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Sperm Transport after Insemination in the Alpine Newt (*Triturus alpestris*, Caudata, Salamandridae)

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The goal of this study was to test if sperm transport to the spermathecae in the Alpine newt (*Triturus alpestris*) requires active co-operation of the female. Artificial insemination of anaesthetised female newts was conducted using spermatophores collected from courting males and with sperm duct contents collected from sacrificed males. Sperm was present in the spermathecae of 9 out of 10 females inseminated with the spermatophores but in only 1 out of 8 females inseminated with sperm duct contents. The females of both groups laid some eggs after insemination, and a portion of these eggs in group of females inseminated with spermatophores were fertilized. However, the number of eggs produced by the females was much lower than typical egg-production in newts. The presence of sperm in the spermathecae of females inseminated with spermatophores and lack of sperm in the spermathecae of females inseminated with sperm duct contents suggests that sperm transport is either induced by the substances present in spermatophores and/or that sperm from the sperm duct is not fully mobile in comparison with sperm from the spermatophores.

Key words: *Triturus*, newts, sperm transport, spermathecae, insemination.

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Internal fertilization characterizes 7 out of 10 families of tailed amphibians (Caudata) (HALLIDAY 1998; SEVER 2002). In families with internal insemination, sperm is stored in exorcine glands, the spermathecae, a structure which may be polyphyletic in origin due to the existence of two basic anatomical types of this organ in tailed amphibians (SEVER 1991; SEVER 1994). Salamandrids have numerous short tubules opening independently into the roof of the cloaca, while in most plethodontids the short tubules open to a common duct connecting them with the cloacal chamber. In the genus *Triturus*, the structure of the spermathecae of the smooth newt (*Triturus vulgaris*) was studied in detail by VERREL and SEVER (1988) and SEVER *et al.* (1999, 2001).

Sperm storage in Caudata facilitates multiple matings and provides the opportunity for sperm competition and cryptic female choice (SEVER & BRIZZI 1998). Polyandry is thought to be an important part of the reproductive biology of tailed amphibians (HALLIDAY 1998). This prediction arose mainly from the observations of courtship in captivity or allozyme data (RAFIŃSKI 1981; HOUCK *et al.* 1985; PECIO 1992; OSIKOWSKI & RAFIŃSKI 2001). Surprisingly, direct molecular evidence confirming polyandry in *Salamandridae* is rare (STEINFARTZ *et al.* 2006).

The reproductive biology of the European newts (*Triturus*), belonging to the family *Salamandridae*, has been intensively studied in the last decades (HALLIDAY 1998). Among them, the Alpine newt, *Triturus* (*Mesotriton*) *alpestris* Laurenti 1768, is one of most extensively researched species (e.g. RAFIŃSKI & OSIKOWSKI 2002; DENOËL *et al.* 2005). As in other *Triturus* species, courtship in the Alpine newt takes place in early spring in small water reservoirs and consists of sequences of stereotypical movements (HALLIDAY 1976; HALLIDAY 1977; PECIO & RAFIŃSKI 1985). The spermatophore is deposited on the bottom of the water reservoir and is picked up by the females' cloacal lips. The spermatophore consists of two parts – a gelatinous base fixing these structures to the bottom of the water reservoir and a sperm containing part (the sperm cap) on top (HALLIDAY 1998). A female newt collects only the sperm cap which becomes lodged into the cloacal orifice. The mechanism of sperm transport from the entrance of the cloacal chamber to the tubules of the spermathecae in *Triturus* is unknown. Detailed knowledge of what happens to sperm after insemination within

the female's reproductive tract is crucial for an understanding of the various aspects of reproductive biology of newts (SEVER *et al.* 1999). One of the most important questions is if the sperm is transported passively by contractions of the smooth muscles of the female's reproductive duct or actively swim through cloaca and the spermathecal tubules. An understanding of this mechanism is needed for future research on sperm competition and cryptic female choice in these amphibians.

Fertilization occurs when sperm is released from the spermathecae onto eggs passing through the cloacal lumen (BOISSEAU & JOLY 1975). Females lay eggs by individually wrapping them in leaves of waterplants. The female may need up to 3 months to lay the whole batch of eggs (BAKER 1992, data for *T. vulgaris*).

The aim of this study was to determine if sperm transport in the Alpine newts from the spermatophore to the spermathecae is possible without the active co-operation of the female and behavioural stimulation during courtship. I also investigated if the possible factor (-s) influencing sperm transport to the spermathecae is present in the sperm duct contents or in the spermatophores.

Material and Methods

The Alpine newts used in the experiment were collected in early spring from a large breeding population in Pucułowski Pond in Gorce Mountains (Carpathians, Southern Poland). The females were caught on land in late April 2003 during migration from their overwintering sites to the breeding pond. They were transferred to the laboratory and were put individually into holding aquaria provided with water and waterplants. Females collected in these circumstances should be uninseminated because newt courtship takes place in water. It has been shown that in the genus *Triturus* sperm do not survive in the spermathecae from one breeding season to the next (VERRELL & SEVER 1988; PECIO 1992; SEVER *et al.* 1999). To ascertain that females were uninseminated, each female was kept alone for 3 days in a glass jar filled with water and containing waterplants for egg laying. Previous observations suggest that under these conditions inseminated females always lay fertilized eggs (PECIO & RAFIŃSKI 1985; OSIKOWSKI & RAFIŃSKI 2001; OSIKOWSKI, unpubl.). If a female had not laid eggs or laid unfertilized eggs only, she was treated as uninseminated and used for further procedures.

Males used in the experiment were collected in the same period of time from the pond using dipnets and kept in water in large plastic containers in groups of 10 individuals. The newts were fed daily with earthworms and crustaceans (*Daphnia* sp.). The temperature in the laboratory was kept at 16°C.

The uninseminated females were divided into two experimental groups. Females of the first group (10 individuals) were artificially inseminated with sperm caps (AI-SC) using the following protocol. Females were anaesthetised by being placed into a 0.1% solution of tricaine methanesulfonate (MS-222). At the same time 2 males and 2 females (individuals not used for further artificial inseminations) were put into a 50 l aquarium with water and a sand covered bottom to induce spermatophores deposition. The males usually quickly began courting the females and laid spermatophores. Freshly laid sperm caps were pipetted with a small glass pipette and immediately put into the opening of the cloacal chamber of an anaesthetised female (1 spermatophore per 1 female). After females had recovered from anaesthesia they were returned into individual holding aquaria with a 20 cm water level and provided with waterplants.

Females of the second group (8 individuals) were artificially inseminated with sperm duct contents (AI-SD). Females were anaesthetised as before. Then a male was sacrificed by a high concentration of MS-222. Sperm ducts were dissected and their contents were pressed out on a glass plate and 2 μl of it (a volume equal to the volume of the sperm cap) was introduced into the opening of the cloacal chambers of anaesthetised females. After the females had awakened they were put back into individual holding pens.

After the artificial inseminations the females of both experimental groups (AI-SC and AI-SD) were kept separately for a period of 7 days in individual aquaria. Every 2 days the waterplants were collected from the aquaria and inspected for the presence of eggs. All eggs found were allowed to develop for 5 days and then the number of developing and non-developing eggs was counted under a stereomicroscope. Non developing eggs were considered unfertilised (PECIO 1992; OSIKOWSKI & RAFIŃSKI 2001).

The females were humanely sacrificed in highly concentrated MS-222 solutions seven days after artificial insemination. Their cloacal regions were dissected and fixed in Bouin's fixative. The tissue material was washed and dehydrated in ethanol and embedded in paraffin. The cross sections (7 μm) were cut with a microtome and stained with hematoxylin-eosin. A series of cross-sections of the cloacal region was then analysed using light microscopy. The number of spermathecal tubules

in each section was counted and the presence or absence of sperm was noted. For each of the females, three consecutive cross sections with the highest number of tubules were used to calculate the average percentage of tubules containing sperm. This value was used to estimate the degree in which the spermathecae were filled with sperm.

Two control groups were also established. In the first group, 4 females captured on land were examined for insemination using the protocol described above. None of these females laid inseminated eggs, and all of them were considered to be uninseminated (C-U). These females were anaesthetised and treated using the protocol of artificial insemination (described above), but only clear water was introduced into their cloacas instead of spermatophores or sperm duct content. After two days the females were killed and cross sections of their cloacae were prepared and analysed for the presence of sperm as described above.

In order to avoid killing many animals, only two females were used as a second control group: females inseminated naturally (C-IN). These females were captured approximately two weeks after the beginning of the breeding season from a pond using a dip-net, anaesthetised as described above, artificially inseminated with clear water and then kept in individual aquaria for one week. During this period their eggs were collected and counted. After one week the females were sacrificed and the presence of sperm in spermathecae was determined using the same procedure as for the females of both experimental groups.

Results

A summary of the results of egg-laying and histological analysis of sperm presence in the spermathecae are shown in Table 1.

Table 1

The egg laying and sperm presence in spermathecae of the Alpine newt

Female	Eggs fertilized	Eggs non-fertilized	Eggs total	% of spermathecal tubules filled with sperm
AI-SP 1	0	0	0	26.9
AI-SP 2	0	0	0	0
AI-SP 3	3	5	8	26.9
AI-SP 4	1	0	1	11.8
AI-SP 5	8	0	8	41.4
AI-SP 6	0	0	0	15.4
AI-SP 7	0	4	4	9
AI-SP 8	0	0	0	23.9
AI-SP 9	0	0	0	14.3
AI-SP 10	7	1	8	17.9
				Mean: 18.75±11.5
AI-SD 1	0	0	0	0
AI-SD 2	0	0	0	0
AI-SD 3	0	0	0	0
AI-SD 4	0	1	1	2.9
AI-SD 5	0	34	34	0
AI-SD 6	0	2	2	0
AI-SD 7	0	2	2	0
AI-SD 8	0	0	0	0
C-U 1	–	–	–	0
C-U 2	–	–	–	0
C-U 3	–	–	–	0
C-U 4	–	–	–	0
C-IN 1	Not tested	Not tested	68	41.7
C-IN 2	Not tested	Not tested	83	26.4

AI-SP: females artificially inseminated with spermatophores
AI-SD: females artificially inseminated with sperm duct content
C-U: control group – females uninseminated
C-IN: control group – females inseminated naturally.

Egg-laying

Five out of 10 AI-SP females laid eggs during the week after artificial insemination. In all cases, however, the number of eggs laid by a single female was lower than 10. Fertilized eggs were found in egg batches of 4 females.

Half (4 of 8) of the AI-SD females laid eggs during this period. None of these females laid fertilized eggs. The control females collected in the pond (C-IN) laid 68 and 83 eggs, respectively. The uninseminated females (C-U) did not lay any eggs.

The presence of sperm in the spermathecae

In the AI-SP group, the presence of sperm in the spermathecae was found in 9 of 10 females. Females of this group had an average of 18.75 % (SD = 11.5) spermathecal tubules containing sperm. Sperm was found in the spermathecae of only one female from the 8 AI-SD females. This female (AI-SD 4) had sperm in one tubule out of 35 (2.9% tubules contained sperm). The proportion of females possessing sperm in the spermathecae significantly differs between females from groups AI-SP and AI-SD (χ^2 with Yates correction, χ^2=9.80; P<0.005). The 4 uninseminated females (C-U) lacked sperm in their spermathecae. Females inseminated naturally (C-IN) had 41.7% and 26.4% of spermathecal tubules containing sperm, respectively.

Discussion

The results of this study indicate that artificial inseminations of anaesthetised females of the Alpine newt did not lead to normal egg-laying. Although half of the females in both experimental groups (AI-SP and AI-SD) laid some eggs and a portion of these eggs in AI-SP group were fertilized, the number of eggs laid was significantly lower than typical egg production by female newts in natural and laboratory conditions. Many observations (e.g. OSIKOWSKI & RAFIŃSKI 2001) indicate that female newts usually produce 200-300 eggs during a single breeding season. The control females inseminated naturally (C-IN) laid 68 and 83 eggs, respectively, during a week. These females were captured approximately 2 weeks after the beginning of the breeding season. Previous observations suggest that maximum egg production by female newts takes place a few days after the first insemination (OSIKOWSKI & RAFIŃSKI 2001; OSIKOWSKI, unpubl.). It is therefore possible that C-IN females laid the majority of their eggs immediately after the first insemination (-s), before being caught, but these females still produced much more eggs than the experimental females during the same period of time. The most likely explanation of this result is that the amount of sperm in the spermathecae of AI-SP and AI-SD females was not sufficient for triggering egg-laying. Information concerning the volume of sperm in the spermathecae should be crucial for the female, allowing her to avoid laying non-fertilized eggs when her sperm reserves are low. It is also possible that females require behavioural stimulation during courtship to trigger egg-maturation and ovulation. The presence of sperm in the spermathecae of females inseminated under anaesthesia indicates that behavioural stimuli is not required for successful sperm transport, but poor egg-laying suggests that such stimuli may be necessary to provide the information that she has already been inseminated.

It is not clear how much sperm is required to fill the spermathecae. SEVER *et al.* (2001) investigated the spermathecae of *T. vulgaris* females inseminated naturally and under laboratory conditions and found that the spermathecae of the 17 individuals tested were not full or crowded with sperm. This agrees with my present findings for the C-IN females which had 41.7% and 26.3% of tubules filled with sperm.

Lack of sperm in spermathecae of the C-U females confirms previous observations that in newts sperm cannot overwinter in the female sperm storage organ (VERRELL & SEVER 1988; PECIO 1992; SEVER *et al.* 1999). This leads to the conclusion that females caught in spring before entering water may be treated as virgin.

The important conclusion arising from this study is that the transfer of sperm from the spermatophores to the spermathecae is more effective in comparison with the sperm from the sperm duct. Sperm was found in the spermathecae of 90% of the AI-SP females in comparison with 12.5% of the AI-SD females. The most likely explanation of this result at this stage of research is that the spermatophores contain the substances inducing passive sperm transport by the reproductive tract of the female or increasing sperm motility. Lack of sperm in the spermathecae of females inseminated with sperm duct content suggests that this hypothetical substance (-s) is not produced by the testes but the cloacal glands involved in spermatophore production. Five types of cloacal glands have been described in *Triturus* (SEVER 1981; SEVER *et al.* 1990). The function of their secretions is not fully understood, but it is likely that they mainly produce substances building the spermatophores. It is well documented that the various substances transferred with sperm to the female may induce favourable responses in female physiology and behaviour in many animal groups (reviewed in

POIANI 2006). The use of chemical or mechanical stimuli during mating to trigger egg maturation, ovulation, sperm transport and other physiological and behavioural processes associated with mating makes adaptive sense for both the males and the females (reviewed in: EBERHARD 1996).

It took less than one hour for the sperm to reach the spermathecal tubules after spermatophore collection by *T. vulgaris* females (SEVER *et al.* 1999). The question if the sperm swim actively into spermathecal tubules or are passively transported by the female reproductive tract is still not resolved. It is possible that both mechanisms act synergistically. In *Notophthalmus viridescens* (Salamandridae) sperm is probably transported passively through the cloacal chamber, but may swim actively into the tubules of the spermathecae (HARDY & DENT 1986). Moreover, naturally inseminated females of this species treated with lidocaine (acting as an antagonist of neuromuscular transmission) had a lower number of sperm in the anterior cloaca in comparison to the females injected with saline. This suggests that sperm may be transported passively through the cloacal chamber while active sperm movement is required for entrance into the tubules of the spermathecae. This prediction is supported by the fact that it is commonly accepted that spermathecal tubules evolved from glands (DENT 1970). The smooth muscles in the walls of spermathecal tubules are more likely to push out the contents out of the tubules than to take them in (HARDY & DENT 1986). My data indicate that sperm are able to enter spermathecal tubules without the behavioural stimuli of the female. If the passive mechanism of transport through the cloacal chamber is correct, the presence of sperm in spermathecae of 9 out of 10 AI-SP in comparison with lack of sperm in spermathecae of almost all AI-SD females clearly indicate that transport is induced by the substances present in spermatophores. On the other hand, the hypothesis on active movement of sperm from the opening of the cloaca to the spermathecal tubules cannot be rejected at this stage of research. In such case lack of sperm in spermathecae of AI-SD females may suggest that sperm from the sperm duct is not fully mobile in comparison with the sperm in the sperm cap.

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