

Different models of tubificid spermatozuogmata

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Abstract

The structure of the spermatozuogmata of different tubificid species belonging to the subfamilies Tubificinae, Limnodriloidinae and Phallo-drilinae was compared to the one of *Tubifex tubifex*. It was concluded that the spermatozuogmata of *Tubificoides* and *Clitellio* (Tubificinae) were very similar to the *Tubifex* ones in having a parallel arrangement of the central fertilizing sperm surrounded by a cortex made of packed atypical spermatozoa. On the contrary, *Kaketio ineri* and *Marcusaedrilus tuber* (Limnodriloidinae) and *Bathydrius formosus* (Phallo-drilinae) show an organization typical for each species but different from that of *Tubifex* mainly in lacking a double sperm line. A more comprehensive definition of the spermatozuogmata is proposed and more attention to the morphology of the sperm bundles when describing new oligochaete species is suggested, since it is possible to use this information for taxonomic and phylogenetic purposes.

Introduction

Even though the presence of sperm aggregates has been known for a long time in oligochaetes, much confusion about their significance and nomenclature persists. As an example we can recall the dispute among Claparède (1861) who described *Clitellio* spermatophores as a new species of opalinid protozoan, *Pachydermum acuminatum*, d'Udekem (1855) who suggested that the filamentous structures observed in the spermathecae of *Tubifex* were glands secreting the wall of the cocoon, and finally, Lankester who, some years later (1870), recognized the real nature of the sperm-ropes in *Clitellio*, *Tubifex*, *Limnodrilus*, *Nais*, and *Stylaria*.

Once the real nature of the sperm aggregates

had been universally acknowledged, the discussion turned to the role played by them in reproductive biology and to terminology. Cook (1971) proposed to call spermatozuogmata the sperm bundles found in the spermathecae of tubificids, whereas the term spermatophores should be confined to 'the structures attached to the body wall and containing sperm' (as in *Bothrioneurum*). Later on, Jamieson (1978) proposed to call spermatophores only those sperm aggregates possessing a chitinous or cellular capsule, independent from their position in the spermathecae or on the body wall, whereas the spermatozuogma should be 'a centripetal or parallel aggregation of spermatozoa, lacking a capsule, implanted in the spermatheca by the concupulant'.

The taxonomic value of the presence of sper-

matophores or spermatozeugmata is under discussion, partly because of the terminological confusion dominating the oligochaetological literature up to the recent years.

The ultrastructural description of *Tubifex* spermatozeugmata by Braidotti & Ferraguti (1982) as an aggregation of fertilizing, eupyrene spermatozoa surrounded by helically wound oligopyrene sperm has induced Erséus (1984) to ask what extent the *Tubifex* model is valid also for other Tubificidae. In other words, if the presence of spermatozeugmata is biunivocally related to the presence of a double sperm line, at least in Tubificinae. Furthermore, Erséus (1982) in his revision of the genus *Limnodriloides* distinguishes between 'spermatozeugmata' and 'sperm bundles' (looser aggregation of sperms) and suggests the potential utility of an electron microscopic study of spermatozeugmata in tubificids.

An extended review is complicated by many technical problems, such as the difficulty in finding mated worms, of obtaining a good fixation of spermatozeugmata within spermathecae, and comparing spermiogenesis with the aspect of mature spermatozoa. Thus, our preliminary results will be confirmed to the description of *Tubificoides* (subfamily Tubificinae), *Kaketio* (subfamily Limnodriloidinae), and *Bathydriulus* (subfamily Phallodrilinae) sperm aggregates, and to some preliminary data on *Marcusaedrilus* (Limnodriloidinae), and *Clitellio* (Tubificinae).

Materials and methods

Specimens of *Kaketio ineri* Righi and Kanner and *Bathydriulus formosus* Erséus were collected at Carrie Bow Cay, on the barrier reef off Belize in the Caribbean Sea, those of *Marcusaedrilus tuber* Erséus were collected in Hong Kong during the Second International Marine Biological Workshop on the Marine Flora and Fauna of Hong Kong and Southern China in 1986. These worms were dissected and immediately fixed in a cacodylate buffered paraformaldehyde-glutaraldehyde mixture in a saturated solution of picric acid.

Clitellio arenarius (Müller) and *Phallodrilus prostatus* (Knoller) were collected near Göteborg, Sweden, *Tubificoides amplivasatus* (Erséus) in the Oresund, Denmark; they were all fixed in phosphate buffered 3% glutaraldehyde. After an overnight washing in the buffer, the worms were post-fixed in buffered 2% solution of osmium tetroxide, dehydrated in a graded ethanol series and embedded in Spurr's resin. Sections were obtained with LKB ultratomes II and V and observed under Hitachi HU 11 ES and Jeol 100 SX electron microscopes.

Observations

Subfamily Tubificinae

Tubificoides amplivastus. The spermatozeugmata of *Tubificoides* are very similar to the *Tubifex* model in being long, club-shaped structures (Figs. 1, 2) built up by an external layer of modified, oligopyrene spermatozoa (Fig. 3), connected by a series of cell junctions (Fig. 6), and surrounding a central core with conventional, fertilizing sperm (Figs. 3, 4). Differences with *Tubifex* concern mainly the mitochondria, which are larger in *Tubificoides*, and the arrangement of the nuclei of the modified line: in *Tubificoides*, in fact, the sperms' heads are often horizontal, perpendicular to the orientation of the fertilizing sperm (Fig. 4). The structure of the junctional complex appears simpler than that of *Tubifex*: in particular, only septate junctions seem to connect the sperm tails (see: Ferraguti *et al.*, 1988) (Fig. 5).

Clitellio arenarius. The overall structure of the spermatozeugmata as reported in the systematic literature (Fig. 7) and visible under the optical microscope (Fig. 8) is suggestive of a similarity with *Tubifex tubifex*. We have electron micrographs only of the spermiogenesis: in the seminal vesicles there are two sperm types (Fig. 9, 11), as in *Tubifex*, distinguished by the same characters: thin and degenerating nucleus, hypertrophic mitochondria, empty acrosomes, and dilated sperm tails are the main features of the atypical spermatozoa (Fig. 10).

Figs. 1–6. Tubificoides amplivasatus. (1) Scheme of a spermatozeugma. (2) Three spermatozeugmata as seen in a whole mounted spermatheca. ($\times 90$). (3) Cross section of a spermatozeugma within the spermatheca as seen under the optical microscope. The axial cylinder (ac) containing the eupyrene spermatozoa and the cortex formed by the oligopyrene ones (c) are clearly visible. ($\times 870$). (4) Cross section of the inner part of the cortex and the periphery of the axial cylinder. Nuclei (n) and mitochondria (m) of the atypical spermatozoa are visible as well as nuclei (N) and tails (T) of the typical spermatozoa. ($\times 22\,000$). (5) In the external portion of the cortex the axonemes show symmetric rays starting from the doublets (arrow). ($\times 22\,000$). (6) A detail of an atypical sperm tail to show the cell junctions (arrow) and the rays. ($\times 60\,000$).

Figs. 7–11. *Clitellio arenarius*. (7) Scheme of a spermatozeugma reproduced from Claparède (1861). (8) Optical micrograph of a cross section of a spermatheca containing three poorly preserved spermatozeugmata clearly of the *Tubifex* type. ($\times 540$). (9) Within the seminal vesicles, under the optical microscope, two sperm lines are recognizable, mainly because of larger tail (t) and smaller nuclear (n) size of the atypical sperm line. Nuclei (N) and tails (T) of the typical line show a different size. ($\times 1200$). (10) The electron microscope shows the aspect of the atypical spermatozoa, similar to the one of *Tubifex* (n = nucleus; m = mitochondria; t = tail). ($\times 17500$). (11) The different aspect of the typical (T) and atypical (t) sperm tails. ($\times 63000$).

Subfamily Limnodriloidinae

Kaketio ineri. In the original description of this species, Righi & Kanner (1979) drew inside the spermathecae, a very peculiar ‘... bundle of spermatozoa with the heads near the opening. The spermatozoa are fastened to the anterior third of a structure (spermatozeugma?) rectangular in outline and not very thick, nearly as long as the ampulla’ (Figs. 14, 15).

When observing *Kaketio* spermathecae, even with an optical microscope but as 1 μm sections

of Spurr embedded material, the morphology of the spermathecae and the spermatozoa is clearly understandable (Fig. 16). The microvilli of the spermathecal epithelium are very long, and form a first layer of the spermathecal contents. The spermathecal fluid, with a somewhat structured appearance, is the second layer, whereas the innermost portion of the spermathecae is occupied by a single ordered aggregation of spermatozoa arranged parallel with the acrosomes outside (Fig. 17), leaning on an enormous proteinaceous structure running the whole length of

Figs. 12–13. *Marcusaedrilus tuber*. (12) In this drawing of the spermatheca some spermatzeugmata (sz) are visible inside the ampulla. (13) A low power micrograph of the whole ampulla confirms the presence of some sperm groups. Note the different diameter of the sperm nuclei. ($\times 4500$).

the spermathecal ampulla (Fig. 18). All the spermatozoa belong to the conventional model for the oligochaetes (i.e. no oligopyrene sperm are found). The acrosome, less than $1\ \mu\text{m}$ long, is composed of an acrosome tube constantly showing a basal, thicker portion and an apical, thinner one. The acrosome vesicle withdrawal is limited to the anterior tenth of the tube. An acrosome rod surrounded by a secondary tube runs inside the thin portion of the tube (Fig. 20). The nucleus is corkscrew-shaped towards the base and straight in the anterior part (Figs. 19, 21). The four clove-shaped mitochondria form a roughly spherical structure (Figs. 21, 22). A short, deeply modified centriolar area (Figs. 22, 21) is followed by a typical oligochaete flagellum with tetragon fibers and glycogen granules (Fig. 23). The proteinaceous structure filling the spermatheca is very peculiar in one aspect: the size and shape of the material composing it are reminiscent of small microtubules which, in the best sections, show a partly ordered disposition of layers (Fig. 18). These kind of 'microtubules' bear a notable similarity with structures already described in the cocoon wall of many oligochaetes (Marcel *et al.*, 1985).

Marcusaedrilus tuber. Observations on the spermatzeugmata of *Marcusaedrilus* are only prelimi-

nary because of technical problems. The spermatozoa appear to be organized in more than one spermatzeugma stored in the ampullae of the spermathecae (Figs. 12, 13). The nuclei of the spermatozoa are arranged parallel within each spermatzeugma and all appear to be of the same size. In the peripheral portion of the ampullae, some spermatozoa are scattered, showing a very large nucleus, suggestive of a dichotomous sperm line (Fig. 13). It should be remembered, however, that there are many descriptions of abnormal spermatozoa in the oligochaetological literature.

Subfamily Phalldrilineae

Bathydrius formosus. The organization of the *Bathydrius* spermatzeugmata is, again, peculiar. The spermatozoa, all apparently of the conventional type, are wound as a skein (Fig. 24), with the acrosomes in the middle (Fig. 26), the nuclei wrapped around and the tails at the exterior (Fig. 28). The single spermatzeugma fills the whole lumen of the spermatheca in the specimens examined (Fig. 25), but two or three spermatzeugmata were observed in the spermathecae of other, whole-mounted individuals examined un-

Figs. 14–23. Kaketio ineri. (14) Scheme of a spermatheca containing the spermatzeugma reproduced from Righi and Kanner (1979). (15) Spermatheca and spermatzeugmata as seen in a whole mounted preparation. The 'Christmas tree' appearance shown in the drawing was probably due to the peculiar package of spermathecal microvilli. ($\times 70$). (16) Longitudinal section of a spermatzeugma within the spermatheca. Under the sperm bundle the prominent proteinaceous structure is visible. Encircled area is enlarged in Fig. 17. ($\times 130$). (17) Low power electron micrograph showing sperm nuclei (n), tails (t), and the proteinaceous

der the light microscope. The spermatozoa are very peculiar for the fact that their acrosome tube is so small as to suggest the possibility that it is absent; an acrosome rod is not visible either (Fig. 29). If these absences were confirmed, *Bathydrius* would be, up to now, the first clitellate lacking an acrosome tube and rod. It must be considered, however, that the whole acrosome is quite unusual in being very small (0.24 μm long) and showing a deep withdrawal of the acrosome vesicle. The nuclei are corkscrew-shaped and highly wound for the whole length (Fig. 26). The five mitochondria (Fig. 32) are very short (Figs. 27, 30) and followed by a small centriolar area and a conventional clitellate axonome (Fig. 31). A dense material embeds the sperm tails and middle pieces (Figs. 27, 28).

Phallosdrilus prostatatus. Observations on *P. prostatatus* spermathecae have confirmed the fact, already known from the literature, that the spermatozoa are stored without any apparent order (Fig. 23). Interestingly, the mitochondria of *Phallosdrilus* sperm (Figs. 34, 35) are exceedingly long not only as compared to the mitochondria of *Bathydrius* but also to the other oligochaetes (length 4.3–5.2 μm ; length: width ratio 12–15.5).

Discussion

From the cytological point of view, the tubificid spermatozeugmata have proven to be a very interesting subject of research: their structure is highly variable within the group, suggesting that the functions of spermatozeugmata in the reproductive biology of tubificids may be more complex than previously thought.

In *Tubifex*, it has been shown that the spermatozeugmata have at least three functions: (1) To

hold together a high number of fertilizing spermatozoa, thus increasing the chances of fertilization (Braidotti & Ferraguti, 1982); (2) To obtain nutrients from the spermathecal fluid to the fertilizing spermatozoa in the axial cylinder (Fleming, 1981); (3) To carry fertilizing sperm to the opening of the spermathecal duct where they will be discharged into the cocoon at the moment of deposition (Ferraguti *et al.*, 1988).

Another possible function, proposed to explain a similar situation found in Lepidoptera, is with regard to gamete competition: a low-cost material (oligopyrene sperm) is injected into the spermathecae of the partner, thus avoiding further copulation with another partner (Silberglied *et al.*, 1984).

In the models of spermatozeugmata described here for other Tubificidae, some of the functions outlined above cannot always be performed: obtaining nutrients from the spermathecal fluid and performing gamete competition is apparently linked to the presence of a double sperm line (Fleming 1981; Silberglied *et al.*, 1984). In *Kaketio*, however, gamete competition can be performed by the mass of proteinaceous material found in the spermatheca together with the spermatozoa, if it is proved that the mass is produced by the 'male' copulant.

In his important book on spermatophores, Mann (1984) writes that 'Spermatophores are of ... the encapsulated type, quite distinct from all those loose aggregates that are represented by so-called sperm-bundles, spermatodesms or spermatozeugmata' and, in another part of the book, 'A large sperm-aggregate designated as spermatozeugma ... lacks a proper capsule and the highly organized manner of sperm assembly which is so characteristic of typical spermatophores'. However, it seems from our observations that the definition by Jamieson (1978) quoted in the intro-

structure (p). Unidentified microorganisms (mi) crowded our specimens of *Kaketio*. The encircled area is enlarged in Fig. 18. ($\times 1000$). (18) A nucleus (n) and a tail (t) lean on the proteinaceous structure which is made by 'microtubules' with an approximate diameter of 18 nm. ($\times 36000$). (19) Straight apical portion of a nucleus with acrosome (a). ($\times 7000$). (20) Detail of two acrosomes. Description in the text. ($\times 52000$). (21) Basal helical portion of a nucleus and short middle piece. ($\times 26000$). (22) Cross section of the four mitochondria and the centriolar area. ($\times 75000$). (23) Cross section of a sperm tail. Note the tetragon fibers (arrowhead), the glycogen particles disposed externally to and between the doublets, and the 'hooks' (arrow) starting from the doublets and directed towards the plasma membrane. ($\times 90000$).

Figs. 24–32. Bathydrilus formosus. (24) Drawing of a spermatozeugma (sz) within a spermatheca. (25) Optical micrograph of a spermatheca containing one skein-shaped spermatozeugma. The acrosomes are in the center, surrounded by the nuclei (n) and the tails (t). ($\times 250$). (26) Acrosome area (a) surrounded by the cork-screw shaped nuclei. ($\times 6300$). (27) Area of the middle pieces. Three mitochondrial groups and one centriolar area are visible, as well as some nuclei and tails. ($\times 20\,000$). (28) Tail area of a spermatozeugma. The tails are embedded in a cementing substance. sw = spermathecal wall. ($\times 6000$). (29) Longitudinal

duction above conforms to the various models of tubificid spermatozeugmata described here. In fact, despite the presence or absence of a double sperm line, the shape of spermatozeugmata appears to be particular for any species studied. We propose, however, that this definition is reworded thus: 'spermatozeugmata are sperm aggregates implanted in the spermatheca by the concopulant, characterized by repetitive order of the spermatozoa and the presence of some sort of cementing agent, but lacking a proper capsule'. With increasing knowledge about the structure of the different models, the spermatozeugmata will become a valuable taxonomic tool.

It is therefore desirable that, when describing species of Tubificidae, taxonomists account for the specific arrangement of the sperm in the spermatheca with as much morphological information as possible. It should not be sufficient to state that 'spermatozeugmata are present', but rather it should be mentioned briefly how the sperm cells are arranged, and the shape of the sperm aggregates and illustration of them should be provided. The latter is possible with a good optical microscope.

Spermatozeugmata will most certainly prove to be increasingly useful for phylogenetic considerations within the Tubificidae, as has been shown to be the case for the ultrastructure of the spermatozoa proper in many oligochaetes (Jamieson *et al.*, 1987).

The present study is, however, preliminary in many respects, and therefore any discussion on its phylogenetic implications should not be accepted as conclusive. As indicated in a holomorphic cladistic analysis by Erséus (1987), the marine subfamily Phallodrilinae appears to be one of the most ancestral groups within the Tubificidae. This appears to be supported by the

relatively simple acrosome in *Bathydrilus*, but this genus is not necessarily a 'typical' member of the Phallodrilinae; e.g., it is the only phallodriline genus known with roundish, skein-like spermatozeugmata. It has not been possible to determine from our material if other phallodrilines have a proper acrosome tube – which has been regarded as a synapomorphy for the oligochaetes as a whole (Ferraguti, 1983); for this reason the poorly developed acrosome tube in *Bathydrilus* can be interpreted in two different ways. Either it represents an ancestral state in the evolution of this tube – the sperm of *Bathydrilus* would then be the most primitive known within the Oligochaeta – or, and perhaps more likely, the acrosome tube has become secondarily reduced in this genus.

The Limnodriloidinae and Tubificinae are closely related and highly apomorph groups within the Tubificidae according to the holomorphic analyses provided by Erséus (1987), a relationship not recognized in an early study (Erséus, 1984). This is corroborated by sperm data; in these two subfamilies spermatozeugmata are common and they are generally of a similar, slender kind. The spermatozeugma of *Kaketio* is apparently rather unique and can be regarded as an autapomorphy for that genus, which in terms of other genital characters is a very specialized limnodriloidine anyway (cf. Righi & Kanner, 1979). The sperm aggregates of *Marcusaedrilus*, with their indication of two sperm lines, appear to be more 'en route' towards the tubificine models. The large genus *Limnodriloides*, which is most closely related to *Marcusaedrilus*, is a mixture of species with sperm being either random, bundled, or truly aggregated as spermatozeugmata, in the spermathecae (cf. Erséus, 1982), also foreshadowing the more advanced tubificine spermatozeugmata. The present findings thus seem to

section of an acrosome on top of a nucleus (n). ($\times 105\,000$). (30) Longitudinal section of a middle piece showing the short mitochondria and the centriolar area (n = nucleus). ($\times 60\,000$). (31) Cross section of a tail, showing the glycogen granules, external to the axoneme, and the 'hooks' starting from the axonemes towards the plasma membrane. ($\times 100\,000$). (32) Cross section of the five clove-shaped mitochondria. ($\times 73\,000$).

Figs. 33–35. Phallodrilus prostates. (33) An optical micrograph of a mated animal spermatheca shows spermatozoa not organized in any particular form. ($\times 500$). (34) Cross section of a group of late spermatids: mitochondria (m) nuclei and tails are visible. ($\times 15\,000$). (35) Longitudinal section of an area similar to the one in Fig. 34. m = mitochondria; n = nucleus. ($\times 15\,000$).

indicate that the Limnodriloidinae, which are exclusively marine, are less apomorphic than the predominantly limnic Tubificinae.

The present study also demonstrates that comparative studies of spermatozuogmata may add to the assessment of phylogenetic relationships even within the subfamily Tubificinae. For instance, the fact that the structure of the junctional complex appears simpler in the spermatozuogmata of *Tubificoides* (marine) than in those of *Tubifex* (limnic) can be used as an argument for a more plesiomorph position of the former genus relative to the latter.

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References

- Braidotti, P. & M. Ferraguti 1982. Two sperm types in the spermatozuogma of *Tubifex tubifex* (Annelida, Oligochaeta). *J. Morph.* 171: 123–136.
- Claparède, E. 1861. Etudes anatomiques sur les annélides, turbellariés, opalines et grégaires observés dans les Hébrides. *Mem. Soc. Phys. Hist. Nat. Genève* 16: 71–164.
- Cook, D. G. 1971. Anatomy. Microdriles in R.O. Brinkhurst and B.G.M. Jamieson (eds.) *Aquatic Oligochaeta of the World*. Oliver & Boyd, Edinburgh: 8–41.
- D'Udekem, J. 1855. Histoire naturelle du *Tubifex* des ruisseaux. *Mém. Courentes et Mém. des Savants Etrangers de l'Acad. Roy. de Belgique* 26: 1–38.
- Erséus, C. 1982. Taxonomic revision of the marine genus *Limnodriloides* (Oligochaeta, Tubificidae). *Verh. naturwiss. Ver. Hamburg (N.F.)* 25: 207–277.
- Erséus, C. 1984. Aspects of the phylogeny of the marine Tubificidae. *Hydrobiologia* 115: 37–44.
- Erséus, C. 1987. Phylogenetic analysis of the aquatic Oligochaeta under the principle of parsimony. *Hydrobiologia* 155: 75–89.
- Ferraguti, M. 1983. Annelida Clitellata in K.G. Adyiodi and R.G. Adyiodi (eds.), *Reproductive Biology of Invertebrates, Vol. II, Spermatogenesis and sperm function*. Wiley, London: 343–376.
- Ferraguti, M., G. Bernadini, G. Melone & R. Dallai 1988. Structure and function of the metachronal wave in *Tubifex tubifex* spermatozuogmata (Annelida, Oligochaeta). *J. Ultrastruct. Mol. Struct. Res.*, 99: 79–95.
- Fleming, T. P. 1981. The ultrastructure and histochemistry of the spermathecae of *Tubifex tubifex* (Annelida: Oligochaeta). *J. Zool. Lond.* 193: 129–146.
- Jamieson, B. G. M. 1978. *Rhyacodrilus arthingtonae*. A new species of freshwater oligochaete (Tubificidae) from North Stradbroke Island, Queensland. *Proc. R. Soc. Qd.* 89: 39–43.
- Jamieson, B. G. M., C. Erséus & M. Ferraguti 1977. Parsimony analysis of the phylogeny of some Oligochaeta (Annelida) using spermatozoal ultrastructure. *Cladistics* 3: 145–155.
- Lankester, E. R. 1870. Remarks on *Opalina* and its contractile vesicles, on *Pachydermon* and annelidan spermaphores. *Quart. J. Micr. Sci.* 10: 143–150.
- Mann, Th. 1984. *Spermaphores*. Springer Verlag, Berlin, 217 pp.
- Marcel, R., C. Cordon & P. Degand 1985. Le cocon d'*Eisenia foetida* Sav. (Annélide Oligochète). Etude ultrastructurale et biochimique. *Arch. Anat. Micr. Morph. Exp.* 74: 181–191.
- Righi, G. & E. Kanner 1979. Marine Oligochaeta (Tubificidae and Enchytraeidae) from the Carribean Sea. *Stud. Fauna Curaçao and Carribean Isl.* 58: 44–68.
- Silberglie, R. E., J. G. Shepherd & J. L. Dickinson 1984. Eunuchs: the role of apyrene sperm in Lepidoptera. *Am. Nat.* 123: 255–265.