young but were never observed mating (Prosser *et al.*, 2002). Long-term sperm storage has been documented in *A. contortrix* and other snakes (Booth & Schuett, 2011) and could result in offspring being sired by males that mated with females in previous seasons. Furthermore, the cryptic coloration and behaviours of many snakes (Shine, 2008), including copperhead, may permit copulation without human detection (Clark *et al.*, 2014), even in well documented and observed populations (Smith & Schuett, 2014).

#### *Bateman's gradients*

As predicted, male copperhead exhibited a statistically significant Bateman gradient, whereas females did not, demonstrating that males in the study population experience a greater benefit from multiple matings (in terms of the number of offspring produced) when compared to females. Three main factors that contribute to this are: (1) a clumped spatial distribution of receptive females in the environment; (2) asynchrony in the timing of female receptivity; and (3) a male-biased OSR influenced by the previous two (Emlen & Oring, 1977; Ims, 1988). A clumped female distribution and asynchrony in the timing of female receptivity allow for multiple matings, as well as for a subset of males in close proximity to aggregated females to monopolize mating. Both contribute to increased variance in male mating success and an elevated sexual selection gradient. Furthermore, a male-biased OSR, with a greater number of sexually active males than females, will promote male competition and elevate the variance in male mating success.

Although Bateman's metrics have been applied to many taxa (e.g. mammals: Clutton-Brock *et al.*, 2006; fish: Jones *et al.*, 2005), few studies have utilized

them to evaluate mating systems in snakes. Robust comparisons among taxa are thus premature (but see Madsen & Shine, 1994; Duvall & Schuett, 1997; Prosser *et al.*, 2002). Furthermore, there is no consensus on the significance of the Bateman paradigm, citing inconsistencies in the original experiment and emergent logical flaws (Tang-Martinez, 2012). However, a multitude of evidence to date supports the Bateman–Trivers postulates (i.e. elevated sexual selection in males; females as a limiting resource for males thus prompting male–male competition), and an elevated variance in male mating and reproductive success has been documented across a wide variety of species (Parker & Birkhead, 2013). Importantly, although the main concern with respect to employing Bateman's metrics is the bias associated with incomplete sampling of open populations (Mobley & Jones, 2012), significant differences among male and female estimates of Bateman's metrics were detected in this population both with traditional calculations and with the maximum-likelihood method for open populations. Furthermore, male and female Bateman's metrics were significantly different from each other, regardless of whether mating success included behavioural observations as well as genotypic parentage data.

#### *Selection on male SVL*

Strong sexual selection is promoted when male phenotypic traits are correlated with access to mates (Gopurenko, Williams & DeWoody, 2007). Given that statistically significant opportunities for selection and sexual selection were found in males, and also that a positive relationship between male SVL and priority-of-access to females has been demonstrated in Copperhead (Schuett, 1996; Schuett, 1997), it is not surprising that a significant correlation emerged in the present study between male SVL and the number of offspring sired. Again, these results are in contrast to those obtained in previous investigations conducted in the study population (Smith & Schuett, 2014). However, our genotypic results, albeit with the removal of one outlier male, are similar to those of other studies investigating the relationship between body size and reproductive success in snakes (Blouin-Demers *et al.*, 2005; Dubey *et al.*, 2009; Ursenbacher, Erny & Fumagalli, 2009). Accurate parentage assignments are essential to correctly estimate male reproductive success and, in this respect, molecular data clearly demonstrate their worth.

#### *Concluding remarks*

The present study contributes to a growing literature that focuses on mating systems and sexual selection, and also emphasizes the importance of genotypic data in estimating paternity and other mating system

parameters (Serbezov *et al.*, 2010) in that correctly developing a parental table requires high confidence in familial relationships. Without genotypic data, estimates of  $I$ ,  $I_s$ , and selection on phenotypic characters important to reproduction depend instead only on observational data that may result in specious inferences as a result of cryptic life histories and other associated factors. Additionally, the present study demonstrates the utility of employing multiple methods for estimating Bateman's metrics, as well as the robust results that emerge when these methods converge on statistically significant differences between males and females. Additional studies on *A. contortrix* and other snakes are clearly warranted to further clarify these, and other, disparities.

#### ACKNOWLEDGEMENTS

We especially thank S. Mussmann who spent numerous hours teaching molecular techniques and analyses to B. A. Levine, and we also thank A. Reynolds and J. Reynolds for laboratory assistance. The Illinois Natural History Survey (Prairie Research Institute, University of Illinois/Urbana-Champaign) is acknowledged for providing a Summer Research Fellowship to B. Levine that yielded the data for the present study. We appreciate the assistance of the W. M. Keck Center for Comparative and Functional Genomics (University of Illinois/Urbana-Champaign) for their quick translation of molecular amplifications into quantitative fragment data. We thank three anonymous reviewers for their comments that helped to improve this manuscript. Additional acknowledgements with regard to individuals, agencies, and NGOs, including sampling and IACUC permits, are provided in Smith *et al.* (2009). The authors declare that there are no conflict of interest.

#### REFERENCES

- Andersson M.** 1994. *Sexual selection*. Princeton, NJ: Princeton University Press.
- Arnold SJ, Duvall D.** 1994. Animal mating systems: a synthesis based on selection theory. *American Naturalist* **143**: 317–348.
- Bateman AJ.** 1948. Intra-sexual selection in *Drosophila*. *Heredity* **2**: 349–368.
- Blouin-Demers G, Gibbs HL, Weatherhead PJ.** 2005. Genetic evidence for sexual selection in black rat snakes, *Elaphe obsoleta*. *Animal Behaviour* **69**: 225–234.
- Boomsa JJ, Fjerdingstad EJ, Frydenberg J.** 1999. Multiple paternity, relatedness and genetic diversity in *Acromyrmex* leaf-cutter ants. *Proceedings of the Royal Society of London Series B, Biological Sciences* **266**: 249–254.
- Booth W, Schuett GW.** 2011. Molecular genetic evidence for alternative reproductive strategies in North American pitvipers (Serpentes: Viperidae): long-term sperm storage and facultative parthenogenesis. *Biological Journal of the Linnean Society* **104**: 934–942.
- Brown WS.** 1993. *Biology, status, and management of the timber rattlesnake (Crotalus horridus): a guide for conservation*. Oxford: Society for the Study of Amphibians and Reptiles.
- Carling MD, Wiseman PA, Byers JA.** 2003. Microsatellite analysis reveals multiple paternity in a population of wild pronghorn antelopes (*Antilocapra Americana*). *Journal of Mammalogy* **84**: 1237–1243.
- Castoe TA, Poole AW, Gu W, de Koning APJ, Daza JM, Smith EN, Pollock DD.** 2010. Rapid identification of thousands of copperhead snake (*Agkistrodon contortrix*) microsatellite loci from modest amounts of 454 shotgun genome sequence. *Molecular Ecology Resources* **10**: 341–347.
- Clark RW, Schuett GW, Repp RA, Amarello M, Smith CF, Hermann HW.** 2014. Mating systems, reproductive success, and sexual selection in secretive species: a case-study of the western diamond-backed rattlesnake, *Crotalus atrox*. *PLoS ONE* **9**: 1–12.
- Clutton-Brock T.** 1988. *Reproductive success: studies of individual variation in contrasting breeding systems*. Chicago, IL: University of Chicago Press.
- Clutton-Brock TH, Hodge SJ, Spong G, Russell AF, Jordan NR, Benett NC, Sharpe LL, Manser MB.** 2006. Intrasexual competition and sexual selection in cooperative mammals. *Nature* **444**: 1065–1068.
- Collet JM, Dean RF, Worley K, Richardson DS, Pizzari T.** 2014. The measure and significance of Bateman's principles. *Proceedings of the Royal Society of London Series B, Biological Sciences* **281**: 20132973.
- Douglas ME.** 1979. Migration and sexual selection in *Ambystoma jeffersonianum*. *Canadian Journal of Zoology* **57**: 2303–2310.
- Dubey S, Brown GP, Madsen T, Shine R.** 2009. Sexual selection favours large body size in males of a tropical snake (*Stegonotus cucullatus*, Colubridae). *Animal Behaviour* **77**: 177–182.
- Duvall D, Schuett GW.** 1997. Straight-line movement and competitive mate searching in prairie rattlesnakes, *Crotalus viridis viridis*. *Animal Behaviour* **54**: 329–334.
- Duvall D, Schuett GW, Arnold SJ.** 1993. Ecology and evolution of snake mating systems. In: Seigel RA, Collins JT, eds. *Snakes: ecology and behavior*. New York, NY: McGraw-Hill, 165–200.
- Emlen ST, Oring LW.** 1977. Ecology, sexual selection, and the evolution of mating systems. *Science* **19**: 215–223.
- Fritzsche K, Arnqvist G.** 2013. Homage to Bateman: sex roles predict sex differences in sexual selection. *Evolution* **67**: 1926–1936.
- Fritzsche K, Booksmythe I.** 2013. The measurement of sexual selection on females and males. *Current Zoology* **59**: 558–563.
- Gopurenko D, Williams RN, DeWoody JA.** 2007. Reproductive and mating system success in the small-mouthed

- salamander (*Ambystoma texanum*) estimated via micro-satellite parentage analysis. *Journal of Evolutionary Biology* **34**: 130–139.
- Gowaty PA, Karlin AA. 1984.** Multiple maternity and paternity in single broods of apparently monogamous eastern bluebirds (*Sialia sialis*). *Behavioral Ecology and Sociobiology* **15**: 91–95.
- Höggren M, Tegelström H. 2002.** Genetic evidence for first-male mating advantage in the adder. In: Schuett GW, Höggren M, Greene HW, eds. *Biology of the vipers*. Eagle Mountain, UT: Eagle Mountain Publishing, 235–242.
- Ims RA. 1988.** The potential for sexual selection: effect of sex ratio and spatiotemporal distribution of receptive females. *Evolutionary Ecology* **2**: 338–352.
- Jellen BC, Aldridge RD. 2011.** Paternity patterns. In: Aldridge RD, Sever DM, eds. *Reproductive biology and phylogeny of snakes*. Boca Raton, FL: CRC Press, 619–644.
- Jones AG. 2009.** On the opportunity for sexual selection, the Bateman gradients, and the maximum intensity of sexual selection. *Evolution* **63**: 1673–1684.
- Jones AG, Rosenqvist G, Berglund A, Avise JC. 2005.** The measurement of sexual selection using Bateman's principles: an experimental test in the sex-role-reversed pipefish *Syngnathus typhle*. *Integrative and Comparative Biology* **45**: 874–884.
- Jones OR, Wang J. 2010.** COLONY: a program for parentage and sibship inference from multi-locus genotype data. *Molecular Ecology Resources* **B10**: 551–555.
- Kissner KJ, Weatherhead PJ, Gibbs HL. 2005.** Experimental assessment of ecological and phenotypic factors affecting male mating success and polyandry in northern watersnakes, *Nerodia sipedon*. *Behavioral Ecology and Sociobiology* **59**: 207–214.
- Levine BA. 2013.** Genetic structure of the copperhead (Viperidae: *Agkistrodon contortrix mokasen*) at its most northern distribution. Master's Thesis, University of Arkansas.
- Lukoscchek V, Avise JC. 2011.** Genetic monandry in 6 viviparous species of true sea snakes. *Heredity* **102**: 347–351.
- Madsen T, Shine R. 1993.** Temporal variability in sexual selection acting on reproductive tactics and body size in male snakes. *American Naturalist* **141**: 167–171.
- Madsen T, Shine R. 1994.** Components of lifetime reproductive success in adders, *Vipera berus*. *Journal of Animal Ecology* **63**: 561–568.
- Mobley KB, Jones AG. 2012.** Overcoming statistical bias to estimate genetic mating systems in open populations: a comparison of Bateman's principles between the sexes in a sex-role reversed pipefish. *Evolution* **67**: 646–660.
- Parker GA, Birkhead TR. 2013.** Polyandry: the history of a revolution. *Philosophical Transactions of the Royal Society of London Series B, Biological Sciences* **368**: 20120335.
- Parker PG, Tang-Martinez Z. 2005.** Bateman gradients in field and laboratory studies: a cautionary tale. *Integrative and Comparative Biology* **45**: 895–902.
- Prosser MR, Weatherhead PJ, Gibbs HL, Brown GP. 2002.** Genetic analysis of the mating system and opportunity for sexual selection in northern water snakes (*Nerodia sipedon*). *Behavioral Ecology* **13**: 800–807.
- R Core Team. 2013.** R: a language and environment for statistical computing. *R Foundation for Statistical Computing*. Vienna, Austria.
- Raymond M, Rousset F. 1995.** GENEPOP (version 1.2): a population genetics software for exact tests and ecumenicism. *Heredity* **86**: 248–249.
- Rivas JA, Burghardt GM. 2005.** Snake mating systems, behavior, and evolution: the revisionary implications of recent findings. *Journal of Comparative Psychology* **119**: 447–454.
- Scharf I, Meiri S. 2013.** Sexual dimorphisms of heads and abdomens: different approaches to 'being large' in female and male lizards. *Biological Journal of the Linnean Society* **110**: 665–673.
- Schuett GW. 1996.** Fighting dynamics of male copperheads, *Agkistrodon contortrix* (Serpentes, Viperidae): stress-induced inhibition of sexual behavior in losers. *Zoo Biology* **15**: 209–221.
- Schuett GW. 1997.** Body size and agonistic experience affect dominance and mating success in male copperheads. *Animal Behaviour* **54**: 213–224.
- Schuett GW, Gillingham JC. 1986.** Sperm storage and multiple paternity in the copperhead, *Agkistrodon contortrix*. *Copeia* **1986**: 807–811.
- Serbzev D, Bernatchez L, Olsen EM, Vollestad LA. 2010.** Mating system and determinants of individual reproductive success in brown trout (*Salmo trutta*) revealed by parentage analysis of an entire stream living population. *Molecular Ecology* **19**: 3193–3205.
- Shine R. 2008.** Tracking elusive timber rattlers with molecular genetics. *Molecular Ecology* **17**: 715–718.
- Shuster SM, Briggs ER, Dennis PA. 2013.** How multiple mating by females affects sexual selection. *Philosophical Transactions of the Royal Society of London Series B, Biological Sciences* **368**: 1–27.
- Shuster SM, Wade MJ. 2003.** *Mating systems and strategies*. Princeton, NJ: Princeton University Press.
- Smith CF. 2007.** Sexual dimorphism, and the spatial and reproductive ecology of the copperhead snake (*Agkistrodon contortrix*). DPhil Thesis, University of Connecticut.
- Smith CF, Schuett GW. 2014.** Male mating success in a North American pitviper is correlated with testosterone levels and movement patterns. *Biological Journal of the Linnean Society* in press.
- Smith CF, Schuett GW, Earley RL, Schwenk K. 2009.** The spatial and reproductive ecology of the copperhead (*Agkistrodon contortrix*) at the northeastern extreme of its range. *Herpetological Monographs* **23**: 45–73.
- Soucy S, Travis J. 2003.** Multiple paternity and population genetic structure in natural populations of the poeciliid fish, *Heterandria formosa*. *Journal of Evolutionary Biology* **16**: 1328–1336.
- Tang-Martinez Z. 2012.** Repetition of Bateman challenges the paradigm. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 11476–11477.

- Thornhill R. 1983.** Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. *American Naturalist* **12**: 765–788.
- Trivers RL. 1972.** Parental investment and sexual selection. In: Campbell O, ed. *Sexual selection and the descent of man*. Chicago, IL: Aldine, 136–179.
- Uller T, Olsson M. 2008.** Multiple paternity in reptiles: patterns and processes. *Molecular Ecology* **17**: 2566–2580.
- Ursenbacher S, Erny C, Fumagalli L. 2009.** Male reproductive success and multiple paternity in wild, low-density populations of the adder (*Vipera berus*). *Heredity* **100**: 365–370.
- Van Oosterhout CV, Hutchinson WF, Wills DPM, Shipley P. 2004.** MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* **4**: 535–538.
- Wade MJ. 1979.** Sexual selection and variance in reproductive success. *American Naturalist* **114**: 742–747.
- Wade MJ, Shuster SM. 2005.** Don't throw Bateman out with the bathwater! *Integrative and Comparative Biology* **45**: 945–951.
- Wang J. 2004.** Sibship reconstruction from genetic data with genotyping errors. *Genetics* **166**: 1063–1079.
- Weatherhead PJ, Prosser MR, Gibbs HL, Brown GP. 2002.** Male reproductive success and sexual selection in northern water snakes determined by microsatellite DNA analysis. *Behavioral Ecology* **13**: 808–815.
- Williams RN, DeWoody JA. 2009.** Reproductive success and sexual selection in wild Eastern tiger salamanders (*Ambystoma t. tigrinum*). *Journal of Evolutionary Biology* **36**: 201–213.

## APPENDIX

Definitions of important mating system and sexual selection terminology used throughout the present study.

*Mating Success* – the total number of mating partners with which copulation occurred for an individual over a specific time period

*Opportunity for Selection (I)* – relative variance in reproductive success; equal to sex-specific variance in reproductive success divided by the square of mean sex-specific reproductive success; *I* will be greater in the sex that has the greater variance in reproductive success; *I* is predicted to be greater in the sex that has ‘cheaper’ gametes and is in greater abundance (often males); also referred to as opportunity for fecundity selection

*Opportunity for Sexual Selection (I<sub>s</sub>)* – relative variance in mating success, where variance is a function of competition for mates; equal to sex-specific variance in number of mates divided by the square of mean sex-specific number of mates; *I<sub>s</sub>* will be greater in the sex that has the greater variance in mating success; *I<sub>s</sub>* is predicted to be greater in the sex that has ‘cheaper’ gametes and is in greater abundance (often males)

*Bateman Gradient (β<sub>ss</sub>)* – sexual selection gradient; equal to the slope of the regression line of reproductive success onto mating success; if β<sub>ss</sub> is equal to 0, reproductive success is not related to mating success (i.e. mating with a greater number of partners does not result in more offspring produced); the sex that has the greater β<sub>ss</sub> will experience a greater fitness benefit by multiple matings; the sex with ‘cheaper’ gametes and that is in greater abundance is expected to have the greater value of β<sub>ss</sub>

*Reproductive Success* – the total number of offspring produced by an individual over a specific time period

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Appendix S1.** A parental table constructed from genotypic parentage analysis of 114 adult Copperhead (*Agkistrodon contortrix*) in a Connecticut, USA, population for the estimation of male and female Bateman's metrics. Genotypic parentage analysis was conducted for 58 females and 56 males. Nineteen of the females and 11 known males produced offspring, and an additional 15 unknown males were inferred as sires.

**Appendix S2.** A parental table constructed from genotypic parentage analysis and behavioural observations of 114 adult Copperhead (*Agkistrodon contortrix*) in a Connecticut, USA, population for the estimation of male and female Bateman's metrics. Genotypic parentage analysis was conducted for 58 females and 56 males. Nineteen of the females and 11 known males produced offspring, and an additional 15 unknown males were inferred as sires. Field data of observed copulations between known individuals are included as matings.