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Yolk proteins in the male reproductive system of the fruit fly *Drosophila melanogaster*: Spatial and temporal patterns of expression

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ABSTRACT

In insects, spermatozoa develop in the testes as clones of single spermatogonia covered by specialized somatic cyst cells (cc). Upon completion of spermatogenesis, spermatozoa are released to the vas deferens, while the cc remain in the testes and die. In the fruit fly *Drosophila melanogaster*, the released spermatozoa first reach the seminal vesicles (SV), the organ where post-testicular maturation begins. Here, we demonstrate the temporal (restricted to the evening and early night hours) accumulation of membranous vesicles containing proteins in the SV lumen of *D. melanogaster*. When SV vesicles were isolated from the semen and co-incubated with testis-derived spermatozoa *in vitro*, their contents bound to the spermatozoa along their tails. The proteins of the SV vesicles were then characterized using 2-D electrophoresis. We identified a prominent protein spot of around 45–47 kDa, which disappears from the SV vesicles in the night, i.e. shortly after they appear in the SV lumen. Sequencing of peptides derived from this spot by mass spectrometry revealed identity with three yolk proteins (YP1-3). This unexpected result was confirmed by western blotting, which demonstrated that SV vesicles contain proteins that are immunoreactive with an antibody against *D. melanogaster* YP1-3. The expression of all *yp* genes was shown to be a unique feature of testis tissues. Using RNA probes we found that their transcripts localize exclusively to the cc that cover fully developed spermatozoa in the distal part of each testis. Temporally, the expression of *yp* genes was found to be restricted to a short period during the day and is followed by the evening accumulation of YP proteins in the cc. Immunohistochemical staining confirmed that cc are the source of SV vesicles containing YPs that are released into the SV lumen. These vesicles interact with spermatozoa and as a result, YPs become extrinsic proteins of the sperm membrane. Thus, we describe for the first time the expression of yolk proteins in the male reproductive system of *D. melanogaster* under physiological conditions, and show that somatic cells of the testes are the source of these proteins.

1. Introduction

A characteristic feature of insect spermatogenesis is clonal development of spermatozoa from single gonioblasts encapsulated within structures called cysts (Dumser, 1980; Friedlander et al., 2005; Phillips, 1970). The walls of these cysts are formed by somatic cyst cells (cc), which serve as the source of signals mediating the differentiation of germ cells during whole spermatogenesis (de Cuevas et al., 1997; Gilboa and Lehmann, 2004; White-Cooper, 2010). The cyst walls also play a crucial role during spermatid individualization that includes the encapsulation of each spermatid by an independent plasma membrane and the elimination of excess sperm cytoplasm (Fabrizio et al., 1998; Noguchi and Miller, 2003). At the end of spermatogenesis, cc surround a fully developed group of spermatozoa (sperm bundle) until they are released from the testis to the vas deferens. When spermatozoa enter the vas deferens, the cc remain in the testis where they die and subsequently undergo fragmentation and phagocytosis by the adjacent testis

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epithelium (Giebultowicz et al., 1997; Gvakharia et al., 2003; Tokuyasu et al., 1972). However, while the developmental role of cc has been described in model insects, such as *Drosophila* and some moths species, their physiological function is still poorly understood. Cyst cells are considered to have somatic equivalents in insect ovarioles: the follicular cells surrounding the oocyte that, apart from their developmental functions, protect the growing oocyte, produce yolk protein (YP) precursors and control oocyte uptake of yolk proteins (vitellogenins) produced in the fat body (de Cuevas et al., 1997; Swevers et al., 2005).

In *Drosophila melanogaster*, spermatozoa released from the testes first enter the paired seminal vesicles (SV) and then are transferred to the single ejaculatory duct (ED). Upon transit via the lumens of these organs, spermatozoa contact compounds of the seminal plasma (SP). The composition of SP varies considerably between the separate segments of the reproductive tract. It is best characterized in the ED due to extensive proteomic analysis of the secretions of accessory glands (that are anatomically connected to the ED) from which many factors affecting spermatozoa maturation are released (Avila et al., 2011; Ayroles et al., 2011). Some of these factors also influence female reproduction when transferred with sperm during mating and their physiological function is the best characterized of all the SP factors (Adams and Wolfner, 2007; Ram and Wolfner, 2007; Ravi Ram and Wolfner, 2007; Wolfner, 2009). The composition of seminal plasma from SV of *D. melanogaster* has yet to be determined, although general conclusions can be drawn based on proteome analysis of the whole SV (Takemori and Yamamoto, 2009), spermatozoa isolated from the SV lumen (Wasbrough et al., 2010) or comparative analysis of proteins from both the whole SV and spermatozoa they contain (Dorus et al., 2006); for a review see (Rettie and Dorus, 2012). Of the >160 different proteins found in the SV, most are involved in the response to toxic and oxidative stress, basic cellular metabolism, proteolysis and cell signaling (Takemori and Yamamoto, 2009). However, none of the identified proteins is directly related to extratesticular sperm maturation. Other studies have focused on sperm plasma membrane glycosidases and their metabolism in the SV of *D. melanogaster* and other fruit fly species (Cattaneo et al., 2002; Intra et al., 2009, 2011; Pasini et al., 1999). These glycosidases, which are thought to be secreted by the SV epithelium and then adsorbed to the sperm surface during their stay within its lumen (Cattaneo et al., 1997), are involved in the modification of sperm surface glycoproteins that affect sperm maturation and also the fertilization process.

SV in *Drosophila* can be regarded as functional homologs of the upper vas deferens (UVD) of other insects, including moths. The UVD serves as a compartment of reproductive ducts where seminal plasma is formed from components that are delivered with sperm from the testes and others that are secreted abundantly by tissues of the UVD wall (Riemann and Giebultowicz, 1991; Riemann and Thorson, 1976). Ultrastructural studies have suggested that the spermatozoa of moths acquire an extracellular coat during their passage through the UVD lumen (Riemann and Giebultowicz, 1992). This coat is composed mostly of glycoproteins produced and secreted in the form of dense granules by the columnar epithelium of the UVD (Bebas et al., 2002). Moreover, the composition of seminal plasma was found to undergo cyclical changes due to the rhythm of sperm release from testes and the rhythm of secretory activity of the UVD, both being controlled by an endogenous peripheral clock present in the male reproductive system (Kotwica-Rolinska et al., 2013; Kotwica et al., 2009). The precise temporal correlation between the release of secretory granules and the appearance of sperm in the UVD lumen suggests that the materials comprising these granules may be involved in sperm maturation. Although the rhythm of sperm release has yet to be characterized in *D. melanogaster*, the presence of a peripheral oscillator in the proximal part of the testis (referred to as the 'testis neck') and the SV has been described in this species, which implies its participation in spermatozoa processing before their release from the testes and during the early stages of their maturation just after they enter the SV lumen (Beaver et al., 2002).

The goal of the present study was to identify protein factors in the SV seminal fluid that may participate in sperm maturation. Temporal analysis of the SV content showed the accumulation of numerous membrane-covered vesicles in their lumen during the evening and early night hours. These vesicles contain proteins that bind to spermatozoa when they are transferred through the SV. Surprisingly, peptides derived from some abundant vesicle proteins originate from the yolk proteins YP1, YP2 and YP3. The *yp1-3* genes appear to be expressed exclusively in the testes. Our findings indicate that YP proteins are produced in the cyst cells of fully developed sperm bundles and are subsequently released together with sperm to the seminal vesicles, where they become extrinsic proteins of the spermatozoa cell membrane.

2. Materials and methods

2.1. Insects

Flies were reared on a yeast-cornmeal-molasses diet at 25 °C in a 12-h light/12-h dark cycle (LD). We used 5-day-old virgin males and, in some experiments, also mated females of the wild type strain Canton-S (CS). Other experiments were performed using the Oregon (OR) wild-type strain maintained in our laboratory and flies caught using a bait of overripe apples in an apple orchard (AO) in the Swietokrzyskie District of Poland. For tissue preparation, insects were dissected 6 times a day (at 4 h intervals), starting at the time the lights went on. In some experiments, tissues were harvested less frequently: three times or only once a day. In the results section the insect dissection times designated as day, evening, early night and night, correspond to 4 h after lights on, 4 h before lights off, at the time of lights off and 4 h after lights off respectively. The orchard flies were captured in the evening and immediately dissected for later analysis.

2.2. Electron microscopy

From 8 to 10 males were dissected 6 times a day. Seminal vesicles (SV) were removed and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.5 (CB) for 2 h at 4 °C. Postfixation was carried out by overnight incubation of the tissues in 1% OsO_4 in CB. Then tissues were dehydrated in an increasing ethanol gradient and propylene oxide, and subsequently saturated with EPON 812 (Electron Microscopy Science, Hatfield PA, USA). Polymerization was performed at 60 °C for 48 h. Ultrathin sections were cut using an ULTRACUT R ultramicrotome (Leica Mikrosysteme GmbH, Wetzlar, Germany) and stained with lead citrate and uranyl acetate (Polanska et al., 2005). The material was analyzed and photographed using a LEO 912AB transmission electron microscope (Carl Zeiss Inc., Oberkochen, Germany).

2.3. Isolation and histochemical analysis of membranous vesicles from SV

Three hundred CS males were dissected in the evening. To obtain samples of seminal fluid, the SV contents were released into 50 μl of Schneider's medium (Lonza, Basel, Switzerland). The SV vesicles (and their remnants) were isolated in two steps: (1) spermatozoa were spun down by centrifugation at 3000 *g* for 10 min; (2) the vesicles were separated from the supernatant by