Histological characterization of sperm storage organs in *Arianta arbustorum* and *Bradybaena fruticum* (Pulmonata: Stylommatophora)

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Abstract. The morphology and physiology of sperm storage organs may provide females with the ability for cryptic female choice, i.e. the ability to influence paternity after mating with different partners. As a first step towards the understanding of physiological processes in the spermathecae of *A. arbustorum* and *B. fruticum* we performed a general histochemical analysis. The findings for both species were practically identical. The concentration of polysaccharides including glycogen increased towards the blind end of the spermathecal tubules. Lipids were more or less evenly distributed in the epithelium along the tubules as well as over the cells. Whether the faint Alcian blue staining of the apical surface of the epithelium is due to mucopolysaccharides needs confirmation. Lipid vesicles were scattered throughout the cytoplasm and were evenly distributed along the tubules. Polysaccharides and lipids have nutritive function. This is certainly true for the highly active epithelium itself, which may also provide nutrients for the stored spermatozoa. However, the exchange of substances between epithelium and spermatozoa has not been observed. At this basic state of histochemical investigation only the distribution of polysaccharides including glycogen along the spermathecal tubules may be interpreted in the context of postmating sexual selection. The increasing concentration of polysaccharides towards the blind end of a spermathecal tubule coincides with the decreasing density and length of the cilia in *A. arbustorum* indicating that the function of the epithelial cells differs along a tubule. The wider, blind end seems to be the main place for sperm storage. As a consequence, one would expect that spermatozoa arriving later or stemming from a second ejaculate would receive less "care". To which extent this may have consequences in sperm competition and to which extent the sperm recipient can control the position of storage of the incoming sperm needs to be tested in future experiments.

Kurzfassung. Histologische Charakterisierung von Spermienspeicherorganen bei *Arianta arbustorum* und *Bradybaena fruticum* (Pulmonata: Stylommatophora). Morphologie und Physiologie von Spermienspeicherorganen können Weibchen kryptische Partnerwahl, d.h. die Fähigkeit die Vaterschaft nach Paarung mit mehreren verschiedenen Partnern zu beeinflussen, ermöglichen. Als einen ersten Schritt zum Verständnis von physiologischen Prozessen in der Spermathek von *A. arbustorum* und *B. fruticum* unternahmen wir eine generelle histochemische Analyse. Die Befunde waren für beide Arten praktisch identisch. Die Konzentration von Polysachariden einschließlich Glycogen nahm zum blinden Ende der Schläuche hin zu. Ob die schwache Alcian-Blau-Färbung der apikalen Oberfläche des Epithels auf Mucopolysacharide zurückzuführen ist, bedarf Bestätigung. Lipid-Vesikel waren im gesamten Cytoplasma verstreut und gleichmäßig entlang den Schläuchen verteilt. Polysacharide und Lipide haben nutritive Funktion. Das betrifft sicherlich das hochaktive Epithel selbst, das möglicherweise auch Nährstoffe für die gespeicherten Spermien bereitstellt. Zu diesem Zeitpunkt kann höchstens die Verteilung der Polysacharide im Zusammenhang mit nach der Paarung stattfindender sexueller Selektion interpretiert werden. Die zunehmende Konzentration von Polysachariden zum blinden Ende der Schläuche hin geht bei *A. arbustorum* mit abnehmender Länge der Cilien des Epithels einher, was darauf schließen lässt, dass sich die Funktion entlang der Schläuche verändert. Das weite, blinde Ende scheint der Hauptort der Speicherung zu sein. Folglich würde man erwarten, dass Spermien, die später eintreffen oder von einem zweiten Ejakulat stammen, weniger „Fürsorge" empfangen. In welchem Ausmaß das Konsequenzen für Spermienkonkurrenz hat und in welchem Ausmaß der Spermienempfänger Kontrolle über die Speicherposition von eintreffenden Spermien hat, bedarf weiterer Experimente.

Key words. Glycogen, histochemistry, lipids, polysaccharides, sexual selection, spermatheca.

Introduction

The ability of females to store conspecific ejaculates over longer periods allows a temporal separation of copulation, fertilization and oviposition (COHEN 1977), and thus may serve as an insurance against the failure of finding a partner in species with low encounter frequencies (e.g., GHISELIN 1969). The recognition that females in most species are promiscuous indicates that sperm storage may also enhance the opportunities for sperm competition by extending the period over which ejaculates from different males compete to fertilize a given set of ova (PARKER 1970; BIRKHEAD & MØLLER 1998; MORELL, 1998).
Male adaptations to sperm competition are widely recognised (SMITH 1984; BIRKHAED & MØLLER 1998). Female adaptations to gain control over fertilization after multiple matings are less obvious, have widely been ignored or were considered to be of secondary importance (KNOWLTON & GREENWELL 1984; BARNETT et al. 1993; EBERHARDT 1996).
Females may influence paternity e.g. through different physiological influences on the survival of sperm from different males. Sperm degradation is a general means to remove old, less fertile sperm from the sperm stores. Thus, sperm storage may comprise a wide spectrum of processes such as nourishment, quiescence and subsequent activation, degradation and resorption of sperm.
Several stylommatophoran gastropods including *Arianta arbustorum* Linnaeus, 1758 and *Bradybaena fruticum* O. F. Müller, 1774 are known to store sperm from several mates in their sperm storage organs, the spermatheca (KHOKHUTKIN 1928; BAUR 1994). *A. arbustorum* has a morphologically complex spermatheca consisting of 2–9 tubules (BAMINGER & HAASE 1999). It has been suggested that the complex structure of the spermatheca allows for differential storage and use of sperm from different mating partners in such a way that cryptic female choice may occur (HAASE & BAUR 1995). In contrast, *B. fruticum* has only a single spermathecal tubule (SCHILEYKO & SCHILEYKO 1992) and thus lacks the possibilities of *A. arbustorum* for differential sperm storage. The ultrastructure of the spermathecal epithelium and surrounding musculature of both species have been investigated with respect to their potential function in postmating sexual selection (BOJAT et al. 2001a, b, 2002). However, little is known about physiological processes and chemical aspects of sperm storage in pulmonate gastropods.
The aim of the present study is a general histochemical analysis of the spermatheca in *A. arbustorum* and *B. fruticum*, as a first step towards the understanding of physiological processes in the sperm storage organs. We analysed the spermathecae of mated and unmated (virgin) individuals of *A. arbustorum* and of sexually mature individuals of *B. fruticum*. We also compared physiological processes in the spermatheca with those in the functionally and ontogenetically linked fertilization chamber, the place of fertilization, which together with the distal part of the hermaphrodite duct and the proximal section of the spermoviduct make up the fertilization pouch (for anatomical details see SCHILEYKO & SCHILEYKO 1992; HAASE & BAUR 1995).

Material and methods

Juvenile and subadult specimens of *A. arbustorum* were collected on Mont Raimeux in the Swiss Jura Mountains (47°18.6'N, 7°25.8'E, 1300 m a.s.l.) in May 2000. Adult, sexually mature individuals of *B. fruticum* were collected in Arlesheim (northern Switzerland: 47°29.6'N, 7°37.8' E, 380 m a.s.l.) in September 2000. All specimens were kept under a light/dark regime of 16/8 hours at 18° C.
The juvenile and subadult individuals of *A. arbustorum* were raised to adulthood in solitary confinement in polystyrol boxes measuring 8.6 x 8.6 x 11.2 cm on soil from their habitat. Hence, they were considered as virgin sexually mature snails. Part of these individuals mated under the above laboratory conditions. Mated snails were placed back into their respective boxes more than three hours after copulation, in order to let them move the received spermatophore into the final position undisturbedly (cf. HOFFMANN 1923). Whether individuals of *B. fruticum* had mated before collecting was controlled later in the histological sections.

For the histological investigations 6 individuals of *A. arbustorum* (4 mated, 2 virgin) and 2 individuals of *B. fruticum* were anaesthetised by immersion in 25 ml tap water containing 2–3 drops of clove tree oil for 2–3 hours at room temperature (BAMINGER & HAASE 1999). Their shells were removed and the fertilization pouches and hermaphrodite ducts dissected. All but two objects were fixed in Carnoy's fixative for one hour. The organs of two mated *A. arbustorum* were fixed in 4% paraformaldehyde in 0.1M PBS (phosphate-buffered saline, pH 7.2). Specimens fixed after Carnoy were embedded in paraplast and serially cross-sectioned at 10 µm. For general histological observations, samples were stained with hematoxylin-eosin. Histochemical characterization on paraplast sections comprised the following tests: PAS (HOTCHKISS 1948) for polysaccharides, BAUER'S (1933) glycogen test, and Alcian blue staining of mucopolysaccharides (STEEDMAN 1950). Paraformaldehyde-fixed objects were embedded in Tissue-Tek. The frozen blocks were sectioned on a Cryocut 1800 (Leica/ Reichert-Jung). The 10 µm thick cross-sections collected on SuperFrost Plus slides were stained with Sudan black B in order to detect lipids (LISON 1934).

Results

In general, our findings were very similar in both species. Therefore we do not distinguish between them in the following description. In mated snails, the PAS reaction detected polysaccharides in the periphery of connective tissue and muscle cells coinciding with the results of the glycogen test. Polysaccharides were differentially distributed in the epithelial cells of fertilization chamber and spermatheca. We distinguished three states: 1) throughout the cytoplasm of the whole cell, 2) apical and peripheral, 3) around the nucleus. Especially in the spermatheca, these states occurred in groups of cells. That is, the epithelium exhibited regions, which differed in physiological activity. Glycogen was detected primarily in the apical region of both epithelia. In the distal region, many cells apparently did not contain glycogen or only in quantities below the sensitivity threshold of our test. Towards the blind end of the spermathecal tubules the glycogen content increased. Glycogen was now also found at the base of the cells or throughout the cytoplasm. The glycogen reserves deposited in the tails of the stored spermatozoa also reacted postively. The distribution of PAS positive substances, which include glycogen, was similar along the spermathecal tubules.

Mucopolysaccharides were practically absent from the fertilization pouch. Rarely, we found single, positively staining cells in the connective tissue. An exception may be the ciliated apical surfaces of the epithelia. They stained very faintly and thus appeared to be covered by mucopolysaccharides. The connective tissue surrounding the fertilization pouch as well as follicles of the albumen gland still attached to the fertilization pouch were definitely Alcian blue positive.

Lipid vesicles were scattered over the whole cytoplasm of the epithelia of fertilization chamber and spermatheca. They were larger in the cells lining the fertilization chamber and there more dense dorsal than ventral. The connective tissue matrix of the fertilization pouch was almost free of lipids. The connective tissue encapsulating the pouch contained again more lipid vesicles.

Histochemically, the fertilization pouch of virgin animals of *A. arbustorum* did not differ much from that of mated individuals. The distribution of the compounds tested for was similar within cells as well as throughout the length of the organ. However, staining was less intensive suggesting that the concentration of polysaccharides including glycogen was lower.

Discussion

The distribution of glycogen within the epithelial cells of both spermatheca and fertilization chamber and along the tubules of the spermatheca is in accordance with our observations of TEM sections (BOJAT et al. 2001b, 2002). However, glycogen makes up only part of the polysaccharides contained in the epithelia. More specific tests will be needed to identify also these compounds. Glycogen is the most important energy reserve in gastropods

(LIVINGSTONE & DE ZWAAN 1993). Therefore, it is not surprising that we detected this compound in the densely ciliated and thus metabolically active transport epithelia.
Whether the epithelial surfaces are really coated with mucopolysaccharides needs confirmation through further, more sensitive tests.
In our previous TEM investigations, we did not specify the vesicles in the cytoplasm of the spermathecal epithelium (BOJAT et al. 2001b, 2002). Our present results suggest that a major part of them contains lipids. In general, lipids appear to play a minor role in the metabolism of stylommatophorans. However, they may be involved in gametogenesis as in other classes of molluscs (VOOGT 1983). Therefore, it seems plausible that the lipids of spermatheca and fertilization chamber have a nutritive function also for the allosperm stored as demonstrated in crustaceans (VOGT & STRUS 1992; LONGO et al. 1998).
Our findings suggest that, apart from maintaining the own metabolism, nutrition of spermatozoa may be an important function of the spermathecal epithelium, although the lack of evidence for uptake of substances of stored spermatozoa in ultrastructural analyses and the rich glycogen reserves in the sperm tails led to the conclusion that sperm might be inactive during storage (GIUSTI & SELMI 1985; BOJAT et al. 2001b). However, prove of nutrient transfer is outstanding. The fact that sperm are capacitated in the spermtheca of both *A. arbustorum* and *B. fruticum* (BOJAT et al. 2001b, 2002) indicates that the spermathecal epithelium has additional functions. More detailed investigations including analyses of enzymatic activity to better understand the interaction of spermatozoa and the spermathecal epithelium are required.
At this basic state of histochemical investigation only the distribution of polysaccharides including glycogen along the spermathecal tubules may be interpreted in the context of postmating sexual selection. The increasing concentration of polysaccharides towards the blind end of a spermathecal tubule coincides with the decreasing density and length of the cilia in *A. arbustorum* (BOJAT et al. 2001b). This indicates that the function of the epithelial cells differs along a tubule and suggests that especially the wider, blind end has the storing function. As a consequence, one would expect that spermatozoa arriving later or stemming from a second ejaculate would receive less "care". To which extent this may have consequences in sperm competition and to which extent the sperm recipient can control the position of storage of the incoming sperm needs to be tested in future experiments.

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