

Multiple paternity in a salamander with socially monogamous behaviour

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Abstract

In the majority of birds and mammals, social monogamy is not congruent with genetic monogamy. No research to date has compared social and genetic monogamy in amphibians. We analysed paternity in clutches of red-backed salamanders (*Plethodon cinereus*), a species in which social monogamy has been demonstrated in the laboratory, and 28% of individuals in the forest are found in male–female pairs in the noncourtship season. We collected 16 clutches of eggs of *P. cinereus* in the southern Appalachian Mountains of Virginia and collected tail clippings from attending mothers. We genotyped embryos and adults at five microsatellite loci in order to analyse paternity of clutches. Most clutches (84.6%) had multiple sires, with two to three sires per clutch. In this study, 25% of clutches had males in addition to females attending eggs. None of the mothers of these clutches were genetically monogamous. All attending males sired some of the offspring in the clutch that they attended (between 9% and 50%) but never sired a majority in that clutch. We conclude that, at least in this population, social monogamy in *P. cinereus* is not concomitant with genetic monogamy.

Keywords: microsatellite, multiple paternity, parentage, *Plethodon cinereus*, salamander, social monogamy

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Introduction

Social monogamy, first distinguished from genetic and mating monogamy by Wickler & Siebt (1983), can be defined as a system in which individual adult males and females form pairs with a specific individual of the opposite sex and not with other individuals of the opposite sex (Mathews 2002). Intersexual pairs are usually considered socially monogamous if they persist for at least one reproductive event or breeding season (*sensu* Baker *et al.* 2004), but longer-term pairs may exist. Social monogamy can, but does not have to, involve paternal care (Gowaty 1996). Social monogamy has been reported from a variety of vertebrate taxa including birds, mammals, reptiles, amphibians, and teleost fish, as well as several invertebrate species (Lack 1968; Komers & Brotherton 1997; Balloux *et al.* 1998; Bull

et al. 1998; Mathews 2002; Taylor *et al.* 2003; Whitman & Cote 2004). Social monogamy may help males ensure paternity of offspring (Trivers 1972; Kokko & Morrell 2005), while females may benefit from securing paternal care for offspring (Kokita & Nakazono 2001) and access to resources in the male's territory that can be used for yolking ova (Gillette *et al.* 2000). Both sexes may benefit from reducing exposure to sexually transmitted diseases (Loehle 1995), and joint defence of a territory (Whitman & Cote 2004). Each of these potential benefits may increase the fitness of socially monogamous individuals.

With the advent of molecular parentage assessment techniques, it has become obvious that social monogamy is not always indicative of underlying genetic mating systems, often due to extra-pair copulations (Hughes 1998). A frequently cited example is Lack's (1968) statement that over 90% of bird species are socially monogamous, while a literature review by Griffith *et al.* (2002) found that genetic monogamy was common in less than 25% of socially monogamous bird species. Whether social monogamy is congruent with genetic monogamy has also been examined

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in mammals, reptiles and fish. In mammals, like birds, social monogamy is not usually congruent with genetic monogamy (Goosens *et al.* 1998; Solomon *et al.* 2004), but in at least some reptiles and fish, social and genetic monogamy are concomitant, with few extra-pair copulations (Jones *et al.* 1998; Taylor *et al.* 2003; Stow & Sunnucks 2004). However, to our knowledge no genetic paternity testing has been conducted to date in any species of amphibian that displays social monogamy.

Amphibians, including salamanders, typically are aggregate breeders, which devote brief periods of time to mating, often at a neutral location (Wells 1977). Aggregate breeding salamanders usually have aquatic larvae and mating strategies such as male-male competition for mates (Gillette 2003, e.g. Duellman & Trueb 1986; Petranka 1998) and female mate choice (e.g. Gabor *et al.* 2000). To our knowledge, genetic paternity testing of aggregate breeding salamanders like newts and ambystomatids has identified multiple paternity in all species studied (e.g. Gabor *et al.* 2000; Jones *et al.* 2002; Garner & Schmidt 2003; Myers & Zamudio 2004; Gopurenko *et al.* 2006; Steinfartz *et al.* 2006).

In contrast to aggregate-breeding salamanders, many species in the Plethodontidae, the most speciose family of salamanders, are wholly terrestrial (Pough *et al.* 2004). To our knowledge, the only paternity test of any plethodontids was of *Desmognathus ocoee*, which found multiple paternity in 96.4% of clutches analysed (Adams *et al.* 2005). However, *D. ocoee* is a desmognathine and has an aquatic larval stage. Desmognathines display only limited territorial behaviour (Mathis *et al.* 1995) and social monogamy has not been reported from this clade. Two other clades of plethodontid salamanders, plethodontines and bolitoglossines, which are abundant in temperate North and Central America, respectively, have wholly terrestrial life cycles and direct developing larvae (Pough *et al.* 2004). Territoriality appears to be widespread among salamanders that are completely terrestrial (Mathis *et al.* 1995). Many plethodontines and bolitoglossines are either territorial or display several characteristics of territorial behaviour (reviewed by Mathis *et al.* 1995). At least one of these territorial species of salamanders, *Plethodon cinereus*, has been shown to exhibit complex social behaviours, including social monogamy, utilizing pheromonal and visual cues (see review in Jaeger & Forester 1993).

The case for social monogamy in *P. cinereus* is based on two lines of evidence. The primary evidence is based on field and laboratory observations that males and females share territories that they defend against members of the opposite sex (Lang & Jaeger 2000). Territories held by individuals of *P. cinereus* have more overlap intersexually than would be expected when compared to a random distribution (Mathis 1991). Jaeger *et al.* (1995) found 28.0% of the adult population of *P. cinereus* ($n = 336$) in male-female pairs while male-male pairs were found only once ($< 1\%$) and no

female-female pairs were found (0%). There were no additional adults of either sex found with opposite sex pairs. These associations between opposite sex pairs are long lasting; they can persist for at least 3 years (Gillette 2003). These opposite sex behavioural associations that preclude additional members of either sex are sufficient to demonstrate social monogamy under currently used definitions (e.g. Davies 1992; Mathews 2002). However, laboratory experiments also provide behavioural support for the hypothesis that pairs of *P. cinereus* display social monogamy, with individuals spending more time near partners with whom they were found cohabitating within the forest compared to novel individuals (Gillette *et al.* 2000). In addition, both males and females exhibit more aggression towards partners that were socially polygamous than towards partners that were socially monogamous (Jaeger *et al.* 2002; Prosen *et al.* 2004, 2006); there were no differences in behaviours towards strangers that were socially polygamous or monogamous strangers. While these behaviours are not necessary components of social monogamy, they do suggest mechanisms for maintaining pair bonds.

Although the occurrence of social or genetic monogamy is not directly linked to parental care per se (Gowaty 1996; Fromhage *et al.* 2005), monogamy is often associated with paternal care (e.g. Møller 1988; Kokita & Nakazono 2001). In *P. cinereus*, nearly all clutches are attended by a female, but the percentage of clutches attended by male-female pairs varies by locality (e.g. Highton & Savage 1961; Friet 1995; reviewed in Petranka 1998). Female attendance of clutches is known to have antipredatory benefits with females (83–91%) actively defending eggs from potentially cannibalistic conspecific adults and possibly protecting eggs from predators such as *Diadophis punctatus* (Highton & Savage 1961; Bachmann 1984). Female attendance also increases the proportion of surviving embryos in the laboratory, potentially by decreasing desiccation of eggs (Heatwole 1961; Highton & Savage 1961). When separated from females, eggs had a greater than 66% decline in survival and hatchlings were smaller (Highton & Savage 1961). Brooding costs to females of *P. cinereus* include time and energy, resulting in decreased future reproductive success as measured by gain of less mass and less production of ova compared to nonbrooding females (Ng & Wilbur 1995; Yurewicz & Wilbur 2004). Male attendance may increase survival of eggs and decrease the time and energy contributed by attending females by increasing moisture available to eggs and potentially defending the eggs that they are attending. However, there have been no experiments to evaluate the costs and benefits of paternal care in *P. cinereus*. While such an evaluation was beyond the scope of this study, we hypothesized that males' attendance of clutches would be greater with a greater proportion of offspring sired in a clutch (*sensu* Møller 1988, 1991; Burke *et al.* 1989; Hatchwell & Davies 1990; Dixon *et al.* 1994). Such a relationship would be expected if males' egg

attendance is a costly behaviour that benefits egg survival (Trivers 1972).

Our objective was to elucidate genetic mating patterns in *P. cinereus* relative to behaviour patterns. Specifically, we wanted to determine if the tendency for intrasexual territoriality and spatial associations of opposite-sex individuals displayed by a socially monogamous salamander resulted in higher rates of single father clutches than are observed in aggregate-breeding salamanders. Within species, alternative mating strategies may be adopted by different individuals (Rubenstein 1980), and while there is no implication that all adults of *P. cinereus* are socially monogamous, the high frequency of male-female pairs (Jaeger *et al.* 1995) and the observation of long-term pair associations (Gillette 2003) in this population makes it a good candidate for assessing the prevalence of genetic monogamy. We also examine our hypothesis that males attending a clutch will provide the majority of paternity in the clutches with which they are associated.

Materials and methods

Sample collection and genotyping

Between 29 June and 20 July 2005, we collected egg clutches of *Plethodon cinereus* ($n = 16$) by lifting and looking beneath cover objects (rocks) in Giles County, Virginia in Jefferson National Forest, within 3 km of Hairpin Turn (37°25'25"N, 80°30'02"W). This area had a large number of rocks suitable for locating clutches of *P. cinereus*. The altitude was approximately 1200 m above sea level, and the site consisted of hardwood forest dominated by red oak (*Quercus rubra*), white oak (*Quercus alba*), and red maple (*Acer rubrum*). We located a female at each nest attending 2–11 eggs ($\bar{x} \pm 1$ SE = 7.06 \pm 0.76). We captured males in addition to females at some nest sites ($n = 4$); we never found multiple males or multiple females at any nest site. We collected all the eggs in each clutch and tail clippings from all females and males found under the same cover object as the clutch. We considered all females and males found within 10 cm of a clutch to be attending that clutch. We removed tail clippings by application of pressure with forceps approximately 0.5 cm from the tips of tails of adults whereby the tail tips subsequently autotomized (*sensu* Adams *et al.* 2005). After collection of tail clippings, we released salamanders adjacent to the cover object from which they had been collected. Snout-vent length (SVL) of each adult was measured using Mitutoyo digital calipers (± 0.01 mm). SVL was used to assign individuals of *P. cinereus* to age classes (*sensu* Saylor 1966). We kept the eggs at 10 °C in Petri dishes (14 \times 1.5 cm) lined with filter paper and moistened with spring water twice a week at Mountain Lake Biological Station (MLBS), Virginia. We placed each tail clipping in a separate 1.5 mL microtube containing 500 μ L

collection buffer (10 mM Tris, 10 mM EDTA, pH 8) and placed it on ice until transported to MLBS where they were frozen and kept at -70 °C.

We dissected embryos out of eggs with a scalpel and forceps, and yolk-filled stomachs were removed (*sensu* Adams *et al.* 2005). DNA was extracted from embryonic tissue and adult tail clippings using a Wizard Genomic DNA Purification Kit (Promega).

We genotyped all individuals at five dinucleotide repeat microsatellite loci. Four of the loci (PcLX16, PcLX23, PcI16, and PcFX08) were amplified by polymerase chain reaction (PCR) following the protocols in Connors & Cabe (2003). PCR amplicons were resolved with an ABI 3130 Automated Sequencer (Applied Biosystems). We sized alleles for these four loci using GENEMAPPER 3.4 software (Applied Biosystems). The fifth locus, PcDX23 (primers HEX/GCAAAACAGCAACAAGACAAC, AACCTTGATGTTGGCAAGG, GenBank Accession no. AY151376) was amplified with similar protocols. Due to a lack of labelled primers suitable for use with the ABI 3130, products from this locus were analysed with a LI-COR 4300 DNA analyser and SAGA software (LI-COR Biotechnology).

Genotypes of the 20 adults collected at the nests and one male found within 0.5 m of one of the nests were used to calculate the number of alleles per locus, allele frequencies, expected and observed heterozygosities, as well as test for Hardy–Weinberg equilibrium at each of the loci and linkage disequilibrium for each pair of loci (GENEPOP version 3.4, Raymond & Roussett 1995).

Parentage analysis

We analysed the genotypes of females and embryos using the computer software GERUD 1.0 to assess the frequency of multiple paternity within each clutch (Jones 2001). Our confidence in female maternity of clutches was based on the inability of GERUD 1.0 to generate output if the attending female genotype was incompatible with any embryo in that clutch. In addition, we calculated with GERUDSIM 1.0 the probability that females in this population would be excluded, using these five loci, as a parent of each embryo in the clutch they were attending (Jones 2001).

The analysis with GERUD 1.0 estimated the minimum number of sires for each clutch of *P. cinereus* and generated potential paternal genotypes. Three clutches with two embryos were excluded from this part of the analysis due to the high potential for underestimating the numbers of sires in clutches with only two embryos. All other clutches had 5–11 embryos. We used maximum likelihood analyses in GERUD 1.0 to identify the proportions of each clutch that could be attributed to each potential paternal genotype. We analysed whether the number of eggs per clutch was related to the number of sires by using correlations in SAS 9.1 (SAS Institute Incorporated 2004). We also performed

Table 1 Variation in five *Plethodon cinereus* microsatellite DNA loci ($n = 21$). Shown are size range of alleles, number of alleles, observed heterozygosity (H_O) and expected heterozygosity (H_E) for each locus

Locus	Size range (bp)	No. of alleles	H_O	H_E
PcLX16	199–211	4	0.565	0.612
PcLX23	166–192	11	0.739	0.727
PcI16sd	144–166	4	0.217	0.202
PcFX08	178–212	13	0.913	0.868
PcDX23	182–222	11	0.783	0.794

correlations of maternal SVL with the numbers of eggs and the number of sires per clutch using SAS 9.1.

We calculated the probability that the estimated number of sires was correct with simulations in GERUDSIM 1.0 (Jones 2001) using the protocols in Adams *et al.* (2005). Genotypes of those males found with females and clutches were compared to the output of paternal alleles generated by GERUD 1.0. Attending males were considered potential sires of an embryo if they possessed the paternal allele present in each locus. The percentage of embryos sired by attending males was estimated for each of the four clutches where attending males were present. We calculated with GERUDSIM 1.0 the probability that we were able to exclude attending males that were not sires of an embryo using these five loci, given knowledge of the maternal genotypes of clutches.

Results

None of the microsatellite loci were found to deviate significantly from Hardy–Weinberg equilibrium, and no pairs of loci exhibited statistically significant linkage disequilibrium in this population. Expected and observed heterozygosities and number of alleles per locus are presented in Table 1.

Females' genotypes were consistent with all embryos in the clutches that they were attending. With no prior knowledge of parentage, the exclusion probability for an individual in this population as a parent of an embryo was 0.8731. Note that exclusion probabilities were higher for attending females if we assume that they must be the mother of all embryos in a clutch. All clutches had at least two embryos and the exclusion probability generated was for each embryo (e.g. exclusion probability was 0.984 for two embryo clutches, 0.998 for three embryo clutches, etc.). Therefore, we were confident that each female was the mother of the clutch that she attended.

Two clutches were found to have one sire, seven clutches were found to have two sires, and four clutches were found to have three sires (Fig. 1). The probabilities of identifying the correct number of sires were nearly 1.0 in clutches with one or two sires, but the probability was lower for clutches

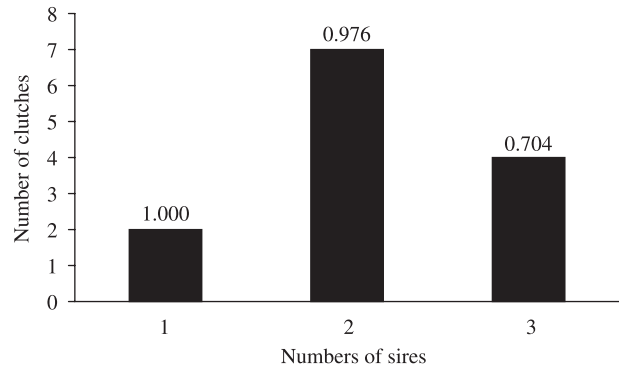


Fig. 1 Numbers of sires in clutches of *Plethodon cinereus* collected in the field ($n = 13$) using five microsatellite loci. The probabilities of correctly identifying the number of sires are shown over the bars.

with three sires (Fig. 1). We were therefore confident in our ability to distinguish female monogamy from polyandry, but slightly less confident in our ability to distinguish between numbers of sires in multiple-sired clutches. The number of eggs was not significantly correlated with the number of sires ($r = 0.5377$, $P = 0.0581$).

Null alleles have a potentially confounding effect on paternity analyses because they lack a priming site for PCR (Jones & Ardren 2003; Myers & Zamudio 2004; Adams *et al.* 2005). When null alleles are present, an individual's genotype that is a heterozygote (e.g. A/null) will be scored as a homozygote (e.g. A/A) for that locus (Jones & Ardren 2003). Null alleles are usually, but not always, detected in populations by significant deviations from Hardy–Weinberg equilibrium. In this study, no maternal null alleles were detected (i.e. no homozygotes were detected in any offspring for alleles not present in the corresponding maternal genotype), nor were there significant deviations from Hardy–Weinberg equilibrium (Table 1). However, offspring of some clutches in this study contained homozygotes for both maternal alleles (three clutches at the LX16 locus and one at the I16 locus), which may be indicative of the presence of a single paternal null allele, not two separate paternal alleles (one for each homozygote) as they were scored. This possible overestimation of the number of paternal alleles present in a clutch may lead to overestimation of numbers of sires (Adams *et al.* 2005). Consequently, we reanalysed the four clutches with potential null alleles using GERUD 1.0, substituting a null allele for the paternal contribution in the appropriate loci of potentially affected embryos. There were no differences in the numbers of sires for any of these four clutches found when these clutches were reanalysed. However, in two of these four clutches, when potential null alleles were included, the maximum-likelihood estimates of the proportions of each clutch that could be attributed to specific paternal genotypes did differ slightly compared to the estimates made without null alleles. The differences were never greater than 14%.

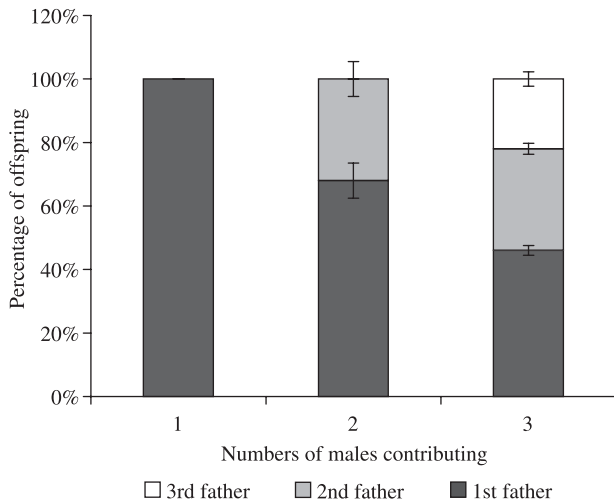


Fig. 2 Mean distribution of paternal contributions within clutches of *Plethodon cinereus* ($n = 13$) as a percentage of offspring ± 1 SE.

On average, in clutches with two sires, one male sired the majority of the offspring, but in clutches with three sires, no one male sired the majority of offspring (Fig. 2). Neither the number of eggs nor the number of sires per clutch was correlated with maternal SVL ($r = 0.3803$, $P = 0.2227$ and $r = 0.5711$, $P = 0.1083$, respectively).

We found an attending male, in addition to a female, at 25% percent of clutches, a number similar to the 28% of *P. cinereus* found in male-female pairs during the noncourtship season by Jaeger *et al.* (1995). The probability of excluding a male as the sire of an embryo was 0.9708 using these five loci. Each attending male was compatible with at least one of the embryos in the clutch that he attended. However, all of the clutches with an attending male had two sires. Attending males sired 38.34% (± 9.84 , $n = 4$) of eggs in the clutches that they attended.

Discussion

Genetic monogamy was found in only 15% of females with clutches. Polyandry was found to be the predominant genetic mating system in this population of *Plethodon cinereus*. Clutches of *P. cinereus* had similar or slightly lower average and maximum number of sires when compared with aggregate-breeding salamander species whose paternity has been quantified in natural conditions (Jones *et al.* 2002; Myers & Zamudio 2004; Adams *et al.* 2005; Gopurenko *et al.* 2006; Steinfartz *et al.* 2006). We note that our probability of correctly identifying the number of sires in clutches with three sires was only about 0.7. It is unclear whether the small number of sires is a function of the small clutch sizes in this species. Although the correlation between clutch size and sire number was not significant, it was high enough to question whether additional clutches would have produced

a significant relationship. The only paternity analysis of another plethodontid salamander, *Desmognathus ocoee*, found slightly higher numbers of sires with an average of 16 eggs per clutch and a maximum of four sires (Adams *et al.* 2005). Similar to data from paternity testing of aggregate-breeding salamanders, our results do not lend support to the hypothesis that genetic monogamy is an important (or common) strategy of *P. cinereus*, in spite of the latter's tendency to exhibit socially monogamous behaviour.

Attending males in this study sired between 9% and 50% of the embryos in the clutches that they attended, and each of these clutches had two sires; they never sired a majority of the embryos. Social monogamy is often suggested as a mechanism for ensuring paternity (Trivers 1972; Kokko & Morrell 2005). In *P. cinereus*, social monogamy does not appear to ensure that the attending male is a father of a majority of a clutch.

Protracted mating seasons and long-term sperm storage are characteristic of some plethodontid salamanders (Adams *et al.* 2005), and may help explain multiple paternity and low percentages of offspring sired by attending males in *P. cinereus*. Individuals of *P. cinereus* breed between October and May, with winter hibernation occurring between December and March (Sayler 1966). This protracted breeding season allows ample opportunity for extra-pair copulations in social monogamous pairs. We note that the attending males found in this study and the male-female pairs found by Jaeger *et al.* (1995) represented only points in time when pairs were found and may not represent long-term pairings. Therefore, females may have formed pairs with multiple males that were separated temporally and the attending males may have been the last mated male and unable to leave egg deposition site after mating due to dry conditions, which can limit movements of *P. cinereus* (reviewed in Petranka 1998). However, this study began in the end of June and eggs are deposited in this area in the end of May or the beginning of June (Yurewicz & Wilbur 2004). There were at least 11 precipitation events between 1 and 29 June 2005 (Liebgold 2006). Therefore, in this study, ambient conditions did not restrict movements of males between mating and egg deposition. More importantly, at least some male-female pairs found within 1 km of our study site persisted for up to 3 years (Gillette 2003).

In some species of birds, there is no relationship between male parentage and male parental care (Yezerinac *et al.* 1996; Hughes 1998; Bouwman *et al.* 2005). This often occurs when the males are unable to assess paternity (Hughes 1998). Despite the extensive use of pheromones for complex social interactions in *P. cinereus*, Peterson (2000) found that females did not distinguish between their own eggs and eggs of other females. Instead, they used an indirect cue, nest site, to locate their clutches. Males of *P. cinereus* may therefore be unable to assess paternity of eggs and only identify either the nest site of a female with which they

mated or the female herself. Although it is possible that the males may not be attending clutches at all but only sharing the same cover object as females (*sensu* Jaeger *et al.* 1998, 2002), this seems unlikely given the potential benefits to eggs from attending adults (Heatwole 1961; Highton & Savage 1961; Bachmann 1984). More importantly, each attending male sired offspring in the clutch that he attended.

One of the many hypotheses attempting to explain why male birds care for broods in which they have low paternity, Yezerinac *et al.* (1996) suggests that there may be a high cost to mistakes and a low cost to a conservative assumption of paternity. For *P. cinereus*, clutches with female attendance have reduced predation and may have reduced risk of desiccation (Heatwole 1961; Highton & Savage 1961). Male attendance may further decrease these causes of mortality. In addition, while females use large fat reserves during the 6–11 weeks of brooding and are emaciated when eggs hatch (Ng & Wilbur 1995), it is unlikely that a male attendant to clutches would have similar costs because a female usually does not feed and remains with her clutch most of the time (Highton & Savage 1961; Ng & Wilbur 1995) whereas males may still forage. In addition, because eggs are brooded in the noncourtship season, there is no loss of matings by males attending clutches. Therefore, there may be a low cost to male attendance of clutches.

In summary, multiple paternity appears to be common in red-backed salamanders, *P. cinereus*, despite the occurrence of social monogamy (Gillette *et al.* 2000; Jaeger *et al.* 2002; Prosen *et al.* 2004). Males that were found attending clutches with females were the sires of some embryos in that clutch but never a majority of embryos. A prolonged breeding season may explain the high frequency of extra-pair copulations, but further research is needed to explore this hypothesis and to perform cost-benefit analyses of male egg attendance despite low paternity in *P. cinereus*.

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This study was conducted in partial fulfillment of Eric Liebgold's Master of Science research exploring mating systems and juvenile movements in the terrestrial and territorial salamander, *Plethodon cinereus*. He is currently a doctoral student at the University of Virginia. Paul Cabe's research focuses on using molecular methods to answer questions about population genetics and evolutionary biology. Robert Jaeger is interested in the behavioural ecology of salamanders. Paul Leberg's research focuses on the application of theory from ecology, evolution and population genetics to questions in wildlife management using molecular and experimental techniques.
