Ultrastructure of spermatogenesis and mature spermatozoa in the flatworm Prosthiostomum siphunculus (Polycladida, Cotylea)

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Abstract
This is the first study investigating spermatogenesis and spermatozoan ultrastructure in the polyclad flatworm Prosthiostomum siphunculus. The testes are numerous and scattered as follicles ventrally between the digestive ramifications. Each follicle contains the different stages of sperm differentiation. Spermatocytes and spermatids derive from a spermatogonium and the spermatids remain connected by intercellular bridges. Chromatoid bodies are present in the cytoplasm of spermatogonia up to spermatids. During early spermiogenesis, a differentiation zone appears in the distal part of spermatids. A ring of microtubules extends along the entire sperm shaft just beneath the cell membrane. An intercentriolar body is present and gives rise to two axonemes, each with a 9 + "1" micro-tubular pattern. Development of the spermatid leads to cell elongation and formation of a filiform, mature spermatozoon with two free flagella and with cortical microtubules along the sperm shaft. The flagella exit the sperm shaft at different levels, a finding common for acotyleans, but so far unique for cotylean polyclads. The Golgi complex produces numerous electron-dense bodies of two types and of different sizes. These bodies are located around a perinuclear row of mitochondria. The elongated nucleus extends almost along the entire sperm body. The nucleus is wide in the proximal part and becomes narrow going towards the distal end. Thread-like chromatin mixed with electron-dense intranuclear spindle-shaped bodies are present throughout nucleus. The general sperm ultrastructure, the presence of intranuclear bodies and a second type of cytoplasmic electron-dense bodies may provide characters useful for phylogenetic analysis.

Keywords: apomorphies; chromatoid bodies; polyclads; spermatogenesis; spermiogenesis

Introduction
The ultrastructural characters of the spermatozoon are known to be useful for interpretation of relationships among Platyhelminthes and have been widely used as criteria for analysis of phylogeny and evolution. Therefore, many studies dealing with spermatogenesis of flatworms have been carried out trying to establish phylogenetic relationships (Euzet et al., 1981; Ehlers, 1985; Hendelberg, 1986; Rohde 1990; Justine, 1991a,b, 1998, 2001; Bä and Marchand 1995; Hoberg et al., 1997).

Traditionally, a distinction between an archoophoran and a neoophoran type of egg in flatworms was made (Hyman, 1951). Despite a large number of studies on male gametogenesis in free-living flatworms (e.g. Hendelberg, 1969, 1974, 1983; Watson and Rohde, 1993; Bä and Marchand, 1995; Watson and Rohde, 1995; Culioli et al., 2006), these were generally limited to investigations of the mature spermatozoon. A smaller number of studies dealing with spermatogenesis have been realised (e.g. Franquinet and Lender, 1973; Rohde and Faubel, 1997; Charni et al., 2010; Liana and Litvaitis, 2010a; Harrath et al., 2012).

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278
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Animals were pre-
Light and TEM microscopy
Adult specimens of
within the Platyhelminthes, making the "Neophora"
polyphyletic (Egger et al., 2015; Laumer et al., 2015),
whereas the "Archoophora" have long been recognised to be
paraphyletic (Ehlers, 1985).
Until now there are about 800 polyclad species described
worldwide (Martin-Duran and Egger, 2012) but investiga-
tions dealing with male gametogenesis are limited to 12
species (8 acotyleans: Imagine zebra, Cryptocelides loveni,
Pleiplana atomata, Idioplana atlantica, Armatothecata
leptalea, Styloplanocera fasciata, Melloplana ferruginea and
Pleiplana japonica; 4 cotyleans: Pseudoceros bicolor,
Phrikoceros mopsus, Enchiridium elvelinae and Boninia
divae; for a review see Thomas, 1970; Hendelberg, 1977;
Liana and Litvaitis, 2007, 2010a, 2010b). Thus, our
knowledge of polyclad spermatogenesis and spermiogenesis
is still quite limited. To increase the number of species studied, we have realised this study in Prosthiostomum
siphunculus. This species (Polycladida: Cotylea: Prosthiost-
oma) is very frequent in the Mediterranean basin and
may even be cosmopolitan (Lang, 1884; Kato, 1937; Riedl,
1959; Gammoudi and Tekaya, 2012, Gammoudi et al., 2012;
Noreña et al., 2014). Reproduction and development of
P. siphunculus were investigated (Lang, 1884; Gammoudi
et al., 2012) and it has recently been used for stem cell and
phylogenic studies (Egger et al., 2009, 2015). However,
nothing is known about the ultrastructural aspects of its
spermatogenesis and sperm structure. The present study
describes the different stages of spermatogenesis and
spermiogenesis in the testes of P. siphunculus, thus providing
additional new data on spermatology of polyclads.

Materials and methods

Sampling

Adult specimens of P. siphunculus were collected from Isola
Palmaria, Italy (44.0479, 9.840696) during spring 2005. For
this study, one specimen was used for sections.

Light and TEM microscopy

Animals were prefixed in 2.5% glutaraldehyde with 0.05%
OsO4 for 10 min on ice, fixed in 2.5% glutaraldehyde in
cacodylate buffer for 1 h at 4°C and post-fixed for 1 h in 1%
OsO4 at 4°C (for a detailed protocol, see Salvenmoser et al.,
2010). After dehydration in a graded ethanol series, the
specimens were embedded in Spurr's resin. Sections were cut
with a Leica ultracut UCT microtome. Semi-thin sections
were stained with methylene blue and AZUR II according
to Richardson et al. (1960) and examined with a Leica
DM5000B light microscope. Images were taken with a Leica
DFC camera using Leica Application Suite version 2.8.1.
Thin sections were contrasted with uranyl acetate and lead
citrate and examined with a Zeiss Libra 120 energy filter
transmission electron microscope. Images were made with the
iTEM software (Olympus) and a TRS 2048 high speed
digital camera (Tröndle, Germany).

Results

Testis morphology

Testes are paired and extend over the body length. They are
scattered in the ventral parenchyma (Figure 1A) forming
rounded follicles (ca. 50 μm in diameter). Each testis follicle
is separated from the surrounding parenchymal tissues by a
basal lamina having a thickness of about 0.4 μm (Figure 2B).
The testis follicles are filled with clusters of male germ cells at
various stages of development (Figures 1B and 2A). Somatic
cells are located at the periphery of the testis in intimate
contact with the basal lamina (Figure 2B). Their flattened
nuclei are lobated and are provided with a prominent
nucleolus (Figure 2B). Aggregates of chromatin are observed
in the nucleoplasm; in the periphery they form a dark con-
tinuous lining beneath the nuclear membrane (Figure 2B).
Maturing spermatozoa are generally located at the periphery of the testis (Figure 2C) and they gradually pass through the
vas deferens (Figures 1C and 2D) into the seminal vesicle.
The vasa deferentia are provided with a thick wall
(Figures 1C and 2D). The latter contains flattened cells
(Figure 2D). Mature spermatozoa were observed in the testis
and in the lumen of the vas deferens (Figures 1C and 2D).

Spermatogenesis

Spermatogonia are spherical or elongated cells (average
diameter is 10.9 μm; minimum measured diameter is
8.9 μm, maximum 13.0 μm, n = 6) which divide mitotically
(Figure 3A). Their nuclei are large (average diameter is
7.3 μm; min. 5.6 μm, max. 9.1 μm, n = 6) containing
granular chromatin with numerous concentrations of
heterochromatin scattered near the nuclear membrane
(Figures 2A and 3B). The latter is surrounded by a relatively
thin rim of cytoplasm where some mitochondria, chromato-
toid bodies and free ribosomes are observed. The nucleus of
primary spermatocytes is spherical in shape and has an
average diameter of 6.9 μm; min. 5.7 μm, max. 7.7 μm (n = 5; Figures 3C–3F). Primary spermatocytes are slightly larger than spermatogonia on average with a diameter of 11.5 μm; min. 11.0 μm, max. 11.9 μm (n = 5). The nucleoplasm is characterised by the presence of synaptonemal complexes indicating the meiotic prophase, which is divided into leptotene, zygotene and pachytene. In the leptotene, the clear distribution between eu- and heterochromatin as seen in a spermatogonium changes and small single spots of heterochromatin appear in the nucleus. Usually, no synaptonemal complexes can be seen in this stage (Figure 3C). In the zygotene, the formation of the synaptonemal complex starts (Figure 3D). In the pachytene, sister chromatids enclose the synaptonemal complexes (Figure 3E), which are connected to the nuclear membrane (Figures 3E and 3F). Each synaptonemal complex is composed of a pair of clear coarse filaments called lateral elements separated by an electron-dense space called central region (Figure 3F, inset). During this stage, the cytoplasm is filled with several mitochondria, chromatoid bodies (Figures 3E and 3F) and ribosomes, rough endoplasmic reticulum and some electron-dense bodies (Figure 3F). At the same time, Golgi complexes of various sizes can be seen in the perinuclear cytoplasm. Chromatoid bodies are observed near the Golgi apparatus (Figure 3F).

We have not unambiguously identified secondary spermatocytes in our sections. Spermatids are connected by intercellular bridges. These structures are delimited by a cell membrane associated with a ring of electron-dense material (Figure 4A). Early spermatids are polarised cells (Figure 4A) characterised by migration of a still spherically shaped nucleus towards the newly formed electron-dense zone of differentiation (Figure 4B). This electron-dense structure marks the distal end of the spermatid. It appears as a small protrusion of cytoplasm where the intercentriolar body (icb) develops (Figure 4C) to support the two flagella. In the spermatid nucleoplasm, the chromatin begins to condense. The
cytoplasm shows an increased number of mitochondria and small electron-dense bodies. Two types of electron-dense bodies are now found in the cytoplasm: a first type with homogeneous density and a second type having two different regions of electron opacities (Figure 4D). This stage represents the initial phase of sperm differentiation or spermiogenesis.

In more advanced stages, spermatids (both cytoplasmic and nuclear compartments) change their spherical shape to become increasingly elongated. The nucleoplasm is provided with condensed chromatin that starts becoming thread like (Figures 4E–4G). Electron-dense bodies and mitochondria become more numerous (Figure 4D). The Golgi complex is strongly developed.

In the following stage, a row of microtubules is located just beneath the cell membrane and extends along the entire sperm shaft. Mitochondria increase considerably in number and form a layer around the nucleus. The latter extends almost throughout the entire sperm shaft. The proximal part of the nucleus is relatively large. Going distally, the elongated nucleus tapers to a very thin extension (Figures 4H and 5C).

**Spermatozoon structure**

The mature spermatozoa of *P. siphunculus* are filiform, biflagellate and provided with elongated nuclei surrounded by an internal layer of mitochondria followed by an external layer of electron-dense bodies (Figure 5A). A row of cortical microtubules underlies the cell membrane (Figure 5A). Through the whole length of the nucleus, intranuclear electron-dense spindle-shaped bodies are present (Figures 5B and 5C).

We follow the proximal–distal terminology of Liana and Litvaitis (2010b), with the flagella exiting at the distal part. The proximal diameter of the spermatozoon is wide and oval (Figures 5B and 6H); the nucleus occupies almost the totality of the shaft. Going distally the nucleus tapers progressively to form a very thin portion near the distal end (Figures 5A and 6F). The same two types of electron-dense bodies as in spermatids are found in the cytoplasm (Figure 5A). Transversal sections along the spermatozoon show that the two flagella extend on opposed sides of the cell and they emerge slightly asymmetrically from the distal end (Figure 6N). The first axoneme exits the shaft.
immediately whereas the second axoneme extends for some small distance inside the shaft (Figure 5D), exiting further from the anterior end (Figure 5E). Each axoneme comprises nine peripheral sets of doublet microtubules arranged in a cylinder around one central unit. The latter contains an electron-dense core, a less dense intermediate zone and fine spokes between the cylinder and doublets (Figure 5F).

Based on the observation of a great number of longitudinal and transversal sections, a diagrammatic reconstruction of mature spermatozoa can be presented (Figure 6).
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Discussion

Spermatogenesis

Contrary to macrostomids, rhabdocoels and triclads where germ cells extend from the peripheral side to the central part of the testis and mature spermatozoa are located near the lumen (Culioli et al., 2006, Charni et al., 2010, Kuales et al., 2011), mature spermatozoa in polyclads are located in the peripheral part of the testis (Liana and Litvaitis, 2010a, present work).

The ultrastructural characters of spermatogenesis and spermatozoa of *P. siphunculus* are similar to those of other described polyclad representatives. Follicular testes are dispersed ventrally. Each follicle is enclosed by a basal lamina and provided with somatic cells and with germ cells at

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**Figure 4** Ultra-thin sections of spermiogenesis. (A) Polarised spermatid after migration of nucleus (n) to one side. Bridges (b) are connecting different spermatids; cb, chromatoid body. Scale bar = 2 μm. (B) Spermatids developing a differentiation zone (large arrows) near n. Scale bar = 2 μm. (C) Intercentriolar body (icb), belonging to the cell with the nucleus on the right. Scale bar = 2 μm. (D) Spermatid cells with cytoplasm elaborating two types of electron-dense bodies eb1 and eb2 produced probably by the well-developed Golgi complex (gc). Several mitochondria (m) are present. Scale bar = 1 μm. (E–G) Different stages of spermiogenesis showing the elongation of the nucleus (n) and cytoplasm. Scale bar for (E) = 2 μm, for (F) = 1 μm and for (G) = 1.5 μm. Inset: detail of n content showing the thread-like chromatin. (H) Sagittal section of mature spermatozoon showing elongated nucleus surrounded by a row of mitochondria (large arrows) and a layer of electron-dense bodies eb1 and eb2. A row of microtubules (arrowheads) is located just beneath the cell membrane and extends along the entire sperm shaft. Scale bar = 2.5 μm.
different stages of development from spermatogonia to spermatozoa. The spermatids are marked by their differentiation zones and intercentriolar bodies. Spermatozoa are filiform and biflagellate, exhibiting a $9 + 1$ axoneme pattern as in most members of the taxon Trepaxonemata (Ehlers, 1985, Justine 2001). A few exceptions were described in digeneans with a $9 + 0$ pattern (Justine and Mattei, 1983b).

**Spermiogenesis**

The beginning of spermiogenesis is marked by the formation of a differentiation zone situated at the periphery of each spermatid, which appears distal to the nucleus as a small protrusion of cytoplasm where the intercentriolar body develops to support the two flagella. This structure was described for flatworms with biflagellate spermatozoa, such as polyclads (Kubo-Irie and Ishikawa, 1983; Liana and Litvaitis, 2010a), prorhynchids (Watson and Rohde, 1993), many rhabdocoels (Culioli et al., 2006), proseriates (Sopott-Ehlers, 1990) and triclads (Franquinet and Lender, 1972; Ishida et al., 1991; Harrath et al., 2014) but is missing in groups with aflagellate spermatozoa, for example, the Macrostomorpha (Willems et al., 2009) and the Prolecithophora (Ehlers, 1988).

**Spermatozoon axoneme**

In acotyleans, one axoneme emerges first from the sperm shaft, and the second axoneme extends at some distance inside the shaft, exiting further from the anterior end. *Styloplanocera fasciata* is an exception within Acotylea because of the same level at which both axonemes exit the sperm shaft (Liana and Litvaitis, 2007). In our work, we found that spermatozoa of the cotylean *P. siphunculus* are provided with two axonemes. The first exits the shaft immediately whereas the second extends for some small distance inside the shaft, which is not the case in all described cotyleans in which both axonemes exit the sperm shaft at the same level at the very anterior end and remain free flagella.
thereafter (Liana and Litvatis, 2007, 2010b). It is interesting to note that a similar feature independently arose in members of cotylean and acotylean polyclads.

In a species of the proposed sister group of Polycladida, Prorhynchidae (named Amplimatricata, Egger et al., 2015), both axonemes exit the sperm shaft subterminally (Watson and Rohde, 1993). Symmetric axonemes may, thus, be a plesiomorphy for Amplimatricata.

**Electron-dense bodies in the spermatozoon cytoplasm**

The spermatozoa of *P. siphunculus* as well as those of most other studied polyclad species contain numerous electron-dense bodies of two different types and mitochondria located around the nucleus (Liana and Litvaitis, 2007, 2010a, 2010b). In *P. siphunculus*, the two different types of electron-dense bodies do not differ much by size but by opacity.
(Figures 4D and 5A). Hendelberg (1977) has shown that the sperm of the acotylean Cryptocelides loveni has two different types of electron-dense bodies, which are formed by the Golgi complex. The large type of electron-dense bodies is abundant in most of the cytoplasmic region and the small electron-dense bodies are located near the nucleus. Only in two polyclad species, Pleioplana atomata and Boninia divae, just a single type of electron-dense bodies in the spermatozoon cytoplasm was reported (Kubo-Irie and Ishikawa, 1983; Liana and Litvaitis, 2010b), similar to a proseriate study (Sopott-Ehlers, 1989). In triclad, the mitochondrion compartment is represented by the fusion of spermatic mitochondria to a unique giant mitochondrion (Silveira and Porter, 1964; Franquinet and Lender, 1972; Ehlers, 1985; Ishida and Teshirogi, 1988; Ishida et al., 1991).

On the other hand, large and/or small electron-dense bodies described in polyclads are lacking in triclad (Franquinet and Lender, 1973; Charni et al., 2010; Harrath et al., 2012) and neodermatans (Ehlers, 1985). In a macrostomorphan, three distinct types of electron-dense bodies in the cytoplasm of the spermatozoon have been reported (Willems et al., 2009), whereas in a prorhynchid there are two types of electron-dense bodies (Watson and Rohde, 1993).

The distribution of electron-dense bodies and mitochondria varies from one polyclad species to the other. Spermatozoa of Pseudoceros bicolor, Phrikoceros mopsus and Boninia divae are provided with electron-dense bodies and mitochondria dispersed in the anterior region (Liana and Litvaitis, 2007, 2010b). This correlates with phylogeny, as these three species belong to the same superfamily Pseudocerotidea Faubel, 1984. On the other hand, these structures are distributed throughout the sperms of E. evelinae and P. siphunculus (Liana and Litvaitis, 2007, this study). The two species belong to the same family Prosthiostomidae of the superfamly, Euryleptoida Faubel, 1984. The separation of pseudocerotids from prosthiostomids is supported by a molecular phylogeny using the large ribosomal subunit (not including Boninia; Rawlinson and Stella, 2012). Therefore, the repartition of mitochondria and electron-dense bodies seems to be a phylogenetically useful character at superfamly or family level.

### Electron-dense bodies in the spermatozoon nucleus

In contrast to acotyleans, in all studied cotylean representatives: P. bicolor, P. mopsus, E. evelinae (Liana and Litvaitis, 2007) and B. divae (Liana and Litvaitis, 2010b) and also in the single studied prorhynchid species, Prorhynchus sp. (Watson and Rohde, 1993), the nucleus extends almost throughout the entire sperm shaft. The same feature is maintained in P. siphunculus. Like in acotylean polyclads, the spermatozoon nucleus of the macrostomorphan M. lignano is rather short (Willems et al., 2009), while it extends almost throughout the entire length in a rhabdocoel (Culioli et al., 2006).

The nucleus content of spermatozoa of triclad is made up of electron-dense chromatins often with helical structure intermingled with another material with electron-light appearance, which was suggested to be residual proteins (Silveira and Porter, 1964; Harrath et al., 2012). In two representatives of the polyclad family Prosthiostomidae, E. evelinae and P. siphunculus, the spermatozoan nucleus contains spindle-shaped bodies (round in transversal sections) whose nature and function is still unknown (Liana and Litvaitis, 2010a). So far, such a structure was not found in other polyclads, in macrostomorphans or in prorhynchids; therefore, we propose the presence of these intranuclear spindle-shaped bodies may be a character of phylogenetic value at family level, but more members of the family should be studied.

In E. evelinae, the distribution of intranuclear electron-dense bodies is restricted to the anterior part, whereas in P. siphunculus, these structures are dispersed throughout the spermatozoon nucleus. The distribution of intranuclear bodies seems to be important at lower systematic levels (distinction between the genera Prosthiostomum and Enchiridium).

### Cortical ring of microtubules

A complete ring of microtubules occurs just beneath the cell membrane and extends along the entire sperm body (Figures 5A, 5D and 6B). The cortical microtubules in spermatozoa of Platyhelminthes are thought to support and protect the spermatozoon’s structure. Their number and disposition vary between taxa, making these structures phylogenetically useful (Justine, 1991a, 1991b, 2001). In fact, in triclad, the distinction between very closely related species could be made by the number and disposition of cortical microtubules. In the freshwater triclad Schmidtea mediterranea, the number of cortical microtubules increases from the anterior extremity of the spermatozoon to the middle region and then decreases to the tail (Harrath et al., 2012). The same feature has been observed in many other freshwater triclad such as Dugesia sicula (Charni et al., 2010). However, in neodermatans, the number decreases from anterior to posterior (Stitt and Fairweather, 1990, Quilichini et al., 2007). They can disappear entirely in some digeneans and monogeneans (Neodermata; Justine and Mattei, 1983a,b). In M. lignano, cortical microtubules are divided into two compartments and the number of cortical microtubules is largest in the middle of the spermatozoan and decreases towards both ends (Willems et al., 2009).

In polyclads, cortical microtubules were described to form a single row beneath the cell membrane (Kubo-Irie and...
and Litvaitis, 2010b). The exception was the cotylean Boninia divae in which the complete sheet of microtubules is doubled in the proximal end of the shaft (Liana and Litvaitis, 2010b). This may have a phylogenetic significance as this taxon was described to share morphological characters (e.g., presence of a Lang’s vesicle, incorporated sperm flagella) with acotyleans (Liana and Litvaitis, 2010b).

Chromatoid bodies
Chromatoid bodies are electron-dense cytoplasmic organelles without any membrane (Meikar et al., 2011). They can be found anywhere within the cytoplasm but often near the nuclear pores or near mitochondria. These components are also referred to as nuage (Yokota, 2012).

In most organisms, chromatoid bodies are germ-cell-specific cytoplasmic structures for RNA processing (Kotaja and Sassone-Corsi, 2007). In flatworms, chromatoid bodies have also been described in neoblast stem cells of triclads (Hori, 1997), polyclads (Sato et al., 2001), macrostomorphans (Ladurner et al., 2008) and ctenulids (Dirks et al., 2012). Here, we describe for the first time chromatoid bodies in male germ cells of a polyclad flatworm (Figures 3B and 3E). Different to mouse, where chromatoid bodies are only found in haploid stages (Kotaja and Sassone-Corsi, 2007), in P. siphunculus they can be observed also in spermatogonia (Figures 3B and 3C) and they persist until the spermatid stage (Figure 4A).

The role of boule in gametogenesis in flatworms
In an interesting study conducted with the macrostomorphian flatworm M. lignano, three boule-like genes were identified and functionally tested. Macbol1 and macbol2 are expressed in the testes, whereas the much shorter macbol3 is expressed in ovaries and developing eggs. Knock-down of macbol1 inhibits differentiation of spermatocytes, whereas knock-down of macbol3 leads to female sterility; knock-down of macbol2 showed no phenotype (Kuales et al., 2011). The involvement of a boule gene in oogenesis is rather unusual in metazoans (Kuales et al., 2011).

We have identified three putative boule genes in the transcriptome of P. siphunculus (Egger et al., 2015), the shortest of which recovers macbol3 as the best BLAST hit (Supplementary File 1), which hints at the possibility of a similar role of this gene in oogenesis as in M. lignano.

Conclusions
P. siphunculus (Polycladida, Cotylea) shares the typical 9+1′ axoneme pattern of spermatozoa with most representatives of the large flatworm taxon Trepaxonemata. During spermatogenesis, chromatoid bodies can be observed in spermatogonia, spermatocytes and spermatids. Mature spermatozoa are elongate and biflagellate, where the flagella exit at different levels from the sperm shaft, a feature previously known from acotylean polyclads but not from cotyleans. As in most other polyclads, two types of electron-dense bodies are found in the spermatozoan cytoplasm, but the specific distribution of the dense bodies along the sperm axis is valuable for systematics. The nucleus extends almost along the full length of the spermatozoon, and the chromatin takes a thread-like shape in association with spindle-shaped electron-dense bodies, similar to another studied species of the family Prosthionostomidae, E. evelinae. Additional studies on spermatogenesis and spermiongenesis in polyclads will provide more clues about possible apomorphic characters and convergencies.

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