Paternal products and by-products in *Drosophila* development

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Tails of fertilizing spermatozoa persist throughout embryogenesis in *Drosophila* species and can be observed within the midguts of larvae after hatching. Throughout development, sperm proteins slowly diffuse or are stripped from the giant sperm tail residing within the embryo's anterior end. The shape and position of the sperm within the embryo are regulated such that, during organ formation, the unused portion of the sperm is enveloped by the developing midgut. This persistent, paternally derived structure is composed of the sperm's mitochondrial derivatives and appears to be defaecated by the larva soon after hatching. These complex sperm-egg interactions may represent mechanisms to avoid intragenomic conflict by ensuring strictly maternal inheritance of mitochondrial DNA (mtDNA).

Keywords: sperm; development; intragenomic conflict; mtDNA; *Drosophila*; fertilization

1. INTRODUCTION

Relative to other animal groups, in *Drosophila* enormous quantities of spermic material are delivered to the egg (Karr & Pitnick 1996) and persist during development, raising intriguing questions about the function and fate of such paternally derived components. Other persistent paternal products include microtubular fragments of mouse sperm axonemes (Simerly *et al.* 1993) and the sperm midpiece mitochondria of humans (Ankel-Simons & Cummins 1996), both of which are detectable up to the 32-cell morula stage, as well as the large mitochondrial derivatives of insect sperm, which have been traced to the blastoderm stage (Perotti 1973, 1975; Friedländer 1980). Evidence that extragenomic, sperm-derived products are essential for development come from two sources: (1) strict paternal-effect mutations resulting in embryonic lethality in *Drosophila melanogaster* (*pal*; Baker 1975) and *ms(3)K81* (Fuyama 1984, 1986a,b; Yasuda *et al.* 1995) and in *Caenorhabditis elegans* (*spe-11*; Browning & Strome 1996); and (2) the male sterility produced in certain intrastrain crosses by the endocellular microbe, *Wolbachia* (Karr 1994; Werren *et al.* 1995). In this system, infected males produce mature sperm that fertilize eggs, but are incapable of zygote formation when mated to uninfected females. We report here a previously unrecognized complexity of interaction between sperm and egg following fertilization in *Drosophila* that culminates in the elimination of the paternally contributed mtDNA.

2. MATERIALS AND METHODS

(a) Immunofluorescence and 3D reconstructions

Sperm within embryos were visualized by indirect immunofluorescence using an anti-sperm antisera, essentially as

described previously (Karr 1991). Three-dimensional (3D) reconstructions were generated by deconvolution from 3D volume sets, ranging from 30–80 µm, using a Princeton Instruments CCD camera. Deconvolutions and 3D reconstructions were performed with PowerMicrotome software (Vaytek, IA, USA). Optical sections were obtained using a Zeiss Axioplan 2 microscope with a 40× C-Apochromatic lens (numerical aperture, 1.2).

(b) Tissue preparation

Midguts were observed following dissection of entire alimentary canals from larvae immediately after they had hatched from their eggs, which had been transferred from oviposition substrate to agar-mollases plates that were free from contact with adult flies. Following dissection into phosphate-buffered saline, fresh alimentary canals were transferred to glycerol for observation at 1000× by differential interference microscopy.

(c) Transmission electron microscopy

Seminal vesicles of reproductively mature males, and intact alimentary canals of recently hatched larvae, were prepared for transmission electron microscopy by separately fixing samples in 2.5% glutaraldehyde, postfixing in osmium tetroxide, staining with uranyl acetate and lead citrate, and processing to plastic by standard procedures for observation using a JOEL 200SX transmission electron microscope. Sperm-derived structures within the midgut were located in these samples following serial thin sectioning and observation in the electron microscope. Specimens were photographed at 3000× (figure 4b), 30 000× (figure 4d,e), and 40 000× (figure 4a,c).

3. RESULTS

Using the polyclonal anti-sperm antibody AST-2 and confocal microscopy, we tracked the fate of the long sperm of *D. melanogaster* (figure 1a) and the giant helical sperm of *D. pachea* (figure 1b) throughout embryogenesis.

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Figure 1. Spermatozoon of *Drosophila melanogaster* (a) and *D. pachea* (b) obtained from a male's seminal vesicle, observed by dark-field (inverted) and phase contrast microscopy, respectively. Regions enclosed by the box have been enlarged $2.6\times$ to show DAPI-stained sperm heads (insets). Scale bar, $100\mu\text{m}$.

Immediately following fertilization, relatively uniform staining is observed along the entire *D. pachea* sperm (figure 2a). Shortly thereafter, regional differences in staining intensity are observed, including regions that appear to be in the process of losing spermic material to the cytoplasm (figure 2b), and by this time dramatic changes in intensity are observed along the helical coils. By the time of blastoderm cellularization, the sperm is observed within the yolk cytoplasm (figure 2c) where it remains until formation of the embryonic digestive tract (figure 2d). Notwithstanding differences in sperm length and structure, essentially identical results were also observed in three species of the *D. melanogaster* group (T. L. Karr and S. Pitnick, unpublished data), six species of the *D. obscura* group (R. R. Snook and T. L. Karr, unpublished data), and four species of the *D. virilis* group (S. Pitnick and T. L. Karr, unpublished data).

Changes in staining intensity during development indicate that some or all of the antigen(s) recognized by the AST-2 antibody are either not uniformly distributed along the sperm tail and/or are differentially degraded. A striking example occurs in *D. pachea* where only a small helical region is observed to be uniformly stained throughout development (figure 2b–d).

The sperm-derived structure was observed in midguts of recently hatched larvae. These structures were only observed in the midgut, never in the fore- or hindguts in 100% (8/8) of *D. melanogaster* larvae (figure 3a) and 93% (13/14) of *D. pachea* larvae (figure 3b). In *D. melanogaster*, the sperm-derived structures were typically broken into numerous pieces of varying length (figure 3a), whereas in *D. pachea* they typically appeared to be a single long helical strand (figure 3b). Therefore, a significant portion of the fertilizing sperm's tail remains intact throughout approximately 23 and 37 hours of embryogenesis at 24°C in *D. melanogaster* and *D. pachea*, respectively. Sperm-derived structures were typically restricted to a short portion of the midgut; only in one individual of each species were pieces of sperm observed to be distributed throughout the midgut. In, respectively, 79% and 58% of *D. melanogaster* and *D. pachea* alimentary canals examined by light microscopy, the sperm remnants were located at a position 80–90% posterior in the midgut.

Although the sperm-derived structure is extraordinarily persistent throughout embryonic development, comparison of *D. pachea* sperm from a male's seminal vesicle with the sperm remnant occupying a larval midgut reveals that numerous sperm tail components, including the plasma membrane and axoneme, have been substantially or completely degraded. Only the paired paracrystalline structures, which are derived from the mitochondria (Rosati *et al.* 1976; Baccetti *et al.* 1977; Hennig 1988) during spermatogenesis, remain essentially intact following larval hatching (figure 4).

Presumably, the sperm tail remnant observed in midguts is defaecated by the larvae soon after hatching. Three hours post-hatching, the sperm-derived structure can still be found, and appears unchanged in the posterior midgut of larvae held without food on agar (*D. pachea*, $N=5$). However, of the larvae permitted to feed for less than one hour after hatching ($N>20$), the entire digestive tract was found to contain food, and no sperm-derived structures were observed. Finally, as most digestion and absorption takes place in the midgut (Chapman 1982), the fact that most sperm remnants were observed in the posterior end of the midgut suggests that they are relatively inert (figure 3).

4. DISCUSSION

Drosophila melanogaster, *D. pachea*, and the other species examined (data not shown) are distantly related, representing approximately 58 Myr of divergence time (Pitnick *et al.* 1995a). They differ in their geographical distribution (Patterson & Stone 1952), resource ecology (Pitnick *et al.* 1995b), sperm morphology (e.g. head length, tail length, and tail shape; figure 1), and in the duration of embryonic development. Despite these differences, observed similarities between these species in the localization and persistence of the sperm throughout embryonic development suggest that the developmental traits examined here are likely to be common to all *Drosophila*. Although only a single sperm tail-specific protein, the DROP-1 proteoglycan (Graner *et al.* 1994), has so far been identified in *Drosophila*, the AST-2 antibody may identify additional sperm tail proteins involved in intracellular sperm–egg interactions.

Figure 2. Fate of sperm throughout embryogenesis. Sperm-derived structure within embryos at pronuclear fusion (a), nuclear cycle 6 (b), cellular blastoderm (c), and gut formation (d) in *D. pachea*, and (e) during dorsal closure in *D. melanogaster*. Arrows indicate: (a-c) regional variations in staining intensity, or the sperm-derived structure in later stages of (d) *D. pachea* or (e) *D. melanogaster*. Arrowheads indicate: (a) the apposed male and female pronuclei; (b) diffuse regions in the immediate vicinity of the sperm; (e) the ectoderm enclosing the amnioserosa (as), which happens during dorsal closure.

Figure 3. Sperm-derived structures (arrowheads) within midguts of fully developed, hatched *D. melanogaster* (a) and *D. pachea* (b) larvae, and one emptied into mounting medium along with gut contents when the midgut of a *D. pachea* larva was breached (c). Scale bar = 15 μ m.

Mitochondria in insect sperm undergo morphological change during spermatogenesis. They aggregate and fuse to form two giant mitochondria, which are arranged as a densely packed sphere, known as the Nebenkern, consisting of multiple layers. During the elongation period, the axoneme is assembled and the spherical mitochondrial derivative unfolds, splits into two parts, and elongates along with the growing axoneme. By the completion of flagellar morphogenesis, the mitochondrial derivatives extend along the length of the sperm tail

(Fuller 1993) and account for 50–90% of the cell volume of insect sperm (Perotti 1973). Our electron microscopy data suggest that only the mitochondrial derivatives persist intact throughout the entirety of embryonic development.

Predominant uniparental inheritance of mtDNA, usually maternal, is typical of most eukaryotic organisms (Alberts *et al.* 1989). However, low levels of paternal mtDNA transmission have been observed in *Drosophila* (Solignac *et al.* 1983; Satta *et al.* 1988; Kondo *et al.* 1990, 1992). These data clearly demonstrate that at low frequency paternal mtDNA can replicate and paternal mitochondria populate the organism. Whereas the mechanism(s) by which this is accomplished is not known, it/they may be related to the extraordinary size of the mitochondrial derivative in *Drosophila*, and thus the substantial amount of paternal mtDNA that enters the egg upon fertilization.

Whereas an ultimate explanation for this pattern of cytoplasmic inheritance, avoidance of lethal intragenomic conflict, has been discussed widely (Cosmides & Tooby 1981; Werren *et al.* 1988; Hurst 1992), except for those species in which the sperm's mitochondria are lost during fertilization (Lambert & Battaglia 1993), the proximate mechanisms for strict maternal inheritance remain a mystery. The structural stability of the sperm's mitochondrial derivative throughout embryonic development, and its sequestration in the midgut, may provide a mechanism by which the embryo actively avoids paternal mtDNA.

Examination of 3D reconstructions of sperm-derived structures reveals species-specific folding and coiling patterns (Karr 1991, 1996; Karr & Pitnick 1996). Some of this interspecific variation is attributable to differences in sperm morphology (see figure 1). The complex nature of sperm–egg interactions illustrated here suggests that the rapid divergence in sperm morphology observed in the genus *Drosophila* (Pitnick *et al.* 1995a,b) has resulted in correlated egg evolution. We postulate that interspecific variation in both extragenomic contributions of sperm to embryogenesis and in egg cytoplasmic factors necessary to coordinate the persistent sperm-derived structure constitute species-specific sperm–egg compatibility traits. If the developmental characters described here are adaptations to limit intragenomic conflict, then we predict that embryos resulting from interspecific fertilizations should exhibit quantifiable deviations in the processing of the sperm tail throughout development, relative to intraspecific fertilizations, that are concomitant with increases in leakage of paternal mtDNA. Although this prediction awaits testing, there is evidence for more frequent leakage of paternal mtDNA in interspecific rather than in intraspecific crosses (Niki *et al.* 1989; Kondo *et al.* 1990). Moreover, intersexual coevolution resulting from selection for gametic compatibility may, under conditions of restricted gene flow among populations, represent a potent, yet unexplored, class of post-zygotic reproduction-isolating mechanisms that has been central to the process of speciation.

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Figure 4. Ultrastructural comparison of *D. pachea* sperm from a male's seminal vesicle, i.e. prior to fertilization (a), with the sperm-derived structure residing within the midgut lumen following larval development (b). The central crystallized and amorphous peripheral regions of the principle (pd) and secondary mitochondrial derivatives (sd) are clearly visible in the sperm cross-section from the seminal vesicle (a), as are the plasma membrane (pm) and axoneme (an). In contrast, following embryonic development the sperm-derived structure in the midgut (b-e) consists of only the crystallized portion of the mitochondrial derivatives (boxes and arrowheads in b-e), identifiable by the characteristic hexagonal array of their rounded subunits in cross-section (c) and the complex banding of the lattice as seen in longitudinal (d) and oblique sections (e). The unusual structure shown in (e) reveals the helical morphology of *D. pachea* sperm persistent in the sperm-derived structure. Scale bar (c, upper left) = 0.2 μm (a, d, e); 2 μm (b); 0.1875 μm (c). (Photographs: D. M. Welker.)

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