

Male reproductive system in the Italian newt *Lissotriton italicus* (Peracca 1898) (Amphibia, Urodela): ultrastructural and morphological study with description of spermiogenesis, spermatozoon and spermatophore

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Received: 2 September 2008 / Revised: 7 January 2009 / Accepted: 14 January 2009 / Published online: 5 February 2009
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Abstract The first morphological and ultrastructural description of testis, Wolffian duct, spermatophore, and spermatozoon of *Lissotriton italicus* is provided. The male reproductive system of this species consists of a pair of monolobed testes, fat bodies, efferent ductules, paired Wolffian duct, and the cloaca. The Wolffian duct is pseudostratified, consisting of an epithelium composed of an alternation of ciliated cells and non-ciliated secretory cells, with scattered basal cells. Melanocytes are found in the basal lamina of the Wolffian duct. Spermiogenesis is of the discontinuous type. In the early spermatid nuclear elongation, flagellar extrusion and development of a large acrosomal vesicle occur. Soon the perforatorium develops and the flagellum elongates. In the late spermatid gradual chromatin condensation and nuclear elongation is noticeable. At the end of spermiogenesis the flagellum develops marginal filament and undulating membrane. The mature sperm of *L. italicus* consists of a distinct head (acrosome, perforatorium, nucleus and nuclear ridge, plus the apical acrosomal barb) and a tail with axoneme, undulating membrane, and axial fiber. Nuclear ridge and apical acrosomal barb are two autapomorphic characters found in the Urodela. Several apomorphic characters for Salamandroidea are also present: elongation of the connecting piece, an apically modified acrosome vesicle, trifoliate axial fiber within the principal

piece, elongate annulus, elongate midpiece, and the gentle merging of the axial fiber/principal piece into the endpiece. The spermatophore of *L. italicus* is formed by a cap consisting of randomly distributed spermatozoa surmounting a short pedicel. A membranelike structure involving the spermatophore is absent.

Keywords Spermiogenesis · Sperm morphology · Wolffian duct · Spermatophore · Amphibia

Introduction

Knowledge of testes, efferent ducts, spermiogenesis and spermatozoal morphology and ultrastructure is indispensable for the study of reproductive biology and is of value for reconstruction of phylogenetic relationships in many animal taxa (Purschke and Jördens 2007; Longo and Trovato 2008; Tavares-Bastos et al. 2008; Tourmente et al. 2008) and in particular for all amphibians taxa (Baker 1965; Wake 1968, 1970; Williams et al. 1984; Zalisko et al. 1984; Brizzi et al. 1992; Lee and Jamieson 1993; Selmi et al. 1997, 1999; Scheltinga and Jamieson 2003a, b, 2006).

In this paper, we present the first morphological and ultrastructural observations on the reproductive system, spermiogenesis, and spermatophores in males of *Lissotriton italicus* (Peracca 1898) to obtain information for a comparative analysis with other species and to reach a conclusion on the phylogenetic relationship of the taxon. This species is endemic in central-southern Italy (Giacoma et al. 1988; Scillitani et al. 2004; Sperone and Tripepi 2005; Brunelli et al. 2007). As is typical of the Salamandroidea, sperm transfer in *L. italicus* takes place by means of spermatophores; they are laid by males and picked up by females. Breeding season occurs in water, from November to May in

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Calabria (Sperone and Tripepi 2005). Embryonic development of *L. italicus* is typical of warm-adapted species that reach metamorphosis in a short time (Tripepi et al. 1998): it is due to the fact that breeding habitats of this species are temporary and dry in late spring.

The purpose of this study is threefold. First, we provide morphological and ultrastructural observations on the male reproductive system, spermiogenesis, spermatozoon, and spermatophore of this species, utilizing techniques of light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Second, we provide further information about reproductive biology of the species. Third, we compare our data with those on morphological and ultrastructural characteristics of sperm and reproductive ducts in other Salamandridae and in urodeles in general.

Materials and methods

Two adult male *L. italicus* were collected monthly in San Vincenzo La Costa (Calabria, Italy) from September 2003 to November 2004. Animals generally were killed and preserved within 24 h of collection. Spermatophores were collected at the end of courtship displays, after their deposition in water from the male cloaca.

Light microscopy (LM)

Tissues prepared for LM were fixed in Bouin's fluid, rinsed in water, dehydrated with ethanol solutions (75, 85, 95, 100%), cleared with xylene, and embedded in paraffin. They were serially sectioned at every 5 μm and stained with H&E. Tissues were examined and photographed with a Leitz Dialux EB20 light microscope.

Scanning electron microscopy (SEM)

Spermatophores, testes, and Wolffian ducts were prefixed for 2 h in cold 3% glutaraldehyde and washed in phosphate buffer (0.05 M, pH 7.5). They were postfixed for 2 h in 1% osmium tetroxide in the same buffer, dehydrated with ethanol solutions (30, 50, 70, 90, 100%), immersed in hexamethyldisilazane, and coated with gold. They were observed and photographed with a Stereoscan DMS 940 Zeiss microscope.

Transmission electron microscopy (TEM)

Small pieces of testis and vas deferens were prefixed for 2 h in cold 3% glutaraldehyde and washed in phosphate buffer (0.05 M, pH 7.5). Then they were postfixed for 2 h in 1% osmium tetroxide in the same buffer, dehydrated with ethanol solutions (30, 50, 70, 90, 100%) soaked in propylene

oxide, and then embedded in Epon-Araldite. Samples were sectioned at 600–900 \AA with LKB Ultratome III ultramicrotome. Ultrathin sections were stained with uranyl acetate in ethanol and lead citrate, coated in an Edwards EM 400 and examined, and photographed with a Zeiss EM 900 electron microscope. Some semithin sections (1–2 μm) were stained with toluidine blue-malachite green-acid fuchsin and observed by light microscopy.

Results

Male reproductive system

The male reproductive system of *L. italicus* consists of a pair of testes, fat bodies, efferent ductules, Wolffian duct and unpaired cloaca. The efferent ductules are arranged as a net connecting each testis, via the upper part of the kidney, to the Wolffian duct.

Each elongated testis is composed of one lobe in which lobules of variable size, here termed loculi, are distinguishable. In the loculi, mature spermatozoa are arranged in characteristic half-moon shaped bundles, the sperm cysts or spermatocysts (Fig. 1a, b). A thin tunica surrounds the testis.

Sertoli cells are in contact with the basement lamella and are easily recognizable for the elongated nucleus with a dark cytoplasm (Figs. 1b, 2c). The testicular loculi communicate with a system of intratesticular ducts passing through connective tissue. Efferent ductules connect each testis with the corresponding Wolffian duct. Only during the breeding period, the Wolffian duct contains sperm randomly distributed throughout the length of the lumen (Fig. 1c, d). Coinciding with the testicular seasonal cycle, the vas deferens shows morphological changes: its diameter is greatest during spermiation when spermatozoa are abundant, whereas a regression is observed at the end of spermiation. Each Wolffian duct is formed by a sequence of constrictions (Fig. 1c). TEM observations also reveal that Wolffian duct is pseudostratified (Fig. 2a), consisting of an epithelium composed of an alternation of ciliated cells and not ciliated secretory cells (containing the large secretory granules), and basal cells which do not extend to the lumen (Figs. 1d, 2a, b). The connective tissue, subadjacent to the duct epithelium, contains fibroblasts, melanocytes, circular smooth muscle, and blood vessels (Fig. 2a). Sperms removed directly from the duct do not undulate, but are immotile and inactive.

Spermiogenesis

Spermiogenesis occurs from late August to September and it is of the discontinuous type as in other amphibians. This process involves nuclear elongation, chromatin condensation,

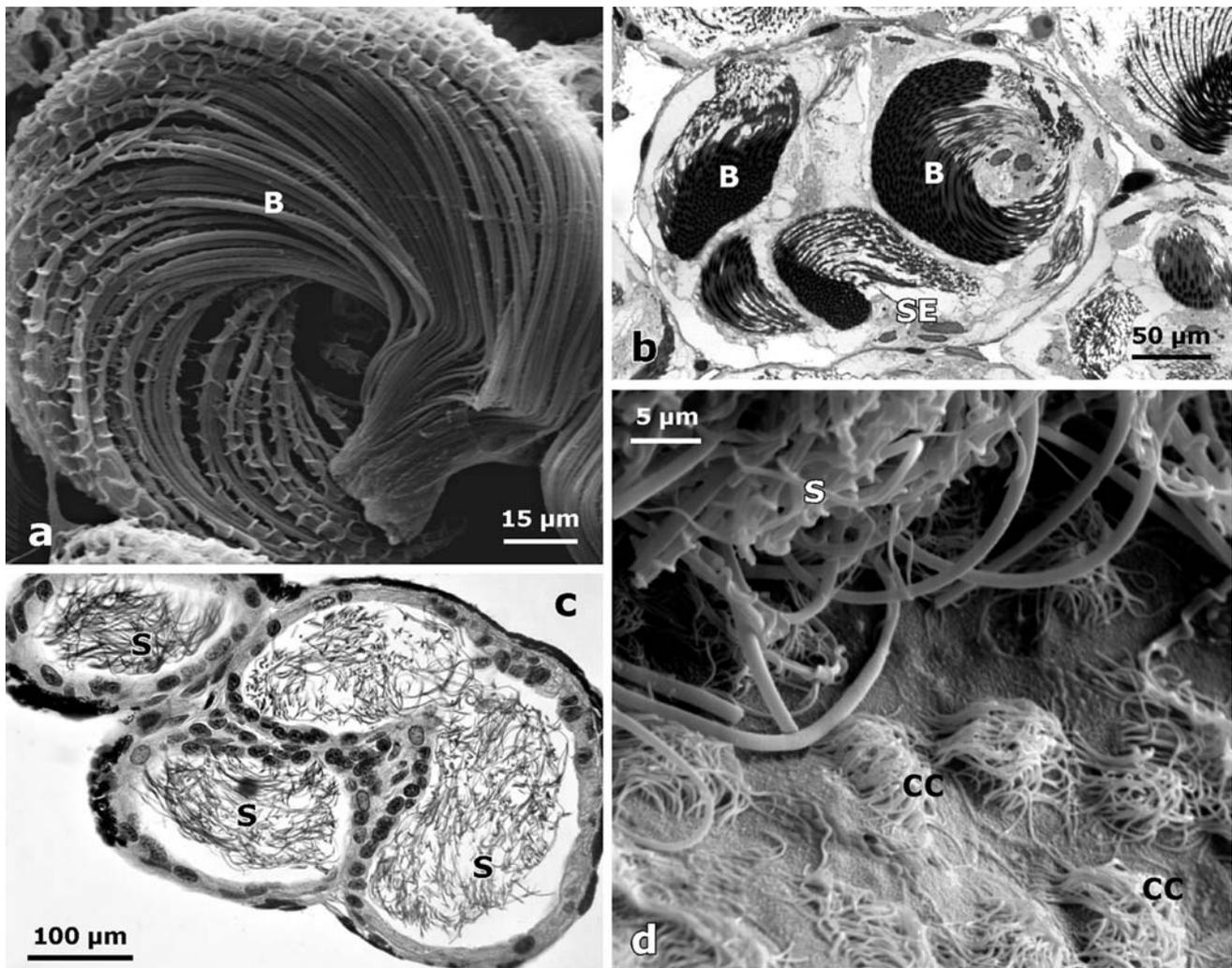


Fig. 1 **a** Scanning electron micrograph (SEM) of a sperm bundle (cyst). **b** Light microscopy (LM). Cyst of spermatozoa in the testis. **c** LM of a section of the Wolffian duct containing spermatozoa. **d** SEM

of lumen of the Wolffian duct, showing spermatozoa and ciliated cells. **B** sperm bundle, **CC** ciliated cell, **S** spermatozoa, **SE** Sertoli cell

acrosome formation, and development of the flagellum with its accessory structures.

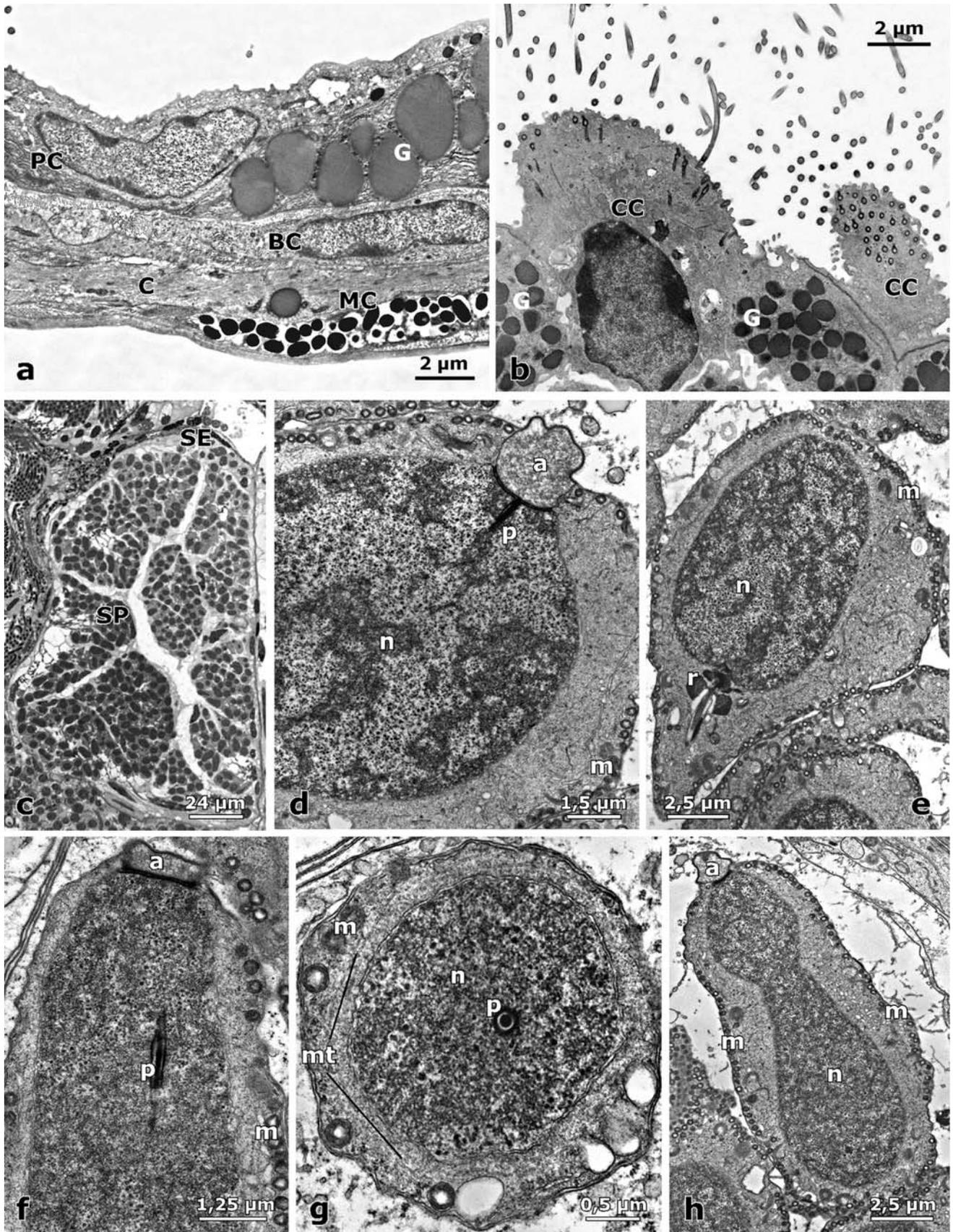
During spermiogenesis germ cells form groups of cells, at the same stage of differentiation, and constitute a spermatocyst (Fig. 2c). Sertoli cell encloses each germinal cyst. Spermatids remain anchored to the numerous cytoplasmic processes of the Sertoli cells during all stages of spermiogenesis (Fig. 3f, g).

In the early spermatid, nuclear elongation and a first flagellar extrusion (Fig. 2e) occur. The flagellum originates near the posterior region of the nucleus; its proximal region is ensheathed by the developing ring, in which two distinct portions can be observed: a granular proximal ring and a distal lamellar ring (Figs. 2e, 3b). The distal ring, which consists of a series of concentric double bands with associated dense particles, contains the distal centriole. Proximal centriole is located in the basal nuclear fossa.

At the same time a large and approximately spheroidal acrosomal vesicle containing small quantities of a flocculent material develops at the anterior end of the nucleus (Fig. 2d, f, h). The nucleus develops a depression at the point of contact between the acrosomal vesicle and the nuclear envelope, where a narrow electron-dense plate intervenes (Figs. 2d–f).

Subsequently, the perforatorium develops and has a cylindrical structure composed of two distinct, homogeneous, concentric layers with different electron-density (Figs. 2f, g, 4f). Anteriorly, the perforatorium is connected to the acrosomal plate but it does not come into contact with acrosome (Fig. 2d).

The flagellum originates from the distal centriole in the posterior vicinity of the nucleus and shows the principal components, marginal fiber, and axial fiber, that at an anterior level are close together, separated by the axoneme



◀ **Fig. 2** Transmission electron micrographs (TEM) of Wolffian duct (a–b) and testis (c–h). **a** Sagittal section of part of the Wolffian duct. **b** Ciliated cells and granulocytes of the Wolffian duct. **c** Spermatozoa, groups of Sertoli cells surrounding spermatids. **d** Longitudinal section of an isodiametric spermatid showing developing acrosome. **e** Longitudinal section of spermatid at commencement of elongation. **f** Longitudinal section of anterior region of elongating spermatid, showing developing acrosome, and perforatorium deeply penetrating the nucleus. **g** Transverse section of spermatid at the same stage as F. **h** Longitudinal section of anterior region of elongating spermatid to one side of the perforatorium. *a* acrosome vesicle, *BC* basal cell, *C* connective tissue, *CC* ciliated cell, *G* granulocyte, *m* mitochondria, *MC* melanocyte, *mt* microtubules, *n* nucleus, *p* perforatorium, *PC* pavement cell, *r* ring or annulus, *SE* Sertoli cell, *SP* Spermatids

(Fig. 3a, b). The axoneme has the usual 9 + 2 arrangement: two central microtubules (singlets) and nine peripheral pairs (doublets) provided with dynein arms (Fig. 3e, h).

In the late spermatid, gradual chromatin condensation occurs (Fig. 3c, f, g). Simultaneously with this condensation, nuclear and cellular elongation is completed. The spermatid is characterized by the presence of perinuclear microtubules and microtubules of the Sertoli cells, the former being arranged circularly around the nucleus and the latter arranged parallel to the long axis of the spermatids (Fig. 3f, g).

Only at the end of spermiogenesis does the acrosome vesicle come into contact with the perforatorium and both structures elongate (Fig. 3d). The flagellum consists of its accessory structures, including the undulating membrane that connects the axoneme, flanked by the marginal filament with the axial rod. The marginal filament is also known as the juxta-axonemal fiber at 8, as lies opposite doublet 8 of the axoneme. Distally, the axial fiber becomes clover-shaped (trifoliate) (Fig. 3e). The endpiece of each spermatid flagellum is constituted by the axoneme and a reduced marginal filament only (Fig. 3h).

Mature spermatozoa

Mature spermatozoa average $360.8 \pm 6.1 \mu\text{m}$ in total length, ($n = 25$, from five different males) with the head occupying a third of the length. SEM and TEM reveal the presence of a nuclear ridge (Fig. 4i) and the acrosomal barb (Fig. 4b, g). The barb, subapical on the head, extends posterolaterally with a length of $2.2 \mu\text{m}$. With the acrosomal vesicle it constitutes the acrosome proper, which with the perforatorium makes up the acrosomal complex (Fig. 4g). The perforatorium is a cylindrical structure beginning from the base of acrosomal barb (Fig. 4e–g) and extends into the anterior end of the nucleus, within the endonuclear canal (Fig. 4h). The perforatorium retains two distinct homogeneous concentric layers with different electron-density (Fig. 4f); it is also enveloped for its entire length by the conical acrosomal vesicle. TEM shows that the head is

made of an acrosome and of a nucleus. SEM shows a tail composed of an axial rod with an undulating membrane coiled around it (Fig. 4a, c).

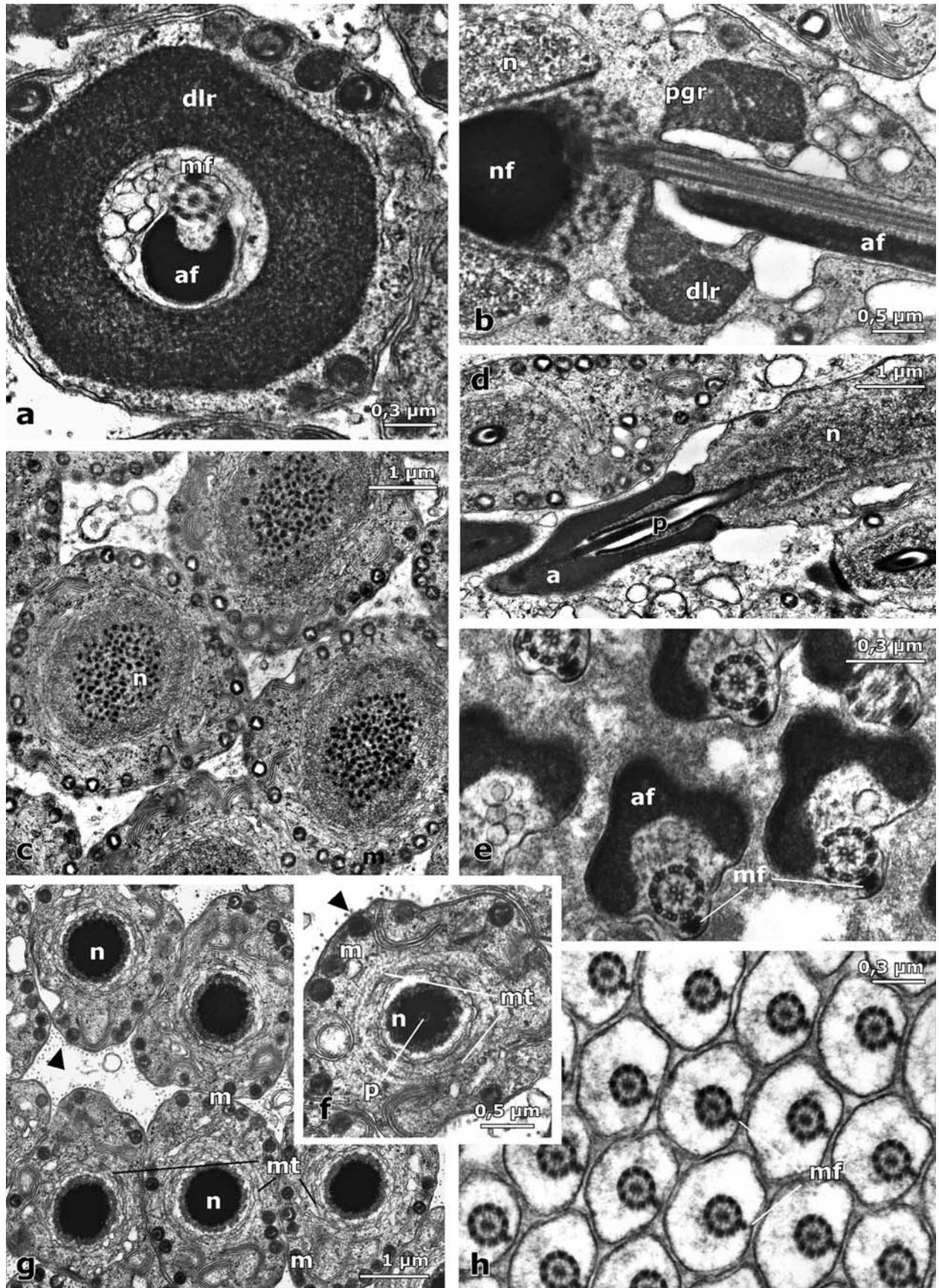
The nucleus is composed of homogeneously electron-dense chromatin and has two terminal cavities or fossae: an anterior one (Fig. 4f, i) constituting the endonuclear canal and containing the posterior region of the perforatorium, and a posterior one (Figs. 4j, 5a, b), the basal nuclear fossa, which contains the connecting piece. The proximal and distal centrioles lie at the caudal end of the nuclear fossa, embedded in a granular matrix (Fig. 5a, proximal centriole). The proximal centriole, from which the axoneme originates, is obliquely oriented with respect to the longitudinal nuclear axis. At the level of the nuclear fossa the chromatin is arranged in a peripheral layer of small particles. The region between connecting piece and tail constitutes the so-called neck (Figs. 4d, 5a). On the nucleus, the nuclear ridge is composed of densely packed parallel microtubules which lie under the nuclear membrane, in contact with the chromatin; in transverse section it has a semicircular appearance (Fig. 4i).

The tail has the components described for the late spermatid: the axoneme, with its marginal filament, connected by the undulating membrane to the axial fiber. In the anterior region of the tail mitochondria accompany the axial fiber, as a halfmoon-shaped border, defining the midpiece. The midpiece thus consists of five components: mitochondria, axial fiber, undulating membrane, axoneme together with a considerable amount of cytoplasm, and the marginal filament (Fig. 5c–e). The axial fiber, in transverse section, is horseshoe-shaped near the head (Fig. 5c). In a distal direction, its thickness decreases and it becomes at first approximately crescentic or halfmoon-shaped (Fig. 5d), then clover-shaped (trifoliate) (Fig. 5f). The tail terminates with a short endpiece. This commences where the axial rod merges with the undulating membrane; it consists only of the axoneme together with the marginal filament, the crescentic shape of which has here become oblong-ovoid. There is a considerable zone of cytoplasm between the doublets and the plasma membrane in the endpiece as also seen in the vicinity of the axial fiber (Fig. 5g).

In the Wolffian duct, sperm preserve all the features described including the acrosomal barb, axial rod and undulating membrane.

Spermatophore

In *L. italicus* fertilization of the eggs occurs in the cloaca of the female. At the end of a complex courtship display, which involves three main phases (orientation display, static display, and retreat display), the male deposits his sperm in a single package, the spermatophore. The female is manipulated in a way that ensures that the spermatophore



◀ **Fig. 3** TEM sections through spermatids. **a** Transverse section through the annulus, axial fiber and axoneme. **b** Longitudinal section through the nuclear fossa (containing the connecting piece) annulus and axial fiber. **c** Transverse section through uncondensed nuclei. Spermatids are not connected by intercellular bridges. **d** Longitudinal section through acrosome vesicle and perforatorium, the latter penetrating the nucleus and the acrosome. **e** Transverse sections through marginal filament (juxta-axonemal fiber) and large axial fiber. **f** Transverse section near the tip of the nucleus, showing the perforatorium. Microtubules fringe the nucleus. **g** Transverse sections of nuclei, microtubules and mitochondria. **h** Transverse sections of developing sperm tails in the posterior region where the axial fiber is absent and the marginal filament (juxta-axonemal fiber) is much reduced. *a* acrosome vesicle, *af* axial fiber, *arrowhead* Sertoli cell microtubules, *dlr* distal lamellar ring, *m* mitochondria, *mf* marginal filament (juxta-axonemal fiber), *mt* microtubules, *n* nucleus, *nf* nuclear fossa, *p* perforatorium, *pgr* proximal granular ring

is taken up into her cloaca and sperm migrate to spermathecae in the roof of the cloaca. The spermatophore of *L. italicus* is characterized by a cap and a short base or pedicel (Fig. 5h). A membranelike structure involving the spermatophore is absent. The central area of the cap consists of free cytoplasmic droplets and fibrous material. Sperms are neither in groups nor aligned, but randomly distributed throughout the periphery of the cap. However, they are less abundant in the center of the cap. Sperms do not undulate at the periphery of the cap on freshly deposited spermatophores, but they are inactive and immotile.

Discussion

Reproductive system

The male reproductive system of *L. italicus* appears to be dissimilar to that of anurans (Bhaduri 1953; Bhaduri and Basu 1957) and urodeles exemplified by *Salamandra* (Lofts 1974; Bergmann 1994) in which the testes are connected to the Wolffian duct by the efferent ductules only via the upper part of the kidney.

The testis of *L. italicus* is externally monolobed and is formed by characteristic internal lobules, here termed loculi, in which mature spermatozoa form half-moon-shaped bundles (spermatocysts). The presence of internal lobules and sperm half-moon shaped bundles is similar to those described by numerous authors for other urodeles (e.g. Pudney et al. 1983; Schindelmeiser et al. 1985; Bergmann 1994; Uribe et al. 1994; Uribe 2003).

As observed in other amphibians (Lofts 1974; Bergmann et al. 1983; Uribe 2003), in the testis of *L. italicus* each Sertoli cell is in contact with a single germ cell throughout spermiogenesis. The synchronous development of a cyst suggests possible interactions between germinal and Sertoli cells: the latter may provide structural and functional support. In particular, microtubules of Sertoli cells seem to

supply structural support for germ cell differentiation (Reed and Stanley 1972; Rastogi et al. 1988; Bào et al. 1991). In addition, Sertoli cells control the position of sperm cells and synchrony of spermatogenetic events during spermiogenesis (Flickinger and Fawcett 1967; Vogl and Soucy 1985).

Few data exist on cell types in urodeles Wolffian ducts: most accounts describe only a mono-layer cubical to prismatic (Lofts 1974; Bergmann et al. 1983), but Uribe (2003) recognized columnar, cuboidal and spherical or ovoid cells.

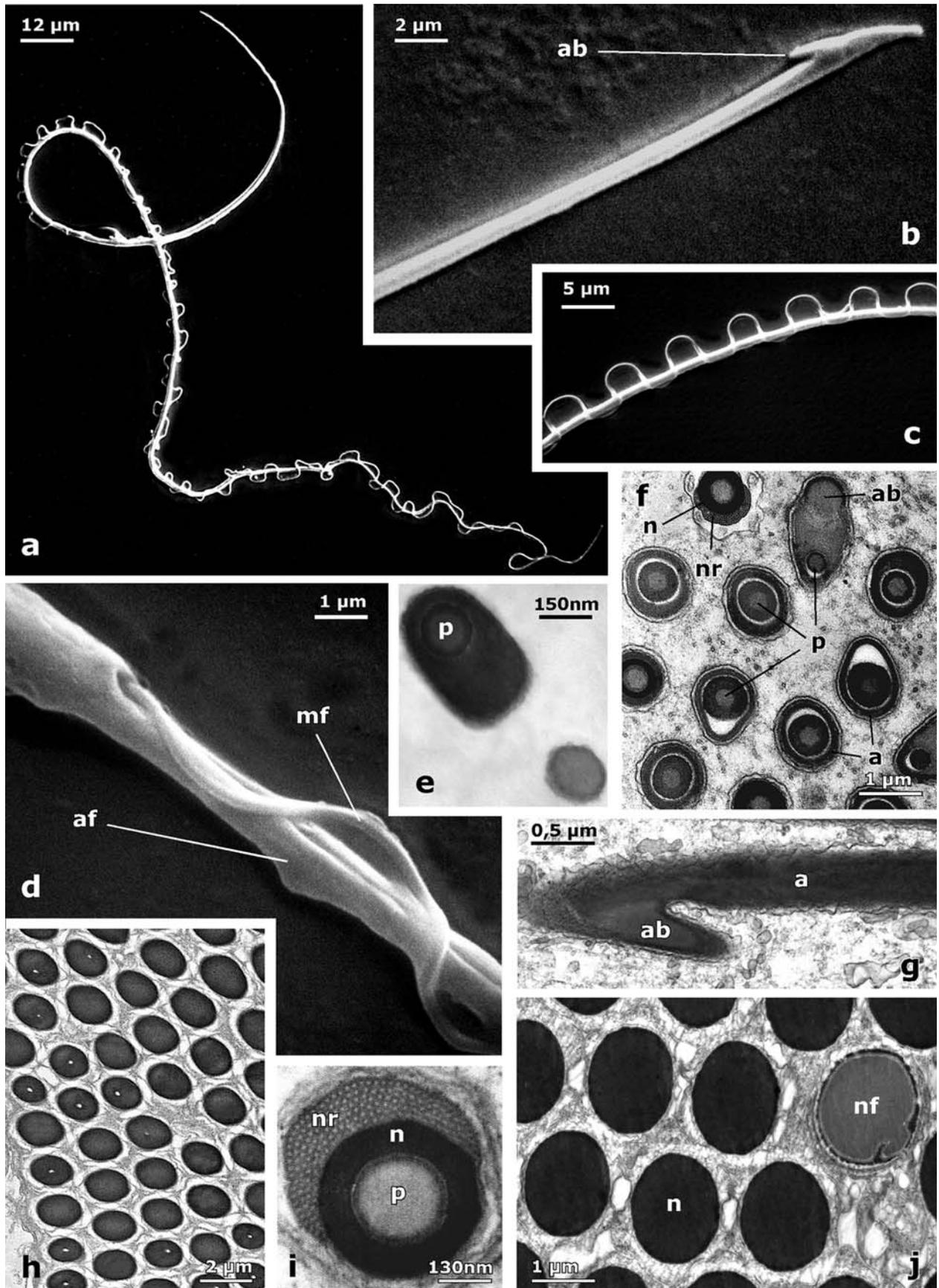
In *L. italicus* our observations allowed us to assert for the first time that the Wolffian duct has a pseudostratified structure with ciliated and non-ciliated secretory cells. The Wolffian duct is not only involved in sperm storage, conservation and maturation, but also in fertilization biology: the secretory activity of non-ciliated cells may provide favorable conditions for sperm storage until breeding and facilitate the transport of spermatozoa. In mammals these cells produce in particular glycoproteins and carbohydrates (Hermo et al. 1991, 1994; Jones 1999). The ciliated cells are probably involved in sperm transport from the testes to the cloaca; according to our observations, in this stage, spermatozoa are non-motile. Similar ciliated cells were described in birds (Baldan et al. 2003) and mammals (Kennedy and Heidger 1979) where sperm immotility in the vas deferens is presumed. Also muscle contractions were possibly involved to the transport of sperm cell towards the proximal regions.

Spermatozoa are present in the Wolffian duct even after complete sperm evacuation from the testes. This suggests that the Wolffian duct may function as a potential sperm supplier for later breeding sessions.

Spermiogenesis

Spermiogenesis of *L. italicus* is of the discontinuous type as observed for most other amphibians (Bào et al. 1991, 2001). It involves nuclear elongation, chromatin condensation, acrosomal formation, and flagellar development. The first two processes induce the conversion of a spherical nucleus with scattered chromatin into a cylindrical nucleus with greatly condensed chromatin (Myles and Hepler 1982). The way in which these events are carried out has been described in many animal taxa (e.g. Fawcett 1958; Bào et al. 1991; Soley 1997; Amaral et al. 1999): in accordance with these works, we observed in *L. italicus* that fine chromatin in the nucleus of the early spermatid is gradually replaced by larger granules to form the typical condensed chromatin observed in the mature sperm.

Unlike anuran sperm (Burgos and Fawcett 1956; Reed and Stanley 1972; Rastogi et al. 1988) but as observed in other Salamandridae (Barker and Biesel 1967), the



◀ **Fig. 4** SEM of mature spermatozoa. **a** Entire spermatozoon. The undulating membrane occupies much of its length. **b** Anterior region with subapical barb typical of salamandroids. **c** Detail of the undulating membrane. **d** Emergence of the axial fiber and the undulating membrane from the centriolar region. TEM of mature spermatozoa. **e** Transverse section of acrosomal barb and perforatorium. **f** Transverse sections of several spermatozoa in the region of the acrosome vesicle, nuclear rostrum and contained perforatorium with, top right, a section through the barb. **g** longitudinal section of the acrosome complex. **h** Transverse sections of many nuclei in or shortly behind the perforatorium region. **i** Transverse section of the nucleus, contained perforatorium and microtubules of the external nuclear ridge. **j** Transverse sections of strongly condensed nuclei and, on the right, a section through the connecting piece within the nuclear fossa. *a* acrosome vesicle, *ab* acrosomal barb, *af* axial fiber, *mf* marginal filament, *n* nucleus, *nf* nuclear fossa, *nr* nuclear ridge, *p* perforatorium

acrosome is developed even in the early spermatid: in this phase it is roundish and is constituted of moderately electron-dense material. However, an acrosome vesicle is illustrated for the isodiametric spermatid of the anuran *Discoglossus* by Sandoz (1970).

Soon the perforatorium associates with acrosome. A perforatorium in spermatids has been observed in many amphibians (Burgos and Fawcett 1956; Baker 1962; Barker and Biesel 1967; Scheltinga and Jamieson 2003a, b, 2006). This structure is formed by actin filaments and plays an important role during the acrosomal reaction and egg penetration (Baccetti et al. 1980).

During spermiogenesis a dense ring of material forms around the distal centriole in early spermatids, as shown for *L. italicus*. Two distinct portion of the ring can be observed. It has been shown that these have distinct fates during late spermiogenesis, forming a granular proximal ring and a distal lamellar ring. It is the distal ring that elongates and forms the annulus (references in Scheltinga and Jamieson 2003a).

An electron-dense annulus in the form of an elongate ring is present in ambystomatid, amphiumid, plethodontid, rhyacotritonid, and salamandrid spermatozoon, beginning within the neck region and extending posteriorly along the axial fiber for the length of the midpiece.

The annulus is located along the margins of the longitudinal groove of the axial fiber in amphiumids, plethodontids, rhyacotritonids, and salamandrids. The elongate form of the annulus in salamanders is an apomorphic character as supported by presence of a plesiomorphically small circular annulus in hynobiid sperm (Scheltinga and Jamieson 2003a).

The flagellum also originates in the early spermatid, but it concludes its development only at the end of spermiogenesis when the undulating membrane is formed and the axoneme and axial rod are separated. During the early phases of development, the flagellum is accompanied by a mitochondrial multilayer.

Spermatozoon

In this paper, we provided for the first time the description of sperm ultrastructure of *L. italicus*. Data collected on sperm morphology confirm the description outlined for the urodele spermatozoa (Baker 1962, 1966; Barker and Biesel 1967; Picheral 1967, 1979; Barker and Baker 1970; Wortham et al. 1977, 1982; Selmi et al. 1999).

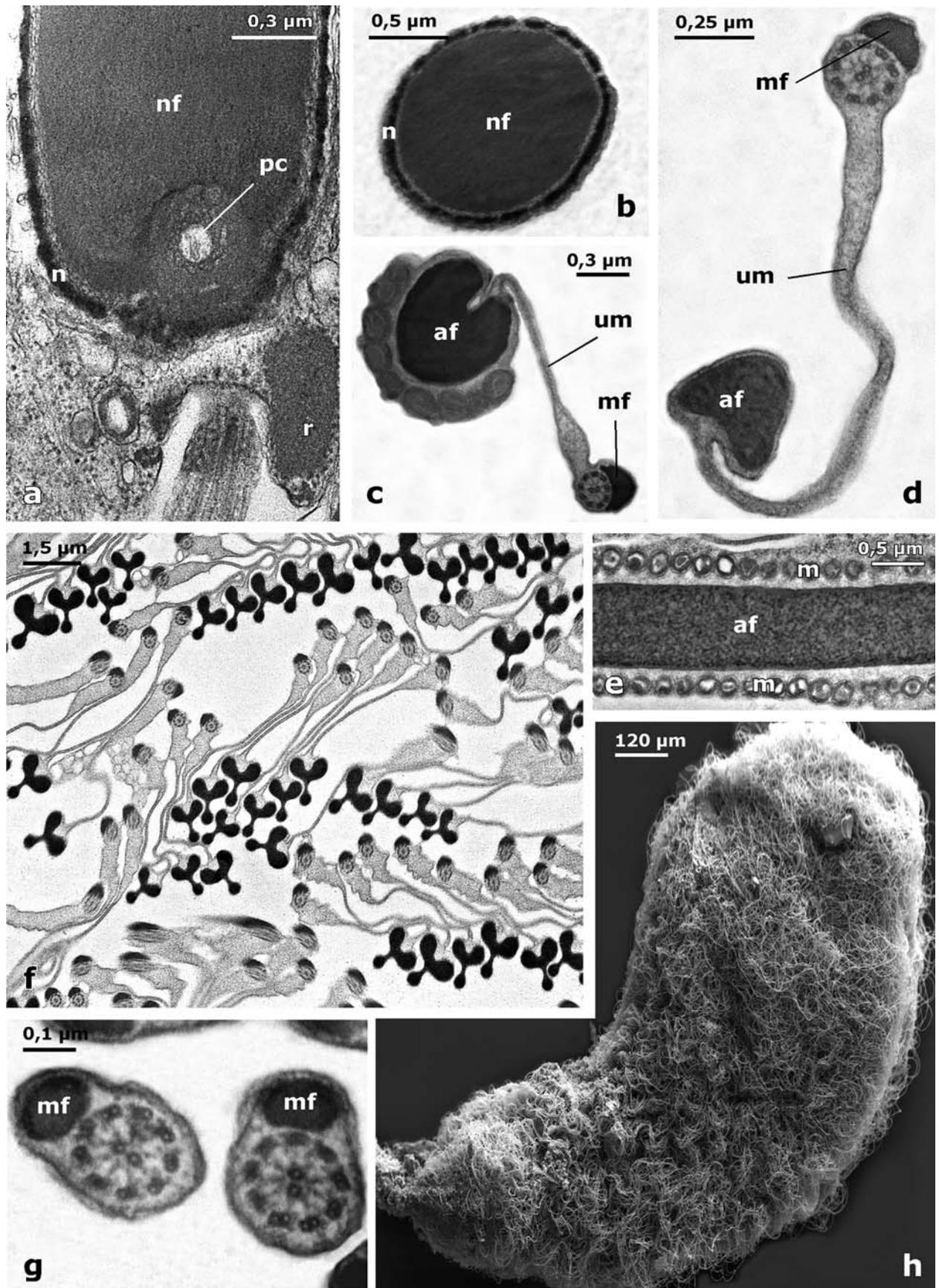
L. italicus differs from other studied newts in that the nuclear ridge in transverse section is semicircular, whereas in *T. carnifex*, *M. alpestris*, *L. vulgaris* (Selmi et al. 1997), *T. cristatus* (Nicander 1970) and *T. helveticus (palmatus)* (Picheral 1979) it is circular.

The general morphology of Urodela spermatozoa, with the exception of those of Sirenidae, is uniform relative to the great diversity seen in Anura and, to a lesser extent, in caecilians. Urodele sperms are elongate and composed of a distinct head (acrosome and nucleus) and a tail with axoneme, undulating membrane, and axial fiber. Those of the Sirenidae (excluded from the Urodela sensu stricto) are distinctive in having two axonemes and undulating membranes running the length of the spermatozoon (references in Scheltinga and Jamieson 2003a).

Of the six species a time belonging to the *Triturus* genus and for which sperm length has been recorded, that of *L. italicus* is closest to *M. Alpestris* and *L. vulgaris*. Recorded lengths, in μm , are: *M. alpestris*, 305–370 (Selmi et al. 1997); *T. carnifex*, 190 (Furieri 1962) or 250–300 (Selmi et al. 1997); *T. cristatus*, ~600 (Falakali et al. 1994); *T. helveticus*, 650 (Picheral 1979); *L. vulgaris*, ~600 (Falakali et al. 1994) or 350–400 (Selmi et al. 1997), and *L. italicus*, 360.8 ± 6.1 (present study). No length data are available for the seventh species, *T. marmoratus*, examined by Fraile et al. (1992). A discussion on some interesting features of the sperm of *L. italicus* follows.

The general structure of the acrosomal complex in *L. italicus* is similar as observed in other urodeles. A barb is absent from the sperm of anurans and caecilians. It is present in salamandroids: ambystomatids, plethodontids, rhyacotritonids, and salamandrids (with the exception of *Taricha granulosa*) and typically consists of a subapically postero-lateral process, as in *L. italicus*. A definite barb is plesiomorphically absent in urodeles, as it does not occur in amphiumids, cryptobranchids and hynobiids, or dicamptodontids. The functional significance of the barb has remained unknown, but it is here suggested that it may function to anchor the sperm at the apex of the testicular sperm cysts or possibly in the cap of the spermatophore, though a role in fertilization cannot be excluded.

A barb of the type seen in *L. italicus* was illustrated by Retzius (1906) for *M. alpestris*, and *T. cristatus* and has been shown to be present in *T. carnifex* by Selmi et al. (1997), using SEM. Recorded lengths, respectively, for the



◀ **Fig. 5** Sections of mature and almost mature spermatozoa. **a** Longitudinal section of the neck region of an almost mature spermatozoon, showing the connecting piece in the nuclear fossa, proximal centriole, annulus and anterior flagellum. **b** Transverse section of the nuclear fossa. The nucleus forms a sheath around the connecting piece. **c** Transverse section of the flagellum. The axial fiber is bordered by mitochondria and connects via the undulating membrane with the axoneme and its marginal filament (juxta-axonemal fiber). **d** Same but posterior to the mitochondrial region. **e** Longitudinal section of the axial fiber and fringing mitochondria. **f** Transverse sections of sperm flagella behind the mitochondrial region, with trifoliate axial fibers. **g** Transverse sections of posterior regions of two flagella. The axial fiber is absent and the marginal filament is reduced, in the absence of an undulating membrane. **h** SEM of the ‘cap’ of a spermatophore with a small portion of the stalk seen at bottom right. *af* axial fiber, *m* mitochondria, *mf* marginal filament, *n* nucleus, *nf* nuclear fossa, *pc* proximal centriole, *r* ring or annulus, *um* undulating membrane

acrosome vesicle and barb of some Salamandridae, in μm , are *M. alpestris*, 10.5, 2.5; *T. carnifex*, 4.6, 2; *L. vulgaris*, 11.5, 2.5 (all Selmi et al. 1997) and *L. italicus*, 10.0, 2.3 (present study).

In *L. italicus* as in other salamanders, the mature sperm nucleus is a very long cylinder narrowing anteriorly. It is circular in transverse section, composed of strongly condensed chromatin. Enclosed within the nuclear membrane, though a non-chromatin structure, is the elongate ‘nuclear ridge’. It is composed of closely adpressed and regularly arranged microtubules orientated parallel to the long axis of the nucleus. The ridge begins at the level of the nuclear shoulders and extends posteriorly along the nucleus. Variation in the circumferential and longitudinal extent of the nuclear ridge or ridges (in hynobiids five separate ridges) is discussed in detail by Scheltinga and Jamieson (2003a). In *L. italicus* the ridge occupies only the anterior fourth.

The presence of a nuclear ridge, though unknown in cryptobranchids, appears to be an autapomorphy of the Urodela as suggested by Selmi et al. (1997) and Jamieson (1999) as it is not seen in the spermatozoa of any other. The role of nuclear ridge is not clear (Scheltinga and Jamieson 2003a).

In *L. italicus*, as in most urodeles, the tail is composed of three regions: midpiece, principal piece, and endpiece. Either midpiece or principal piece may be absent in some salamanders (see Scheltinga and Jamieson 2003a). As in *L. italicus*, the midpiece occurs along the anterior portion of the tail and contains the marginal (juxta-axonemal) fiber associated with doublet 8, axoneme, undulating membrane, axial fiber, and mitochondria. The principal piece occurs posterior to the midpiece, containing the same structures with the exception of mitochondria. A relatively short endpiece consisting of only the juxta-axonemal fiber and axoneme occurs at the free end of the tail. The structure of the spermatozoon tail is highly distinctive of amphibians, the unilateral location of the undulating membrane and its axial fiber being synapomorphic conditions for the Lissamphibia relative to the bilateral condition in dipnoan fish (Jamieson

et al. 1993; Jamieson 1995, 1999; Scheltinga and Jamieson 2003a).

The axial fiber in *L. italicus*, as in other urodele sperm lies on the same side of the axoneme as doublets 2, 3, and 4. It extends from the base of the connecting piece or nuclear fossa to the beginning of the endpiece, progressively changing in shape, nature, and size posteriorly. It is absent from all but the most anterior portion of the endpiece.

Running the length of the axoneme, and closely associated with doublets 7, 8, and 9, in *L. italicus* as in other salamanders, is a longitudinal fiber, the juxta-axonemal fiber at 8. Posteriorly, the juxta-axonemal fiber decreases in size; however, it is often enlarged again within the endpiece, though not in *L. italicus*.

For most of the length of the *L. italicus* sperm tail a well developed undulating membrane (cytoplasmic sheath) occurs between the axoneme (near doublets 2, 3, and 4) and the axial fiber. The lateral extent (distance between axoneme and axial fiber) varies between 0.25 and 4.2 μm . The maximum recorded width (lateral extent) of the undulating membrane of *Triturus* spermatozoa is as follows: *M. alpestris*, *T. carnifex*, and *L. vulgaris*, 3.0 μm (Selmi et al. 1997); *L. italicus* 1.9 μm (present study). The posterior end of the undulating membrane defines the beginning of the endpiece (Scheltinga and Jamieson 2003a).

In conclusion, spermatozoa of *L. italicus* shows two autapomorphic characters for the Urodela: (1) the presence of the nuclear ridge (unknown in cryptobranchids) and; (2) the unilateral modification of the anterior acrosome vesicle; e.g., as a barb, as well as having several internal unique characters.

The spermatozoa of salamandrids including *L. italicus* and other Salamandroidea (ambystomatids, amphiumids, dicamptodontids, plethodontids, proteids, rhyacotritonids) possesses several apomorphic characters for Salamandroidea, including: elongation of the connecting piece, an apically or subapically modified (hooked, knobbed, or barbed) acrosome vesicle (unknown in proteids), trifoliate axial fiber within the principal piece (absent in rhyacotritonids), elongate annulus (secondarily lost in dicamptodontids and unknown in proteids), elongate midpiece (secondarily lost in dicamptodontids), and the gentle merging of the axial fiber/principal piece into the endpiece.

Spermatophore

Among urodele spermatophores, three different microstructures are known: (1) in Ambistomatidae the central area of the cap is surrounded by a membranelike structure, distorted near the top of the stalk; sperm are concentrated in groups at the cap surface with heads directed outwards and tail inward (Russel et al. 1981); (2) in *Notophthalmus viridescens*, *L. vulgaris* and *Cynops pyrrhogaster* the membranelike

structure is rounded; in *N. viridescens* sperm are concentrated on the periphery in coiled groups with heads and tails in register, and heads directed inward (Zalisko et al. 1984); in *L. vulgaris* and *C. pyrrhogaster* sperm are randomly distributed throughout the periphery of the cap and they are less abundant in the center of the cap (Zalisko et al. 1984); (3) in *Pleurodeles waltl* the membranelike structure is absent and spermatozoa are distributed randomly throughout the periphery of the cap (Zalisko et al. 1984). The spermatophore of *L. italicus* has a simple structure in which sperm are randomly distributed throughout the periphery of the cap. Probably, as suggested for *L. vulgaris* (Zalisko et al. 1984), a proteinaceous matrix, formed by mucoproteins and glycoproteins, holds together spermatozoa.

The structure of the spermatophore of *L. italicus* is similar to that described for *P. waltl* for the absence of the membranelike structure and to that described for *P. waltl*, *L. vulgaris*, and *C. pyrrhogaster* for the random distribution of sperm throughout the periphery of the cap. It differs greatly from the spermatophores of *Ambystoma texanum*, *A. tigrinum*, and *N. viridescens*.

Taxonomic variations in sperm distribution in the spermatophore cap suggests that each arrangement enhances efficiency of sperm transfer to the female and is related to courtship behavior, to differences in site of spermatophore deposition, and to amount of the cap retained in the female's cloaca during courtship. In *L. italicus*, as in other salamandrids and ambystomatids that court under water, females squat over the spermatophore, inserting the cap into the cloaca, rise and move away, usually leaving the base and a part of the cap attached to the substratum. The random distribution of sperm throughout the periphery of the cap enhances a rapid removal of sufficient number of spermatozoa. After spermatophore is taken up into the cloaca of the female, sperm become motile and migrate to spermathecae in the roof of the cloaca.

Acknowledgments This work was supported by Aspromonte National Park (Calabria, Italy), and carried out with the approval of the "Ministero dell'Ambiente e della Tutela del Territorio (Direzione per la protezione della Natura)" permit number 2004/30911. We thank Enrico Perrotta for technical assistance.

References

- Amaral MJL, Fernandes AP, Bào SN, Recco-Pimental SM (1999) Ultrastructural study of the spermiogenesis in three species of *Physalaemus* (Anura, Leptodactylidae). *Biocell* 23:211–221
- Baccetti B, Bigliardi E, Burini AG (1980) The morphogenesis of vertebrate perforatorium. *J Ultrastruct Res* 71:272–287. doi:10.1016/S0022-5320(80)90079-9
- Baker CL (1962) Spermatozoa of Amphiumae: spermateleosis, helical motility and reversibility. *J Tenn Acad Sci* 37:23–39
- Baker CL (1965) The male urogenital system of the *Salamandridae*. *J Tenn Acad Sci* 40:1–5
- Baker CL (1966) Spermatozoa and spermateleosis in the Salamandridae with electron microscopy of *Diemictylus*. *J Tenn Acad Sci* 41:2–25
- Baldan C, Orsi AM, Viotto MJ, Baraldi-Artoni SM, Stefanini MA (2003) Morphology of the vas deferens of the budgerigar (*Melospittacus undulatus*). *J Submicrosc Cytol Pathol* 35(1):77–82
- Bào SN, Dalton GC, De Oliveira SF (1991) Spermiogenesis in *Odonotophrynus cultripes* (Amphibia, Anura, Leptodactylidae): ultrastructural and cytochemical studies of proteins using E-PTA. *J Morphol* 207:303–314. doi:10.1002/jmor.1052070307
- Bào SN, Calazans Vieira GH, De Paiva Fernandes A (2001) Spermiogenesis in *Melanophryniscus cambaraensis* (Amphibia, Anura, Bufonidae): ultrastructural and cytochemical studies of carbohydrates using lectins. *Cytobios* 106(S2):203–216
- Barker KR, Baker CL (1970) Urodele spermateleosis: a comparative electron microscope study. In: Baccetti B (ed) *Comparative spermatology*. Accademia Nazionale dei Lincei, Rome, pp 81–84
- Barker KR, Biesel JJ (1967) Spermateleosis of a salamander *Amphiuma tridactylum* Cuvier; a correlated light and electron microscope study. *Cellule* 67:91–118
- Bergmann M (1994) The morphology of the testis in *Salamandra salamandra* (L.). *Mertensiella* 4:75–82
- Bergmann M, Schindelmeiser J, Greven H (1983) The glandular tissue in the testis of *Salamandra salamandra* (L.) (Amphibia, Urodela). *Acta Zool* 64:123–130
- Bhaduri JL (1953) A study of the urogenital system of *Salientia*. *Proc Zool Soc Bengal* 6:1–111
- Bhaduri JL, Basu SL (1957) A study of the urogenital system of *Salientia*. Part I. Ranidae and Hyperoliidae of Africa. *Ann Mus R Congo Belg Sci Zool* 55:1–56
- Brizzi R, Calloni C, Delfino G (1992) Male cloacal region of *Triturus italicus* (Peracca) with references to the cloacal anatomy and reproductive patterns of the *Salamandridae*. *Archivio italiano di Anatomia ed Embriologia* 97:121–138
- Brunelli E, Perrotta I, Bonacci A, Tripepi S (2007) The balancers of *Triturus italicus*: an ultrastructural approach. *Ital J Zool (Modena)* 74(2):135–142. doi:10.1080/11250000701263497
- Burgos MH, Fawcett DM (1956) An electron microscope study of spermatid differentiation in the toad, *Bufo arenarum*, Hensel. *J Biophys Biochem Cytol* 2:223–240
- Falakali B, Alpagut N, Çevik E (1994) Spermatozoon morphology in two species groups of *Triturus* (Salamandridae): Urodela living around Izmir. *Turk J Zool* 18:245–249
- Fawcett DW (1958) The structure of the mammalian spermatozoon. *Int Rev Cytol* 7:195–234. doi:10.1016/S0074-7696(08)62688-1
- Flickinger C, Fawcett DW (1967) The junctional specializations of Sertoli cells in the seminiferous epithelium. *Anat Rec* 158(2):207–221. doi:10.1002/ar.1091580210
- Fraile B, Sáez FJ, Codesal J, Paniagua R (1992) Characterisation of secondary spermatocytes in the marbled newt (*Triturus marmoratus*). *J Anat* 180:81–88
- Furieri P (1962) Osservazioni sullo spermatozoo di *Triturus cristatus carnifex* (Laurenti), studio al microscopio elettronico. *Monit Zool Ital* 68:90–102
- Giacoma C, Picariello O, Puntillo D, Rossi F, Tripepi S (1988) The distribution and habitats of the newt (*Triturus*, Amphibia) in Calabria (Southern Italy). *Monit Zool Ital* 22(NS):449–464
- Hermo L, Wright J, Oko R, Morales CR (1991) Role of epithelial cells of the male excurrent duct system of the rat in the endocytosis or secretion of sulfated glycoprotein-2 (clusterin). *Biol Reprod* 44:1113–1131. doi:10.1095/biolreprod44.6.1113
- Hermo L, Oko R, Morales CR (1994) Secretion and endocytosis in the male reproductive tract: a role in sperm maturation. *Int Rev Cytol* 154:105–189. doi:10.1016/S0074-7696(08)62199-3
- Jamieson BGM (1995) Evolution of tetrapod spermatozoa with particular reference to amniotes. In: Jamieson BGM, Ausio J, Justine JL (eds) *Mémoires du Muséum national d'Histoire naturelle*,

- vol 166, Advances in Spermatozoal Phylogeny and Taxonomy Paris, pp 343–358
- Jamieson BGM (1999) Spermatozoal phylogeny of the Vertebrata. In: Gagnon C (ed) The male gamete: from basic science to clinical applications. Cache River Press, Vienna, pp 303–331
- Jamieson BGM, Lee MSY, Long K (1993) Ultrastructure of the spermatozoon of the internally fertilizing frog *Ascaphus truei* (Ascaphidae: Anura: Amphibia) with phylogenetic considerations. *Herpetologica* 49:52–65
- Jones RC (1999) To store or to mature spermatozoa? The primary role of the epididymis. *Int J Androl* 22:57–67. doi:10.1046/j.1365-2605.1999.00151.x
- Kennedy SW, Heidger PM Jr (1979) Fine structural studies of the rat vas deferens. *Anat Rec* 194:159–180. doi:10.1002/ar.1091940111
- Lee MSY, Jamieson BGM (1993) The ultrastructure of the spermatozoa of bufonid and hylid frogs (*Anura, Amphibia*): implications for phylogeny and fertilization biology. *Zool Scr* 22(3):309–323. doi:10.1111/j.1463-6409.1993.tb00361.x
- Lofts B (1974) Reproduction. In: Lofts B (ed) Physiology of the Amphibia, vol II. Academic Press, New York, pp 107–218
- Longo G, Trovato M (2008) Ultrastructure of seminal receptacle and sperm storage in *Armadillidium granulatum* Brandt (Isopoda: Oniscidea). *Ital J Zool (Modena)* 75(2):113–123. doi:10.1080/11250000701866075
- Myles DG, Hepler PK (1982) Shaping of the sperm nucleus in Marsilea: a distinction between factors responsible for shape generation and shape determination. *Dev Biol* 90:238–252. doi:10.1016/0012-1606(82)90373-6
- Nicander L (1970) Comparative studies on the fine structure of vertebrate spermatozoa. In: Baccetti B (ed) Comparative spermatology. Accademia Nazionale dei Lincei, Rome, pp 47–55
- Picheral B (1967) Structure et organisation du spermatozoïde de *Pleurodeles waltlii* Michah (Amphibien, Urodele). *Arch Biol (Liege)* 78:193–221
- Picheral B (1979) Structural, comparative, and functional aspects of spermatozoa in urodeles. In: Fawcett DW, Bedford JM (eds) The Spermatozoon. Urban and Schwarzenberg, Baltimore, pp 267–287
- Pudney J, Canick JA, Mak P, Callard GV (1983) The differentiation of Leydig cells, steroidogenesis, and the spermatogenic wave in the testis of *Necturus maculosus*. *Gen Comp Endocrinol* 50:43–66. doi:10.1016/0016-6480(83)90241-1
- Purschke G, Jördens J (2007) Male genital organs in the eulittoral meiofaunal polychaete *Stygocapitella subterranea* (Annelida, Parergodrilidae): ultrastructure, functional and phylogenetic significance. *Zoomorphology* 126(4):283–297. doi:10.1007/s00435-007-0047-z
- Rastogi RK, Bagnara JT, Iela L, Krasovich MA (1988) Reproduction in the Mexican leaf frog. *Pachymedusa dacnicolor*. IV. Spermatogenesis: a light and ultrasonic study. *J Morphol* 197:277–302. doi:10.1002/jmor.1051970304
- Reed SC, Stanley HP (1972) Fine structure of spermatogenesis in the South African clawed toad *Xenopus laevis*. Daudin. *J Ultrastruct Res* 41:277–295. doi:10.1016/S0022-5320(72)90070-6
- Retzius G (1906) Die Spermien der Amphibien. *Biologische Untersuchungen. Neue Folge* 13:49–70
- Russel LD, Brandon RA, Zalisko EJ, Martan J (1981) Spermatophore of the salamander *Ambystoma texanum*. *Tissue Cell* 18:609–621. doi:10.1016/0040-8166(81)90031-8
- Sandoz D (1970) Etude cytochimique des polysaccharides au cours de la spermatogenese d'un amphibien anoure: le discoglosse *Discoglossus pictus* (Oth.). *J Microsc (Paris)* 9:243–262
- Scheltinga DM, Jamieson BGM (2003a) The mature spermatozoon. In: Sever DM (ed) Reproductive biology and phylogeny of Urodela. Science Publishers, Enfield, pp 203–274
- Scheltinga DM, Jamieson BGM (2003b) Spermatogenesis and the mature spermatozoon: form, function and phylogenetic implications. In: Jamieson BGM (ed) Reproductive biology and phylogeny of Anura. Science Publishers, Enfield, pp 119–251
- Scheltinga DM, Jamieson BGM (2006) Ultrastructure and phylogeny of caecilian spermatozoa. In: Exbrayat JM (ed) Reproductive biology and phylogeny of Gymnophiona. Caecilians Science Publishers, Enfield, pp 247–274
- Scillitani G, Carafa M, Scalera R, Tripepi S (2004) Conservation and biology of *Triturus italicus* in Italy (*Amphibia, Salamandridae*). *Ital J Zool (Modena)* 71(Suppl. 1):45–54
- Selmi MG, Brizzi R, Bigliardi E (1997) Sperm morphology of salamandrids (*Amphibia, Urodela*): implications for phylogeny and fertilization biology. *Tissue Cell* 29(6):651–664. doi:10.1016/S0040-8166(97)80041-9
- Selmi MG, Brizzi R, Bigliardi E (1999) The contribution of spermatology on phylogeny of Plethodontids (*Amphibia, Urodela*). *Rivista di Idrobiologia* 38(1/2/3):1–7
- Soley JT (1997) Nuclear morphogenesis and the role of the manchette during spermiogenesis in the ostrich (*Struthio camelus*). *J Anat* 190:563–576. doi:10.1046/j.1469-7580.1997.19040563.x
- Sperone E, Tripepi S (2005) Ecological preferences of the Italian newt *Triturus italicus* (Peracca, 1898) in Calabria. *Russ J Herpetol* 12(suppl.):269–271
- Tavares-Bastos L, Colli GR, Bão SN (2008) The evolution of sperm ultrastructure among Boidae (Serpentes). *Zoomorphology* 127(3):189–202. doi:10.1007/s00435-008-0062-8
- Tourmente M, Giojalas L, Chiaraviglio M (2008) Sperm ultrastructure of *Bothrops alternatus* and *Bothrops diporus* (Viperidae, Serpentes), and its possible relation to the reproductive features of the species. *Zoomorphology* 127(4):241–248. doi:10.1007/s00435-008-0067-3
- Tripepi S, Rossi F, Peluso G (1998) Embryonic development of the newt *Triturus italicus* in relation to temperature. *Amphib-reptil* 19:345–355. doi:10.1163/156853898X00016
- Uribe MCA (2003) The testes, spermatogenesis and male reproductive ducts. In: Sever DM (ed) Reproductive biology and phylogeny of Urodela. Science Publishers, Enfield, pp 183–202
- Uribe MCA, Gómez Rios G, Brandon RA (1994) Spermatogenesis in the urodele *Ambystoma dumerilii*. *J Morphol* 222:287–299. doi:10.1002/jmor.1052220306
- Vogl AW, Soucy LJ (1985) Arrangement and possible function of actin filament bundles in ectoplasmic specializations of ground squirrel sertoli cells. *J Cell Biol* 100:814–825. doi:10.1083/jcb.100.3.814
- Wake MH (1968) Evolutionary morphology of the caecilian urogenital system, Part I. The gonads and fat bodies. *J Morphol* 126:291–332. doi:10.1002/jmor.1051260303
- Wake MH (1970) Evolutionary morphology of the caecilian urogenital system, Part II. The kidneys and urogenital ducts. *Acta Anat (Basel)* 75:321–358. doi:10.1159/000143456
- Williams AA, Brandon RA, Martan J (1984) Male genital ducts in the salamanders *Eurycea lucifuga* and *Eurycea longicauda*. *Herpetologica* 40:322–330
- Wortham JWE Jr, Brandon RA, Martan J (1977) Comparative morphology of some plethodontid salamander spermatozoa. *Copeia* 1977:666–680. doi:10.2307/1443165
- Wortham JWE Jr, Murphy JA, Martan J, Brandon RA (1982) Scanning electron microscopy of some salamander spermatozoa. *Copeia* 1982:52–60. doi:10.2307/1444267
- Schindelmeiser J, Bergmann M, Greven H (1985) Cellular differentiation in the urodele testis. In: Duncker HR, Fleischer G (eds) Functional morphology in vertebrates. Proceedings of the 1st international symposium on vertebrate morphology. Gustav Fischer Verlag, Stuttgart, pp 445–447
- Zalisko EJ, Brandon RA, Martan J (1984) Microstructure and histochemistry of Salamander spermatophores (*Ambystomatidae, Salamandridae* and *Plethodontidae*). *Copeia* 3:739–747. doi:10.2307/1445158