

Artur Osikowski · Jan Rafiński

Multiple insemination increases reproductive success of female Montandon's newt (*Triturus montandoni*, Caudata, Salamandridae)

Received: 7 January 2000 / Revised: 13 September 2000 / Accepted: 7 October 2000

Abstract The adaptive significance of multiple matings for females is a matter of much controversy. In insects, supplying the female's sperm reserves with portions of fresh spermatozoa may be the main function of multiple matings. This simple explanation may also be applied to other animals which produce large numbers of eggs over prolonged periods of time. We tested the fertility insurance hypothesis in Montandon's newt (*Triturus montandoni*, Amphibia, Salamandridae). *T. montandoni* females are inseminated internally by spermatophores they have picked up, and subsequently lay eggs fertilized by spermatozoa released from the spermatheca. We compared the reproductive success of singly and multiply inseminated females of Montandon's newt in the laboratory. Multiply inseminated females laid more eggs and had a lower percentage of non-developing eggs than females who mated only once. Our data suggest that remating increases the reproductive success of multiply inseminated females by replenishing sperm reserves in the spermatheca or by supplying females with fresh portions of spermatozoa with high fertilizing capacity.

Keywords Multiple matings · Polyandry · *Triturus montandoni* · Newts

Introduction

The benefits of multiple matings to females have been avidly discussed but are still not fully understood (e.g., Eberhard 1996; Birkhead and Parker 1997; Birkhead and Møller 1998; Brooks and Jennions 1999). Polygyny clearly allows males to increase their reproductive success by siring more offspring (Bateman 1948). In con-

Communicated by A. Mathis

A. Osikowski · Jan Rafiński (✉)
Department of Comparative Anatomy, Institute of Zoology,
Jagiellonian University, ul. Ingardena 6, 30060 Kraków, Poland
e-mail: rafin@zuk.iz.uj.edu.pl
e-mail: osik@zuk.iz.uj.edu.pl
Tel.: +48-12-6336377, Fax: +48-12-6336377

trast, the number of offspring left by a female is not expected to correlate with the number of matings. In most species, the number of sperm in a single ejaculate is several orders of magnitude larger than the number of ova produced by a single female (Parker 1992). Every mating entails costs for the female (summarized by Stockley 1998), e.g., loss of time and energy, risk of predation, risk of infection by sexually transmitted diseases, and a reduction in parental care by male breeding partners because of their lower likelihood of paternity. However, females of many species actively solicit copulations with more than one male even within a single breeding season (Olsson et al. 1994; Eberhard 1996; Zeh 1997). Strict genetic monogamy by females may be rare (Eberhard 1996). With the exception of certain cases, for example where males simply force copulations on females, one would therefore expect females to gain some benefits from multiple matings.

Potential benefits of multiple mating for females can be either direct (e.g., food, acquisition of nutrients from the seminal fluid, paternal care, fertility insurance) or indirect (genetic, arising from the production of genetically variable or high-quality progeny) (reviewed by Birkhead and Parker 1997). When females obtain direct benefits, the explanation for polyandry is straightforward and often supported by direct evidence (Parker 1992). The hypothesis of genetic advantages through increased fitness of the progeny is much more controversial (Parker 1992; Newcomer et al. 1999). Increased offspring viability of multiply inseminated females has been found in some reptiles and arthropods (Madsen et al. 1992; Olsson et al. 1994; Watson 1998), but the mechanisms responsible are not clear. Zeh (1997) proposed that through polyandry, females could avoid fertilization by sperm that are genetically incompatible with her eggs.

The simplest and most widespread explanation for the evolution of female multiple mating is the hypothesis of fertility insurance (Parker 1970). In species in which females produce a large number of eggs which are deposited over a prolonged time, one insemination may be in-

sufficient to provide enough sperm with full fertilizing ability for the entire period of egg-laying. Even when the number of sperm in one ejaculate is large enough to fertilize all the females' eggs, the sperm may leak from the storage organs with time (Childress and Hartl 1972) and/or may lose their fertilizing capacity. Observational data for some species indicate that multiple mating is necessary to ensure an adequate sperm supply and full fertility. Pyle and Gromko (1978) and Gromko et al. (1984) reported that a single copulation in *Drosophila* does not ensure continuous fecundity. Ridley (1988) concluded that repeated matings may be universally necessary for full fecundity and fertility in insects. Yet in other groups for which multiple matings have been documented, there have been surprisingly few tests of the fertility insurance hypothesis (Birkhead and Parker 1997).

The fertility insurance hypothesis may also hold for vertebrates with internal fertilization, in which females store sperm and produce large numbers of eggs during a prolonged breeding season, i.e., some internally fertilized fishes and most tailed amphibians (Urodela). In 90% of urodelan species, fertilization is internal (Halliday 1998) and sperm are stored in a sperm storage organ – the spermatheca. The spermatheca consists of many thin-walled tubules which open to the cloacal chamber where fertilization takes place (Sever 1992). In several urodelan species (e.g., most members of the Salamandridae), eggs are laid one by one over an extended period of time. The number of sperm released from spermathecal tubules each time an egg is laid may be high (Sever et al. 1999), potentially depleting sperm reserves before all the eggs have been laid. On the other hand, a prolonged egg-laying period may accompany a decrease in the fertilizing capacity of the sperm stored in the spermatheca. Under such circumstances, multiple matings may restore the depleted spermatozoa reserves and/or provide a supply of fresh sperm with full fertilizing capacity.

Here we present the results of a study on the influence of multiple matings on egg production, duration of the egg-laying period, and egg development in Montandon's newt (*Triturus montandoni*). The reproductive behavior of Montandon's newt has been described by Pecio and Rafiński (1985). Mating takes place in spring and early summer in ponds and other small water reservoirs. As in other newt species, *T. montandoni* females are inseminated internally by sperm caps they pick up from spermatophores deposited by males on the bottom of ponds during a complex courtship. Females are courted by males in a sequence of stereotyped movements. A receptive female moves in the direction of a courting male which then turns away from the female, and the male deposits a spermatophore only when touched on the tail tip by a female. To transfer the sperm to her cloaca, a female has to follow a male closely. Both spermatophore deposition and sperm transfer require co-operation of the female, ruling out forced insemination in this species. After insemination, sperm are stored in the females' sperm storage organs (spermathecae) and are used for

fertilization of eggs laid during the egg-laying period. In *T. montandoni*, egg-laying occurs over 2–3 months during which time females produce 100–250 eggs (Juszczak 1987). Eggs are laid one by one and carefully wrapped in the leaves of waterplants.

A prolonged breeding season and egg-laying period in Montandon's newt together with a high frequency of encounters with sexually active males create opportunities for females to mate with several males. Through analysis of allozymic variation of progeny collected from females inseminated in nature, Rafiński (1981) reported a high level of multiple paternity in the Alpine newt, *T. alpestris*. Alpine and Montandon's newts have very similar reproductive strategies, so *T. montandoni* females probably also mate with more than one male during one breeding season. In the laboratory, *T. montandoni* remate easily.

The aim of our study was to test the hypothesis that polyandry of *T. montandoni* females provides increased reproductive success measured as the number of eggs produced during the breeding season and percentage of developing eggs. We compared the duration of the egg-laying period, number of eggs produced, and percentage of non-developing eggs for singly and multiply (two, three, or four times) mated females.

Methods

Both male and female Montandon's newts were collected from the same site in the Gorce Mountains (Carpathians, southern Poland) in early spring 1999. We collected females on land before they entered water where mating takes place. Females were kept singly in water in small holding aquaria (18×25×20 cm) provided with waterplants where some laid fertilized eggs. Males were caught either on land or in water, and then kept in water in groups of ten individuals in plastic boxes (40×30×20 cm). All animals before and during the experiment were fed small earthworms and crustaceans. The air temperature in the laboratory was controlled at 16°C and a 12/12 h dark/light regime was maintained.

Females that did not lay fertilized eggs within 3 days were considered to be uninseminated and were divided into two groups. In the first group ($n=27$) females were mated two, three, or four times at intervals of 3 days, each time with a different male. In the second group ($n=22$), females were inseminated only once and were then paired with a courting male for at least 1 h at the same intervals as the females of the first group, but sperm transfer was prevented (referred to below as pairing). Thus the only difference between the two groups was in the number of inseminations. Females of the first group were allowed to pick up as many sperm caps as they wanted during each mating trial. The number of sperm transfers was also not limited for the first mating of females in the second group. No female picked up more than three sperm caps during any single mating trial. We allowed each pair to court for 1 h. If insemination was not successful (i.e., the sperm cap did not attach to the female's cloaca) during this period, we discontinued the experiment and treated that female as non-receptive in that encounter. Our previous observations had suggested that female Montandon's newts lose their receptivity when kept for more than 2 weeks in the laboratory. We decided to mate pairs only during the first 10 days following the first insemination. In the group of multiply inseminated females, the period from the first to the last mating was 6.8 ± 3.1 days (mean \pm SD). All matings were arranged in pairs in aquaria (50×30×25 cm) with the bottom covered with sand. The animals were not measured to minimize harassment and the pairs were assigned randomly as to size, weight, and condition. Most males were used only once; in the few cases where a male

was used a second time, the interval between matings was at least 2 days.

After mating, females were put again singly in holding aquaria provided with waterplants. Eggs laid by females were collected at intervals of 2–6 days, counted, and kept in water in glass jars. Eggs were allowed to develop for 4 days and then the number of developing and non-developing eggs was counted in each egg collection under a stereomicroscope. The end of the egg-laying period was established when a female had stopped laying eggs for more than 7 days.

To test the effects of the number of inseminations and the phase of the egg-laying period on the proportion of non-developing eggs, a two-way ANOVA was used on arcsin-transformed data. After transformation, the distribution of the data did not deviate from normality. An ANOVA was also used to test differences in egg number produced by singly versus multiply mated females because these data also did not deviate significantly from normality. For all other statistical tests, non-parametric statistics were used. All statistical tests were two-tailed.

Results

Duration of egg-laying period and the number of eggs laid

There was no significant difference in the duration of the egg-laying period between the females inseminated multiply (mean±SD=25.5±3.3 days) and singly (26.5±3.5 days) (Mann-Whitney *U*-test, $Z=1.18$, $P=0.24$). However, multiply inseminated females produced significantly more eggs than females inseminated once (one-way ANOVA, $F_{1,47}=7.00$, $P<0.05$; Fig. 1).

For both multiply and singly mated females, we used a sign test to determine whether the number of eggs produced increased after successive pairings. If courtship itself influenced egg-laying, we would expect an increased number of eggs deposited in both groups. To eliminate the influence of the variable number of days between egg collections for some females, we included only those females for which the same time interval had elapsed between consecutive egg collections (2 days for second pairing and 3 days for third pairing). In both groups of females, the number of eggs laid between the second and third pairing increased significantly (sign test: $n=20$, $Z=3.35$, $P<0.001$ for multiply inseminated females; $n=13$, $Z=2.21$, $P=0.027$ for singly-inseminated females). Between the third and fourth pairings, the number of eggs produced decreased significantly in both groups of females (sign test, $n=15$, $Z=2.07$, $P=0.039$ for multiply inseminated females; $n=10$, $Z=2.21$, $P=0.027$ for singly inseminated females).

Proportion of viable eggs

The percentage of non-developing eggs deposited by multiply inseminated females was lower (5.1±5.1%) than that deposited by females inseminated singly (11.3±12.0%). To test if the percentage of non-developing eggs changed with time, the egg-laying period of each female was divided into two phases of the same du-

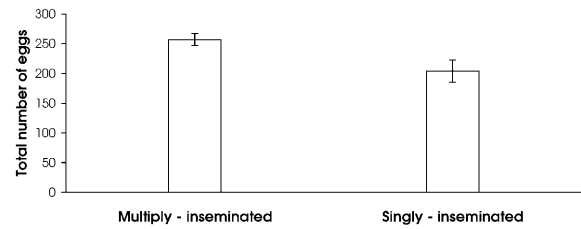


Fig. 1 Total number of eggs (mean±SE) in clutches produced by multiply and singly mated females

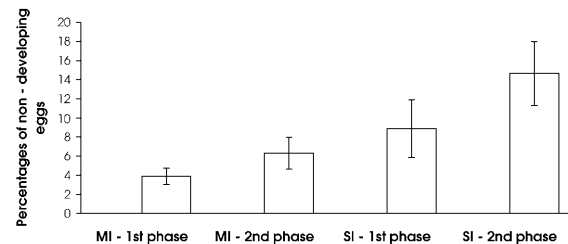


Fig. 2 Percentages (mean±SE) of non-developing eggs in the first and second phase of the egg-laying period in multiply (*MI*) and singly (*SI*) inseminated females

ration (Fig. 2). The percentage of non-developing eggs in both groups of females was lower in the first than in the second phase (3.9±4.3% and 6.3±8.5%, respectively, in multiply inseminated females; 8.9±13.5% and 14.6±14.9%, respectively, in singly inseminated females). A two-way ANOVA showed that both main effects were significant (number of inseminations: $F_{1,88}=9.212$, $P=0.003$; egg-laying phase: $F_{1,88}=4.058$, $P=0.047$) whereas their interaction was not ($F_{1,88}=0.499$, $P=0.482$). In the second phase of the egg-laying period, females inseminated singly had a higher percentage of non-developing eggs than females inseminated multiply (Mann-Whitney *U*-test, $Z=2.59$, $P<0.01$), whereas in the first phase of the egg-laying period, there were no differences in the percentages of non-developing eggs laid by singly and multiply inseminated females (Mann-Whitney *U*-test, $Z=1.43$, $P=0.154$).

Discussion

Our data indicate clearly that multiple mating increases the reproductive success of female Montandon's newts, in agreement with the fertility insurance hypothesis (Birkhead and Parker 1997). First, multiply inseminated females produced significantly more eggs than females inseminated only once. This difference did not result from a longer duration of the egg-laying period in this group, since no significant difference in the egg-laying period between singly and multiply inseminated females was found. In our experiment, singly mated females were confronted repeatedly with courting males, but these females produced fewer eggs than females inseminated several times. Therefore, increased egg production

by multiply inseminated females resulted from multiple inseminations and not courtship itself. In both singly and multiply inseminated females, the number of eggs laid increased 2–3 days after the second pairing. Although this increase could have resulted from a stimulating effect of courtship itself, the number of eggs produced after the third mating declined in both groups. For the few females inseminated for the fourth time, no increase in egg production was observed. Thus the increase in number of eggs produced after the second mating might have been caused by a general tendency for laying more eggs at the beginning of the egg-laying period and not by the pairing itself. Our independent data collected from the Alpine newt (*T. alpestris*) confirm that courtship itself does not induce egg-laying (A. Osikowski and J. Rafiński, unpublished data).

Two different but not exclusive mechanisms may be responsible for the overall larger number of eggs produced by multiply inseminated females. First, ovulation could be triggered by chemical substances present in the sperm cap, and declining levels of these substances in singly mated females may result in lower numbers of ovulations and/or smaller numbers of eggs per ovulation. In some insects, biologically active substances in the spermatophores may induce ovulation (for a review see Eberhard 1996). As far as we know, no data on the presence of biologically active substances in sperm caps of tailed amphibians have been reported (Mann 1984; Halliday 1998). Second, a larger number of eggs produced by multiply inseminated females may result from physical stimulation which may occur when the sperm cap enters the cloacal chamber, or later on, during sperm storage inside the spermathecal tubules. A decrease in the volume of sperm in these tubules during egg-laying may be a signal for the female to stop ovulating because of the risk that ovulated eggs might not be fertilized. After a remating, the pressure of fresh sperm and accompanying fluid on the walls of the spermathecal tubules may induce ovulation to restart.

Singly inseminated females not only produced fewer eggs but also laid a higher proportion of non-developing eggs than females inseminated multiply. The simplest explanation is that depletion of the sperm reserves and/or loss of fertilizing capacity with time caused this higher rate of deposition of non-fertilized eggs. In *T. vulgaris*, a species phylogenetically closely related to *T. montandoni* (Rafiński and Arntzen 1987; Zajc and Arntzen 1999), some females inseminated only once at the beginning of the egg-laying period also laid unfertilized eggs at the end of the breeding season (Pecio 1992).

Both singly and multiply inseminated females produced more non-developing eggs during the second phase of the egg-laying period, but this was especially pronounced for females inseminated singly. The latter had a higher percentage of non-developing eggs in the second phase of the egg-laying period than females inseminated multiply. We did not determine if the non-developing eggs were fertilized or not, but our earlier observations on different species of the genus *Triturus* indicated that most non-developing eggs were not fertilized.

In the genus *Triturus*, sperm are transported from the cloacal chamber into the spermathecal tubules where sperm are stored during the egg-laying period (Sever et al. 1999). In the smooth newt (*T. vulgaris*), the spermatheca consists of 40–60 simple, blind tubules penetrating the surrounding tissue and opening independently to the roof and side walls of the cloaca (Verrel and Sever 1988). In Montandon's newt, spermathecal structure is very similar except that the number of tubules is larger (own unpublished data). The sperm are released from the tubules when eggs pass from the oviduct to the cloacal chamber (Hardy and Dent 1986). Though the number of sperm in a single sperm cap is high enough to ensure the fertilization of all the eggs laid in one breeding season (Waights 1998), the higher proportion of non-developing eggs during the latter stages of the egg-laying period may result from a declining number of sperm. The volume of sperm present in one sperm cap relative to the volume of the spermatheca is not known for this or other species of the genus *Triturus*. Hardy and Dent (1986) compared the volumes of the sperm cap and the spermathecal lumen in the red-spotted newt (*Notophthalmus viridescens*) and estimated that only a small portion of sperm enters the cloacal chamber and moves to the spermatheca tubules; the rest is expelled from or leaks out of the vent. A similar situation may exist in the genus *Triturus*, since the posterior portion of the sperm cap sticks out of the female's cloaca for at least 1 h after insemination (Sever et al. 1999; own observations). It is highly unlikely that sperm are released one by one from spermathecal tubules during egg-laying. Indeed, in *T. vulgaris*, tangled masses of sperm were observed external to spermathecal orifices during the egg-laying period (Sever et al. 1999).

Eggs of Urodela do not possess blocks against polyspermy, so more than one sperm usually penetrate each egg (Elinson 1986). The mean number of sperm penetrating one egg in *T. vulgaris* was estimated to be 4.2, but up to 100 sperm were found in a single egg (Waights 1998). This indicates that, in Urodela, the number of sperm required for fertilization may be significantly higher than in other animals in which physiological polyspermy does not occur, and therefore sperm reserves may be quickly depleted.

A second possible mechanism responsible for a higher percentage of non-developing eggs in clutches produced by singly mated females may be a decline in the fertilizing ability of spermatozoa stored in spermatheca. Variance in the duration of sperm storage in Urodela is large (Halliday 1998). In *T. vulgaris*, sperm are not stored from one year to the next (Verrel and Sever 1988; Pecio 1992). Our observations suggest that sperm stored in spermatheca do not survive over the winter in Montandon's newt. Females collected on land in early spring do not lay fertilized eggs until they have been inseminated.

Transmission electron microscopy observations of spermathecal tubules of *T. vulgaris* indicated that there may be some sperm degeneration and spermophagy

even a few days after mating (Sever et al. 1999). In *N. viridescens*, spermiphagy occurs from the beginning of the sperm storage period (Sever et al. 1996). Given rapid sperm degeneration, multiple insemination may be necessary to supply a female with fresh sperm with full fertilizing ability.

Acknowledgements We thank J. Radwan and J.M. Szymura for useful comments about our work and W. Babik for his help in statistical analysis. J. Nowak assisted in animal care. We also thank the communicating editor and two referees for their comments which greatly helped in preparing the final version of the manuscript. The experiments described in this paper were conducted in accordance with local and federal laws in Poland.

References

- Bateman AJ (1948) Intra-sexual selection in *Drosophila*. *Heredity* 2:349–368
- Birkhead TR, Møller AP (1998) Sperm competition and sexual selection. Academic Press, London
- Birkhead TR, Parker GA (1997) Sperm competition and mating systems. In: Krebs JR, Davies NB (eds) *Behavioural ecology: an evolutionary approach*, 4th edn. Blackwell, Oxford, pp 121–145
- Brooks R, Jennions MD (1999) The dark side of sexual selection. *Trends Ecol Evol* 14:336–337
- Childress D, Hartl DL (1972) Sperm preference in *Drosophila melanogaster*. *Genetics* 71:417–427
- Eberhard WG (1996) *Female control: sexual selection by cryptic female choice*. Princeton University Press, Princeton, NJ
- Elinson RP (1986) Fertilization in amphibians: the ancestry of the block to polyspermy. *Int Rev Cytol* 101:59–100
- Gromko MH, Newport MEA, Kortier MG (1984) Sperm dependence of female receptivity to remating in *Drosophila melanogaster*. *Evolution* 38:1273–1282
- Halliday T (1998) Sperm competition in amphibians. In: Birkhead TR, Møller AP (eds) *Sperm competition and sexual selection*. Academic Press, London, pp 465–502
- Hardy MP, Dent JN (1986) Transport of sperm within the cloaca of the female red-spotted newt. *J Morphol* 190:259–270
- Juszczyk W (1987) *Amphibians and reptiles of Poland* [in Polish]. PWN, Warsaw
- Madsen T, Shine R, Loman J, Håkansson T (1992) Why do female adders copulate so frequently? *Nature* 372:230
- Mann T (1984) *Spermatophores*. Springer, Berlin Heidelberg New York
- Newcomer SD, Zeh JA, Zeh DW (1999) Genetic benefits enhance the reproductive success of polyandrous females. *Proc Natl Acad Sci USA* 96:10236–10241
- Olsson M, Madsen T, Shine R, Gullberg A, Tegelström H (1994) Can female adders multiply? Reply. *Nature* 369:528
- Parker GA (1970) Sperm competition and its evolutionary consequences in insects. *Biol Rev* 45:525–568
- Parker GA (1992) Snakes and female sexuality. *Nature* 355:395–396
- Pecio A (1992) Insemination and egg laying dynamics in the smooth newt, *Triturus vulgaris*, in the laboratory. *Herpetol J* 2:5–7
- Pecio A, Rafiński J (1985) Sexual behaviour of the Montandon's newt, *Triturus montandoni* (Boulenger) (Caudata: Salamandridae). *Amphibia-Reptilia* 6:11–22
- Pyle DW, Gromko MH (1978) Repeated mating by female *Drosophila melanogaster*: the adaptive importance. *Experientia* 34:449–450
- Rafiński JN (1981) Multiple paternity in a natural population of the alpine newt, *Triturus alpestris* (Laur). *Amphibia-Reptilia* 2:282
- Rafiński J, Arntzen JW (1987) Biochemical systematics of the Old World newts, genus *Triturus*: allozyme data. *Herpetologica* 43:446–457
- Ridley M (1988) Mating frequency and fecundity in insects. *Biol Rev* 63:509–549
- Sever DM (1992) Comparative anatomy and phylogeny of the cloacae of salamanders (Amphibia: Caudata). IV. Salamandridae. *Anat Rec* 232:229–244
- Sever DM, Rania LC, Krenz JD (1996) Annual cycle of sperm storage in spermathecae of the red-spotted newt, *Notophthalmus viridescens* (Amphibia: Salamandridae). *J Morphol* 227:155–170
- Sever DM, Halliday T, Waights V, Brown J, Davies HA, Moriarty EC (1999) Sperm storage in females of the smooth newt (*Triturus v. vulgaris* L.). I. Ultrastructure of the spermathecae during the breeding season. *J Exp Zool* 283:51–70
- Stockley P (1998) Sexual conflict resulting from adaptations to sperm competition. *Trends Ecol Evol* 12:154–159
- Verrel PA, Sever DM (1988) The cloaca and spermatheca of the female smooth newt, *Triturus vulgaris* L (Amphibia: Urodela: Salamandridae). *Acta Zool (Stockh)* 69:65–70
- Waights V (1998) *Spermatophore production and sperm utilisation in the smooth newt, Triturus v. vulgaris*. PhD thesis, The Open University, Milton Keynes
- Watson PJ (1998) Multi-male mating and female choice increase offspring growth in the spider *Neriene litigiosa* (Linyphiidae). *Anim Behav* 55:387–403
- Zajc I, Arntzen JW (1999) Phylogenetic relationships of the European newts (genus *Triturus*) tested with mitochondrial DNA sequence data. *Contrib Zool* 68:73–81
- Zeh JA (1997) Polyandry and enhanced reproductive success in the harlequin-beetle-riding pseudoscorpion. *Behav Ecol Sociobiol* 40:119–126