Ultrastructure of spermatid development within the testis of the Yellow-Bellied Sea Snake, *Pelamis platurus* (Squamata: Elapidae)

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ABSTRACT

Little is known about spermatid development during spermiogenesis in snakes, as there is only one complete study in ophidians, which details the spermatid ultrastructure within the viperid, *Agkistrodon piscivorus*. Thus, the following study will add to our understanding of the ontogenic steps of spermiogenesis in snakes by examining spermatid maturation in the elapid, *Pelamis platurus*, which were collected in Costa Rica in 2009. The spermatids of *P. platurus* share many similar ultrastructural characteristics to that described for other squamates during spermiogenesis. Three notable differences between the spermatids of *P. platurus* and those of other snakes is a round and shorter epinuclear lucent zone, enlarged caudal nuclear shoulders, and more prominent 3 and 8 peripheral fibers in the principal and endpieces. Also, the midpiece is much longer in *P. platurus* and is similar to that reported for all snakes studied to date. Other features of chromatin condensation and morphology of the acrosome complex are similar to what has been observed in *A. piscivorus* and other squamates. Though the spermatids in *P. platurus* appear to be quite similar to other snakes and lizards studied to date, some differences in subcellular details are still observed. Analysis of developing spermatids in *P. platurus* and other snakes could reveal morphologically conserved traits between different species along with subtle changes that could help determine phylogenetic relationships once a suitable number of species have been examined for ophidians and other squamates.

Introduction

Over the last 20 years, large numbers of ultrastructural studies have produced sizeable volumes of published data on the spermatozoa of Squamata. These data have focused on morphological facets that may be beneficial in phylogenetic deduction because gamete ultrastructure has been considered a source for non-traditional character matrices.\textsuperscript{1-9} However, given the large number of snake species, descriptions of spermatozoa ultrastructure in the Ophidia are still limited at best,\textsuperscript{10} and complete descriptions of the entire process of spermiogenesis within snakes are rare.,\textsuperscript{9,10} There are a few reports that focus on specific parts of spermiogenesis in snakes,\textsuperscript{11-15} but to our knowledge the only comprehensive spermiogenic study in ophidians that describes the complete ultrastructural ontogeny of acrosome development, flagellar formation, elongation, and condensation of the spermatid DNA concerned the Cottonmouth (*Agkistrodon piscivorus*),\textsuperscript{10} which is a member of the Viperidae. There also exists a comparative account of spermiogenesis ultrastucturally between *Agkistrodon contortrix* (though incomplete for the Copperhead) and its sister taxon *A. piscivorus*,\textsuperscript{9} and an earlier study that determines all the major events of spermatid development in the Rock Python, *Python sebae*.\textsuperscript{16} However, the latter spermiogenic study is based entirely on drawings. There are no studies to date that describe the complete steps of spermiogenesis in Elapidae. Furthermore, there is only one study that details the spermatozoal microanatomy within elapids and was performed on *Oxyuranus microlepidotus*\textsuperscript{17}. The steps of spermiogenesis should show similar morphological structures to that observed within functional spermatozoa.\textsuperscript{10} However, because of the inconsistencies of the descriptions of spermatid morphologies and incomplete data for all the phases of spermiogenesis within snakes and other squamates,
determination of parallel support for ultrastructural features within the spermatid and spermatozoa and the implications of spermiogenic data in phylogenetic inference is not possible at the present time. Because spermiogenesis provides the potential to identify many more morphological characters that could be used in combinations with those known for mature spermatozoa, comparative studies between species, particularly in snakes, might aid in nontraditional/nonmolecular phylogenetic analysis and aid our understanding of spermatozoal maturation. Spermiogenic development at the ultrastructural level may also provide valuable data as a histopathological tool in studies of how pesticides affect the process of spermatogenesis within amniotic testes. Indeed, ultrastructural abnormalities have been identified in spermatogenesis, particularly during spermiogenesis, upon pesticide exposure in mammals.

The purpose of this study is to record the ultrastructural ontogeny of spermiogenesis within the Yellow-Bellied Sea Snake, *Pelamis platurus*. The Yellow-Bellied Sea Snake is in the family Elapidae, a group of over 340 venomous species with a cosmopolitan distribution. *P. platurus* has the widest distribution of any snake, and is completely marine. Their large geographic range and aquatic lifestyle may allow this snake to serve as a model species for histopathological studies on the impact heavy metals and pesticides have on testis and spermatogenesis. The results of this study will be compared with the ultrastructure of spermatid development in *Agkistrodon piscivorus* and *A. contortrix*, to the spermatozoa morphologies of the South American Rattlesnake, *Crotalus durissus*, to Bothrops alternatus and *B. diporus*, to the elapid, *Oxyuranus microlepidotus* and to the spermatozoa of other colubrid snakes. Results from this study can subsequently be combined with ultrastructural data of spermiogenesis not only within snakes, but also to that of other squamates.

**Materials and methods**

**Animal collection**

Adult male *Pelamis platurus*, (n = 3) were collected on July 10, 2009 12 km off the Playa del Coco in Golfo de Papagayo (Guanacaste, Costa Rica). The snakes were collected in dip nets by hand and stored in large containers containing seawater. Snakes were sacrificed within 12 hours of capture via an intraperitoneal injection of sodium pentobarbital as approved by the Institutional Animal Care and Use Committee at Southeastern Louisiana University. The testes and reproductive tracts were removed and fixed in Trump’s fixative (EMS, Hatfield, PA, USA). The testes were then cut into transverse sections and stored under refrigeration (4°C) until embedding. Whole specimens were deposited in the vertebrate collections at Southeastern Louisiana University. All other required permits for collection, euthanasia, tissue extraction, and storage were obtained prior to collection from the Ministerio del Ambiente y Energia of the Costa Rican government.

**Tissue preparation**

Testicular tissues from the 3 specimens were cut into 2–3 mm blocks and washed twice with cacodylate buffer (pH 7.0) for 15 min each. They were then post-fixed in 2% osmium tetroxide for 2 h, washed with cacodylate buffer (pH 7.0) 3 times for 15 min., dehydrated in a graded series of ethanol solutions (70%, 85%, 95% X2, 100% X 2), and cleared with 2 15 min washes of propylene oxide. Each piece of testis was then gradually introduced to epoxy resin (Embed 812, EMS, Hatfield, PA, USA) (2:1 and 1:1 solutions of propylene oxide:epoxy resin). Tissues were then placed in pure Embed 812 for 24 h. Fresh resin was prepared and the tissues were embedded in small beam capsules, and subsequently cured for 48 h at 70°C in a Fisher isotemperature vacuum oven (Fisher Scientific, Pittsburg, PA). Sections (90 nm) were obtained by use of a diamond knife (DDK, Wilmington, DE) on an LKB automated ultramicrotome (LKB Produkter AB, Bromma, Sweden). Sections were then placed on copper grids and stained with uranyl acetate and lead citrate.

**Ultrastructural analysis**

The samples were examined under a JEOL JEM-1200EX II transmission electron microscope (JEOL Inc., USA). Micrographs were obtained for representative spermatids and structural components associated with spermiogenesis via a Gatan 785 Erlangshen digital camera (Gatan Inc., Warrendale, PA, USA). The micrographs were then analyzed and composite plates were assembled using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA).
Results

Developing germ cells within the *Pelamis platurus* testis undergo mitosis, meiosis, and spermiogenesis within the germinal epithelium as observed in all amniotes. The spermatids, which undergo spermiogenesis, are located apically within the germinal epithelium in association with the lumen of the seminiferous tubule (Fig. 1, spermatids). Once spermiogenesis is concluded, mature spermatozoa are shed to this lumen for transport to the excurrent ducts of the testis. The beginning of spermiogenesis is marked by the amassing of round spermatids within the apical seminiferous epithelium of *P. platurus*. Haploid step one spermatids begin the production of the acrosomal vesicles via juxtapositioned Golgi apparatus and their transport vesicles (Fig. 2A, Ga; D, black arrows), which dominate these spermatids’ cytoplasms near the apices of the nuclei. The acrosomal vesicle does not make contact with the nuclear membrane (Fig. 2A, Av) and a small diffuse acrosomal granule (Fig. 2A, black arrow) is located within the lumen of the vesicle. The spermatid cytoplasm also contains numerous mitochondria (Fig. 2A, Mi) and a prominent accumulation of protein known as nuage (Fig. 2A, Ng). Later in the early round spermatid stage, the acrosomal vesicle (Fig 2B, Av) has made contact with the nucleus and the acrosomal granule (Fig. 2B, black arrow) is positioned basally within the vesicle near the nuclear surface. Also, the centrioles (proximal and distal) are situated on the caudal pole (opposite the acrosome complex) of the nucleus (Fig. 2C, Pc and Dc) and the beginning of the principal piece (Fig. 2C, Pp) of the flagellum starts to form.

Once the acrosomal vesicle engages the nuclear surface, it begins to increase in size and the large prominent acrosomal granule remains basally positioned (Fig. 3A,C,D, black arrows). The acrosomal granule sits on the inner membrane of the acrosomal vesicle. The vesicle and granule increase in size presumably from merging transport vesicles (Fig. 2D, black arrows) of the Golgi complex. As the acrosomal complex increases in size, it also causes the nuclear fossa to enlarge and deepen.
resulting in a large indentation on the apical nuclear membrane (Fig. 3A, C). A prominent subacrosomal space (Fig. 3A,C,D, Sa) is already forming during this early stage of development between the apical nuclear membrane and the inner acrosome vesicle membrane. Within the subacrosomal space, a dark electron dense protein accumulation can also be visualized just below the acrosomal granule, which is known as the subacrosomal granule (Fig. 3C, white arrow). The flagellum continues to elongate and mitochondria start to associate themselves with the future midpiece (Fig. 3B, Mi). Toward the climax of the round spermatid stage, the most distal part of the nucleus starts elongation and chromatin begins to condense in a filamentous fashion (Fig. 3D, Nu). The acrosomal complex has enlarged to a point of flattening the apical surface of the nucleus and the acrosomal granule reaches its maximum size (Fig. 3D, Av, black arrow).

As nuclear elongation progresses, acrosomal vesicle formation has terminated, the vesicle begins to envelop the apical nucleus by moving caudally along its lateral edges, and the acrosomal granule starts to diffuse into the lumen of the vesicle (Fig. 4A, D, Av, Ag). The subacrosomal space begins to segregate into the epinuclear lucent zone, the acrosomal lucent ridge, and a subacrosomal granular cone (Fig. 4D, black arrowhead, Ar, and Sa). Chromatin condensation continues and the chromatin condenses in a spiral fashion (Fig. 4B, *) leaving pits of chromatin free areas within the nucleoplasm (Fig. 4A,B, white arrows). As the chromatin further condenses, the nucleus begins to stain more intensely (Fig. 4C,D, Nu). Large conspicuous nuclear shoulders (Fig. 4C, Ns) form on either side of the well-developed caudal nuclear fossa (Fig. 4C, Nf).

At the late elongation stage, the acrosomal vesicle envelops the entire apex of the nucleus (Fig. 5A, A1).
Figure 3. Late stages of round spermatid development in Pelamis platarus. (A) The acrosomal vesicle (Av) has made a deep indentation into the apical nucleus (Nu). The acrosomal granule (black arrow) is now located basally within the acrosome. A prominent subacrosomal space (Sa) develops under the acrosomal vesicle. (B,C) The centrioles (Pc, Dc), which are housed in the caudal nuclear fossa (Nf) continue to contribute to the growing principal piece (Pp). Mitochondria (Mi) start to relocate to the area next to the caudal distal centriole. Nu, nucleus; Sa, subacrosomal space; Av, acrosomal vesicle; black arrow, acrosomal granule; white arrow, subacrosomal granule. (D) At the end of the round spermatid stage, the caudal end of the nucleus begins to elongate and chromatin condenses in a fibrous fashion (Nu). The acrosomal granule (black arrow) has reached its maximum size within an acrosomal vesicle (Av) that has collapsed on to the apical nuclear surface. Sa, subascrosomal space. Bar = 2 μm for all micrographs.

superficial to the nucleus, and has constricted it into
the nuclear rostrum (Fig. 5A, E, Nr). Within the acro-
some, a prominent basal plate forms and gives rise to
the perforatorium (Fig. 5A; C, black arrowhead; E, Pe,
Bp). Juxtaposed to the nuclear membrane of the ro-
strum a continuous band of protein accumulation
(Fig. 5A; D, E, F, Sa) spans the entire length of the sub-
acrosomal space/cone. Located within the cranial sub-
acrosomal space is a well-developed and round-
shaped epinuclear lucent zone (Fig. 5D,E, white arrow,
Ep). During late elongation, the microtubules of the
manchette also develop and start to constrict the
nuclear width of elongating spermatids (Fig. 5A, Ma).
Though both parallel and circum-cylindrical microtu-
bules form, the parallel microtubular network is more
prominent and complex in its array around the con-
densing nucleus (Fig. 5G, Ma). Small nuclear lacunae
are also visible within the nucleus proper (Fig. 5G,
white arrowhead).

As the elongating spermatids complete conden-
sation of the chromatin and overall maturation,
their flagella differentiate into the 4 major
components of the mature spermatozoa. The proximal neck region (Fig. 6, Ne) contains the proximal centriole (Fig. 6, Pc) connected to the caudal portion of the nucleus within the nuclear fossa (Fig. 6, white arrow). The distal centriole (Fig. 6, Dc) extends away from the proximal...
The fibrous sheath blocks begin immediately after the neck region at mitochondria tier 1. There are roughly between 43–49 centric rings of mitochondria around the extremely elongated midpieces of the *P. platurus* terminal step spermatids. Most of these mitochondria appear round in sagittal and tubular to round in cross-sections of the flagellum/midpiece (Fig. 6. Mi). Peripheral fibers are associated with all the microtubule triplets of the distal centriole (Fig. 6A, Pf), which only continue into the midpiece, principal piece, and endpiece at microtubule doublets 3 and 8 (Fig. 6B, C,D, Pf). The fibrous sheath surrounds the axoneme of the midpiece and principal piece and is absent in the endpiece (Fig. 6B,C,D, Fs).

**Discussion**

Presently, only one other ultrastructural study describes the entire developmental process of spermiogenesis within an ophidian, *Agkistrodon piscivorus*. Several other studies reveal bits and pieces about spermionic morphologies during spermatogenesis in snakes and a comparison of spermionic development has been preliminarily completed between the
viperid sister taxa *Agkistrodon piscivorus* and *A. contortrix*. However, within Elapidae, only one study exists that details spermatozoa ultrastructure, and no data are available presently that provides information on spermatid ultrastructure in elapids. Because of the limited comparative data available within ophidians, caution must be the practice when drawing morphological conclusions or the phylogenetic potential of ultrastructure of spermatids between species of ophidians. Nevertheless, there are several common ultrastructural features of spermiogenic germ cells observed between these species of snakes studied to date. This supports the overwhelming data that suggest most of the major changes occurring during spermiogenesis are highly conserved in reptiles and other amniotes. Thus, it is not surprising that many of the spermiogenic features within *Pelamis platurus* are also observed within the spermatozoa of the other snakes studied to date.

During acrosome formation in *Pelamis platurus* and in the other snakes, the acrosomal vesicle develops from transport vesicles budding from the Golgi apparatus, which merge to form an intact vesicle before contact is made with the nuclear membrane. This is comparable to what was reported in other squamates. Also, the acrosomal granule is seen within this vesicle before nuclear contact, but in contrast to the basal positioned granule described in *S. lateralis*, the granule is centrally located and then migrates to its basal position once the acrosome makes contact with the nucleus, much like that of and *Agkistrodon piscivorus*. The granule’s largest growth phase occurs in this basal position within the acrosome of *P. platurus, A. piscivorus,* and most other squamates. In contrast, the acrosomal granule is not present in *A. contortrix* until after the vesicle makes contact with the nuclear membrane.
emerges where the acrosome is seated on the nucleus, which is a characteristic of spermiogenesis in vertebrates. The remaining steps of acrosome development within *P. platyrus* parallel that detailed for *A. contortrix*, *A. piscivorius*, and other squamates such as: a prominent subacrosomal space, multilaminar membranes, and the acrosomal lateral shoulders that envelop the apical nucleus. P. platyrus round spermatids lack the 2 Golgi apparatus and rough endoplasmic reticulum association with the acrosomal vesicle observed during acrosome formation in *Agkistrodon contortrix* and *A. piscivorius*. Ferreira and Dolder also noted a distinct endoplasmic reticulum association near the acrosome during the developing round spermatid stage within *Iguana iguana*, which has also been seen in some sceloprine lizards.

In early elongation, it is observed that the acrosome complex in *Pelamis platyrus*, like *Agkistrodon piscivorius* and *A. contortrix*, is highly compartmentalized, similar to that of most of the squamates studied recently. This extensive compartmentalization most likely assists in the release of hydrolytic enzymes that digest the outer layers of the ovum during fertilization. The compartments found within the *P. platyrus* acrosome are: the subacrosomal space/cone, perforatorium, acrosomal vesicle, and the outer Sertoli cell membrane layers, which are all comparable to the squamates studied to date. Further stratification is apparent within the subacrosome space and includes the epinuclear lucent within *Pelamis platyrus* and both *Agkistrodon piscivorius* and *A. contortrix*. This lucent zone within *P. platyrus* appears shorter and more bulbous like that in *Oxyuranus microlepidotus* spermatozoa. This is different than other snakes where the lucent zone is elongated. The acrosome lucent ridge in *P. platyrus* is more conspicuous and better developed between the subacrosomal protein cone and the inner acrosomal membrane of all the other snakes studied to date, including *O. microlepidotus*. The other major structure that is under-developed or absent in *P. platyrus* are the acrosomal (or sometimes called nuclear) shoulders present where the acrosome meets the terminal end of the nuclear rostrum caudally. The other snakes studied to date have these shoulders present during the elongation phase of spermiogenesis or within their mature spermatozoa.

As elongation progresses in *Pelamis platyrus*, the spermatid nuclei are relocated apically within the cytoplasm and come in contact with the cell membranes of the developing spermatids as described in *Agkistrodon piscivorius* and *A. contortrix*. This contact of the apical nucleus with the cell membrane has been suggested to cause acrosome collapse and migration laterally of the acrosome complex of other squamates, the rearrangement of cellular organelles caudally, and may benefit in the removal of cytoplasm later in maturation in reptiles and other amniotes.

At the end of elongation and while elongates are condensing, the manchette becomes visible in both transverse and sagittal sections in *Pelamis platyrus* spermatids. These microtubule arrays were hypothesized to aid in the elongation of the spermatid nucleus. Parallel microtubules far outnumber the circum-cylindrical fibers in *P. platyrus* similar to that described in *Agkistrodon* species. This absence of circum-cylindrical microtubules is also prominent in *Scincella lateralis* and completely absent in *Anolis lineatopus*, which have more robust-bodied spermatozoa than that of other squamate species. The nuclear bodies of *P. platyrus* do appear to have wider diameters than many other squamates, which have both types of microtubule during elongation. During DNA condensation, the chromat in *P. platyrus* condenses in a spiral fashion, which was not documented in some squamates, but was noted by Ferreira and Dolder in the lizard *Tropidurus itambre*, in some *Sceloporus* species, in the snake *Cerastes cerastes*, and in Cottonmouths, which resulted in large areas of open nucleoplasm. These open areas of nucleoplasm continue as lacunae within the body of fully condensed nuclei in maturing spermatids in *P. platyrus*, which is similar to *Bothrops* spermatozoa and *A. contortrix* elongating spermatids, but seem to have been lost in *A. piscivorius* spermatids, *Oxyuranus microlepidotus*, and many other snake spermatozoa. Also during condensation and elongation, uniform translucent areas are seen on either side of the caudally located nuclear fossa where the flagellum attaches to the nuclear body of the developing spermaticid. These open spaces are more pronounced in the *P. platyrus* than that of other ophidians and squamates studied to date.

As flagellar differentiation progresses, large numbers of mitochondria become present in the posterior portion of the *Pelamis platyrus* spermatid cytoplasm and are associated with the flagellum, which is
consistent with other amniotes. However, there are more than 40 concentric rings of mitochondria that run down the *P. platurus* midpiece to the annulus in sagittal section and they appear round to occasionally tubular in transverse section of the midpiece. These very long midpieces are considered a synapomorphy for snake spermatids and spermatozoa. Dense bodies are present along with functional mitochondria within the midpiece of the *P. platurus* and *Oxyuranus microlepidotus* spermatozoa, which is similar to that described for *Crotalus durissus* but in contrast to *Agkistrodon* and to *Bothrops* species, where dense bodies are absent from the midpiece. Typically 10–11 mitochondria/dense bodies are contained within each concentric ring of *P. platurus* midpieces. This appears to be similar to *Agkistrodon* species and *Oxyuranus microlepidotus* spermatozoa but different from the visual data presented in other previously studied snakes, which seem to run from 12–16 mitochondria per ring. The proximal and distal centrioles are found within the *P. platurus* spermatid’s short neck and have little to no pericentriolar material as described in all other snake spermatids and spermatozoa to date. The neck region within *P. platurus* does have a well-developed dense collar and the axoneme microtubule triplets within the centrioles and the microtubule doublets of the midpiece have dark staining peripheral fibers associated with all 9 sets. The peripheral fibers located near microtubule doublets 3 and 8, which continue into the distal parts of the flagellum, are considered a synapomorphy for squamates and Lepidosauria and are also observed within the mid, principal, and endpieces of *P. platurus*. The principal and endpieces in *P. platurus* are highly conserved and similar to all the squamates studied to date. The axoneme has the 9 + 2 microtubule doublet organization as described for all vertebrates. The principal piece is surrounded by the fibrous sheath similar to the midpiece in *Agkistrodon*; however, the peripheral fibers 3 and 8 in *P. platurus* flagella extend throughout the mid, principal, and endpieces and are more robust in size than that documented in other snakes to date, including *Oxyuranus microlepidotus* spermatozoa.

Much of the morphological data presented here for *Pelamis platurus* corroborates the microanatomy of the mature spermatozoa described for the elapid *Oxyuranus microlepidotus* and the pitvipers *Crotalus durissus*, *Bothrops*, and with that of the spermatid descriptions for *Agkistrodon piscivorus* and *A. contortrix*. Presently, Cunha et al. and Tourmente et al. studies represent the only morphological data for spermatozoa within Viperidae and the Oliver et al. data on *O. microlepidotus* spermatozoa is the only study done on elapid spermatozoa. There are several key differences seen within the spermatids of *P. platurus* that are not previously described for snakes. The late elongating spermatids of the *P. platurus* have larger caudal nuclear shoulders and a shorter, more rounded epinuclear lucent zone than that described in other snakes. Also in *P. platurus* the peripheral fibers 3 and 8 appear much more conspicuous throughout the principal piece and are still readily visible in the endpiece, which is not the case for the other snakes studied to date. Lastly, the *P. platurus* lacks the acrosomal shoulders at the terminal ends of the acrosome where the nuclear rostrum meets the wider nuclear body. Interestingly, *P. platurus* spermatids differ from the *O. microlepidotus* spermatozoa and thus may provide important comparative histological data between 2 elapids. *O. microlepidotus* spermatozoa vary from *P. platurus* spermatids in that they lack a basal plate, nuclear lacunae, and peripheral fibers 3 and 8 in their endpieces, which are all observed within the Yellow-bellied Sea Snake spermatids. These differences suggest that these ultrastructural traits may be important modifications between species that could have phylogenetic implications or are just developmental or stages differences between the mature spermatozoa and the ontogenic process of spermiogenesis. Providing conclusive reasons for why these differences exist between species within Elapids cannot be answered until more species within Elapidae and other ophidians are studied during the process of spermiogenesis and spermatozoal maturation.

This study of spermatid ultrastructure in *P. platurus* represents only the second comprehensive study of spermatid development across the entire spermiogenic stage of spermatogenesis in snakes. Although development of spermatozoa in Squamata appears conserved in many aspects, morphological differences are observed, and combining data from spermiogenesis and spermatozoa morphology may provide insight into the evolutionary relationships of Ophidia and other squamate taxa. Unfortunately, insufficient data are available to reconstruct a comprehensive understanding of spermiogenesis in reptiles, especially in a robust phylogenetic context. Future spermiogenic data
within species of Ophidia may help provide sufficient morphological detail to attempt a viable phylogenetic analysis of snake taxa and aid the molecular phylogenetic data that are currently available. This study does provide solid ultrastructural data on the features of spermatid formation and provides standard histological information for potential histopathological studies regarding spermatogenesis and the effects of aquatic pesticides on the process of sperm development within an aquatic snake species.

**Disclosure of potential conflicts of interest**
No potential conflicts of interest were disclosed.

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**References**


