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Techniques of Immunofluorescence and Confocal Microscopy Applied to the Study of Syngnathids Gonadal Structure and Development and to the Dopaminergic control of the Reproduction in Teleosts

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Abstract

Reproduction is an indispensable function for the perpetuation of the species and is perfomed with extraordinarily diverse reproductive strategies (adaptations that improve the chances of fertilization and/or increase the survaival rate of offsprings), each of wich is under control of a sophisticated network of regulatory signals. The array of resporductive strategies among teleosts are extraordinarily diverse and, among them, those of syngnathids are peculiar. The Syngnathidae are a small family of brackish and freshwater species. They have attracted attention for decades due to their unique morphology, remarkable camouflage ability and the distinctive phenomenon of male pregnancy. Females deposit eggs, on or into a male incubation area, on the tail (subfamily Urophori) or on the trunk (subfamily Gastrophori). Syngnathids Mediterranean species only belong to *Syngnathus, Hippocampus* and *Nerophis* genera. Very little is known about the male reproductive biology and on the nervous system control on the reproductibe cycle of these fish. The aims of this work are therefore to clarify: 1) relevant aspects of male reproduction in both internal and external brooder species, such as those belongin to *Syngnathus* and *Hippocanous* (Urophori) and *Nerophis* genera (Gastrophori); 2) characterize the Dopaminergic system in adult and young males of the pipefish Syngnathus abaster. The aims have been achieved using different techniques of light, confocal and electron microscopy.

Riassunto

La riproduzione è una funzione indispensabile per la conservazione della specie ed è attuata attraverso differenti strategie riproduttive controllate da una sofisticata rete segnali di regolatori. Nei teleostei troviamo strategie riproduttive straordinariamente differenti, tra queste, quelle dei Singnatidi sono particolarmente peculiari. I Syngnathidi sono una piccola famiglia d'acqua dolce, marina e salmastra. Sono particolarmente studiati per la loro affascinante morfologia, per la straordinaria capacità di mimetizzarsi e per il fenomeno della gravidanza maschile. Le femminile depositano le uova all'interno di un area incubatrice, sulla coda (sottofamiglia Urophori) o sulla superficie ventrale del tronco (sottofamiglia Gastrophori) dei maschi. Nel Mediterraneo troviamo le specie appartenenti ai generi *Syngnathus*, *Hippocampus* e *Nerophis*. Le informazioni a carico della biologia riproduttiva maschile e del controllo del sistema nervoso sul ciclo riproduttivo di questi pesci sono molto scarse. Gli obiettivi di questo lavoro sono quindi chiarire: 1) gli aspetti rilevanti della riproduzione maschile in entrambe le specie a fecondazione interna ed esterna, in particolare nei generi *Syngnathus*, *Hippocampus* (Urophori) e *Nerophis* (Gastrophori); 2) caratterizzare il sistema dopaminergico in maschi adulti e giovani del pesce ago *Singnathus abaster*. Gli obiettivi di questo lavoro sono stati raggiunti utilizzando tecniche di microscopia ottica classica, confocale ed elettronica.

Introduction

The general aim of my PhD thesis is to contribute to the knowledge of the male reproductive biology of some teleostean species belonging to the family Syngnathidae, studying the maturation of both male gonads and gametes and how the nervous system, and particularly the dopamine, acts in the various phases of the reproductive cycle.

Reproductive Biology

The family Syngnathidae (pipefishes, seahorses and seadragons) are well known for their highly specialized morphology, and the diversity of morphological forms. This exceptional morphological variation is reflected in the current taxonomy of the group: 14 of the 54 currently recognized syngnathid genera are monotypic (Froese and Pauly 2010), and the majority of genera are composed of fewer than three species.

The small teleostean family is also known for the remarkable adaptations for paternal care. The female deposits eggs directly onto a specialized brooding area or into a pouch of the male body (Breder and Rosen 1966). This evolutionary innovation ensures males complete confidence of paternity (Jones and Avise 1997; Jones *et al*., 1998, 1999), but at a level of paternal investment that exceeds that of most other vertebrates (Breder and Rosen 1966). The brooding structures vary in complexity in five steps, from: (1) a simple unprotected ventral area for gluing, (2) individual membranous egg compartments, (3) protection of eggs in a pouch with pouch plates, (4) bilateral pouch folds that grow together into a closed pouch, to (5) the most complex and completely enclosed brooding pouch of seahorses (Dawson 1985). There is a further significant difference among species in that brooding may occur on the tail (Urophori: A-type) or on the abdomen (Gastrophori: B-type)

(Herald 1959). Among the Gastrophori, brooding structures only vary in complexity from step 1 to step 3 (Dawson 1985).

Syngnathidae has traditionally been included as a member of the order Gasterosteiformes, which includes 11 families in two suborders: the Gasterosteoidei, with the Hypoptychidae, Gasterosteidae and Aulorhynchidae; and the Syngnathoidei, with the Indostomidae, Aulostomidae, Fistulariidae, Macroramphosidae, Centriscidae, Pegasidae, Solenostomidae and Syngnathidae (c.f. Wilson and Orr 2011).

Studies based on morphological characters have proposed monophyly of the order Gasterosteiformes and suggested sister groups based on weak evidence, and while the close relationships of super families is well supported, the relationships among these family pairs remains unclear (Pietsch, 1978; Johnson and Patterson 1993; Orr, 1995; Keivany and Nelson 2006).

In contrast, studies based on molecular data clearly refute the monophyly of the Gasterosteiformes, placing gasterosteoids close to the cottoid–zoarcoid lineage (Imamura and Yabe 2002), excluding the Indostomidae, and placing both groups distant from syngnathoids. In addition, while the sister group of syngnathoids remains unknown and the relationships among syngnathoid lineages are poorly resolved, the family Solenostomidae (ghost pipefishes) has been faithfully recovered as the sister group of the Syngnathidae in both morphological and molecular analyses (c.f. Wilson and Orr 2011).

The evolutionary relationships among members of the family Syngnathidae have been resolved with greater confidence. According to Herald (1959), the Syngnathidae diverged early in its evolution into tail (Urophori) and trunk-brooding (Gastrophori) species. Following the development of a rudimentary form of male brooding in both these lineages, brood-pouch complexity evolved in parallel in the Gastrophori and Urophori, resulting in the fully enclosed pouch of the seahorse and the highly developed brooding structures found in

some gastrophorine species. Herald (1959) suggested that the brooding structures of urophorine pipefish with partially enclosed brood pouches could be further subdivided into monophyletic lineages according to their method of closure (inverted, semi-inverted, overlapping and everted) and proposed a multistage model by which the fully enclosed pouch of the seahorse was derived from pipefish ancestors with an everted brooding structure. Molecular data obtained performing mtDNA analyses (Wilson *et al.,* 2001, 2003; Wilson and Orr 2011) supported the evolutionary model of the family characterized an early divergence of the two groups, trunk- and tail-brooding lineages, (Urophori and Gastrophori). Those data, however, suggested that several major pouch types within each of these lineages had independent evolutionary origins, challenging the phylogenetic model proposed by Herald (1959). mtDNA sequence data also supported a close evolutionary relationship between *Syngnathus*and *Hippocampus*, contradicting Herald's (1959) theory on the origin of the seahorse brood pouch. The most up-to-date phylogenetic tree of the family Syngnathidae, based on mtDNA sequence data, is provided in the paper of Wilson and Orr (2011).

The members of the family Syngnathidae inhabit coastal tropical and temperate warm waters of seas and oceans and also occur in river estuaries. They have an elongated body entirely covered by bone plates connected to each other to form belts (rings). Only 294 of the 558 nominal species of syngnathids are presently considered to be valid (Froese and Pauly 2010).

Syngnathids are present in the Mediterranean sea with only three genera: Nerophis, *Syngnathus* and *Hippocampus*. The genus *Syngnathu*s comprises six species, i.e. *Syngnathus acus*, *S. abaster*, *S. tenuirostri*s, *S. phlegon*, *S. typhle*, *S.tenionotus*, whereas the other two genera are represented with only two species each, *i.e. Nerophis ophidion, N. maculatus, Hippocampus hippocampus, and H. guttulatus* (Dowson 1986). *Hippocampus fuscus,*

immigrant from the Red Sea, and *Syngnathus rostellatus,* immigrant from the Atlantic Ocean have been newly recorded (Gokoglu *et al.,* 2004).

Seahorses are exclusively marine species, whereas pipefishes, especially those belonging to the genus *Syngnathus*, inhabit sea and brackish waters, and in some case, even freshwaters (i.e. *S. abaster*).

The high level of adaptability to different habitats of the *Syngnathus* species is closely related to the high and particular plasticity of their body structure, which determines different local morphotypes (D'Ancona 1934; Tortonese 1970). To this, morphological plasticity may be attributed the numerous controversies on the systematic of this genus. A variable number of Mediterranean species (from sixteen to nine) was reported during the 19th century (Kaup 1856; Duméril 1870). A more accurate revision of the genus based on the analysis of 11 different morphological characters recognized in the Mediterranean and Black sea, 10 species only (D'Ancona 1934).

In the syngnathids studied to date, males of species with less complex brooding structures (e.g., *Nerophis ophidion*) is suggested spend less energy on their young than do those brooding embryos in enclosed pouches with placenta-like structures (Berglund *et al.,* 1986; Masonjones 2001; Carcupino *et al.,* 1997, 2002). According to Wilson *et al.* (2003), it is important to recognize that parental expenditures are not necessarily equivalent to parental investment, because expenditures such as parental guarding may not necessarily carry a fitness cost. However, in many cases, time and energy expenditures may be positively correlated and, in general, a large expenditure will often carry larger costs and therefore represent a higher parental investment. If increasing pouch complexity results in a general increase in male parental investment relative to females, male pregnancy predisposes males to limit female reproductive success; sexual selection may then operate more strongly on females and female sexual signals may evolve (sex-role reversal). Sex-role reversal, which

is found in fishes, insects, amphibians and birds (Oring and Lank 1986; Simmons 1995; Berglund and Rosenqvist 2003), is characterized by intense mating competition among females, which should also favor the evolution of sexual dimorphism, where females are larger and more colorful than males. On the contrary, conventional sex role, which represents the most common pattern in nature, is characterized by male – male competition and female choice, and is traditionally viewed as being due to females limiting the reproductive potential of males.

Although several pipefishes are sex-role reversed (e.g., *Nerophis ophidion*, *Stigmatopora nigra*, *Syngnathus typhl*e), with females that are more vividly colored and striped than males, some other species retain conventional sex roles (e.g., *Hippichthys penicillus*) (see Rosenqvist and Berglund 2011). One notable exception to this pattern is the genus *Hippocampu*s, in which, although these species have the highest degree of pouch development, sex-role reversal has not yet been documented (Vincent and Sadler 1995; Kvarnemo *et al.,* 2000; Masonjones and Lewis 2000).

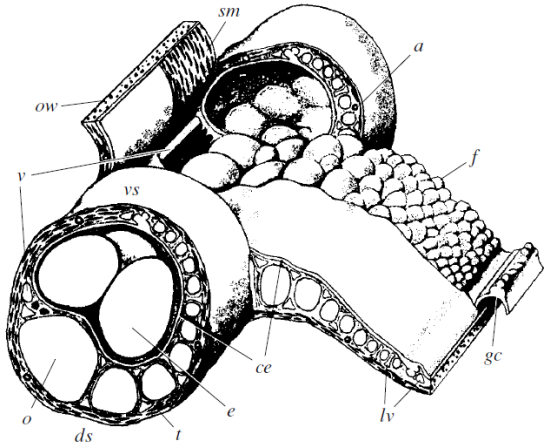
All species however show elaborate courtship behaviour involving only one partner (monogamy) or multiple partners (polygamy). Genetic analyses have discovered a wide variety of genetic mating patterns in pipefishes and seahorses (Jones and Avise 1997a, b, 2001; Jones *et al.,* 1999; Wilson 2006). The broad-nosed pipefish *Syngnathus typhle*, for example, exhibits multiple mating by males as well as by females. Hence, *S. typhle* is characterized by a polygynandrous mating system, where both sexes mate multiply over the course of a single pregnancy. In a related American species, the gulf pipefish *Syngnathus scovelli,* males received eggs from only one female per pregnancy, whereas females mated with several males, a classic case of polyandry (Jones and Avise 1997a). Classical polyandry may also be the most common pattern found in Gastrophori pipefishes, as for instance, *Nerophis ophidion* (McCoy *et al.,* 2001). In contrast, some syngnathids exhibit true genetic

monogamy such as the seahorse *Hippocampus angustus* (Jones *et al.,* 1998). A mating system strictly monogamous, which may occur in most seahorses, at least within each brooding period (Foster and Vincent 2004), may also be present in some pipefish species, as reported in *Hippichthys penicillus, Corythoichthys haematopterus* (Watanabe *et al.,* 1997; Matsumoto and Yanagisawa 2001; Sogabe and Yanagisawa 2007).

Although in synagnthids, male parental investment seems bigger relative to females, *H. abdominalis* and *H. erectus* oocytes are relatively large compared to the eggs of the majority of other marine teleost species (Pankhurst and Conroy 1987; Selman *at al.,* 1999; Poortenaar *et al.,* 2004). Large egg size is generally reflected in low fecundity and a more significant investment in parental care.

Syngnathids are also unique among teleosts in their ovarian structure. The ovary consists of a rolled follicular sheet, which has stem cell compartments, called the germinal ridge, running along the entire length of the edge of the follicular sheet (Wallace and Selman 1981; Begovac and Wallace 1987; Selman *et al.,* 1991). Oocyte development starts at the germinal ridge, and developing follicles are arranged in sequence according to their development (Begovac and Wallace 1988). Distinct differences in ovarian structure have been reported in syngnathids. Some species have a single germinal ridge at one edge of the follicular sheet, with the most advanced follicles at the opposite edge, referred to as the mature edge. This type of ovary has been reported only in three species of congeneric pipefish, *Syngnathus typhle* and *S. scovelli.* and *S. schlegeli* (Begovac and Wallace 1987; Sogabe and Ahnesjo 2011; Sogabe *et al.,* 2013), and one species of the sister genus *Hippichthys* (*Hippichthys spicifer*; see Ishihara and Tachihara 2009). Other species, such as *Hippocampus erectus*, *Corythoichthys haematopterus*, *Nerophis ophidion*, and *Urocampus nanus*) have two germinal ridges, one at each edge of the follicular sheet, with the most advanced follicles situated midway along the follicular sheet between the

edges (Selman *et al.,* 1991; Sogabe *et al.,* 2008, 2012; Sogabe and Ahnesjo 2011). Furthermore, it has been reported that the mode of egg production varies among syngnathids in relation to differences in ovarian structure. In species with a single germinal ridge, oocyte maturation occurs asynchronously, and the number of mature eggs increases continuously over time (i.e., the asynchronous type; Begovac and Wallace 1988; Sogabe and Ahnesjö 2011). On the other hand, in species with two germinal ridges, oocyte maturation occurs in group(s), and mature eggs are produced in batches or at one time before spawning (i.e., the group-synchronous type; *Sogabe et al.,* 2008, 2012; Sogabe and Ahnesjo 2011; Sogabe *et al.,* 2013). The mode of egg production has important implications for how eggs are spawned, and thus a close link between ovarian structure, mode of egg production, and mating pattern (i.e., spawning frequency of females) (Sogabe *et al.,* 2008; Sogabe and Ahnesjö 2011).



Scheme of the structure of the ovary in the pipefish *Syngnathus scovelli* (from Begovac and Wallace, 1987). (*a*) Arteriole: (*v*) vein; (*vs*) ventral side; (*gc*) germinal ridge; (*ds*) dorsal side; (*lv*) lynphatic vessel; (*sm*) smooth muscle fibers; (*o*) oocyte; (*ce*) coelomic epithelium; (*ow*) ovarian wall; (*t*) theca; (*f*) follicles; (*e*) egg.

The male reproductive apparatus of syngnathids still largely remains a mystery to investigators, and, at present, conflicting data are reported for both testis and sperm morphology.

Testes of restricted lobular and unrestricted tubular type have been reported in *Microphis brachyurus lineatus* (Kaup) (Miranda-Marure *et al.,* 2004), *Syngnathus abaster* Risso and *Syngnathus acus* L. (Carcupino *et al.,* 1999) and *Phyllopteris taeniolatus* (Forsgren and Young 2008) respectively. In addition in the last two species, the germinal epithelium has been reported to be organized in the typical spermatocysts (Carcupino *et al*., 1999), whereas in *Syngnathus schlegeli* Kaup these structures seem to be absent, or at least, difficult to be recognized (Watanabe *et al*., 2000).

Large droplets-containing cells characterize the testis of several syngnathid species. These cells, however, are reported to have different localization. In *S. schlegeli*, they appear to be localized in the germinal epithelium only (Watanabe *et al*., 2000), whereas in *S. abaster* and *S. acus* they have been also observed inside the lumen, where they have been interpreted as germinal cells in different developmental stages, associated to a semicystic spermatogenesis (Carcupino *et al*., 1999).

The functional sperm of most syngnathids examined up to now have been categorized as introsperm type (Watanabe *et al*., 2000; Van Look *et al*., 2007; Biagi *et* al., 2008; Dzyuba *et al.,* 2008), which has elongated head and long flagellum, and is typical of internal fertilizing fish (Jamieson 1991). This type of sperm is the unique type found in a brackish water population of *S. Abaster, S. acus* and *N. ophidion* (Carcupino *et al.,* 1999; Ah-King *et al.,* 2006). Only one type of sperm has also been reported in *M. brachyurus* lineatus. In this species however, sperm seem to be of the aflagellate type (Miranda-Marure *et al.,* 2004). *Syngnathus schlegeli* and *Hippocampus kuda*, seem to have two sperm types: functional sperm of introsperm type and no functional sperm of aquasperm type (Watanabe *et al.,* 2000;

Van Look *et al.,* 2007). The latter, which is typical of external fertilizing fish (Jamieson 1991), is characterized by a large spherical head. Three different morphotypes of sperm, differing in flagellum length, head length and head wide, have been reported in a freshwater population of *S. abaster* (Dzyuba *et al.,* 2008). Among them however, only the longest seem to be functional.

To shed light on these topics, I have analysed the male gonad and mature sperm morphology of six syngnathid species; five internal brooder species, i.e. *Syngnathus abaster, S. acus*, *S. tenionotus*, *S. typhle*, and *Hippocampus guttulatus*, and one external brooder species, i.e. *Nerophis ophidion*, using different light and electron microscopic techniques. I particularly intended to ascertain in all above mentioned species:

(i) the testis structure, and spermatogonia localization,

(ii) the germinal epithelium organization,

(iii) the morphology and localization of Leydig cells

(iv) the spermatogenetic process and sperm structure

(v) the presence of polymorphic sperm.

Dopaminergic control of reproduction

Dopamine is one of the major neurotransmitters of the nervous system. Only some of its functions are conserved among different vertebrate groups, and this is reflected in the anatomical aspects of DA systems in the brain of different mammal taxa (Yamamoto and Vernier 2011). In mammals, DA-containing neurons are located in two midbrain nuclei, the ventral-tegmental-area (VTA) and the substantia nigra (SNc). In these areas, they are

involved in the control of several behavioral processes, such as learning and memory (Wise 2004; Hyman *et al.,* 2006), social behavior (Young *et al.,* 2011; O'Connell and Hofmann 2011), and the selection of motor programs (Joshua *et al.,* 2009; Vidal-Gadea *et al.,* 2011). VTA plays a pivotal role in the reward pathway while SNc is involved in the extrapyramidal control of movements.

In mammals, DA neurons are located in the mesencephalon and into the basal diencephalon, have a common developmental origin. In contrast, dopaminergic cell groups are not located in the midbrain of teleost, which makes establishing functionally similar cell groups between the mammalian mesencephalic dopaminergic neurons and dopaminergic cell groups in teleostes exceedingly difficult (Wullimann and Mueller 2004; O'Connell and Hofmann 2011; Yamamoto and Vernier 2011).

The neuroendocrine control of reproduction is regulated in fishes, as in mammals, by the hypotalamus-pitituary-gonadal axis. Neuroendocrine cells, localized in the hypothalamus, synthetize the Gonadotropin-Release-Hormone (GnRH) (Yu *et al.,* 1991), which stimulates the release of gonadotropins (GtH) from the pituitary gland (Kobayashi *et al.,* 1997). In most teleostes species, Dopamine (DA) modulates pituitary activity (Chaang *et al.,* 1983; Peter and Fryer 1983) by inhibiting GtH release (Dufour *et al.,* 2010). In teleostes DA-releasing neurones are located mainly in three areas: the olfactory bulb, the diencephalon and the telencephalon. DAergic neurons, which are involved in the regulation of pituitary function, are localized in the preoptic area (POA) (Fremberg *et al.,* 1977), as shown in *Acipenser sturio* and in some condroittis (Adrio *et al.,* 2002; Meek *et al.,* 1989).

The POA is a region between telencephalon and diencephalon and in this area we can recognize one magnocellular and one parvocellular nuclei. Most of the studies on the role of DA in fish brain are focused on the control on gonadal development (Hernandez-

Rauda *et al.,* 1999), and on sexual differentiation (Gagnè and Blaise 2003). DA activity changes with the development and the reproductive cycle and it is probably controlled by environmental cues as well as by endogenous signals (Dufour *et al.,* 2010).

A phenomenon particularly well studied in many fish (*Astatotilapia burtoni*) is the remarkable plasticity of GnRH secreting neurons in the POA, respect with the social environment. Males exhibit two distinctive phenotypes based on social status, correspond with the soma size of GnRH neurons in the POA (Francis *et al.,*1993). This mechanism of socially induced cell size change provides the potential for relatively quick adaptive changes in the neuron-endocrine system.

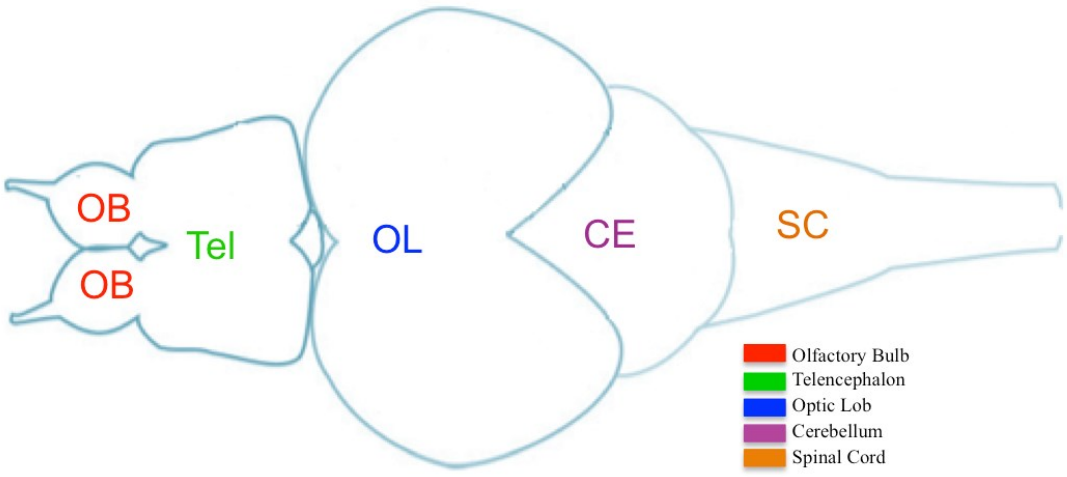


Fig. Brain organization in fish

The present thesis aimed at investigating, in the POA of *S. abaster*: 1) the existence of a DA inhibitory tone on the endocrine reproductive axis, and 2) if there are modifications in morphometric parameters of DAergic neurones (as shown for GnRH ones in the same area) in correlation with sexual maturity. To better understand where dopamine acts for regulating the reproductive process, in this species I have determined the distribution of the

dopaminergic cells (by tyrosine hydroxylase immunohistochemistry), and I have verifty if these cells suffer alterations analysing morphometric parameters (Area, Perimeter, Circularity and cell perfield).

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Testis structure, spermatogenesis and sperm morphology in pipefishes of the genus *Syngnathus*

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Abstract

Biagi F., Piras F., Farina V., Zedda M., Mura E., Floris A., Franzoi P., Fausto A.M., Taddei A.R. and Carcupino M. 2014. Testis structure, spermatogenesis and sperm morphology in pipefishes of the genus *Syngnathus*. —*Acta Zoologica* (Stockholm) **00**: 000–000.

Testes, spermatogenesis and sperm morphology have been analysed in four species of the *Syngnathus* genus. All species show testes of unrestricted lobular type, characterized by a single germinal compartment, with central lumen, and an external tunica albuginea. The spermatogenesis occurs throughout a process of semicystic type, in which germinal spermatocysts open precociously, so germ cells complete maturation in the testis lumen. Amongst them, aflagellate and flagellate multinucleate cells are recognizable. This type of spermatogenesis may be therefore related to the reduced number of simultaneously mature sperm produced by syngnathids. Only one type of mature sperm has been identified in all examined species. It is always a monoflagellate cell, characterized by an elongated head. Elongated head has generally been correlated with the internal fertilization and/or to the production of spermatophore. As this is not the case of syngnathids, a possible function to explain the particularly elongated head of syngnathids is discussed.

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Introduction

The array of reproductive strategies amongst teleosts is extraordinarily diverse and, amongst them, those of syngnathids are peculiar. In this teleosts family, eggs are laid by the females on or into the male brooding structure, which has, according to the species, a different localization and extraordinarily diverse morphology (see Wilson and Orr 2011). Egg fertilization is assumed to take place after their deposition, but how sperm move to reach the eggs has not been completely understood yet.

In fish, adaptations for sperm transfer have involved the modification of the testis and sperm morphology (Burns *et al.* 1995, 2000), as well as the transformation of male fins or other body regions into intromittent organs (Meisner 2005; Burns and Weitzman 2006). The male reproductive apparatus of syngnathids still largely remains a mystery to investigators, and, at present, conflicting data are reported for both testis and sperm morphology.

Testes of restricted lobular and unrestricted tubular type have been reported in *Microphis brachyurus lineatus* (Kaup) (Miranda-Marure *et al.* 2004), *Syngnathus abaster* Risso and *Syngnathus acus* L. (Carcupino *et al.* 1999), respectively. In addition, in the last two species, the germinal epithelium has been reported to be organized in the typical spermatocysts (Carcupino *et al.* 1999), whereas in *Syngnathus schlegeli* Kaup, these structures seem to be absent, or at least, difficult to be recognized (Watanabe *et al.* 2000).

Large droplets-containing cells characterize the testis of several syngnathid species. These cells, however, are reported to have different localization. In *S. schlegeli*, they appear to be localized in the germinal epithelium only (Watanabe *et al.* 2000), whereas in *S. abaster* and *S. acus*, they have been also observed inside the lumen, where they have been interpreted as germinal cells in different developmental stages, associated with a semicystic spermatogenesis (Carcupino *et al.* 1999).

The functional sperm of most syngnathids examined up to now have been categorized as introsperm type (Watanabe

et al. 2000; Van Look *et al.* 2007; Biagi *et al.* 2008; Dzyuba *et al.* 2008), which has elongated head and long flagellum, and is typical of internal fertilizing fish (Jamieson 1991). This type of sperm is the unique type found in a brackish water population of *S. Abaster*, *S. acus* and *N. ophidion* (Carcupino *et al.* 1999; Ah-King *et al.* 2006). Only one type of sperm has also been reported in *M. brachyurus lineatus*. In this species, however, sperm seem to be of the aflagellate type (Miranda-Marure *et al.* 2004). *Syngnathus schlegeli* and *Hippocampus kuda* seem to have two sperm types: functional sperm of introsperm type and non-functional sperm of aquasperm type (Watanabe *et al.* 2000; Van Look *et al.* 2007). The latter, which is typical of external fertilizing fish (Jamieson 1991), is characterized by a large spherical head. Three different morphotypes of sperm, differing in flagellum length, head length and head wide, have been reported in a freshwater population of *S. abaster* (Dzyuba *et al.* 2008). Amongst them, however, only the longest seems to be functional.

To shed light on these topics, we analysed the male gonad of four internal brooder species belonging to the *Syngnathus* genus, using different light and electron microscopic techniques. We particularly intended to ascertain: (i) the testis structure and spermatogonia localization, (ii) the germinal epithelium organization, (iii) the morphology and localization of Leydig cells, and (iv) the modality of spermatogenesis and sperm structure.

Materials and Methods

Samples

Twenty-eight adult males belonging to the pipefishes species *Syngnathus abaster* (n = 24), *S. tenuirostris* (n = 3), *S. typhle* (n = 4) and *S. acus* (n = 4) were analysed.

S. abaster, the easiest species to be sampled, were collected in two Mediterranean lagoons (Cabras lagoon, in western Sardinia and Venice lagoon, Italy). *S. tenuirostris and S. typhle* were both collected in Venice lagoon. *S. acus* were collected in the sea near Alghero (Sardinia). All samples of the above species were captured during the reproductive period (May–September).

Different analyses with different purposes were used. In particular, for the description of the general organization of the testes, the following analyses were performed:

Histological techniques. Eight entire reproductive apparatuses (two for each species) were dissected, fixed in aqueous Bouin's fixative, dehydrated in a graded ethanol series, cleared in Bioclear and finally embedded in paraffin wax. Sections (5 μm) were stained with Mayer's Hemalum and Eosin (Mazzi 1977) and observed with a Zeiss Axiophot light microscope (ZEISS, Oberkochen, Germany).

In an attempt to observe a larger number of mature spermatozoa, hardly observable in thin sections of paraffin-embedded samples, some thicker cryosections were tested. Additional testes obtained from two males of *S. abaster* were fixed in 4% paraformaldehyde (pH 7.4) at 4 °C. Then, samples were washed in 0.4 M Sorenson's phosphate buffer (PBS) at least for 1 h (three changes of 20 min each). The testes were then transferred in 30% sucrose solution for 1 day. Afterwards, sections (20 μm thick), obtained using a cryostat (Microm Cryo-Star HM 560, Walldorf, Germany), were placed on electrostatically charged slides (Superfrost Plus, Menzel-Gläser) and stained with Haematoxylin and Eosin. After staining, all preparations were dehydrated in ethanol, mounted in Canada balsam and observed with a Zeiss Axiophot light microscopy. Digital images were obtained using a Canon Power Shot camera G2.

Transmission electron microscopy (TEM) for testis ultrastructure. Testes of both *S. abaster* and *S. acus* (two for each species) were dissected and fixed for 2 h in 4% paraformaldehyde-5% glutaraldehyde buffered with sodium cacodylate (0.1 M, pH 7.2). Specimens were then rinsed overnight in the same buffer, postfixed for 1 h in 1% osmium tetroxide buffered with sodium cacodilate, dehydrated gradually in ethanol and embedded in Epon 812 resin. Semi-thin and thin sections of 1 μm and 80 nm thick, respectively, were cut with a Reichert Ultracut ultramicrotome. Semi-thin sections were stained with toluidine blue and then photographed with a Canon Power Shot G6 camera, mounted on Zeiss Axiophot light microscope. Thin sections were stained with uranyl acetate and lead citrate, observed and photographed with a Jeol Tem 1200 EX II transmission electron microscope (JEOL, Tokyo, Japan).

Fluorescent microscopy. To evidence the muscular fibres of the tunica albuginea, muscle actin was labelled with Rh-phalloidin. Two testes of *S. abaster* were removed in phosphate-buffered saline (PBS) and fixed for 1 h at 4 °C with freshly prepared 4% paraformaldehyde in 0.1 M PBS, pH 7.4, thoroughly rinsed and permeated with Triton X-100. F-actin filaments were stained for 40 min at room temperature with Rh-phalloidin (Molecular probes, Eugene, Ore, USA) diluted 1 : 100. Nuclei were stained for 5 min with the nuclear dye DAPI (1 mg/mL in PBS, SIGMA). After final rinsing in PBS for 5 min, the samples were mounted in a mixture of 5% PBS + 90% glycerol + 5% propyl gallate and observed in a LEICA DMI 6000B microscope connected to a TCS SP5 confocal scanning system equipped with laser 405 DIODE (UV), ARGON, HeNe 543, HeNe 633. Video images were collected using ′10 and ′40 lenses.

For the luminal cells and sperm morphology, the following analysis was used:

Light microscopy. To analyse the different types of cells free in the testicular lumen, two testes for each species were dissected and kept in acetic orcein for 24 h, then gently squashed and observed with a Zeiss Axiophot light microscope.

Moreover, to obtain isolated luminal cells, testes of further 10 males of *S. abaster* and one of *S. typhle* were isolated and open with forceps. A small aliquot (20 μL) of 5% glutaraldehyde-fixed luminal cells was applied onto a coverslip precoated with polylysine (1 mg mL-1, Sigma P1274) and allowed to air-dry. Next, luminal cells adhering to the coverslip were stained with toluidine blue 0.1% in aqueous solution and observed with a Zeiss Axiophot light microscope (Zeiss). A pool of 60 polyflagellate cells, obtained from different slides of a single *S. abaster* specimen, were analysed to estimate the flagella numbers.

Scanning electron microscopy (SEM). The external morphology of cells free in the testicular lumen, including mature sperm, was analysed using an aliquot (20 μL) of formalin-fixed luminal cells obtained from testes of 4 *S. abaster* males and applied onto a coverslip precoated with polylysine. Next, luminal cells adhering to the coverslips were dehydrated using a graded ethanol series (5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% and 100% ethanol) and critical-point dried (Bal-Tec CPD 030) Critical Point Dryer, gold-palladium coated and observed with (JEOL JSM 5200; Jeol) scanning electron microscope.

Fluorescent microscopy. To evidence both the cytoplasmic and flagella microtubules, aliquots (20 μL) of luminal cells obtained from the testes of three males of *Syngnathus abaster* were applied onto a coverslip precoated with polylysine and allowed to dry. Then, the sample was fixed in methanol for 5 min at −20 °C, permeated in acetone for 5 min at −20 °C and rinsed in PBS. Fixed cells were then pre-incubated with PBS contains 1% BSA (bovine serum albumin) for 20 min, incubated in monoclonal anti-α-tubulin antibody, clone DM 1A (Sigma), diluted 1 : 400 for 1 h in the dark, then rinsed three times on PBS for 5 min, post-incubated in an anti-mouse-TRITH antibody (Sigma) diluted 1 : 200 for 1 h in the dark. Nuclei were stained for 5 min with DAPI. After final rinsing in PBS for 5 min, the samples were mounted in a mixture of 5% PBS + 90% glycerol + 5% propyl gallate and observed in a LEICA DMI 6000B microscope connected to the confocal system.

Morphometry. To study the nuclear morphometry of the different luminal cells types, two samples of *S. abaster* processed with acetic orcein (see above) were analysed. Thirty nuclei per each cellular type were measured.

Furthermore, to study the morphometry of the spermatozoa, such as the length of both the head (including the nucleus and the midpiece) and flagellum, intact spermatozoa, stained with toluidine blue (see above) and obtained from the two species, *S. abaster* (n = 200, 20 sperm per males) and *S. typhle* (n = 17, obtained by a single male) were analysed. Abnormal, broken or difficult to measure spermatozoa were discarded.

Digital images of the different luminal cell types and mature spermatozoa were acquired with a digital camera Nikon DS-FI1 connected with the control unit DS-L2 and mounted on an optical microscope Nikon Eclipse 80i (Nikon, Shinjuku, Japan). The measurements were made using the program Tpsdig2.

Results

The morphology of the testes was similar in all examined species. The paired testes were semi-translucent organs characterized by a large central lumen and a thin wall (Fig. 1A–C). The latter consisted of the germinal epithelium and a thin vascularized tunica albuginea (Figs 1A–C, 2, 3A).

In all examined species, the tissue of the tunica albuginea was continuous and did not pierce inside the organ, where intergerminal compartments were not observed. (Fig. 1A). The tunica albuginea had about the same thickness as the germinal epithelium and was characterized by connective tissue, rich in muscle fibres and blood vessels. (Figs 1A–C, 2). Fluorescent microscopic analysis on *S. abaster* testes showed that the muscle fibres were disposed in two layers: the internal layer, characterized by fibres oriented perpendicularly to the main axis of the testis, formed a continuous muscular sheath, and the external layer, with only few longitudinal fibres, formed an irregular weft (Fig. 2).

Moreover, between the tunica albuginea and the germinal epithelium, testes of all species had small groups of globular cells resting on the external surface of the basal membrane were visible (Fig. 1B). These cells, analysed in *S. abaster* and *S. acus* using the transmission electron microscopy, had rounded nuclei with one or two large nucleoli. Their cytoplasm was rich in both mitochondria with tubular crests and vesicles of smooth endoplasmic reticulum (Fig. 1D–E).

In all examined species, the germinal epithelium had the typical organization in spermatocysts, which were formed by germ cells enveloped by Sertoli cells, resting on the basal membrane (Figs 1A–C, 3). Sertoli cells were elongated and flat cells characterized by small nuclei of irregular shape (Figs 1B, 3). The transmission electron microscopic analysis on *S. acus* and *S. abaster* testes showed that, in both species, the Sertoli cells cytoplasm appeared more electron-dense than that of germ cells and contained mitochondria, both rough and smooth endoplasmic reticulum, free ribosomes and microvilli-like projections of the surface facing the lumen (Fig. 3B). Moreover, Sertoli cells of the same spermatocysts were joined together with tight-like junctions (Fig. 3B). Along the entire length of the testis, spermatocysts contained spermatogonia and primary spermatocytes (Figs 1A–C, 3). The latter were recognizable by the presence of numerous synaptonemal complexes (Fig. 3C–D). Neither secondary spermatocytes nor spermatids were observed.

In all examined species, inside the testicular lumen, large spherical cells and spermatozoa were embedded in a fibrous-like secretion (Figs 1A–C, 4–7). Two types of spherical cells,

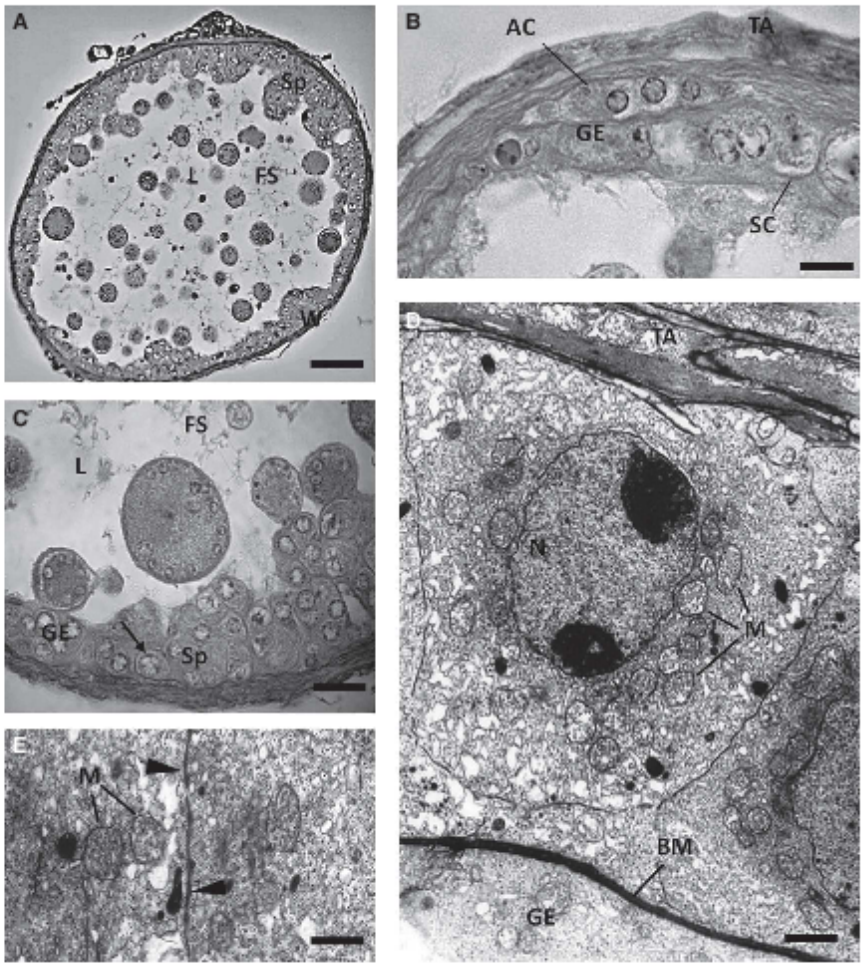

Fig. 1—**A–C**. Light micrographs of *Syngnathus temminckii* testis. —**A**. Transversal section showing large central testicular lumen and thin wall. —**B–C.** High magnification of the testis wall showing germinal epithelium, tunica albuginea and albuginea's basal cell. —**D–E**. Transmission electron micrographs of *Syngnathus acus* testis.—**D**. Albuginea's basal cell. —**E**. High magnification of adjacent albuginea's basal cells. (AC) albuginea's basal cell, (arrow) spermatogonia, (arrowheads) cellular junctions, (BM) basal membrane, (FS) fibrous secretion, (GE) germinal epithelium, (L) testis lumen, (M) mitochondria, (N) nucleus, (SC) Sertoli cell, (Sp) spermatocytes, (TA) tunica albuginea, (W) wall. Scale bar: **A** = 88 μm, **B** = 26 μm, **C** = 18 μm, **D** = 186 nm, **E** = 70 nm.

aflagellate and flagellate cells, were recognizable. In *S. abaster*, both cellular types were variable in size (diameter of 20 μm in average) and showed one, two or more nuclei (Figs 1A,C, 3, 4–6).

The aflagellate cells had the cytoplasm rich in droplets of different size and density. This made the observation of the nuclei difficult, which were well recognizable using a fluorescent nuclear dye (Fig. 4A,B). All aflagellate cells had nuclei (n = 30) of the same size (9 ± 0.64 μm) and morphology, and they occupied most of the cell volume (Fig. 4A,B). In contrast, the flagellate cells showed a less amount of cytoplasmic globules and had nuclei localized at the periphery (Figs 4C–F, 5, 6). These cells were unquestionably developing spermatids, and their nuclei were always characterized by a well-developed nuclear fossa, to which corresponded an emerging flagellum (Figs 4C–F, 6). Mono-, bi- and multiflagellate cells were visible (Figs 4C–F, 5–6). Of 60 flagellated cells, obtained by different slides of the same specimen of *S. abaster* and analysed by light microscopy, only 2% had a single nucleus, 32% showed two nuclei, about 6% had more than ten nuclei, and the remaining 60% had a variable number of nuclei, from 3 to 10.

In all examined species, on the basis of nuclear morphology, young, intermediate and late spermatids were distinguished. In *S. abaster*, young spermatids had spherical nuclei of 7.3 ± 1.09 μm in diameter, with one or two spherical dots of nucleolus-like appearance (Fig. 6A). Zones of condensed chromatin were particularly visible at the periphery of the nucleus and in the nuclear fossa region (Figs 4D, 6A).

The intermediate spermatids had smaller nuclei of 3.19 ± 0.57 μm, still spherical in shape and similar morphology, but inside them the nucleolus-like dots were no more evident (Fig. 6B,C). The late spermatids had nuclei of subspherical shape (3.59 ± 0.36 μm) and chromatin uniformly condensed (Fig. 6D,E).

Mature sperm were measured in *Syngnathus abaster* and *S. typhle* only (Figs 4G, 7). In both species, they were elongated monoflagellate cells with flagella of several tens of microns (54.82 ± 3.32 μm, n = 200) in *S. abaster* and (58.37 ± 2.7 μm, n = 17) in *S. typhle*. They were characterized by a cylindrical head occupied for most of its length by the nucleus of 3.25 ± 0.14 μm (n = 200) in *S. abaster* and of 3.35 ± 0.29 μm (n = 17) in *S. typhle*. A short midpiece was evident (Fig. 7A–C, insert).

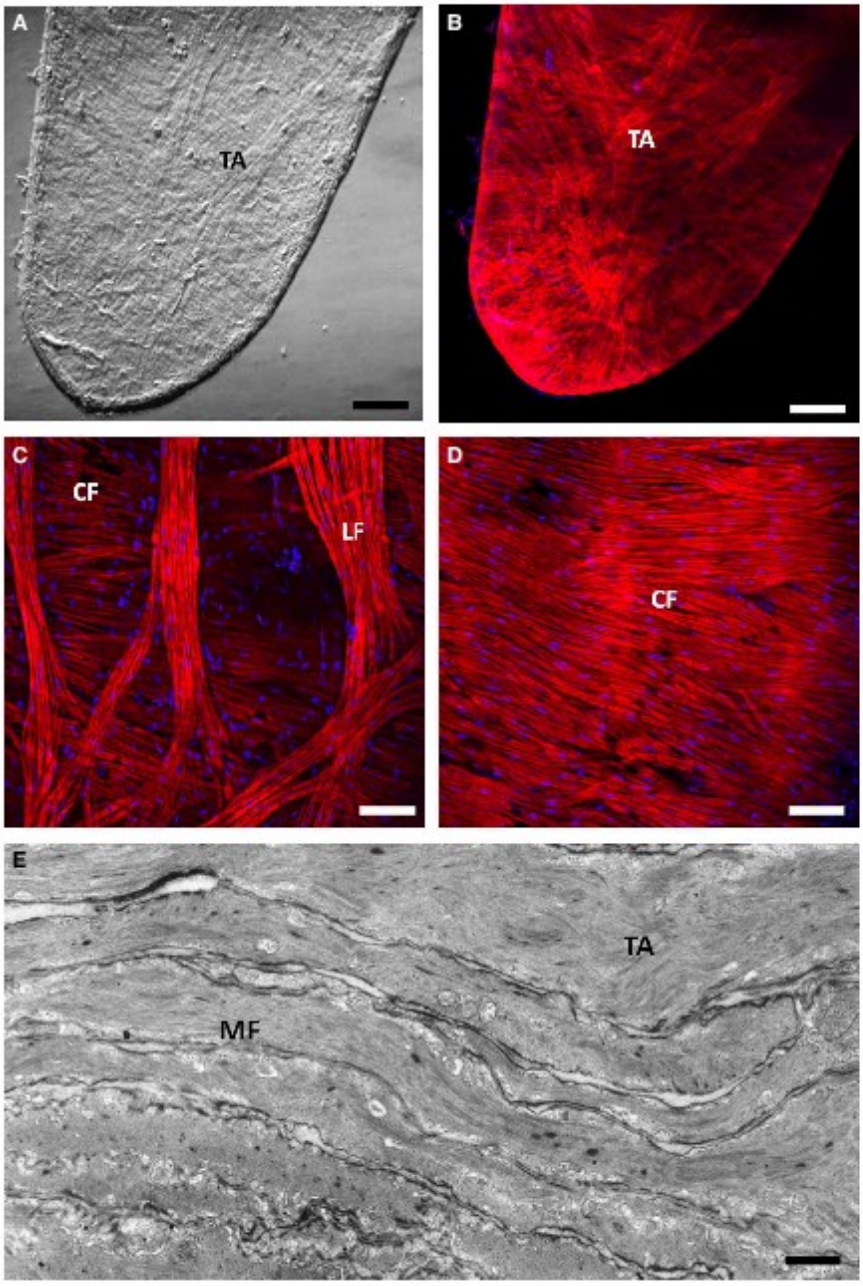

Fig. 2—A. Light micrographs of the apical portion of *Syngnathus abaster* testis. —**B**. Confocal laser Scanning Microscopy (CLSM) images of the same portion of testis showing the F-actin localization in the tunica albuginea muscular sheaths. —**C–D**. High magnification of the same testis portion showing the doubles fluorescent stain with nuclei in blue and F-actin filament in red. —**C**. Note the different arrangement of muscular fibres in the two internal and external sheaths. —**E**. Transmission electron micrograph of muscle fibres characterizing the tunica albuginea of *S. acus* testis. (CF) circular muscular fibres, (LF) longitudinal muscle fibres, (MF) muscle fibres, (TA) tunica albuginea. Scale bar: **A–B** = 71 μm, **C–D** = 18 μm, **E** = 120 nm.

Discussion

In all syngnathid species here analysed, the testis atypically consists of a single and continuous germinal compartment surrounded by a single and continuous somatic compartment. Each testis appears as a tubular organ characterized by a unique testicular lumen surrounded by two concentric layers, the thick tunica albuginea and the germinal epithelium, separated by the basement membrane. In all other vertebrates, from fish to mammals (see Schulz *et al.* 2010), the testis has a more complicated organization being the germinal compartment generally arranged in numerous germinal units, amongst which numerous intergerminal extensions of somatic compartment are present.

The tunica albuginea of syngnathids testes contains blood vessels, connective cells and numerous smooth muscle fibres. Moreover, small groups of cells are visible in close contact with the external surface of the basal membrane. Large cells surrounded by connective tissue have been also seen in *M. brachyurus lineatus*, but they were interpreted as primary spermatogonia (Miranda-Marure *et al.* 2004). However, we retain that both their localization and cytoplasmic characteristics, such as mitochondria with tubular crests and abundant vesicle of smooth endoplasmic reticulum, are consistent with the interstitial Leydig cells.

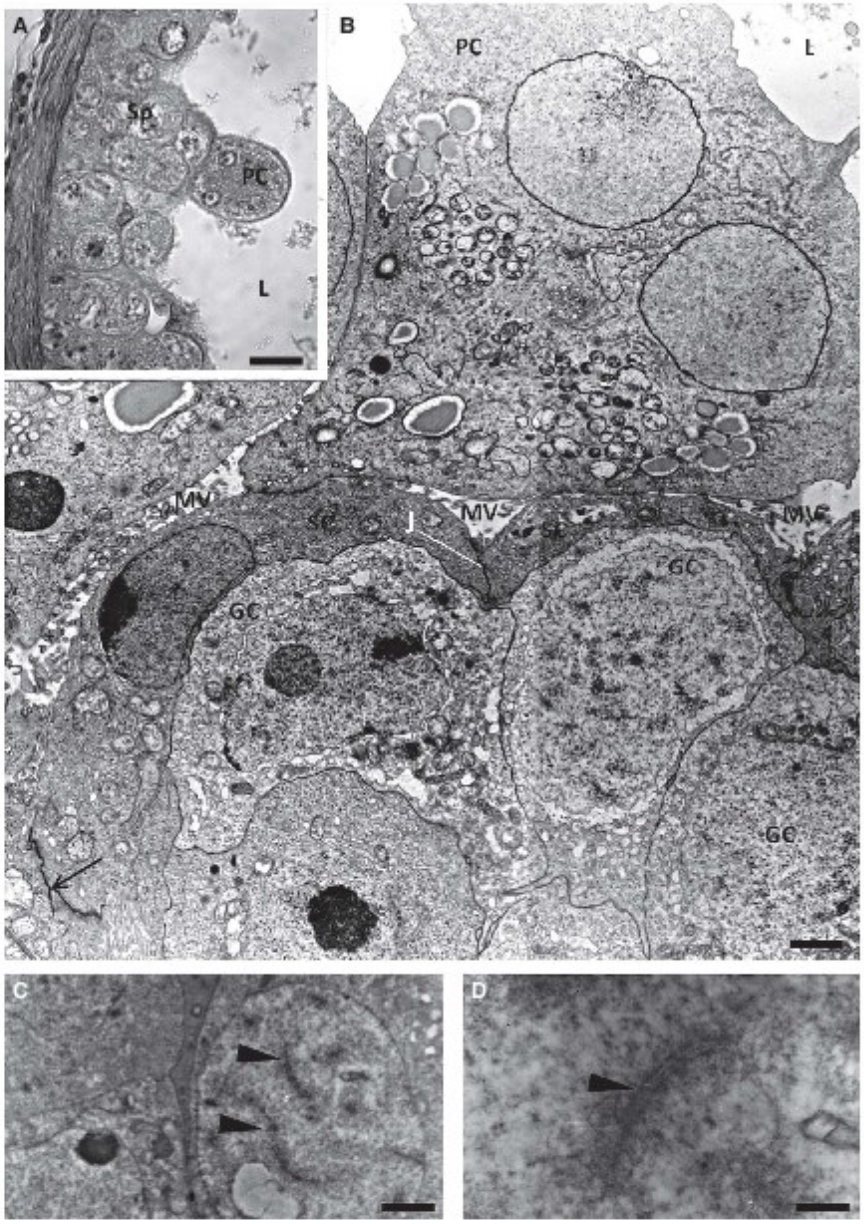

Fig. 3—**A**. Light microscopy image of *Syngnathus abaster* testis showing spermatocysts containing spermatocytes and polynucleated cells inside the lumen. —**B**. Transmission electron micrograph of *Syngnathus acus* spermatocysts with germ cells enveloped by Sertoli cells. —**C**. Transmission electron micrograph of primary spermatocytes of *S. acus*. —**D**. High magnification of synaptonemal complex. (Arrow) basal membrane, (arrowheads) synaptonemal complex, (GC) germ cells, (J) tight-like junctions, (L) lumen, (MV) microvilli-like projections, (PC) polynucleated cell, (SC) Sertoli cells, (Sp) spermatocytes. Scale bar: **A** = 16 μm, **B** = 1 μm, **C** = 130 nm, **D** = 30 nm.

Because of the presence of an atypical permanent lumen, the syngnathids testis was defined as belonging to the tubular type (Carcupino *et al.* 1999). However, according to the better elucidated definition reported in Parenti and Grier (2004), testes of tubular type are only those in which 'the germinal compartments do not terminate at the testis periphery, but form highly branched, anastomosing loops or tubules'. Instead, testes of lobular type are those in which 'the germinal compartments may form anastomosing networks proximally, but distally they extend to the periphery of the testis and terminate blindly'. On this basis, testes of the syngnathids here analysed, must be included in the lobular type, as are formed by a single germinal compartment, without forming any kind of anastomosing networks and terminating blindly.

Spermatogonia are clearly distributed along the entire length of the testis, so we may confirm that also in these representatives of Syngnathidae, the testis organization is of the unrestricted lobular type, typically found throughout the Neoteleostei, except for the atherinomorphs (Parenti and Grier 2004).

Both the light and electron microscopic analyses definitely demonstrate that at least in *Syngnathus* species, the germinal epithelium has the typical tripartite organization, in which germ cells surrounded by Sertoli cells form spermatocysts resting on the basal lamina. Sertoli cells are easily recognizable from germ cells because of their elongated and flat shape, small nuclei of irregular shape and their capacity to form tight-like junctions with adjacent Sertoli cells belonging to the same spermatocysts.

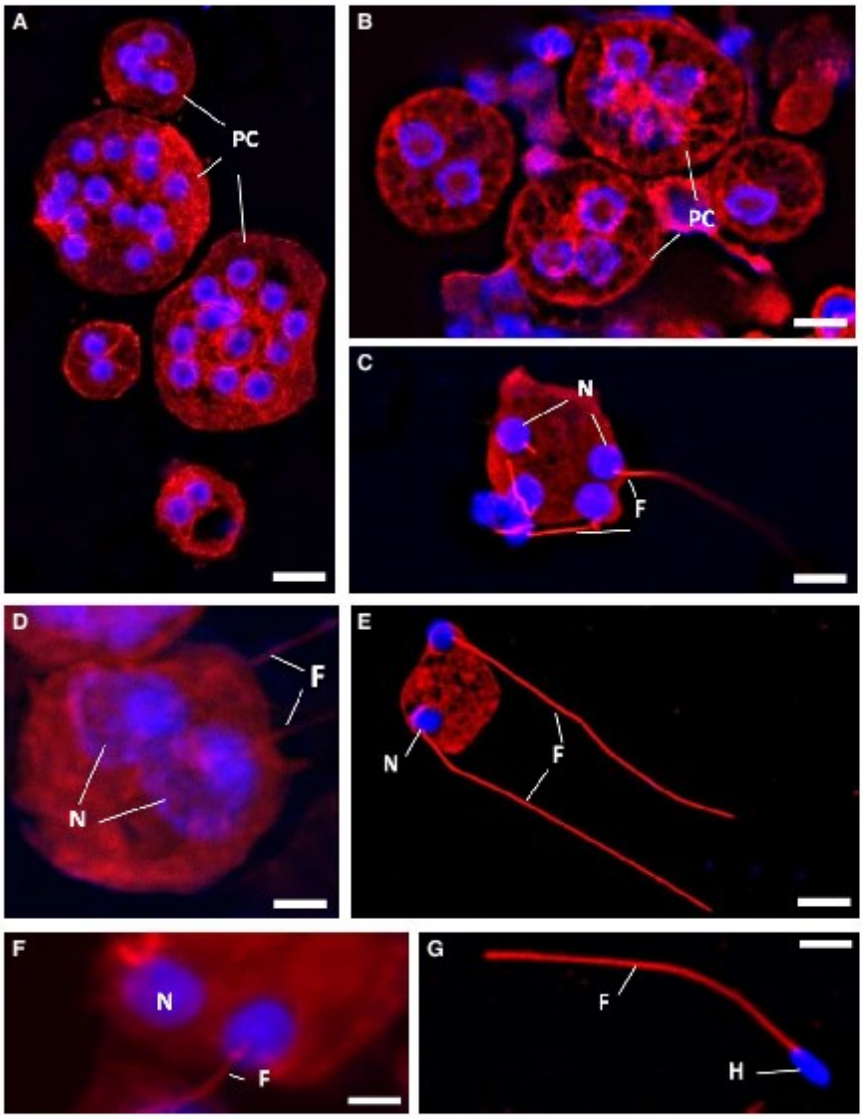

Fig. 4—Confocal laser scanning microscopy (CLSM) images of luminal cells obtained from *Syngnathus abaster* testes showing the dual fluorescent stain with nuclei in blue and microtubules in red. —**A–B**. Aflagellate polynucleated cells. —**C**. Polynucleated spermatids showing nuclei localized at the periphery of the cells and emerging flagella. —**D–F**. Spermatids in different developmental stages. —**D**. Young spermatids with nuclei characterized by zones of condensed chromatin particularly visible at the nuclear periphery and in the fossa region. —**E**. Intermediate spermatids with more condensed nuclei. —**F**. Late spermatids with nuclei of subspherical shape. —**G**. Mature sperm with cylindrical head. (F) flagella, (H) head, (N) nuclei, (PC) polynucleated cells. Scale bar: **A** = 15 μm, **B** = 3 μm, **C** = 9 μm, **D** = 2 μm, **E** = 9 μm, **F** = 2 μm, **G** = 3 μm.

In agreement with previous data (Carcupino *et al.* 1999), in the germinal epithelium of all reproductive males here analysed, the spermatocysts only contain spermatogonia and primary spermatocytes. Developing spermatids are recognizable by both the presence of flagella emerging from a deep nuclear fossa and nuclei characterized by different degrees of chromatin condensation. They are only visible inside the testis lumen together with matures sperm. These data confirm that, at least in *Syngnathus* pipefishes, the spermatogenetic process is of semicystic type.

Generally, the spermatogenesis of bony fish is cystic, and therefore, the process takes place entirely inside the cyst, when the cyst breaks the spermatozoa are released into the lumen. In contrast, in the semicystic type, the cyst ruptures at the spermatocyte or spermatid stage, so germ cells only partly develop inside the cyst (Mattei and Mattei 1978). This type of spermatogenesis may result in asynchronous maturation of spermatids, thereby reducing the number of simultaneously mature sperm.

Semicystic spermatogenesis was described for the first time in some Gobiesocidae (Mattei and Mattei 1978) and is currently known in several teleostean groups, such as Blenniidae (Lahnsteiner and Patzner 1990; Lahnsteiner *et al.* 1990; Giacomello *et al.* 2008), Opistognathidae (Manni and Rasotto 1997), Channidae (Srivastava and Singh 1994), Gobiidae (Mazzoldi 2001), the Ciprinodontiformes *Fundulus heteroclitus* (Selman and Wallace 1986), the Ophidiiformes *Ophidion sp.* (Mattei *et al.* 1993) and *Ophidion barbatum* (Hernández *et al.* 2005), the Lofiiformes *Lophiomus setigerus* (Yoneda *et al.* 1998a), some Scorpeniformes (Muñoz *et al.* 2002; Sàbat 2002; Sàbat *et al.* 2009), the Siluriformes, *Malapterurus electricus* (Shahin 2006), the Caraciformes, *Acestrorhynchus lacustris* (Bazzoli and Godinho 1991), *Bryconops affinis* (Andrade *et al.* 2001) and *H. marginatus* (Magalhaes *et al.* 2011), and the

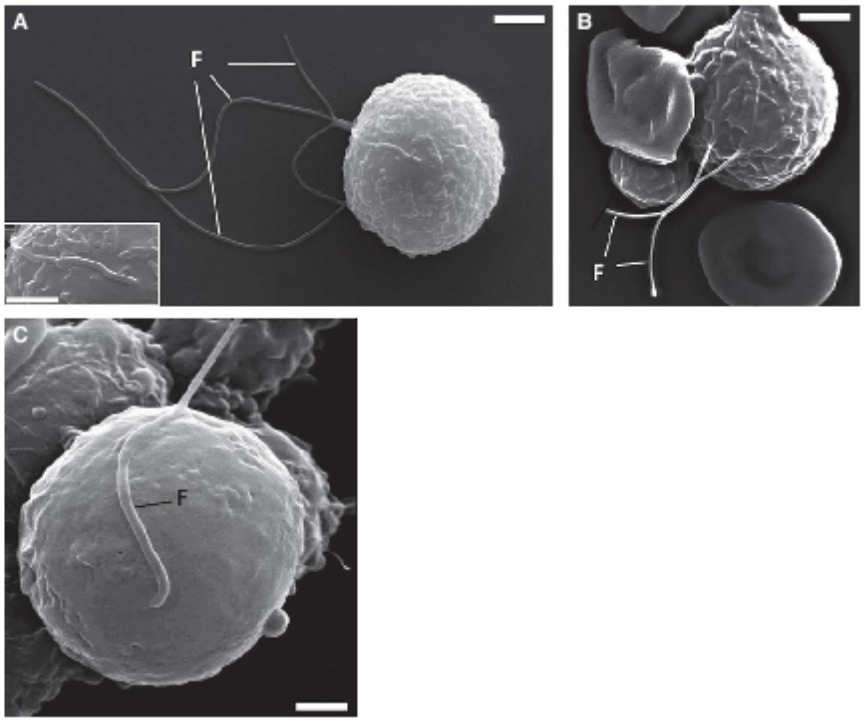

Fig. 5—Scanning electron microscopy images of flagellate cells obtained from *Syngnathus abaster* testis lumen. —**A–C.** Mono-, bi- and multiflagellate spermatids. (F) flagella**.** Scale bar: **A** = 2.7 μm, Insert = 1.3 μm, **B** = 1 μm, **C** = 1 μm.

Pleuronectiformes, *Solea senegalensis* (García-López *et al.* 2005).

Several hypotheses have been formulated regarding the functional significance of semicystic spermatogenesis. This type of spermatogenesis leads to a reduced number of simultaneously mature sperm, and it has been interpreted as one of the possible mechanisms evolved to reduce the cost of sperm production. This is the case of species, such as syngnathids, performing male parental care, in which the energies devoted to reproduction cannot all be invested by the male in sperm production. (Manni and Rasotto 1997; Giacomello *et al.* 2008). This mechanism seems therefore to be crucial in those species in which, a small ejaculate size is justified by their low fecundity, monogamous mating system, absence of sperm competition and presence of male parental care, such as some Opistognathidae (Rasotto *et al.* 1992; Marconato and Rasotto 1993) and Gobiidae (Mazzoldi 2001). However, the asynchronous maturation of spermatids, thereby reducing the number of simultaneously mature sperm, related to a spermatogenetic process of the semicystic type, has been hypothesized to be also useful in those species showing a promiscuous mating system and/or a long-lasting egg deposition (see some blennies). In such species, the males are forced to parcel out their sperm expenditure during mating into several successive ejaculates, releasing only limited portions of sperm at each ejaculation (Giacomello *et al.* 2008). All syngnathid species have very small ejaculate size, low fecundity, male parental care, absence of sperm competition, and their mating system varies from monogamy to different types of polygamy (Wilson *et al.* 2003; Sanna *et al.* 2008).

It should be also mentioned that the semicystic spermatogenesis has been associated to other functions. According to Muñoz *et al.* (2002), in some species (see *Ophidion marginatum*, *Lophiomus setigerus* and *Scorpena notata*) that lay eggs within a gelatinous mass, such as syngnathids, (Fahay 1992; Yoneda *et al.* 1998b), this type of spermatogenesis could be in some way related to the secretion of abundant thick seminal fluid, having function in keeping the spermatozoa together, facilitating fertilization of the whole egg mass. In other species, such as Blenniidae, the male gonads exhibit a peculiar organization, consisting of a strictly spermatogenetic part (testicular lobules or testis) and a glandular part (testicular gland) (Lahnsteiner *et al.* 1990; Richtarski and Patzner 2000). These species show spermatogenesis of the semicystic type and a high interspecific variability associated with the level of semicystic spermatogenesis (with spermatids released at different stages of maturity) (Lahnsteiner and Patzner 1990) and with the relative development of the two gonad parts (Richtarski and Patzner 2000). Species in which spermatids are released at a very late stage of maturation have a reduced testicular gland, which has been supposed to be exclusively related to the seminal fluid production. In contrast, species where spermatids are released at an earlier stage of maturation, have more developed testicular gland involved in both seminal fluid production and metabolic supply of germ cells (Giacomello *et al.* 2008). In relation to this last aspect, it should not be forgotten that in the semicystic spermatogenetic process, Sertoli cells cannot regulate and support the metabolites transfer towards the developing germ cells, when the latter are free inside the

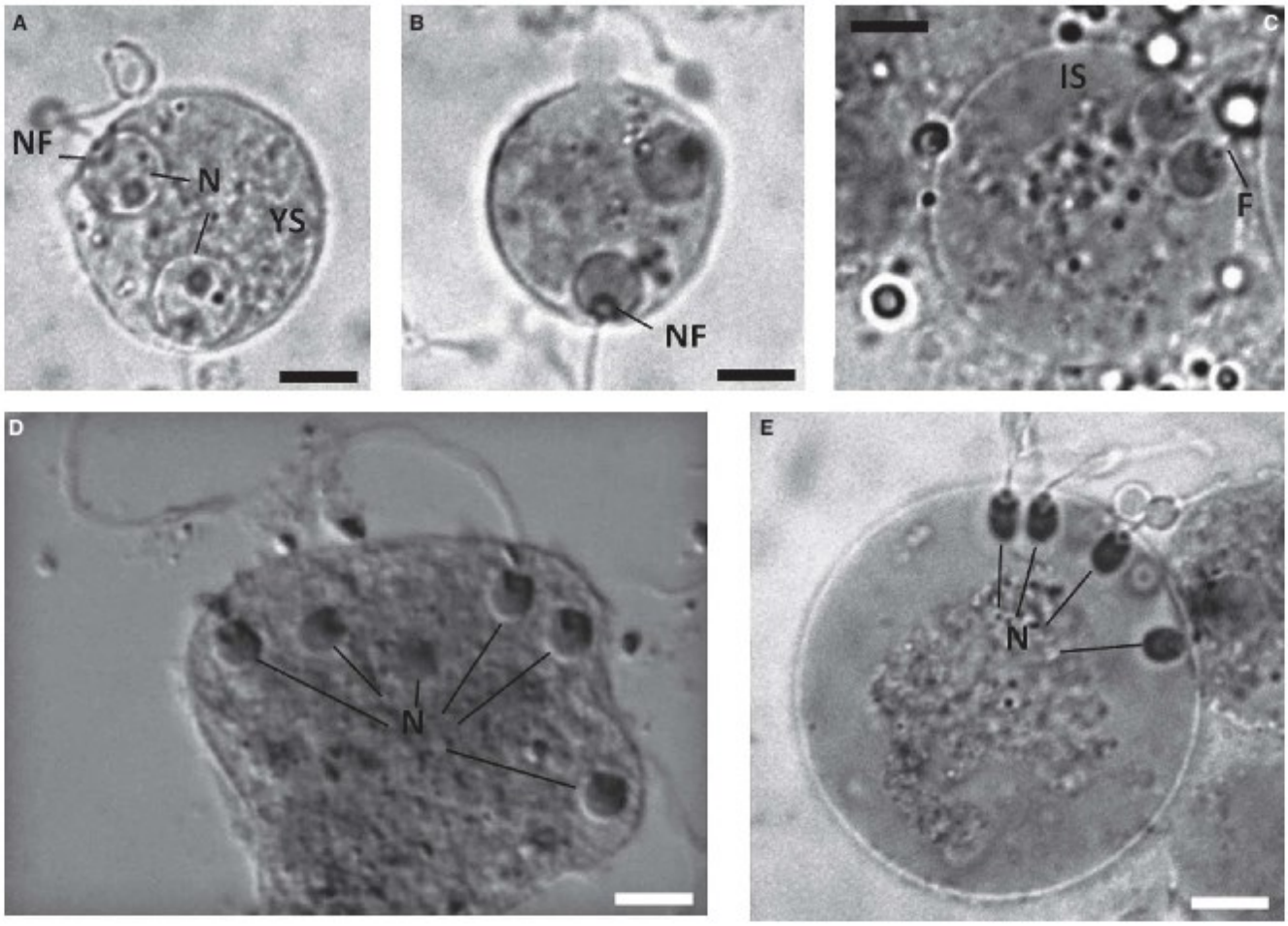

Fig. 6—A–E. Light microscopy images of *S. abaster* spermatids stained with acetic orcein at different developmental stages. –**A.** Young spermatids. —**B–C.** Intermediate spermatids; —**D–E.** Late spermatids. (F) flagella, (IS) intermediate spermatids, (N) nucleus, (NF) nuclear fossa, (YS) young spermatids. Scale bar: **A** = 11 μm, **B** = 10 μm, **C** = 12 μm, **D** = 10 μm, **E** = 12 μm.

lumen. So, their metabolic demands depend on their stage of maturity at the moment of the cyst rupture.

In the typical semicystic spermatogenesis, spermatocytes and/or spermatids are released after the cytokinesis, amongst isogenetic germ cells, is completed. Therefore, germ cells advance individually through spermiogenesis inside the lumen. As previously documented in *Syngnathus abaster* and *S. acus* (Carcupino *et al.* 1999), and here confirmed in the same and in other species of the same genus, developing germ cells inside the lumen are mono- and, more frequently, polynucleate and polyflagellate cells. Individualization of mature sperm seems to occur at the end of spermiogenesis, so the cytokinesis seems to be abolished or at least delayed. Moreover, together with developing spermatids and mature spermatozoa, another type of polynucleated cells is present inside the testicular lumen of *Syngnathus* species. These cells are aflagellate and characterized by a large amount of cytoplasmic droplets. Large droplets-containing cells have also been reported in *S. schlegeli* testis (Watanabe *et al.* 2000). In this species, however, this kind of cells seems to be only recognizable inside the germinal epithelium. In contrast, in the reproductive males here analysed, these cells are frequently observed free into the testicular lumen, but they are also recognizable coming out from the epithelium. On the basis of these data, the large droplets-containing cells of *S. schlegeli* testis are likely of the same cellular type reported here. The apparent discrepancy in their localization may only be due to a different developmental stage of the males analysed.

A smaller amount of droplets of different size and electron density may be also recognizable in the cytoplasm of developing polynucleated and flagellate spermatids, so the present data seem to support the hypothesis (Carcupino *et al.* 1999) that aflagellate cells may be germ cells. These cells could therefore represent the youngest germ cells released inside the lumen at the spermatocyte or very early spermatid stage, after having accumulated a large amount of material in form of droplets. These droplets progressively reduce in size and number during the germ cell maturation.

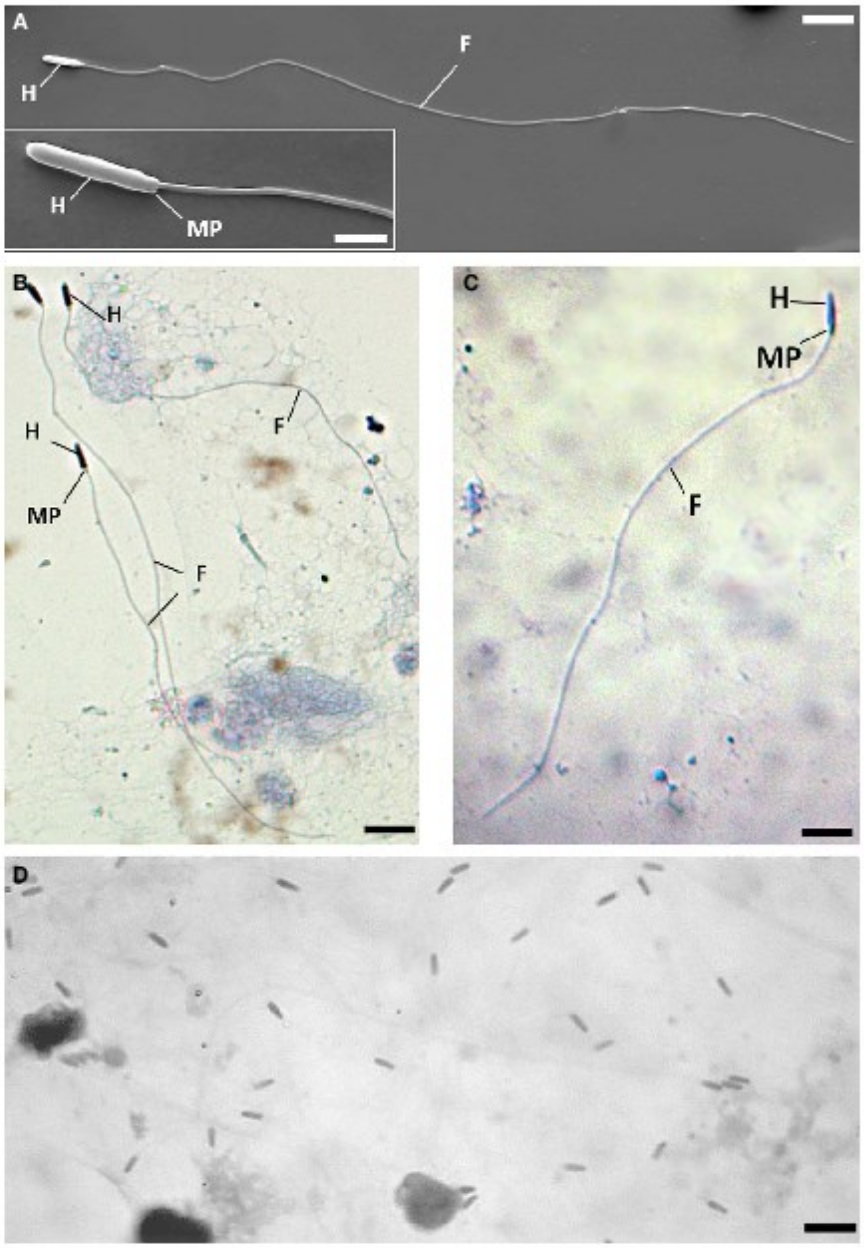

Fig. 7—**A**, Insert. Scanning electron micrographs of mature sperm of *Syngnathus abaster*. —**B**–**C**. Mature sperm stained with toluidine Blue of and *S. abaster* and *S. typhle* respectively. —**D**. Cryosection of *S. abaster* testis showing several sperm inside the lumen. (F) flagellum, (H) head, (MP) short mid-piece. Scale bar: **A** = 7.5 μm, Insert = 1.3 μm, **B** = 5 μm, **C** = 2.4 μm, **D** = 6 μm.

No glandular structures have been identified in the testis and along the sperm ducts of *Syngnathus* species (data not shown). Consequently, we consider that the large amount of droplets accumulated in the cytoplasm of germ cells could have a dual role in: i) the formation of an abundant and fibrous seminal fluid, which could have the function for trapping the very low number of mature sperm produced by syngnathids (Watanabe *et al.* 2000; Van Look *et al.* 2007; Biagi *et al.* 2008; Dzyuba *et al.* 2008), avoiding sperm loss during mating and ii) the metabolic supply of the developing germ cells, which are released in a very early spermatogenetic stage.

In the light of these two possible functions, it could be also interpreted the functional significance of the delayed cytokinesis observed in the semicystic spermatogenesis of *Syngnathus* species. An early cytoplasmic division amongst isogenetic cell could lead to a reduction in the amount of cytoplasm and organelles. Consequently, the production and accumulation of material useful for the energy requirements of each germ cell and the production of a sufficient amount of seminal fluid may be endangered.

In agreement with our previous data (Carcupino *et al.* 1999), only one type of sperm has been recognized in all *Syngnathus* species here analysed. The data on mature sperm morphology previously reported in Syngnathidae, such as the aflagellate sperm in *M. brachyurus lineatus* (Miranda-Marure *et al.* 2004) and the dimorphic flagellate sperm in *S. schlegeli* and *Hippocampus kuda* (Watanabe *et al.* 2000; Van Look *et al.*

2007) that are conflicting, may be therefore due to the semicystic spermatogenesis, which determines the simultaneous presence of different kinds of flagellate and aflagellate cells in the testicular lumen.

The most particular feature of mature sperm is the elongated shape of the head, a morphology that has also been observed in some Blenniidae (Lahnsteiner *et al.* 1990) in *Lepadogaster lepadogaster* (Mattei and Mattei 1978), and in *Ophidion barbatum* (Hernández *et al.* 2005), all species with external fertilization and semicystic spermatogenesis. In general, spermatozoa with elongated heads are related to internal fertilization (Jamieson and Leung 1991), an explanation that does not match any of the mentioned species. According to Burns *et al.* (1995), the elongated nucleus may also facilitate the storage of the spermatozoa in the testicular ducts. Nevertheless, in the specific cases of *O. barbatum* (Hernández *et al.* 2005) and *Syngnathus* species (data not shown), no packaging of spermatozoa was observed. Because neither have these species internal fertilization nor spermatophores production, a third possibility to explain the particularly elongated head of sperm could be their need to cross through the gelatinous mass of maternal origin to reach the eggs.

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II

Intra- and inter-males variability of mature sperm traits analysed in two brackish water populations of the pipefish Syngnathus abaster (Syngnathidae)

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Running head: Sperm morphometric variability in *S. abaster*

Abstract

Sperm cells are highly diversified in animals and considerable research effort has focused on variation in sperm morphology among species. Surprisingly little is known about intraspecific variation in sperm morphology. We analysed within- and between-male variation in mature sperm traits in two brackish water populations of the pipefish Syngnathus abaster. Four morphometric parameters, such as the width and length of the head (including nucleus, and midpiece), length of flagellum and total sperm length were taken into account. The differences in all morphometric parameters analysed between populations were not statistically significant. Moreover, the MDS analysis shows that: i) the two populations seem to be indistinguishable based on their spermatozoa and ii) there is not polymorphism, being sperm not distinguishable into discrete classes both within a single male and between males of each populations. The latter datum does not seem to support the presence of polymorphic sperm in syngnathids. Both populations, however, exhibit a high variation in all sperm traits, both among individual sperm within an ejaculate and among males within each population. The relationship between sperm traits variability and the low selection pressure, determined by the absence of postcopulatory sexual selection (i.e absence of sperm competition) is discussed.

Introduction

Commonly, male gametes in one species are unified in a single morph-type, but the production of multiple morphs within a species occurs, to some extent, in both animals and plants (Till-Bottraud *et al.* 2005). The phenomenon of the production of multiple discrete morphological classes in male gametes within a single species is termed sperm/pollen polymorphism (heteromorphism).

In the animal kingdom, sperm polymorphism can be distinguished in the following categories: heteromorphic sperm *sensu stricto*, which are regularly produced through a constant developmental process during spermatogenesis, and aberrant sperm, which are irregularly formed by some errors at certain stages during spermatogenesis (Tachi 1990; Swallow and Wilkinson 2002). The former are called "parasperm",in opposition to "eusperm" (functional sperm) (Healy and Jamieson 1981; Jamieson 1987), and the seconds are called "deformed sperm". Both parasperm and deformed sperm are generally non-fertile sperm. According to Hayakawa (2007), deformed sperm must be clearly distinguished from parasperm and the main differences between them can be seen in the more regular process of development and in a lesser variability of morphological traits of the parasperm respect to deformed sperm.

The occurrence of parasperm has been widely confirmed in invertebrates: Annelida (Oligochaeta), Rotifera, Pogonophora, Mollusca, and Arthropoda (Jamieson 1987; Swallow and Wilkinson 2002; Hayakawa 2007),whereas, there are few reports concerning parasperm in vertebrates, being, most of the polymorphic spermatozoa reported, apparently abnormal spermatozoa (Van der Horst *et al.* 2011).

Among teleosts, parasperm are reported only in few species, including some species of Syngnathidae family, in which the semen contained, together with functional sperm (eusperm), parasperm. Teleost parasperm varies in morphology among species. In cardinal fish (Apogonidae) and in a marine cottoid, (*Blepsias cirrhosis*), parasperm possessed two flagella, (Mattei and Mattei

1984; Lahnsteiner 2003 and Fishelson *et al.* 2006; Hayakawa 2007), whereas in another non copulatory marine cottoid (*Hemilepidotus gilbert*) and in a marine sculpin (*Cottus kazika*), parasperm are unflagellate cells (Hayakawa 2007; Daisuke *et al.* 2010).

Syngnathidae is a small fish family characterised by extremely specialized paternal care; males take care of the embryos carrying eggs on the ventral surface of the body (external brooders), or inside a specialized brooding cutaneous organ (internal brooders), commonly referred as the brood pouch, (Herald 1959; Carcupino *et al.* 1997, 2002; Watanabe *et al.* 1999; Monteiro *et al.* 2005).

The functional spermatozoa of all syngnathids examined up to now (Carcupino *et al.* 1999; Watanabe *et al.* 2000; Ah-King *et al.* 2006; Van Look *et al.* 2007; Biagi *et al.* 2008; Dzyuba *et al.* 2008; Biagi *et al.* 2014) may be considered belonging to the "introsperm type" (according to the classification of Jamieson (1991). This sperm type is a mono-flagellate cell with an elongated nucleus, and it is typical of internal fertilizing fishes (Jamieson 1991). In the testis lumen of *Syngnathus schlegeli* and *Hippocampus kuda* (Watanabe *et al.* 2000; Van Look *et al.* 2007), eusperm are seen to be free together with another kind of sperm, similar to those categorised as "aquasperm" type. The acquasperm are characterized by a spherical head and are typical of external fertilizing fish (Jamieson, 1991). Moreover, three different morphotypes, differing in flagellum length, head length and head wide, have been reported in a freshwater population of *Syngnathus abaster* (Dzyuba *et al.* 2008).

It should be pointed out that clear criteria to identify parasperm must be used in studies of parasperm in fish. Parasperm of teleosts need to be recognized by comparing cells in the cyst and within the semen, because irregularly shaped cells in the seminal fluid could be either parasperm or normal spermatids (Hayakawa 2007). Some teleosts, including syngnathids, at least those belonging to the *Syngnathus* genus (Carcupino *et al.* 1999; Biagi *et al.* 2014) and *Phyllopteryx taeniolatus* (Forsgren and Young 2009), show a spermatogenetic process of the semicystic type. This type of spermatogenesis results in an asynchronous maturation of spermatids, with the

simultaneous presence of germ cells at different stages, inside the testis lumen (Mattei 1993; Hayakawa 2007; Biagi *et al.* 2014).

Because the semicystic spermatogenesis, at least in *Syngnathus schlegeli*, the acquasperm may not be parasperm but, more likely, developing spermatids, as it has been also admitted by Watanabe *et al.* (2000). However, Dzyuba *et al.* (2008) reject this hypotheses for the shorter spermatozoa of *Syngnathus abaster*. These authors asserted that "…the sperm head dimensions were not so high variable as might have been expected if the short spermatozoa were actually developing spermatids".

The aim of the present study is to shed light on this topic examining the variation in sperm morphometric within male, within a population (between males) and between two brackish water populations of *Syngnathus abaster*.

Materials and Methods

Samples

Adult males of *Syngnathus abaster* were collected in two Mediterranean lagoons (Cabras pound, in western Sardinia and Venice lagoon, Italy). All samples (N=12 for Sardinian population, and N= 10 for Venice population) were captured during the reproductive period (May-September, 2013).

Sperm morphology

Sperm morphology was analysed using both Light (LM) and Scanning Electron microscopy (SEM).

For LM analysis, aliquots (20 µl) of un-fixed seminal fluid, obtained from testes of one male for each population, were placed on slides and immediately observed with a Zeiss Axiophot light microscope using the Nomarski Interference Contrast (NIC).

For SEM analysis, aliquots (20 µl) of formalin-fixed seminal fluid, obtained from testes of one male of the Sardinian population, were applied onto a coverslip pre-coated with poly-lysine. Next, germinal cells adhering to the coverslips were dehydrated using a graded ethanol series (5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 95 and 100% ethanol) and critical point dried in a Critical Point Dryer (Bal-Tec CPD 030), gold-palladium coated and observed with a scanning electron microscope(JEOL JSM 5200).

Sperm morphometric

Aliquots (20 µl) of seminal fluid, fixed in 5% glutaraldehyde, was placed on slides pre-coated with poly-lysine, stained with toluidine blue to 0.1% in aqueous solution and allowed to dry to air before being analysed to obtain digital images and related measures, using an optical microscope. Digital images were acquired with a digital camera Nikon DS-fi1 connected with the control unit DS-L2 and mounted on an optical microscope Nikon Eclipse 80i. The measurements were made using the program Tpsdig2.

Four morphometric parameters, such as the width and length of the head (including the nucleus, and the midpiece), the length of the flagellum and the total sperm length, were taken into account. The last one was calculated as the sum of head and flagellum length. Twenty intact sperm were analysed for each male (N= 10 for Sardinian population and N=9 for Venice population). Sperms damaged, or difficult to measure, were discarded. As a standardized measure of variation, the coefficient of variation (CV = (SD/mean)×100) were used, symbolising as CVbm the between-male CV in mean sperm traits and CVwm the mean within-male CV in sperm traits. This was done for all males in all populations (table 2). Given that the CV of small samples tends to be underestimated, the formula of CVbm was adjusted as suggested by Sokal and Rohlf (1995) and by Laskemoen *et al.* (2007) in the following way: $CV_{adj} = CV \times (1 + (1 / 4N))$.

Statistical analyses

The averages of all parameters in the two populations were compared using the t-Student. Moreover, a metric multi-dimensional scaling (MDS) to examine the differences in sperm population was carried out using the PRIMER statistical package version 6.0. Differences within a single male were estimated analysing 30 sperm, obtained by a single male randomly chosen among Sardinian males. Differences within each *S. abaster* populations were estimated analysing 200 sperm for the Sardinian population, and 180 for the Venice population. Differences between populations were estimated analysing 200 sperm obtained pooling 100 sperm, randomly selected, for each population.

Results

The morphological analysis showed that mature and normal sperm of both population analysed were very similar. All analysed mature sperm were mono-flagellate cells characterized by a cylindrical head, entirely occupied by the nucleus, followed by a short midpiece, particularly visible in the SEM images (Fig. 1A-C). Due to semicystic spermatogenesis, full-developed sperm and developing spermatid are both free in the testis lumen (Fig. 1 D). The latter, are uni- and more frequently multinucleated cells, with a unique flagellum emerging from each nucleus (Fig. 1D-E). Moreover, several abnormal sperm, such as those having a very short flagellum were observed, but their unusual appearance (Fig. 1 F) was clearly related to a damage during handling (i.e. the flagellum appeared clearly broken, because lacking of its terminal end).

The morphometric analysis of full-developed germ cells evidenced that the differences in the average value of the four sperm parameters analysed in the two populations were not statistically significant (Table 1). The average values of three characters (width and length of the head and length of the flagellum) measured in all males of the two populations (Figs. 2-4), show a variation range equal:

i) from 0.68 to 0.85 µm for the average width of the head in the Sardinian population and 0.63-0.76 µm in the Venice population (Fig. 2);

ii) from 3.02 to 3.52 µm for the average length of the head in the Sardinian population and 3.06 - 3.87 µm in the Venice population (Fig. 3);

iii) from 51.04 to 60.72 µm for the average length of the flagellum in the Sardinian population and 51.67-57.24 µm in the Venice population of (Fig. 4).

Moreover, the total length of the spermatozoa in both populations showed a range of sizes (minimum-maximum) of 45-80 µm for the Sardinian population and 50-75 µm to that of Venice, although, most of mature sperms analysed had total length between 55 and 65 µm in both populations (Fig. 5).

The variability of all analysed sperm traits were high in both population, as shown by the respective coefficients of variation within and between males calculated in both population (differences were not statistically significant) (see table 1).

Finally, as shown by the MDS analysis, sperm population within a single male, as well as those within each population and between populations were not structured in discrete groups or classes (Figs. 6, 7).

Discussion

Data reported in the present paper show that the differences in the average values of all morphometric parameters analysed between the two brackish water populations of *Syngnathus abaster* and tested with the t-Student were no statistically significant ($0.1 < p \leq 0.9$). Moreover, the stress values (the measure for a goodness-of-fit statistic) of all the MDS analyses here performed are $0.1 <$ Stress values ≤ 0.2. These values, which correspond to a good representation of the data without a real danger of bad interpretation, allow as to assert that: i) the two populations seem to be indistinguishable on the base on their spermatozoa (stress value = 0.16) and ii) there is no polymorphism, being sperm not distinguishable into discrete classes either within a single male (stress value = 0.13) or within each populations (stress values = 0.14 and 0.2 for Sardinian and

Venice populations respectively). The latter datum does not seem to support the presence of polymorphic sperm, classified in three different groups, reported in the freshwater population of the same species *S. abaster* (Dzyuba *et al.* 2008).

First, the two studies differ in the methods performed to obtain measurable sperm. We used fixed spermatozoa, whereas Dzyuba *et al.* (2008) used living cells. In addition, we have measured mature (apparently fully formed) and intact sperm (sperm with all their three portions clearly visible, i.e. nucleus, midpiece and flagellum) only, whereas Dzyuba *et al.* (2008) measured all sperm when it was possible to obtain clear images. Because these methodological differences, spermatozoa with very short flagellum were discarded in our study, whereas Dzyuba *et al.* (2008) included them in their data set, despite the analysis on motility of these sperm had a negative outcome. This could explain, for example, the high difference in the minimum value of total sperm length obtained in the two studies; 45 and 55 μm in our study for Sardininan and Venice population respectively, and 11 μm for the Ukrainian freshwater population. However, of the three morph-types showed by the Dzyuba *et al.* (2008), only the spermatozoa with flagella longer than 40 μm were mobile and most mobile sperm were longer than 60 μm. The latter datum seems to support our criteria in choosing of mature and intact spermatozoa only, most of which had total length between 55 and 65 μm in both populations.

Second, the two studies differ in the number of sperm measured per male. In our study, we measured 20 sperm per male for each of 10/9 males per population, whereas Dzyuba *et al.* (2008) measured a pool of only 93 spermatozoa obtained by 14 sperm samples. Although the authors did not specify the numbers of sperm per each males, their average was less than 10 sperm per male. Because of that, in our study the variability in sperm traits both within male and between males of the same population has been estimated, whereas, Dzyuba *et al.* (2008) estimated only the variability between males.

Last, but not least, the two studies use a different form of *S. abaster* species. We have analyzed the of brackish water form whereas Dzyuba *et al.* (2008) used the freshwater one. This species lives in both brackish water and freshwater, as well as seawater, and even if marine and freshwater forms appear morphologically identical, there is still not unequivocal genetic evidence that they are the same species.

Apart from the considerations listed above, there is more than one difficulty in considering the different types of sperm reported in syngnathids species as heteromorphism:

i) generally, parasperm clearly differ from eusperm in both morphology and /or size. On the contrary, the various morphs reported in *S. abaster* by Dzyuba *et al.* (2008) are much overlapped, making unambiguous categorization of each sperm class difficult, thus assessing their respective function and potential adaptive significance problematical.

ii) As mentioned in the introduction, the spermatozoa of acquasperm type, reported in the seminal fluid of *Syngnathus schlegeli* and *Hippocampus kuda* (Watanabe *et al.* 2000; Van Look *et al.* 2007) could be either parasperm or, more likely, developing spermatids. The semicystic spermatogenetic process causes a precocious release from the spermatocysts of germ cells, which complete their development within the lumen of testis (Carcupino *et al.* 1999. Biagi *et al.* 2014). Semicystic spermatogenesis leads to a reduced number of simultaneously mature sperm, and this has been interpreted as one of the possible mechanisms evolved to reduce the cost of sperm production. This is particularly true in those species that, like syngnathids, perform male parental care. In these species, it has been speculated that the male cannot invest all the energies devoted to reproduction in sperm production (Manni and Rasotto 1997; Giacomello *et al.* 2008). However, no data on the occurrence of semicystic spermatogenesis in *Hippocampus* species are known, so that further studies on the spermatogenetic process in these syngnathids are needed to clarify the nature of parasperm reported in *H. kuda*.

iii) The reason why parasperm are produced has not been fully elucidated, and their relationship to the reproductive mode is uncertain. However, the most likely function for invertebrates parasperm has been hypothesized to be correlated to competition, i.e. parasperm may be available for males to overcome sperm competition. Sperm competition occurs between sperm from two or more males to fertilize ovulated or spawned eggs from a single female (Parker 1970). Therefore, sperm emitted from multiple males compete each other for eggs. It is predicted that sperm competition may result in elimination or displacement of eusperm from previous males, blocking or destruction of the nonkineusperm (soldier sperm; Kura and Nakashima 2000), and preventing or delaying further mating of females. A possible role correlated to sperm competition, has been also reported by Hayakawa (2007), for the unflagellate parasperm in a non copulatory marine cottoid. It has been experimentally demonstrated that parasperm of this teleost accumulate at the boundary surface of an egg mass where ovarian fluid contacts seawater and that parasperm lumps are obstacles to the late-arriving sneakers eusperm.

Males of all syngnathid species (with or without a brood pouch) have exclusive paternity of their young, so no sperm competition seems to be present (Avise *et al.* 2002; Ah-King *et al.* 2006). Lack of sperm competition is also supported by the very low sperm concentration (much lower than estimated for other fish species) (Van Look *et al.* 2007; Biagi *et al.* 2008; Dzyuba *et al.* 2008). The functional sperm : egg ratio has been estimated to be about 70 : 1 in the brackish water form of *S. abaster* (Biagi *et al.*, 2008) lower, 191 : 1 in freshwater form of the same species (*S. abaster*) (Dzyuba *et al.* 2008) and even much lower 5 : 1 in *Hippocampus kuda* (Van Look *et al.* 2007). These values are numerous orders of magnitude lower than estimated in the zebrafish (*Danio rerio*) (48000 : 1), which was considered to have one of the lower sperm concentration in fish (Stockley *et al.* 1996).

Due to both the low sperm concentration and high reproductive efficiency of males (most of the carried eggs are fertilized) (personal observation), it could be speculated that all sperm,

produced by Syngnathidae, must be functional and free of errors. Despite this, both populations of *S. abaster* here analysed exhibit a high variation in all sperm traits, both among individual sperm within an ejaculate and among males. However, sperm variability is not only associated to heteromorphism or the spermatogenetic errors. Recently, comparative studies of both passerine birds and some teleost species have showed that the variations are also present in normal spermatozoa within a species within each male of the same species. These types of variations are negatively associated with the risk of sperm competition (Calhim *et al.* 2007; Thünken *et al.* 2007; Immler *et al.* 2008; Kleven *et al.* 2008; Lifjeld *et al.* 2010). Sperm design is commonly assumed to be under male and hence diploid genetic control (Beatty 1970; Parker and Begon 1993; Birkhead *et al.* 2005). Yet, there is increasing evidence that haploid genes of the sperm are expressed and may influence the sperm design and function (Joseph and Kirkpatrick 2004; Immler 2008). The expression of the haploid set of genes of the sperm may be one explanation for the observed variation among sibling sperm, as sibling sperm differ genetically by 50% on average. The optima for sperm design under diploid and haploid control may differ (Parker and Begon1993), however, under increased selection pressure for example, due to intense postcopulatory sexual selection we expect the optima under diploid and haploid scenario to be more similar. In addition, under intense selection, variation in design among sibling sperm is expected to be reduced due to directional selection. This can be interpreted as evidence of stronger stabilizing selection on males producing an optimal sperm type when the risk of sperm competition increases. Parker (1998) emphasized this principle by mentioning that the production of high quality error free spermatozoa is costly and that there will be selection against it if the costs are not equal to or out-weighed by the benefits (fertilizing the oocyte). Thus, in the absence of sperm competition, there may be little benefit in investing energy on the quality of sperm production (Bauer e Breed 2006)

In conclusion, we consider that the high variability in syngnathids sperm traits does not relate to the phenomenon of polymorphism *sensu stricto*, but rather to the variation in design

among sibling functional sperm due to a low selection pressure determined by the absence of postcopulatory sexual selection, i.e absence of sperm competition.

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Figure legends

Figure 1. Full-developed sperm, developing spermatids and damaged sperm of *Syngnathus abaster*. A-B. Scanning electron micrographs of mature sperm obtained by a male of Sardinain population. C. Toluidine Blue stained mature sperm obtained by fixed seminal fluid of one male of Venice population. D-E. Nomarski interference contrast micrographs of developing spermatid with two (D) and four (E) nuclei and flagella, obtained by un-fixed seminal fluid of one male of Venice population. F. damaged toluidine Blue stained mature sperm obtained by fixed seminal fluid of one male of Sardinian population. Flagellum (F), nucleus (N), sperm midpiece (MP). Polynucleated cells (PC), spermatozoon (Sp). Scale bar: **A**= 3µm; **B**= 1,8µm, **C**= 10µm, **D**= 4µm, **E**= 8,3µm, **F**=4,5µm

Figure 2. Head width of *Sungnathus abaster* sperm calculated in each male of both Sardinian (n= 10 specimens) and Venice (N=9) populations. Twenty spermatozoa were measured for each male. The mean and standard deviation are indicated by symbol (oblique strikethrough circle and black

diamond) and vertical bars, respectively. Within each population, individual males are ordered by increasing mean values.

Figure 3. Head length of *S. abaster* sperm calculated in each male of both Sardinian (n= 10 specimens) and Venice (N=9 specimens) populations. Twenty spermatozoa were measured for each male. The mean and standard deviation are indicated by symbol (oblique strikethrough circle and black diamond) and vertical bars, respectively. Within each population, individual males are ordered by increasing mean values.

Figure 4. Flagellum length of S. abaster sperm calculated in each male of both Sardinian (n= 10 specimens) and Venice (N=9 specimens) populations. Twenty spermatozoa were measured for each male. The mean and standard deviation are indicated by symbol (oblique strikethrough circle and black diamond) and vertical bars, respectively. Within each population, individual males are ordered by increasing mean values.

Figure 5. Histogram represented the frequency distribution of the sperm total length in both populations. (N= 200, in Sardinian population and N= 180 in Venice population).

Figure 6. MDS plots regarding within a single Sardinian male (N= 30) (**A**) and within Sardinian population sperm data (N= 200) (**B**).

Figure 7. MDS plots regarding Venice population (**A**) (N= 180) and Sardinian and Venice populations sperm data (N = 200 obtained pooling 100 randomly selected sperm from each population) (**B**).

Table 1. Descriptive statistics of sperm morphometric of the two analysed *Syngnathus abaster* populations. Data derived from 200 spermatozoa from Sardinian population and 180 from Venice population (20 spermatozoa per specimens); all measurements are in µm, and are referred to mean ± Standard deviation, t-Student and coefficient of variation (CV) calculated for all parameters within (CVwm) and between males (CVbm) of each population. Coefficient of variation between males were calculated as SD/mean x 100 and adjusted for sample size following the formula (CV X(1+ (1/4n))) (Sokal and Rohlf 1995).

Sperm traits	*S. abaster* Sardinian N= 10	*S. abaster* Venice N=9	t-Student
Head width (HW)	0.75±0.12 µm	0.70±0.12 µm	t= 1.66; p= 0.113
Head length (HL)	3.27±0.26 µm	3.25±0.14 µm	t= 4.52; p= 0.38
Flagellum length (FL)	55.13±6.35 µm	54.15±4.28 µm	t= 2.64; p= 0.676
Tot. sperm length (TL)	58.4±6.44 µm	57.55±7.49 µm	t= 1.26; p= 0.22
HW CV_{wm}	13.56±3.56 µm	15.22±3.37 µm	t= 0.639; p= 0.53
HW CV_{bm}	16.4	17.61	
HL CV_{wm}	7.07±1.66 µm	7.73±1.73 µm	t= 0.218; p= 0.83
HL CV_{bm}	8.15	4.43	
FL CV_{wm}	9.36±5.04 µm	7.17±2.38 µm	t= 0.377; p= 0.71
FL CV_{bm}	11.81	8.13	
LT CV_{wm}	8.89±4.71 µm	6.84±2.21 µm	t= 0.284; p= 0.78
LT CV_{bm}	11.3	13.37	

Table 1

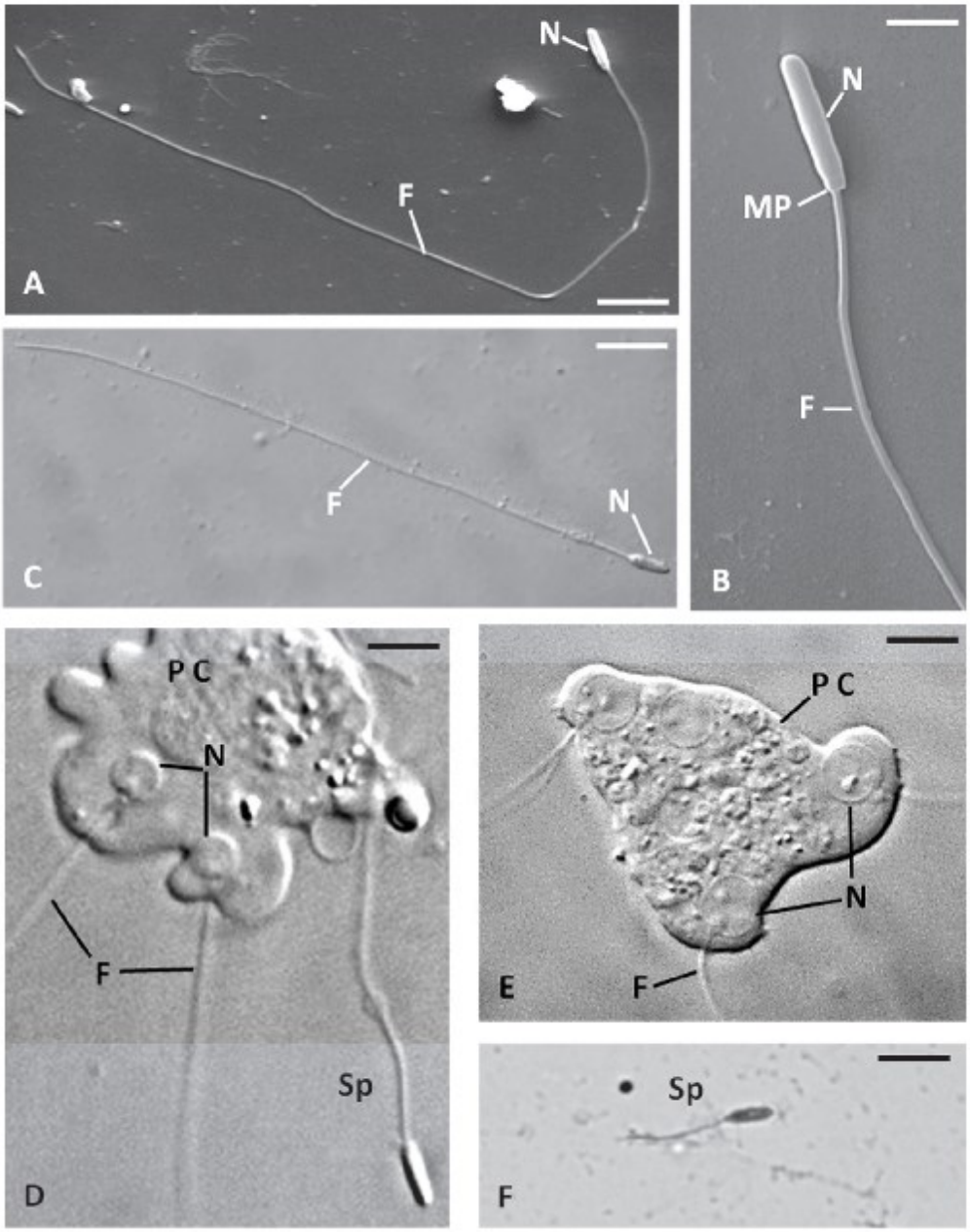
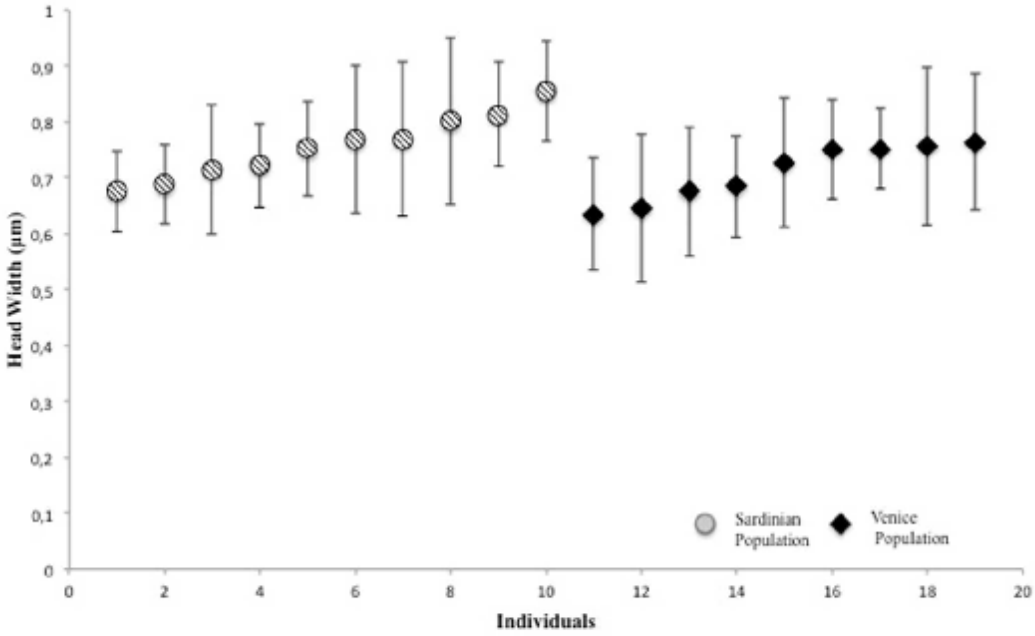


Fig. 1



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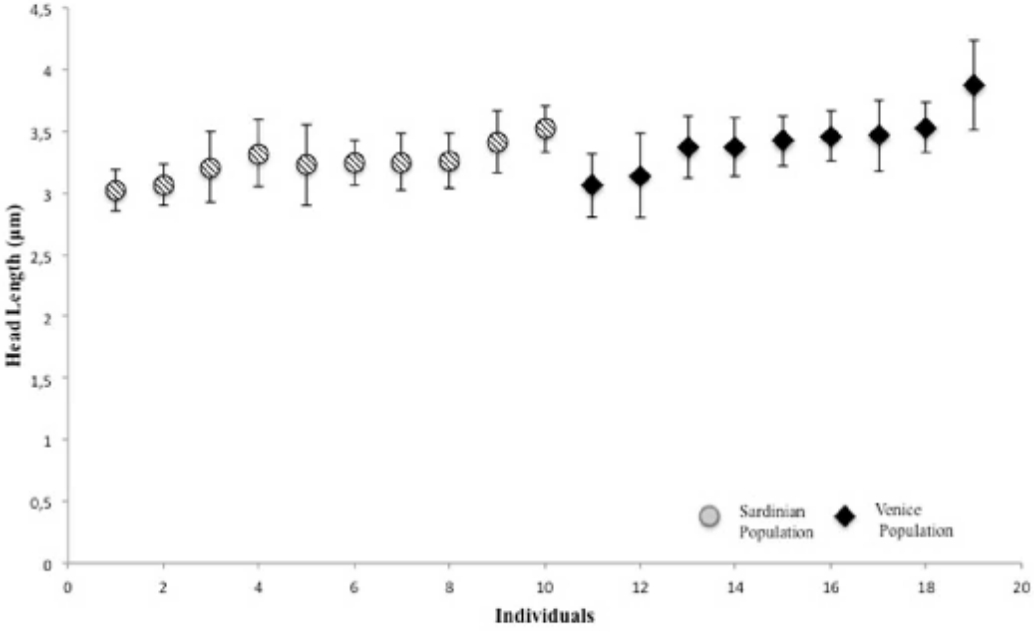

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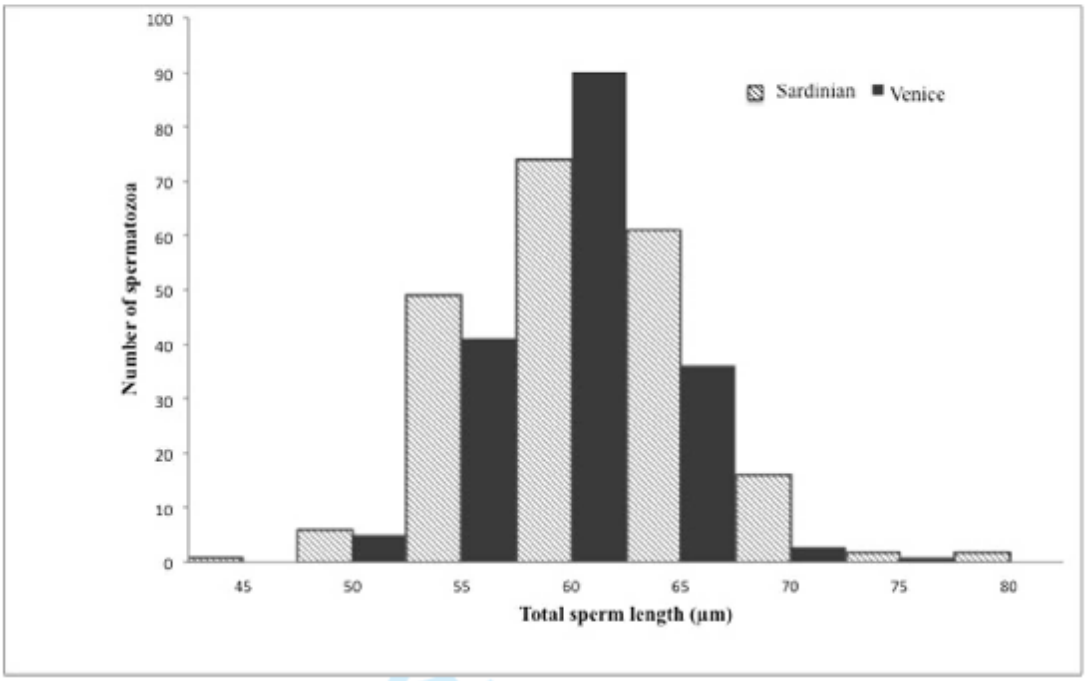

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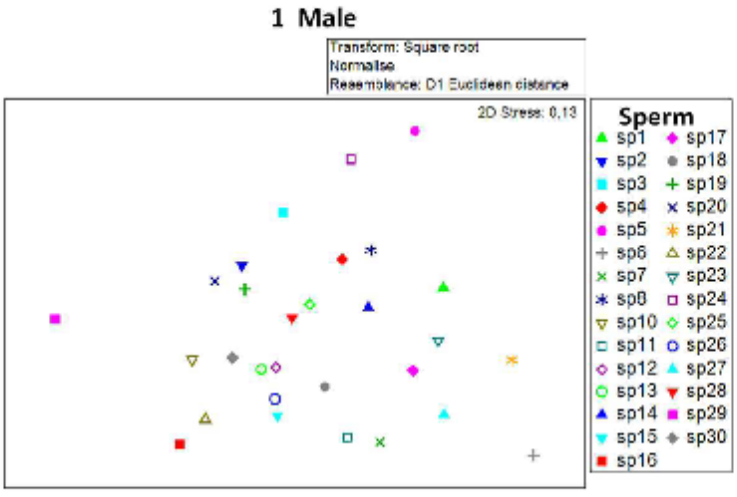
1 Male
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Sperm
sp1 sp17
sp2 sp18
sp3 sp19
sp4 sp20
sp5 sp21
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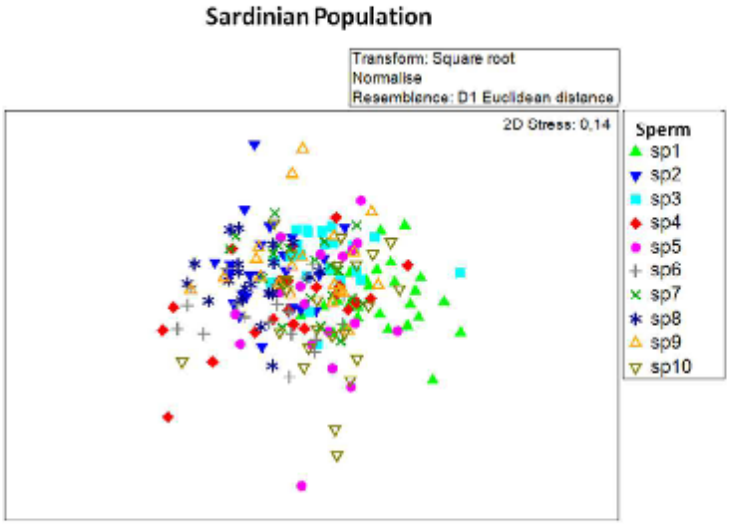
sp13 sp28
sp14 sp29
sp15 sp30
sp16

A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Sperm
sp1
sp2
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sp7
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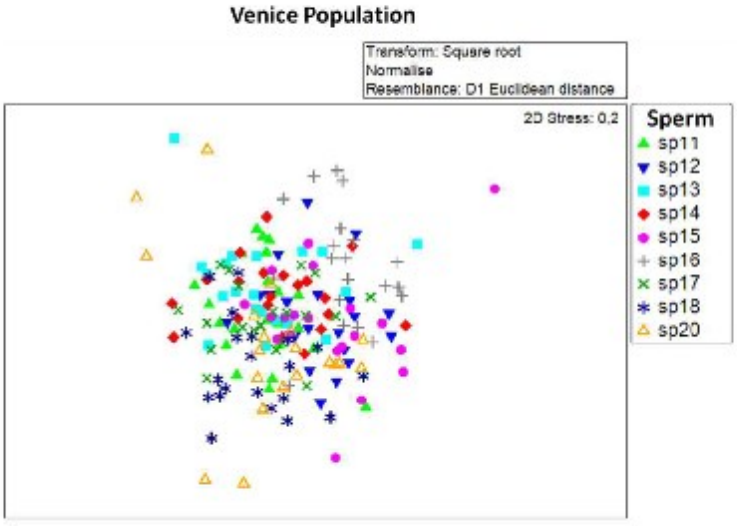
sp9
sp10

B

Fig. 6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Normalise
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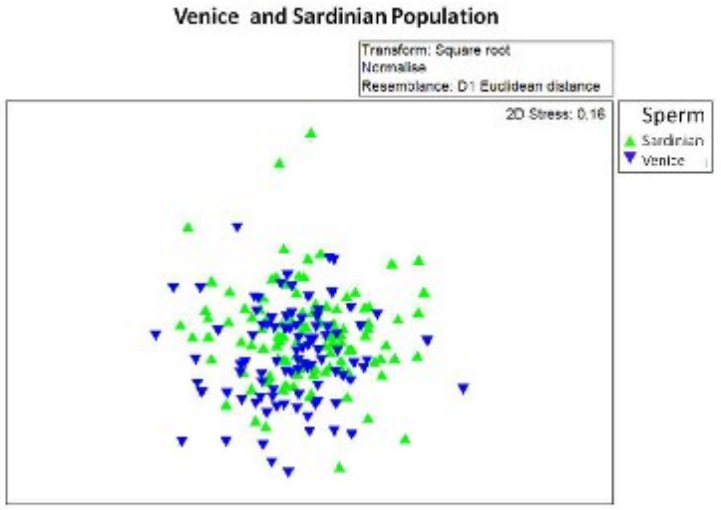
Sperm
sp11
sp12
sp13
sp14
sp15
sp16
sp17
sp18
sp20

A


Venice and Sardinian Population
Transform: Square root
Normalise
Resemblance: D1 Euclidean distance
2D Stress: 0.16
Sperm
Sardinian
Venice

B

Fig. 7

III

Male gonads morphology, spermatogenesis and sperm ultrastructure of the seahorse *Hippocampus guttulatus* (Syngnathidae).

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Running head: Semicystic spermatogenesis in *H. guttulatus*

Abstract

Testes morphology, spermatogenetic process and mature sperm ultrastructure were analysed in adult males of *Hippocampus guttulatus* using both light and transmission electron microscopy. The *H. guttulatus* testis is organized in a single large germinal compartment, with a central lumen and an external tunica albuginea. In germinal epithelium, spermatcysts only contain spermatogonia and primary spermatocytes. Inside the testis lumen, together with mature sperm, two types of large mono-nucleate cells were recognizable. The first type was constituted by aflagellate cells with cytoplasm rich in droplets; the second type was represented by mono-flagellate cells with cytoplasm containing a less amount of droplets. Both types of cells were interpreted as developing germ cells precociously released inside the testis lumen, where their maturation was completed. Functional sperm consisted of three distinct portions; the cylindrical head, the midpiece and the flagellum. These and previous data about the same topic reported on other syngnathids species were compared and discussed.

Introduction

Seahorses and their close relatives pipefishes and seadragon (Family Syngnathidae) occupy a very interesting position in the field of reproductive biology of bony fishes. Several interesting features characterize Syngnathids. They show peculiar parental care, with male pregnancy (Breder and Rosen 1966); have heterogeneous mating system varying from monogamy to different types of polygamy, associated with conventional or inverted sex roles) (c.f. Jones et al. 1999) and display an atypical organization of both female and male gonads (Begovac and Wallace 1987, 1988; Selman et al. 1991; Carcupino et al. 1999; Sogabe et al. 2008; ; Sogabeet al. 201, Biagi et al. 2014).

Numerous seminiferous lobules or tubules, which are connected to the main sperm duct via an efferent duct system generally characterize the teleost testes (Parenti and Greir 2004; Schulz et al. 2010). The efferent duct system collects and, sometimes stores, the spermatozoa. The syngnathids testes lack an of efferent duct system. Testes, at least several species belonging to the *Syngnathus* genus, i.e. *Syngnathus abaster*, *S. typhle*, *S. tenuirostris*, *S. acus*, are constituted by a single seminiferous compartment of unrestricted lobular types, which continue into a sperm duct. The two sperm ducts converge posteriorly to form a single main duct, which run parallel to the urethra and open independently in the apex of a urogenital papilla. The latter is located caudal to the anus, hidden by numerous skin folds arranged radially to the anal opening (personal observation). Two sperm ducts, originating from the last portion of the testis and converging posteriorly to form a single main duct which open in an urogenital papilla, also characterized the reproductive systems of other syngnathids species such as *Nerophis ophidion* and *Hippocampus guttuatus*, although the male gonad organization in these species has not be analysed in details. However, at least in *Hippocampus kuda*, testes have a morphology similar to that reported for the *Sygngnathus* species (Laksanawimol 2004).

In the testis of both non–brooding and brooding males of *H. kuda*, examined during the reproductive season, spermatogonia and primary spermatocytes werefound along the entire length of the testis, while secondary spermatocytes and spermatids were only found inside the lumen. This seem to confirm that in syngnathids of *Hippocampus* genus, as well as those of *Syngnathus*, the testis organization is of unrestricted lobular type and the spermatogenetic process is of the semicystic type (Carcupino et al. 1999; Biagi et al. 2014).

This type of spermatogenesis is currently known in few species, belonging to several teleostean groups (Selman and Wallace 1986; Bazzoli and Godinho 1991; Mattei et al. 1993, Manni and Rasotto 1997; Yoneda et al. 1998, Carcupino et al. 1999, Giacomello et al. 2008; Srivastava and Singh 1994; Andrade et al. 2001; Mazzoldi 2001, Muñoz et al. 2002; Sàbat 2002; Laksanawimol 2004; García-López et al. 2005; Hernández et al. 2005; Shahin 2006; Sabat et al. 2009; Magalhaes et al. 2011). It consists of a precocious open of the germinal cysts, causing an asynchronous maturation of spermatids and the simultaneous presence of germ cells at different developmental stages inside the testis lumen. Moreover, in *Syngnathus* species, developing germ cells inside the lumen are mono- and, more frequently, poly-nucleate and poly-flagellate cells. Individualization of mature sperm seems to occur at the end of spermiogenesis, so the cytokinesis seems to be abolished or at least delayed (Carcupino et al. 1999; Biagi et al. 2014). Poly-nucleate developing germ cells has not been reported in *H. kuda* (Laksanawimol 2004).

Syngnathids are also known to produce a very low number of sperm. The functional sperm : egg ratio has been estimated to be about 191 : 1 in *S. abaster* (Dzyuba et al. 2008) and even much lower 5 : 1 in *Hippocampus kuda* (Van Look et al. 2007). These values are numerous orders of magnitude lower than estimated in the zebrafish (*Danio rerio*) (48000 : 1), which was considered to have one of the lower sperm concentration in fish (Stokley et al.

1996). Probably due to this low sperm concentration, no sperm were observed in the testis lumen of both non-brooding and brooding male of *H. kuda* by Laksanawimol (2004), whereas dimorphic sperm have been reported in the same species by Van Look et al. (2007). In the latter study, however no data on sperm ultrastructure were shown. Sperm polymorphism was also reported in a freshwater population of *Syngnathus abaster* (Dzyuba et al. 2008), which belong to a monophyletic lineage within the urphorine subfamily including *Syngnathus* and to *Hippocampus* speies (Wilson and Orr 2011). Recently we have demonstrated that in two population the brackish water form of the same species *S. abaster* mature sperm are great variable in their morphometric traits, but they can not be distinguished in different morphotypes (Piras et al. sumitted).

The aim of this paper was to analyse the male gonad morphology, the spermatogenetic process and both sperm traits and ultrastructure in the seahorse *Hippocampus guttulatus*. Results obtained could be also contribute to shed light to the presence of sperm polymorphism in *Syngnathids*.

Materials and Methods

Sampling

Four adult male of *Hippocampus guttulatus* were sampled from Venice lagoon (Veneto), during the reproductive period (May-September 2013). Alive specimens, delivered to the laboratory within 3 h, were sacrificed by exposure to the anaesthetic 3-aminobenzoic acid ethyl ether (MS-222, Sigma-Aldrich) for 10 min, and then processed for microscopic analysis.

Light microscopy:

Two specimens were dissected to extract the testes, which were fixed in aqueous Bouin's fixative, dehydrated in a graded ethanol series, cleared in Bioclear and finally embedded in paraffin wax. Sections (5µm) were stained with Eosine and Emallume di Mayer (Mazzi 1977) and processed for the morphological analysis using a Zeiss Axiophot light microscope.

Gonads, dissected by a further one male, were gently open in order to obtain aliquots of seminal fluid containing cells eventually free inside the testicular lumen. Aliquots of 20 µl each were seminal fluid fixed in glutaraldehyde 5%-fixed were placed on poly-lysine coated coverslips (1 mg mL-1; Sigma P1274) and air-dried. Then, samples were stained with Toluidine Blue 0.1% in aqueous solution and analysed with a Zeiss Axiophot light microscope.

Transmission electron microscopy (TEM):

Two additional male gonads were fixed for 2h in 4% paraformaldehyde-5% glutaraldehyde buffered with sodium cacodylate (0.1M and pH 7.2). Specimens were then rinsed overnight in the same buffer, post-fixed for 1h in 1% osmium tetroxide buffered with sodium cacodylate. After dehydrating in an ethanol series, sample were embedded in Epon 812 resin. Thin sections, of about 80 nm thick, were cut with a Reichert Ultracut ultramicrotome and stained with stained with uranyl acetate and lead citrate. Samples were examined and photographed with a Jeol Tem 1200 EX II transmission electron microscope.

Morphometry:

Intact spermatozoa (N = 20), obtained from the gonads of one male stained with toluidine blue (see above) were analysed in order to study spermatozoa morphometric, such as head length (including nucleus and midpiece) and the length of the flagellum. Abnormal, broken or difficult to measure spermatozoa were discarded.

Digital images of mature spermatozoa were acquired with a digital camera Nikon DS-FI1 connected with DS-L2 control unit and mounted on an optical microscope Nikon Eclipse 80i. The measurements were made using the program Tpsdig2.

Results

The paired testes were semi-translucent organs (Fig. 1A) adhering to the abdominal cavity by extensions of the mesenteries. Each testis was characterized by a large central lumen and a thin wall (Fig. 1B). The latter consisted of the germinal epithelium and a vascularized fibrous capsule consisting of connective tissue rich in muscle fibres (Fig.1B-E). The tissue of the capsule is continuous and does not enter the organ interior where inter-germinal compartments were not observed. (Fig. 1B).

The germinal epithelium showed the typical organization of spermatocysts formed by germ cells enveloped by Sertoli cells, resting on the basal membrane (Fig. 1 C-E). Along the entire length of the testis, the germinal epithelium contained spermatocysts inside which spermatogonia and primary spermatocytes were easily recognizable (Fig. 1 C-D). Developing spermatids and mature sperm were never observed inside the spematocysts.

Two types of large mono-nucleate cells, aflagellate and flagellate cells, together thin mature spermatozoa were observed inside the testis lumen (Figs. 1F, 2-4). The aflagellate cells had irregular shape, characterized by numerous cytoplasmic protrusions and round nuclei with a large eccentric spherical nucleolus (Figs 1F, 2 A-B). Their cytoplasm was rich in rough endoplasmic reticulum, Golgi complexes and droplets of different size, and density. (Figs. 2B, 3A,B). Cells of similar appearances were visible emerging from the surface of the germinal epithelium facing the central lumen (Figs. 1C, E-F, 2A). Some of these cells appeared unquestionable to be spermatids; in their cytoplasm a forming flagellum was recognizable (Fig. 2C-D)

On the contrary, the flagellate cells showed a less amount of cytoplasmic droplets. Inside the nucleus, the nucleolus was no more evident and in the cytoplasm, large cisterns of rough endoplasmic reticulum and all typical components of the future sperm midpiece were often visible. They were mitochondria, surrounding a cytoplasmic anal, and an emerging flagellum, running inside the cytoplasmic canal. Patches of electron-dense material begin to accumulate in close association to the inner membrane of the cytoplasmic canal (Fig. 3B, insert, C).

Mature sperm of *Hippocampus guttulatu*s (Fig. 4) are anacrosomal and mono-flagellate sperm of several tens of microns in length (51.35 ±3.68 μm, n = 20). The spermatozoa consisted of three distinct portions; the head, the midpiece and the flagellum. The head was cylindrical in shape and entirely occupied by the nucleus. (2.78 ±0.19 μm, n = 20) (Fig. 4A). At the basal end of the nucleus, a deep nuclear fossa was present, and inside it, both the basal and the distal centrioles were localized. The midpiece was clearly marked under the nucleus by two mitochondrial rings. These latter were housed inside a cytoplasmic collar, which was separated from the first portion of the flagellum, by a deep so-called cytoplasmic canal (Fig-4B-D). The plasma membrane of the collar lining the canal appeared closely associated to a sheath of electron-dense material arranged in a ring-like structures regularly spaced. The flagellum, had an internal "9+2" axoneme, originating from the distal centriole, surrounded by the plasma membrane which form two lateral fins (Fig. 4E).

Discussion

Like other syngnathids (Carcupino et al. 1999; Biagi et al. 2014), the seahorse *Hippocampus guttulatus* here analysed, shows testis atypically constituted by a single and continuous germinal compartment, surrounded by a single and continuous somatic compartment. Each testis appears as

a tubular organ characterized by a unique testicular lumen surrounded by two concentric layers, the tunica albuginea and the germinal epithelium, separated by the basement membrane.

The germinal compartments, which extends to the periphery of the testis and terminates blindly, appeared formed by a germinal epithelium having the tripartite organization, typical of teleost testis; i.e. germ cells are surrounded by Sertoli cells forming spermatocysts, which rest on the basal lamina. Inside spermatocysts, spermatogonia are clearly distributed along the entire length of the testis. Based on these data, the testis organization in *H. guttulatus* may be attributed to the unrestricted lobular type, typically found throughout the Neoteleostei, including other syngnathids (Biagi et al. 2014; Laksanawimol 2004), except for the atherinomorphs (Parenti and Grier 2004).

In agreement with previous data on syngnathids testes (Carcupino et al. 1999, Biagi et al. 2014), the germinal spermtocysts of all reproductive males here analysed, only contain spermatogonia and primary spermatocytes. Inside the testis lumem developing spermatids, which are always mono-nucleate cells, identifiable by both the presence of the flagellum emerging from a deep nuclear fossa, and nuclei characterized by different degrees of chromatin condensation, are only visible inside the testis lumen together with matures sperm. The spermatogenetic process may therefore attributed to the semicystic type. In the semicystic spermatogenesis, the cysts rupture at the spermatocyte or spermatid stage, so germ cells only partly develop inside them, producing an asynchronous maturation of spermatids and thereby reducing the number of simultaneously mature sperm (Mattei and Mattei 1978).

Because developing spermatids of *H. guttulatus* are always mono-nucleate cells, the semicystc spermatogenetic process seems to have the typical features, i.e. spermatocytes and/or spermatids are released after that cytokinesis among isogenetic germ cells is completed. Therefore, germ cells advance individually through spermiogenesis inside the

lumen. In contrast, in the *Syngnathus* species, the cytokinesis seems to be abolished or at least delayed. In fact, as previously documented in *Syngnathus abaster* and *S. acus* (Carcupino et al. 1999), and recently confirmed in the same and in other species of the same genus (Biagi et al. 2014), developing germ cells inside the lumen of these species are mono- and, more frequently, polynucleate and polyflagellate cells. A possible functional significate of the delayed cytokinesis in *Syngnathus* species will be discuss below.

The semicystic spermatogenesis was interpreted as a possible mechanism, evolved several time in different teleost taxa, to reduce the cost of sperm production. Therefore, it seems to be crucial particularly in those species in which, a small ejaculate size is justified by their low fecundity, monogamous mating system, and absence of sperm competition and presence of male parental care (Rasotto et al. 1992; Marconato and Rasotto 1993; Mazzoldi 2001). This is also the case of *H. guttulatus*, which has small ejaculate size, low fecundity, male parental care, absence of sperm competition, and mating system strictly monogamous (for references c.f Sanna et al. 2008; Wilson et al. 2003).

Moreover, together with developing spermatids and mature spermatozoa, another type of mono-nucleate cells are present inside the testicular lumen of *H. guttulatus*. These cells are aflagellate and characterized by a large amount of cytoplasmic droplets. Large droplets-containing cells were also reported in several species of the *Syngnathus* genus, such as *S. schlegeli* (Watanabe et al. 2000), *S. abaster, S. acus, S. tenuirostris* and *S. typhle* (Carcupino et al. 1999; Biagi et al. 2014), although in these species these cells are, as well as developing spermatids, polynucleated cells. In both reproductive males of *H. guttulatus* and *Syngnathus* species, however the aflagellate cells are frequently observed either free into the testicular lumen and coming out from the epithelium. Moreover, like in the *Syngnathus* species mentioned above, a smaller amount of droplets of different size and electron-density may be

also recognizable in the cytoplasm of developing spermatids of *H. guttulatus*. All these data support the hypothesis first reported by Carcupino et al. (1999), and recently reformulated by Biagi et al. (2014), that these aflagellate cells represent the youngest germ cells released inside the lumen at the spermatocyte or a very early spermatid stage, after having accumulated a large amount of material in form of droplets. These droplets progressively reduce in size and number during the germ cell maturation.

It was speculated that the large amount of droplets could be involved: (i) in the formation of an abundant and fibrous seminal fluid, having the function to trap the very low number of mature sperm produced by syngnathids, avoiding sperm loss during mating, (ii) in the metabolic supply to the developing germ cells, which are released in a very early spermatogenetic stage (Biagi et al. 2014).

As regards of the first possible function, it must be said that in some teleost species (such as *Ophidion marginatum* and *Lophiomus setigerus*), which lay eggs in a gelatinous mass (Fahay 1992; Yoneda et al 1998b) like syngnathids, the semicystic spermatogenesis was thought to be in somehow related to the secretion of abundant thick seminal fluid. The latter was reported to act in maintaining sperm together and facilitating fertilization of egg mass (Muñoz et al. 2002). Whereas, concerning to the second function, it should not be forgotten that in the semicystic spermatogenetic process, Sertoli cells cannot regulate and support the metabolites transfer towards the developing germ cells, when the latter are free inside the lumen.

If the functions of large amount of droplets accumulated in the cytoplasm of syngnathids developing germ cells are the same, why are these cells in *Syngnathus* species polynucleate? The answer to this question is not so easy. The delayed cytokinesis observed in the semicystic spermatogenesis of *Syngnathus* species, was recently speculated to be

correlated to the need in limiting the reduction of cytoplasm and organelles. An early cytoplasmic division among isogenetic cells could endangered the production and accumulation of a sufficient amount of material employed either in the energy requirements of each germ cell and the production of the seminal fluid (Biagi et al. 2014). This hypothesis seems not be supported by the absence of polynucleate cells in *H. guttulatus.* A possible explanation of these different data, could be sought in a less need in *H. guttulatus* in producing a large amount of fibrous seminal fluid. Although *H. guttulatus* has lower concentration of sperm respect the *Syngnathus* species, it has a closed pouch and a monogamous mating system. Because of that *H. guttulatus* males mate much less frequently respect *Synagnthus* species and not release sperm for a long period of time. These features could reduce the loss of sperm during fertilization. In fact, the *Syngnathus* species apparently have a larger amount of sperm, but they have a semi-closed pouch and a polygamous mating system.

According to the ultrastructural analysis of all types of flagellated cells recognizable inside the testis lumen of *H. guttulatus*, we have identified only one type of mature sperm. They are characterized by an elongated head, completely occupied by a nucleus with condensed chromatin, a short midpiece characterized by two mitochondrial rings surrounding the first portion of the axoneme, and a long flagellum. The latter datum does not seem to support the presence of dimorphic sperm reported in *H. kuda* (Van Look et al. 2007). In this latter species, type 1 spermatozoa, which were considered the only sperm type taking part in fertilization, seem to have similar morphology and morphometric traits of *H. guttualtus* sperm. The *total sperm* length is 51.35 ±3.68 µm (mean ± standard deviation, N = 20) in *H. guttulatus* and 49.3 µm (median length of flaglellum N = 44) in *H. kuda*. The head length is 2.78 ±0.19 µm (N = 20) in *H. guttulatus* and 3.7 µm (median length, N = 44) in *H. kuda.* On the contrary, type 2 spermatozoa of *H. kuda* , which were interpreted a remnant population

of the primitive externally fertilizing sperm type (aquasperm) no taking part in fertilization, were reported to have a very large spherical head.

However, the difference between our study and that one of Van Look and co-authors (2007) could have been arisen by several reasons. First, the two studies differ in the methods performed to obtain measurable sperm. We used fixed spermatozoa, whereas Van Look et al. (2007) used living cells. Second, we have only measured mature (apparently fully formed) and intact sperm (sperm with all their three portions clearly visible i.e. nucleus, midpiece and flagellum), whereas Van Look et al. (2007) measured all sperm, for which it was possible to obtain clear images. This could explain, for example, the high difference in the minimum and maximum values of flagellar length obtained in the 2 studies; 47.68 and 61.49 µm in our study for *H. guttulatus*, and 6.3 and 69.3 µm for *H. kuda*.

Last, but not least, the spermatogenetic process in *H. ku*da is not known. In fact, if *H. kuda* has, like *H. guttutalus* and other syngnathids species (i.e. *Syngnathus abaster*, *S. typlhe*, *S. tenuirostris*, *S. acus* and *Phyllopteris taeniolatus*) (Biagi et al. 2014; Forsgren and Young 2008) a spermatogentic process of semicystic type, it should be very likely that the type 2 sperm are developing spermatids. Moreover, both the simultaneous presence of flagellate and aflagellate cells inside the testis lumen, determined by the semicystic spermatogenesis, and the difficulty to see the thin flagellum in the histological sections, may have induced other authors to interpret these cells as aflagellate spermatozoa. This could be the case of Miranda-Marure et al. (2004) for the aflagellate sperm reported in *Microphis brachyurus lineatus*.

Functional sperm with elongated head similar in morphology to those of *H. guttulatus* and *H. kuda* were also reported in other syngnathids species, such as *Syngnathus abaster*, *S. typhle*, *S. tenuirostris*, *S. acus* and *Nerophis ophidion* (Carcupino et al. 1999; Ah-king et al. 2006; Biagi et al. 2014 and Piras et al. submitted). A similar type of sperm are also present

in some Blenniidae (Lahnsteiner et al. 1990) in *Lepadogaster lepadogaster* (Mattei and Mattei 1978), and in *Ophidion barbatum* (Hernandez et al. 2005), all species with external fertilization and semicystic spermatogenesis.

In general, spermatozoa with elongated heads are related to internal fertilization (Jamieson and Leung 1991), an explanation that does not match any of the all abovementioned species. According to Burns et al. (1995), the elongated nucleus may also facilitate the storage of the spermatozoa in the testicular ducts. Nevertheless, in the specific cases of *O. barbatum* (Hernandez et al. 2005), and syngnathids species (data not shown) no packaging of spermatozoa were observed. On the base of these data, and according to the hypothesis first formulated by Biagi et al. (2014) a third possibility to explain the elongated head of syngnathids sperm could be related to their need to cross through the gelatinous mass of maternal origin to reach the eggs.

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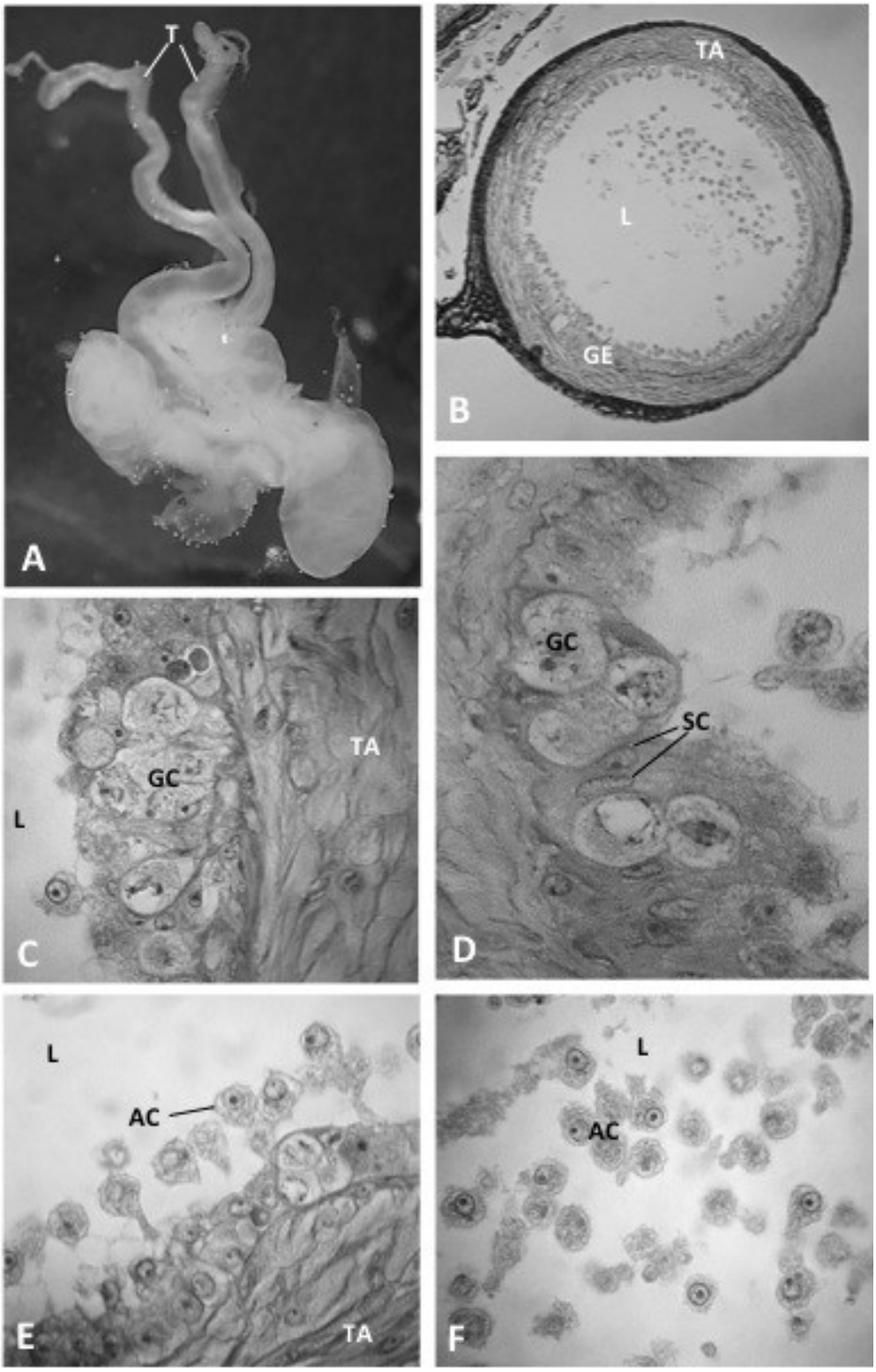

Figure 1. A. Entire reproductive apparatus of *Hippocampus guttulatus* mature male showing paired testes of uniform external morphology. **B. T**ransverse paraffin sections of testis appearing as a hollow tube. **C-E**. High magnification of sections obtained by the same

testis reported in B, showing: (i) spermatocysts formed by germ cells (spermatogonia and spermatocytes) enveloped by Sertoli cells; and aflagellate mono-nucleate cells protruding from the germinal epithelium. **F**. Aflagellate mono-nucleate cells free inside the lumen. Aflegellate cells (ac); germ cells (gc); germinal epithelium (ge); Sertoli cells (Sc); tunica albuginea (ta); testis (t).

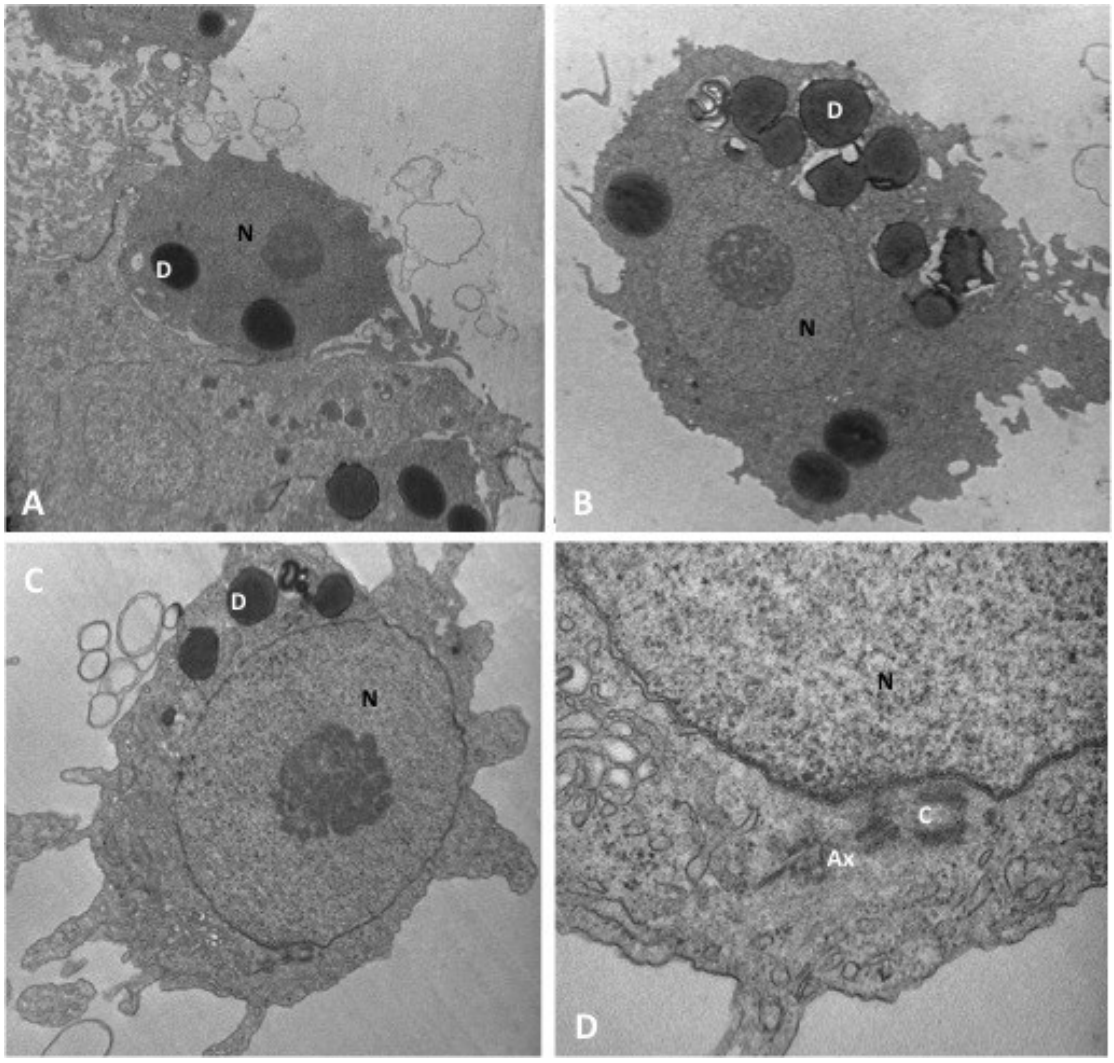

Figure 2. Transmission electron micrographs of both aflagellate cells protruding from the germinal epithelium (**A**) and free inside the testicular lumen (**B-C**) in *Hippocampus guttulatus* testis. High magnification of the centrioles region of the same cells reported in figure **C,** showing both distal and proximal centriole and the forming axoneme**.** Axoneme (ax); centrioles (c); cytoplasmic droplets (d); nucleus (n).

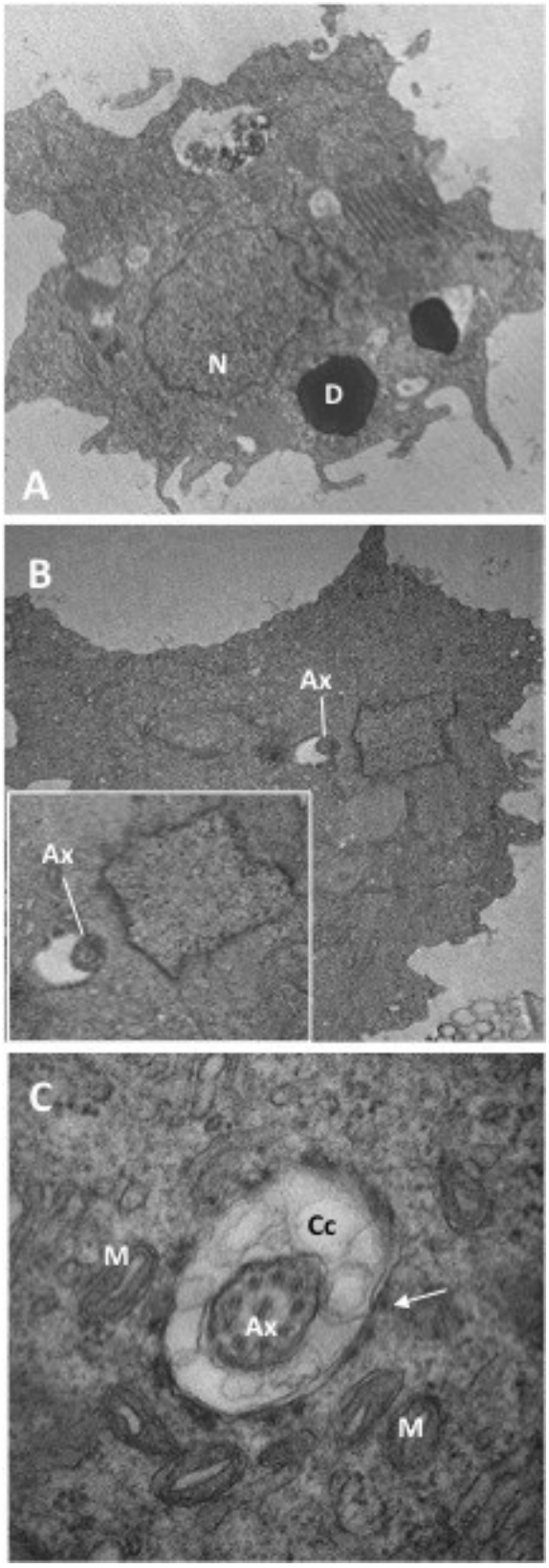

Figure 3. A-C. Transmission electron micrographs of developing spermatids of *Hippocampus guttulatus* testis showing: (i) less amount of cytoplasmic droplets; (ii) nuclei

lacking of nucleolus; (iii) cytoplasmic canal inside which run the flagellum; spots of electron-dense material accumulating on the plasma membrane, facing the cytoplasmic canal; (iv) small rounded mitochondria accumulating in the future region of the cytoplasmic collar. Spots of electron-dense material (arrow); axoneme (ax); cytoplasmic canal (cc); cytoplasmic droplets (d); mitochondria (m); nucleus (n).

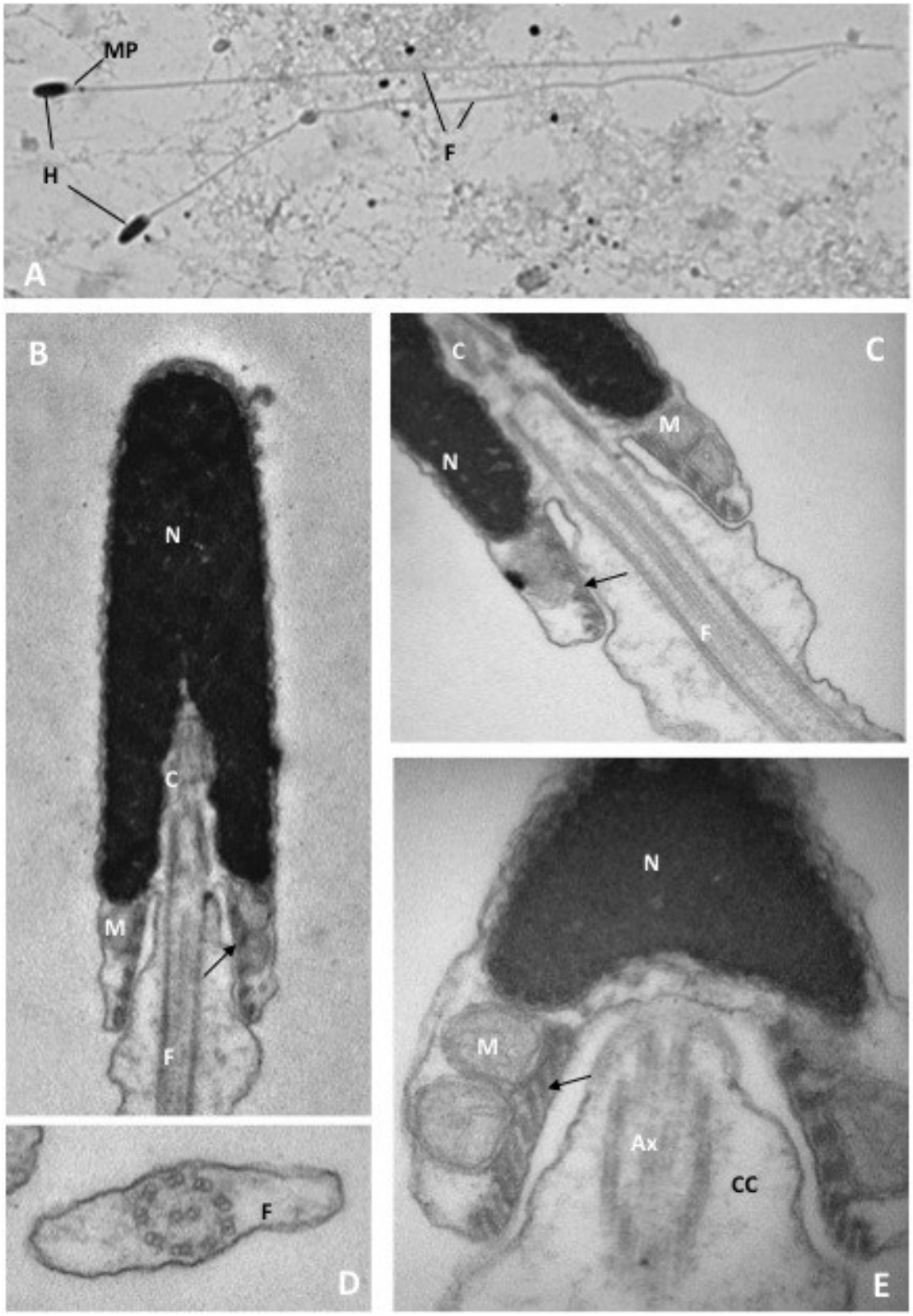

Figure 4. **A**. Light microscopic image of *Hippocampus guttulatus* mature sperm. **B-E.** Transmission electron micrographs of mature spermatozoa showing: (i) elongated and

anacrosomal sperm heads, with condensed nucleui; (ii) deep nuclear fossa, with two centrioles; (iii) "9+2" axoneme originating from distal centriole; (iv) short midpiece characterized by the cytoplasmic collar occupied by two rings of mitochondria; (v) the cytoplasmic canal; (vi) numerous rings of electron-dense material between the mitochondria and the internal plasma membrane of the collar. Spots of electron-dense material (arrow); axoneme (ax); cytoplasmic canal (cc); sperm head (h); midpiece (mp); mitochondria (m); nucleus (n).

IV

Morphology and ultrastructure of the male gonad in the straight-nosed pipefish *Nerophis ophidion* (Syngnathidae).

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Running head: Semicystic spermatogenesis in *H. guttulatus*

Abstract

Testes morphology, spermatogenetic process and mature sperm ultrastructure were analysed in adult males of *Nerophis ophidion* using both light and transmission electron microscopy. *N. ophidion* testis consists of two regions clearly marked a different morphology, which correspond to germ cells proliferation and maturation respectively. The first portion was characteried by a unique germina compartment bordered by a tunica albuginela. The surface of the germinal epithelium appeared increased by numerous invaginations of the tunica albuginea, which conferred to this portion an incomplete compartmentalized appearance. The germinal spermatocysts contained spermatogonia and spermatocytes only. The second portion appeared larger of the first one consists of a thin wall and a large central lumen occupied by numerous free large cells and mature sperm. The large cells, free inside the lumen, were unquestionably developing spermatids, confirming that *N. ophidion,* as well as the other examined syngnathids species, has a semicystic spermatogenesis. In germinal epithelium, spermatcysts only contain spermatogonia and primary spermatocytes. Inside the testis lumen, together with mature sperm, two types of large mono-nucleate cells were recognizable. The only one type of functional sperm observed were formed by three distinct portions: the cylindrical head, the midpiece and the flagellum. These and previous data about the same topic reported on other syngnathids species were compared and discussed from the point of view of phylogenetic relationships within the family Syngnathidae.

Introduction

Seahorses, pipefish and seadragons are fishes belonging to the family Syngnathidae, which includes over 295 species (Froese and Pauly 2011). Syngnathids have attracted attention for decades due to their unique morphology, remarkable camouflage ability and the distinctive phenomenon whereby males give birth to live young. Syngnathids are also unique among teleosts in both female and male gonads structure.

In most teleosts, oogonia are scattered singly or in oogonial nests throughout the ovary, there is no sequential arrangement of oocytes and ovarian lamellae are usually present (Begovac and Wallace 1987; Selman et al. 1991).

The syngnathids ovary atypically consists of a rolled follicular sheet, which has stem cell compartments, called the germinal ridge, running along the entire length of the edge of the follicular sheet (Wallace and Selman 1981; Begovac and Wallace 1987; Selman et al. 1991). Oocyte development starts at the germinal ridge, and developing follicles are arranged in sequence according to their development (Begovac and Wallace 1988). Distinct differences in ovarian structure have been reported in syngnathids. Some species have a single germinal ridge at one edge of the follicular sheet, with the most advanced follicles at the opposite edge, referred to as the mature edge. This type of ovary has been reported only in three species of congeneric pipefish, *Syngnathus typhle* and *S. scovelli.* and *S. schlegeli* (Begovac and Wallace 1987; Sogabe and Ahnesjo 2011, Sogabe et al. 2013), and one species of the genus *Hippichthys* (*Hippichthys spicifer*; see Ishihara and Tachihara 2009). Other species, such as *Hippocampus erectus*, *Corythoichthys haematopterus*, *Nerophis ophidion*, and *Urocampus nanus*, have two germinal ridges, one at each edge of the follicular sheet, with the most advanced follicles situated midway along the follicular sheet between the edges (Selman et al. 1991; Sogabe et al. 2008, 2012a; Sogabe and Ahnesjo 2011). Furthermore, it

has been reported that the mode of egg production varies among syngnathids in relation to differences in ovarian structure. In species with ovary characterized by a single germinal ridge, oocyte maturation occurs asynchronously, and the number of mature eggs increases continuously over time (i.e., the asynchronous type; Begovac and Wallace 1988; Sogabe and Ahnesjö 2011). On the other hand, in species with two germinal ridges, oocyte maturation occurs in group(s), and mature eggs are produced in batches or at one time before spawning (i.e., the group-synchronous type; Sogabe et al. 2008, 2012a; Sogabe and Ahnesjo 2011; Sogabe et al. 2013). The mode of egg production has important implications for how eggs are spawned, and thus a close link between ovarian structure, mode of egg production, and mating pattern (i.e., spawning frequency of females) (Sogabe et al. 2008; Sogabe and Ahnesjö 2011).

Syngnathids testis also shows an atypical structure compared to those of other teleost and vertebrates. In vertebrates, from fish to mammals (see Schulz et al. 2010), the testis consists of two main compartments, the somatic and the germinal compartment. In fish, the germinal compartment houses the germinal epithelium delineated by a basement membrane and it generally contains numerous germinal tubules or lobules. The somatic compartment contains steroidogenic Leydig cells, blood/lymphatic vessels and connective cells, and forms the intertubular or interlobular tissue. According to Parenti and Grier (2004), teleosts testes of tubular type are only those in which "the germinal compartments do not terminate at the testis periphery, but form highly branched, anastomosing loops or tubules". Instead, testes of lobular type are those in which "the germinal compartments may form anastomosing networks proximally, but distally they extend to the periphery of the testis and terminate blindly". Anastomosing tubular testis characterizes basal osteichthyans, including basal teleosts, whereas a lobular testis characterizes higher teleosts. The lobular testis type has been proposed as a diagnostic or synapomorphic character of the Neoteleostei (Parenti and Grier

2004). In addition, the lobular testis could be divided into two types based on distribution and arrangement of spermatogonia. The atherinomorph testis has a restricted distribution of spermatogonia at the distal ends of lobules, "restricted testis type". In contrast to the "un-re restricted testis type" or the ''perciform testis type,'' so-called because of its initial description in fishes at one time classified in the order Perciformes, such as the striped mullet, *Mugil cephalus* spermatogonia are distributed along the lengths of testis lobules. Surveys of gonad morphology during the past two decades have confirmed the presence of the unique, restricted testis type in atherinomorphs (*viz.*, Grier and Collette 1987; Grier and Parenti 1994; Downing and Burns 1995). The lobular testis type is therefore, a diagnostic or synapomorphic character of the Neoteleostei and the restricted lobular type of testis is diagnostic of atherinomorph fishes.

Data on syngnathids testis structure most concer species belonging to the trunk-brooding lineage, Urophori (Wilson and Orr 2011), with most emphasis to the two most species-rich genera in the family, *Hippocampus* (*H. guttulatus* and *H kuda*) (Piras et al. present PhD. thesis) and *Syngnathus* (*S. abaster*, *S. typhle*, *S. tenionotus*, *S. acus*, *S. schlegeli*) (Carcupino et al. 1999; Watanabe et al. 2000; Biagi et al. 2014). Although in all above mentioned species, testis belongs to the "perciform type", it however atypically consists of a single and continuous germinal compartment surrounded by a single and continuous somatic compartment. Due to this structure, syngnathids testis lacks of an efferent duct system, which in teleost testes generally connects the multiple lobules/tubules to the main germinal duct.

The only exception to this model within the urophorine lineage seems to be the welly dragon *Phyllopteris taeniolatus*, in which multiple germinal compartments of tubular type have been reported (Forsgren and Young 2008). This latter species however, belongs to a lineage distantly connected to the monophyletic lineage formed by *Hippocampus* and *Syngnathus* within the Urophorine subfamily (Wilson et al. 2003; Wilson and Orr 2011).

Phyllopteris taeniolatus, however, as well as *Syngnathus* and *Hippocampus* species, has a spermatogenesis of the semicystic type.

In addition, in the only representatives species of the second lineage of the family (gastrophorine subfamily), *Microphis brachyurus,* testis has been reported to belong to the restricted lobular type characterized by a cystic spermatogenesis. Moreover it consists of two regions differing in both length and morphological appearance (Miranda-Marure et al. 2004).

Because, the differences in the ovarian structure among syngnathids species has been demonstrated not directly due to phylogenetic relatedness, we intend to verify if this assumption is also true for testis structure. The aim of this paper is therefore to analysed the testis structure, the spermatogenetic mode and spermatozoa ultrastructure in the straight nosed pipefish *Nerophis ophidion* (L. 1758), a syngnathids species occupying a basal position within the Gastrophorine lineage (Wilson et al. 2001, 2003; Wilson and Orr 2011). The straight nosed pipefish is a marine species that inhabit algal zone or eel-grass beds (*Zostera marina* and the Mediterranean *Posidonia oceanica*) along the coastal zone from 2 to 15 meters depth. The straight nosed pipefish is commonly found along the west coast of Sweden and in the southern Baltic Sea, to a lesser extent in the Gulf of Bothnia. Elsewhere the distribution extends from central Norway south along the coast (including the British Isles) to Morocco and continues into the Mediterranean Sea and Black Sea. It is not distributed, however, along the North Sea coast between Denmark and the English Channel.

Adults spawn in May to August. It feeds on small crustaceans and fish fry. The male broods the embryos attached to their abdomen where paternity is ensured despite brooding of embryos outside of the male's body. Each male broods eggs from a single female, for each brood, but females may deposit eggs on several males (Dawson 1986; Froese and Pauly 2010)

Materials and Methods

Samples

Seven adult male brooding eggs of *Nerophis ophidion* were sampled from Venice lagoon (Veneto), during the reproductive period (July 2013). Alive specimens, delivered to the laboratory within 3 h, were sacrificed, by exposure to the anaesthetic 3-aminobenzoic acid ethyl ether (MS-222, Sigma-Aldrich) for 10 min, and then processed for microscopic analysis.

Light microscopy

Two specimens were dissected to extract the testes, which were immediately gently squashed onto a glass slide and freshly observed under a Zeiss Axiophot light microscope (ZEISS, Oberkochen, Germany).

Four additional testes, obtained dissecting two further males, were fixed in aqueous Bouin's fixative, dehydrated in a graded ethanol series, cleared in Bioclear and finally embedded in paraffin wax. Sections (5µm) were stained with Eosine and Emallume di Mayer (Mazzi 1977) and processed for the morphological studies with the same Light microscope

Transmission electron microscopy (TEM):

One adult male were dissected and the gonads were fixed for 2 h in 4%paraformaldehyde-5% glutaraldehyde buffered with sodium cacodyl ate (0.1 M, pH 7.2). Specimens were then rinsed overnight in the same buffer, post-fixed for 1 h in 1% osmium tetroxide buffered with sodium cacodilate, dehydrated gradually in ethanol and embedded in Epon 812 resin. Thin sections of 80 nm thick were cut with a Reichert Ultracut ultramicrotome, stained with uranyl acetate and lead citrate, observed and photographed with a Jeol Tem 1200 EX II transmission electron microscope (JEOL, Tokyo, Japan).

Morphometry:

Aliquots (20 µl) of seminal fluid, obtained by the testes of one male, were fixed in 5% glutaraldehyde, applied onto coverslips precoated with polylysine (1 mg mL-1, Sigma P1274) and allowed to air-dry. Next, cells adhering to the coverslip were stained with toluidine blue 0.1% in aqueous solution and observed with a Zeiss Axiophot light microscope (Zeiss). Digital images were acquired with a digital camera Nikon DS-fi1 connected with the control unit DS-L2 and mounted on an optical microscope Nikon Eclipse 80i. The measurements were made using the program Tpsdig2.

Four morphometric parameters, such as the width and length of the head (including the nucleus, and the midpiece), the length of the flagellum and the total sperm length, were taken into account. The last one was calculated as the sum of head and flagellum length. Twenty intact sperm were analysed. Abnormal, broken or difficult to measure spermatozoa were discarded.

Results

The testes were two elongated translucent bodies located in the coelomic cavity below the intestine. Each testis appeared divided into two parts of different morphology. The apical part, amounting to about a third of the length of the entire gonad, was thin and when observed under the stereomicroscope showed an irregular and compartmentalized appearance with transparent areas interspersed with areas of more dense and latescent appearance (Figs.1A, 2A). The remaining part of the gonad was thicker and had a more homogeneous and translucent appearance (Fig.1A, 2A). In the transverse and longitudinal sections, the apical third showed a uniform morphology for its entire length. It was characterized by a compact appearance for the lack of an evident central lumen. The gonadal tissue was made up of a compact germinal epithelium bordered by a thin vascularized tunica albuginea. This latter entered inside the organ forming inter-germinal septa. (Fig.2 B-E). The germinal epithelium

had the typical organization in spermaticysts, resting on the basal membrane, and consisting of germ cells enveloped by Sertoli cells. Spermatocysts contained spermatogonia and spermatocytes. Developing spermatids and mature sperm were not never observed inside the cysts (Fig.2 B-E). The gonadal lumen in this portion seems to be totally absent or reduced in the form of fissures occupied by a fat-like secretion (fig.2 B-E).

An abrupt narrowing marks the transition between the first and the second part of the testis (Fig. 3). No particular feature has been seen in this poorly extended transition portion. Immediately below of the norrowing, the second portion of the testis appeared as a hollow tube, consisting of a thin wall and a large central lumen occupied by numerous free large cells and mature sperm, all immersed in a fibrous secretion (Figs 3). The wall appeared constituted by the tunica albuginea of same appearance of the preceding testicular portion, whereas the germinal epithelium was not more evident. It appeared to be substituted by a mono-layered somatic epithelium with cells of cubic shape, characterized by a compact cytoplasm and oblong-shaped nucleus, and basally located (Fig. 3).

Proceeding caudally, the cytoplasm of the somatic cells progressively appeared less compact and characterized by a several vacuoles (Fig 4A, C). These latter, when observed at the transmission electron microscope, appeared different in size and full of a fine granular material (Fig 4B, D). More caudally, the somatic cells cytoplasm showed signs of disruption, probably caused by an olocrine-like secretory pattern (Fig. 4 E-F).

In all examined males, inside the testicular lumen of the second testicular region, large spherical cells and spermatozoa embedded in a fibrous-like secretion were observed (Figs 3, 4A-C,). Two types of spherical cells, aflagellate and flagellate cells were recognizable (Fig 5). Most of the aflagellate cells were mononucleate cells, although few polynucleate cells were recognizable (Fig 5D). Their nuclei, which appeared of a uniform

morphology characterized by dispersed chromatin and one spherical dots of nucleolus-like appearance, occupied most of the cell volume (Fig.5B). Their cytoplasm was rich in droplets of different size and density (Fig. 4B, 5A).

In contrast, the flagellate cells showed a less amount of cytoplasmic globules, and had nuclei of different appearances (Figs 5B, C, 6, 7*)*. These cells were unquestionably developing spermatids, which can be categorized in young, intermediate and late spermatids on the base of their nuclear shape, degree of both chromatin condensation and midpiece formation. The young spermatids had spherical nuclei with one or two spherical dots of nucleolus-like appearance, like those showed by the aflagellate cells (Fig. 6A). They are however characterized by small spots of condensed chromatin, which appeared to be concentred in a nuclear region closed to external cytoplasmic centrioles, from which the axoneme takes place. No mitochondria were recognizable in proximity of the axoneme (Fig. 6A).

In a more advanced stage of development respect to the preceding one, the nuclei had an irregular shape; the nucleoli were not more evident, and the nuclear fossa begin to be evident (Fig. 6B). The deep invagination of the nuclear membrane appeared also marked by the accumulation of condensed chromatin. Inside the nuclear fossa, the centrioles localized, and small mitochondria began to accumulate around the first portion of the axoneme. The sperm collar with its canal began to develop (Fig. 6B). The intermediate spermatids had smaller nuclei, ovoid in shape and chromatin condensed in larger spots uniformly distributed in the nuclear volume, although it appeared more concentrated in correspondence of the nuclear fossa (Fig. 6C-D). The late spermatids had little smaller ovoid nuclei with chromatin almost uniformly condensed (Fig. 7A). Moreover in this stage, the amount of cytoplasm surrounding the nucleus is much lower of the preceding stage (Fig. 7A).

The mature spermatozoa consisted of three distinct portions; the head, the midpiece and the flagellum (Figs 7-8). The head was cylindrical and slightly curved in shape and it was entirely occupied by the nucleus. (2.78 ±0.19 μm, n = 20) (table 1). At the basal end of the nucleus, a deep nuclear fossa is present, (Fig 7 B-. The midpiece was clearly marked under the nucleus by mitochondrial rings. These latter were housed inside a cytoplasmic collar, which was separated from the first portion of the flagellum, by a deep so-called cytoplasmic canal (Fig- 7D). The plasma membrane of the collar lining the canal appeared closely associated to a sheath of electron-dense material arranged in a ring-like structures regularly spaced (Fig 6D, 7D,E). The flagellum, had an internal "9+2" axoneme, originating from the distal centriole, surrounded by the plasma membrane which form two lateral fins (Fig. 7F).

Discussion

N. ophidion testis consists of two regions clearly marked by: (i) an narrowing of separation and, (ii) a different morphology, which can be attributed to a different functional implication. The first portion is characterized by the absence of an evident central lumen and consists of a single germinal compartment bordered by a thin vascularized tunica albuginea. The surface of the germinal epithelium appears increased by numerous invaginations of the tunica albuginea, which confers to this portion an incomplete compartmentalized appearance. The germinal epithelium had the typical organization in spermatocysts resting on the basal membrane, and consisting of germ cells enveloped by Sertoli cells. Spermatocysts contained spermatogonia and spermatocytes. Developing spermatids and mature sperm are not never observed inside the cyst.

The second portion of the testis appears larger of the first and has a hollow tube appearance. It consists of a thin wall and a large central lumen occupied by numerous free

large cells and mature sperm, all immersed in a fibrous secretion. No germinal cysts are recognizable. The large cells free inside the lumen are unquestionably developing spermatid, confirming that *N. ophidion* as well as the other syngnathids species examined (Carcupino et al. 1999; Forsgren and Young 2008; Biagi et al. 2014) has a semicystic spermatogenesis. The wall of this portion appears simply constituted by the tunica albuginea, of the same appearance of the preceding one, and by a mono-layered somatic epithelium. Due to these morphological differences, the two testis portions may be considered as the regions of germ cells proliferation and maturation respectively.

This testis organization is in some way similar to that reported by Miranda-Marure et al. (2004) in the other gastrophorine *Microphis brachiurus*. In both species the testes are divided into two different regions, although testes in this latter species seem to greatly differ in both the germinal compartment morphology and spermatogenetic mode. *M. brachiurus* testis has been reported to be of the restricted lobular type with cystic spermatogenesis. Unfortunately, no images of testis have been published by Miranda-Marure and co-authors (2004) and the description of the two testicular regions is not detailed. Therefore, on the base of the absence of clear images and a poor description, it is impossible to compare the different results obtained in our and their study. However, it should be taken into account that, according to Parenti and Grier (2004), the lobular testis type is proposed as a diagnostic or synapomorphic character of the Neoteleostei, whereas the restricted lobular type of testis is diagnostic of atherinomorph fishes. Leaving aside these considerations, at least the gonadal subdivision in two regions seem to be a character shared only by species of the gastrophorine subfamily.

No subdivision are reported in the testis of Urophorine species examined up to now, such as those of the genera *Syngnathus* (Carcupino et al. 1999; Biagi et al. 2014),

Hippocampus (Piras et al. present PhD thesis) and *Phyllopteryxs* (Forsgren and Yuong 2008). In *Syngnathus* and *Hippocampus* species however, the testis lacks of any kind of compartmentalization, and invaginations of the tunica albugiea are not present at any level of the testis. The close similarity of the testis structure in these two syngnathids genera seems to support molecular data, which have recently shown that *Hippocampus* and *Syngnathus* are closely related, constituting a monophyletic lineage within the subfamily (Wilson et al. 2003; Wilson and Orr 2011). On the other hand, compartmentalization is reported in the testis of *Phyllopteryxs taeniolatus* (Forsgren and Yuong 2008). Moreover in this species, testis is described to be "formed by an intricate system of interconnecting seminiferous tubules". According to the better elucidated definition reported in Parenti and Grier (2004), teleosts testes of tubular type are only those in which "the germinal compartments do not terminate at the testis periphery, but form highly branched, anastomosing loops or tubules". Instead, testes of lobular type are those in which "the germinal compartments may form anastomosing networks proximally, but distally they extend to the periphery of the testis and terminate blindly". From the histological images published by Forsgren and Young (2008) seems that also the testis of *P. taeniolatus* consist of a unique compartment, inside which the surface of the germinal epithelium is increased by invagination of the tunica abuginea.

Although the testis structure in representative of other families belonging to the suborder Syngnathoidei, to which the family Syngnathideae is ascribed, are poorly investigate, a testis structure characterized by a single germinal compartment seems to be a synapomorphic character of the Syngnathidae family. In *Fistularia commersonii* (one of the four species belonging to the single genus within the family Fistulariidae, ascribed together with Syngnathidae to the same suborder) the male gonad is reported to have an unrestricted lobular organization, with spermatogenic lobules forming an anastomosing network proximally to the sperm ducts.

Moreover, another synapomorphic character of the Syngnathidae family seems to be the spermatogenetic process of the semicystic type. The only exception seems to be the gastrophorine *M. brachiurus.* As mentioned above, the lack in this species of testis images obtained with histological or other microscopic techniques does not permit a corrected comparative analysis. However, the mature sperm of *M. brachiurus,* observed free into the testicular lumen, are reported to be spherical aflagellate cells very similar in morphology to the spermatocytes recognizable inside the cysts. Both flagella and nuclei, the latter characterized by different shape and different degree of chromatin condensation (typical chances of the spermatids development) are not clear visible using the classical histological techniques. Therefore, further analyses, performed with more appropriate techniques, could clarify if the aflagellate sperm of *M. brachiurus* are really mature germ cells or developing spermatids, which undergo to the differentiation process inside the testicular lumen by virtue of the semicystic spermotogenesis.

In the typical semicystic spermatogenesis, spermatocytes and/or spermatids are released after the cytokinesis among isogenetic germ cells is completed. Therefore, germ cells advance individually through spermiogenesis inside the lumen. This is also the case of most of the analized syngnathids, such as species of *Hippocampus*, *Nerophis* and *Phyllopterix*, in which developing spermatid are mononucleted cells. In the contrary, the developing germ cells inside the lumen of *Syngnathus* species are mono- and, more frequently, polynucleate and polyflagellate cells. Individualization of mature sperm seems, in these species, to occur at the end of spermiogenesis, so the cytokinesis seems to be abolished or at least delayed (Carcupino et al. 1999; Biagi et al. 2014). Because of this feature, in all syngnathids species, in which spermatids typically develop individually, this process may be considered of the "asynchronous type". On the other hand, in the *Synagnthus* species, in which developing spermatids are grouped in large symplastic cells with nuclei of different

developmental stages, the semicystic spermatogesis may be chategorized as new type of semicystic spermatogenesis never reported in other teleost here called “discontinuous and group-synchronous type”. In all cases however, the semicystic spermatogenesis leads to a reduced number of simultaneously mature sperm, and it has been interpreted as one of the possible mechanisms evolved to reduce the cost of sperm production. This mechanism seems therefore to be crucial in those species in which, a small ejaculate size is justified by their low fecundity, monogamous mating system, absence of sperm competition and presence of male parental care, such as some Opistognatidae (Rasotto et al. 1992; Marconato and Rasotto 1993) and Gobiidae (Mazzoldi 2001). A spermatogenetic process of the semicystic type, has been hypothesized to be also useful in those species showing a promiscuous mating system and/or a long-lasting eggs deposition (see some blennies). In such species, the males are forced to parcel out their sperm expenditure during mating into several successive ejaculates, releasing only limited portions of sperm at each ejaculation (Giacomello et al. 2008). All syngnathid species have very small ejaculate size, low fecundity, male parental care, absence of sperm competition, and their mating system varies from monogamy to different types of polygamy (Sanna et al. 2008; Wilson et al. 2003; Rosenqvist and Berglund 2011).

From this point of view is arduous to speculate a possible function of the two types of semicystic spermatogenesis occuring in syngnathids, particularly if this aspect is considered in a larger contest of the reproductive biology of these fishes, such as ovarian structure, mode of egg production, and mating pattern. It has been recently demonstrated that synagnthids species, which mate polygynandrously (*i.e.* males brood eggs from several females at the same time and females transfer eggs to several males within a short time span; Berglund et al. 1989; Jones et al. 1999) polyandruosly, such as *Syngnathus* species, have ovary of asynchronous type characterized by several stages of developing follicles originating from a single germinal ridge. In contrast, others syngnathids, such as messmate

pipefish *Corythoichthys haematopterus*, big-belly seahorse *Hippocampus abdominalis*, lined seahorse *Hippocampus erectus,* which mate monogamously with a stable pair bonding (Matsumoto and Yanagisawa 2001; Foster and Vincent 2004), and barhead pipefish *Microphis leiaspis* and *Nerophis ophidion,* which have polyandrous mating system, have ovary of group-synchronous type, with grouped developing follicles originating from two germinal ridges. Moreover, recent data suggest that the timing and number of ovulations are variable even among species having similar kinds of ovarian structures. In *C. haematopterus*, females extrude all mature eggs as a single sheet to transfer them to the male's brood pouch (Matsumoto and Yanagisawa 2001). After spawning, females *C. haematopterus* are unable to spawn a new clutch for 10–19 days (Sogabe et al. 2007). Females of *Hippocampus* are also unable to replenish mature eggs for a long period of time (9–45 days) after spawning (Foster and Vincent 2004). In *N. ophidion*, females are unable to prepare mature eggs at least for the first 3 days after spawning, due to the group-synchronous egg production. The number of mature eggs in the ovarian lumen increases each time they ovulate. Females should be able to deposit small numbers of eggs when they finish the first ovulation. If receptive males are not available, females continue to produce another batch of eggs. *C. haematopterus* and *Hippocampus* species are distantly connected within the subfamily urophorine and both species are even more distant to *Nerophis ophidion*, which is a member of the minor subfamily gastrophorine. All these three species have ovary of group-synchronous type. This has been interpreted as a demonstration that the similarity of ovarian structure between *Corythoichthys*, *Hippocampus* and *Nerophis* is not directly due to phylogenic relatedness, but constrained more or less by the mating type.

About the two different semicystic spermatogeneic modality showed by syngnathids, and here proposed as "discontinuous and group-synchronous type semicystic type" and "asynchronous semicystic type" it may be speculated that they are related, as well as the

mode of egg production, to the mating type. In particular, the so called "discontinuous and group-synchronous semicystic type" may represent a modality able to reduce the loss of mature sperm, which could seriously affect the reproductive success of those species, such as some of the *Syngnathus* genus, characterized by a very low sperm concentration and polygynandrous mating system within a short time span. In contrast, the " asynchronous semicystic type", which occurs in those species in which males, like the females, mate much less frequently and not release sperm for a long period of time, (i.e. the monogamous *Hippocampus guttulatus* and the polyandrous *Nerophis ophidion*) the sperm loss is less probable, less important and less compromising the reproductive success, even in the presence of a very low concentration of spermatozoa.

According to the ultrastructural analysis of all types of flagellated cells recognizable inside the testis lumen of *N. ophidion*, we have identified only one type of mature sperm. They are characterized by an elongated head, completely occupied by a nucleus with condensed chromatin, a short medpiece characterized by two mitochondrial rings surrounding the first portion of the axoneme, and a long flagellum. These data seems to confirm the presence in all examined syngnathids species of only one type of mature sperm. Moreover, the ultrastructure organization of mature sperm is very similar in all examined species (Carcupino et al. 1999; Biagi et al. 2014; Piras et al. present PhD. Thesis). The small differences recognized only regard the nuclear size and shape, which slender and thinner in Syngnathus species respect to *Hippocampus* and *Nerophis*. Moreover, the sperm head of the latter species is atypically slightly curved.

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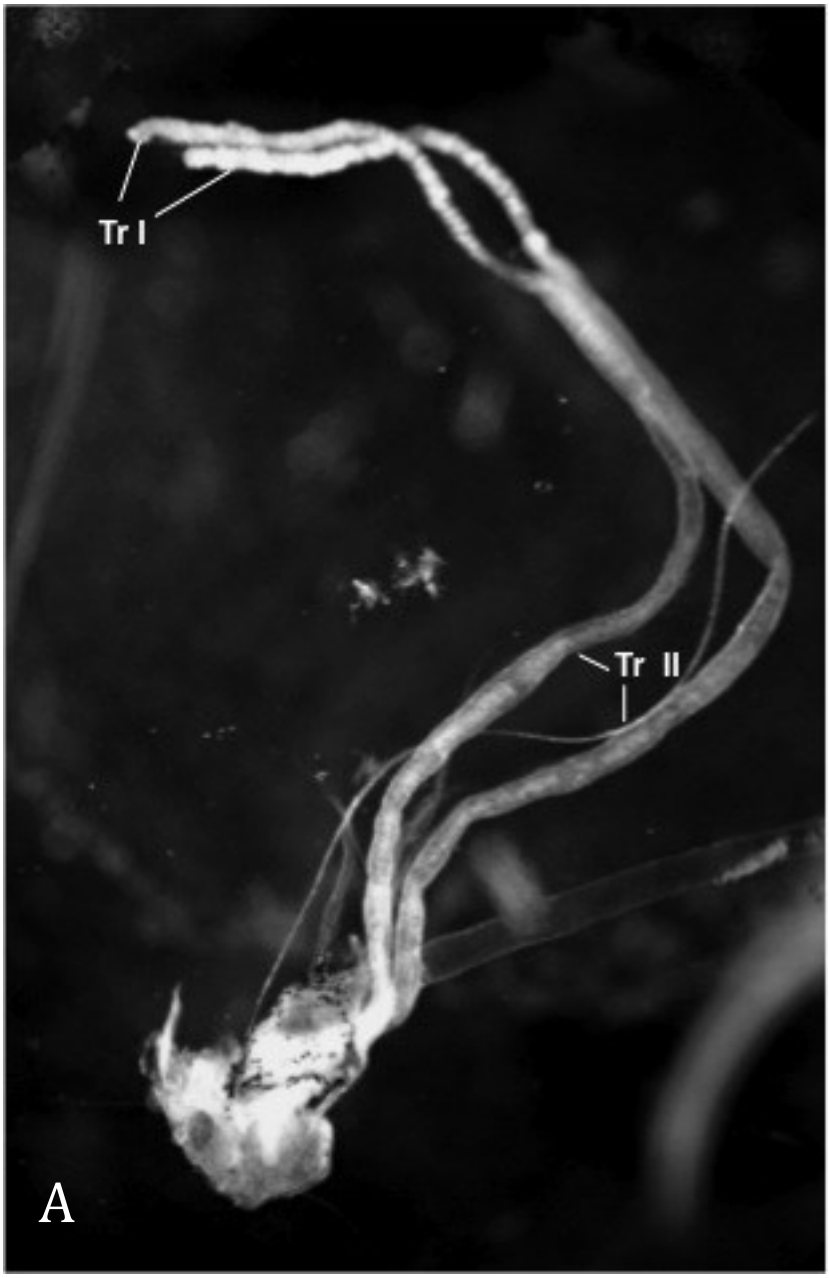

Figure 1. Entire reproductive apparatus of *N. ophidion* male with testes characterized by two different region. The apical third (Tr I) with an irregular and compartmentalized appearance with transparent areas interspersed with areas of more dense and latescent appearance, and the remaining part of the gonad (Tr II) thicker and with more homogeneous and translucent appearance.

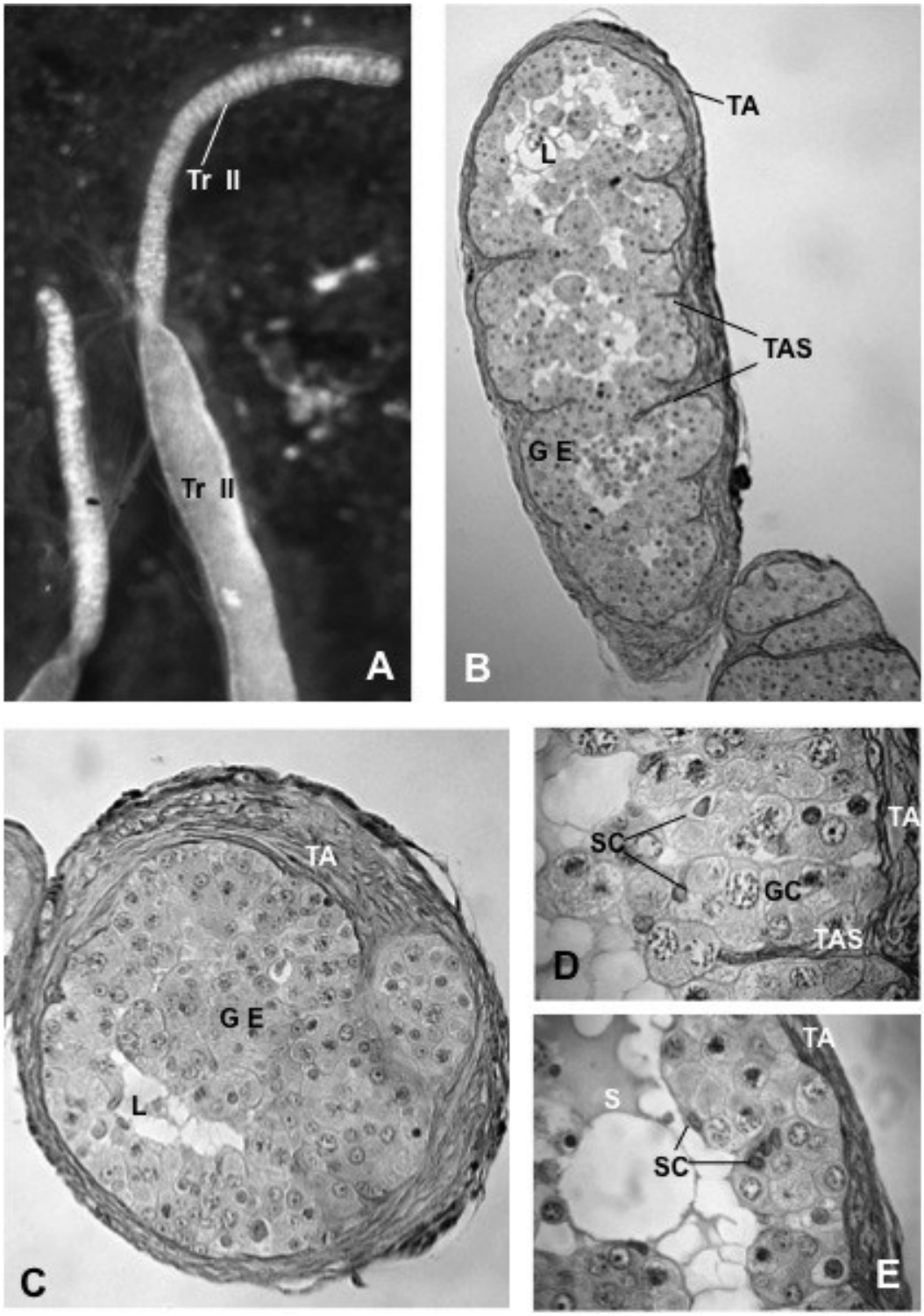

Figure 2. A. High magnification of a single testis of another male showing two different region. **B-C**. Sagittal (**B**) and **t**ransverse paraffin sections of the apical testicular region showing the compartmentalization due to tunica albuginea septa and the compact germinal epithelium. **D-E**. High magnification of transverse sections showing spermatocysts formed be germ cells (spermatogonia and spermatocytes) enveloped by Sertoli cells. Germinal

epithelium (Ge); germ cells (Gc); lumen (L); fat-like secretion (S); Sertoli cells (Sc); tunica albugine (TA); tunica albuginea septa (TSA); apical testis region (Tr I); caudal testis region (Tr II).

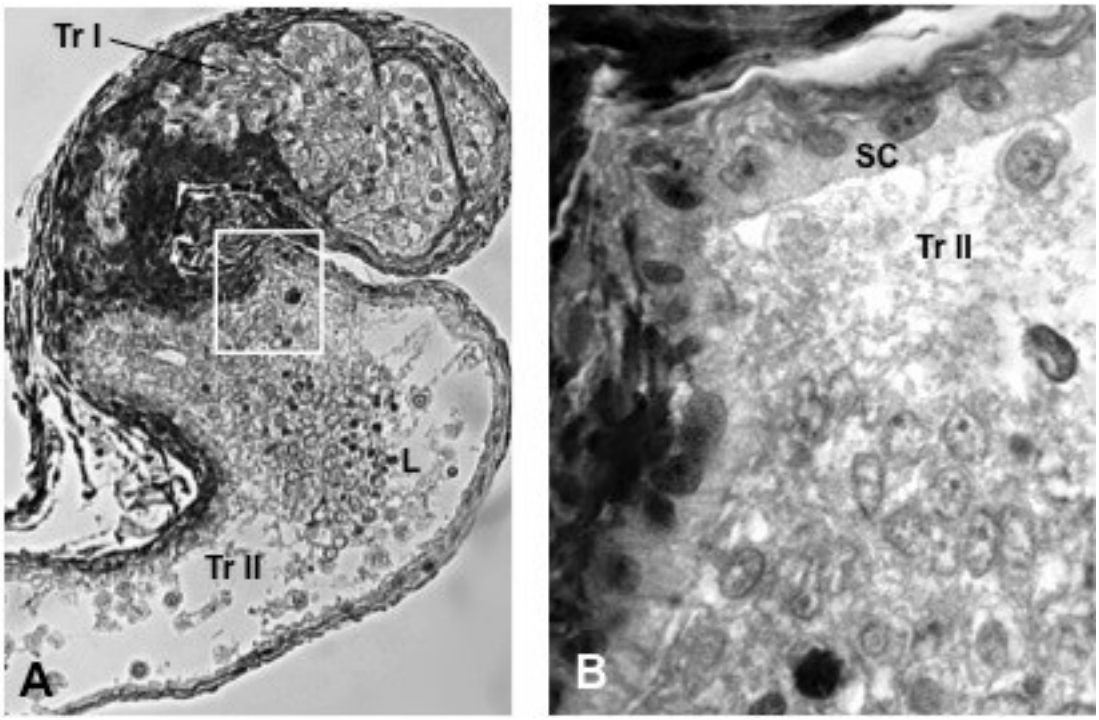

Figure 3. A. Sagittal paraffin section of *N. ophidion* testis showing the abrupt narrowing marks the transition between the first and the second part of the testis and the beginning of second testis region. **B**. High magnification of the same section reffered to the area marked by the white rectangle. Lumen full germinal cells immersed in granular secretion (L); Somatic epithelial cells (SC); apical testis region (Tr I); caudal testis region (Tr II).

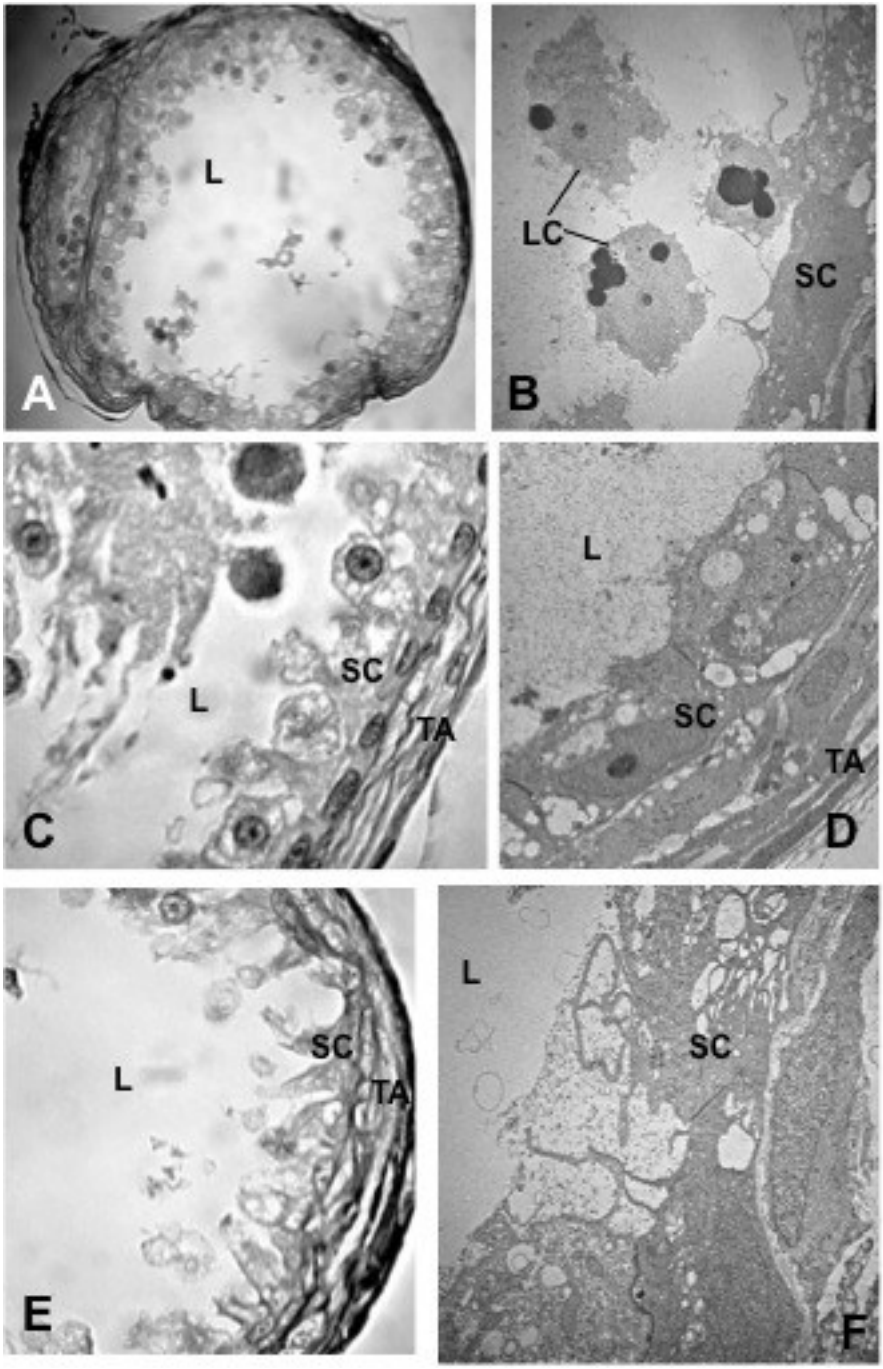

Figure 4. A, C. Paraffin sections of *N. ophidion* testis showing the the second part of the testis and the second testis region immediately below the narrowing. **B, D**. Transmission electron micrographs of the same testis portion showed in **A** and **C**. **E-F**. Ligh (**E**) and transmission electron (**F**) of a mor caudal portion of the secon testicular region. Lumen full germinal cells immersed in granular secretion (L); Somatic epithelial cells (SC); apical testis region (Tr I); caudal testis region (Tr II).

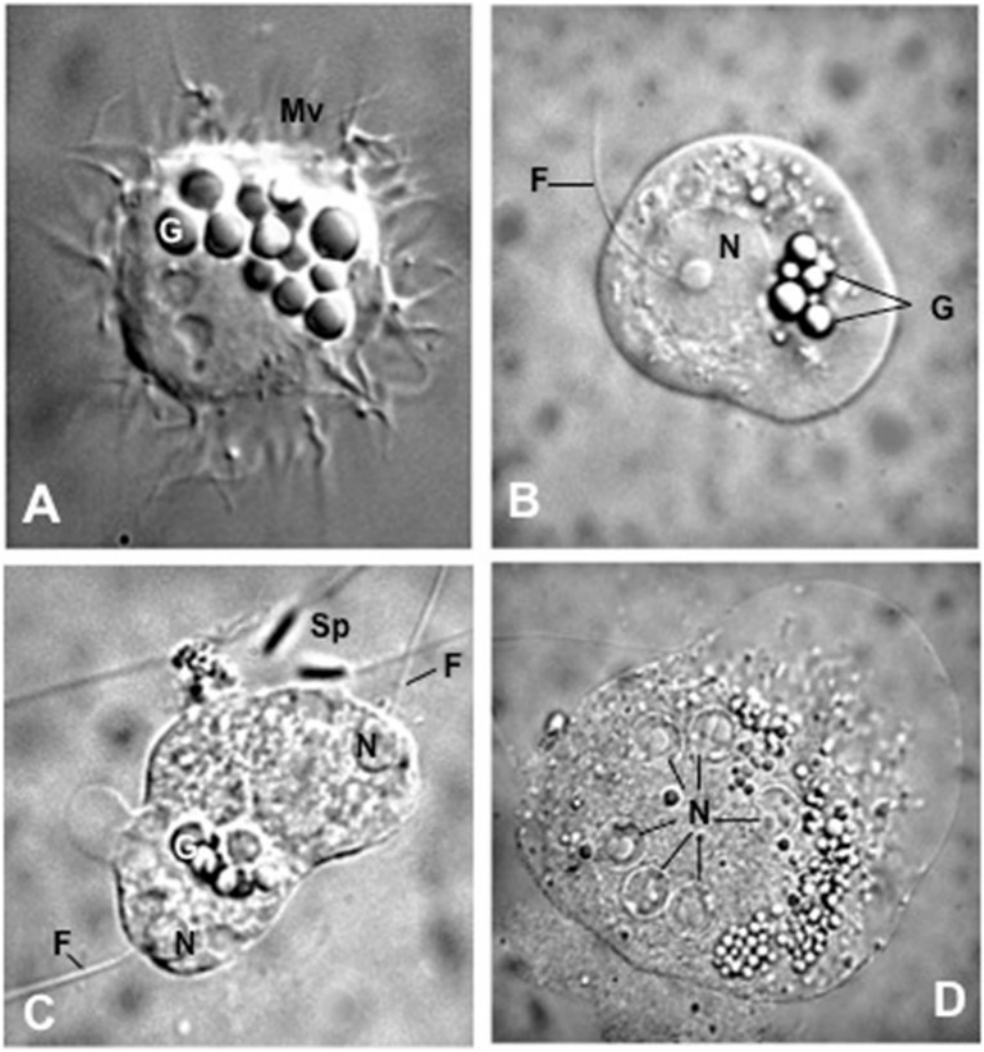

Figure 5. Unfixed and unstained cells free inside the testis lumen of *N. ophidion* testis. **A**. aflagellate cell with numerous microvilli-like projection and cytoplasm rich in droplets. **B-C.** Flagellate cells with less amount of cytoplasmic droplets. **D**. Polynucleate cells rarely observed in side the testiculat lumen. Cytoplasmic droplets (D); flagella (F); nuclei (N); mature sperm (Sp)

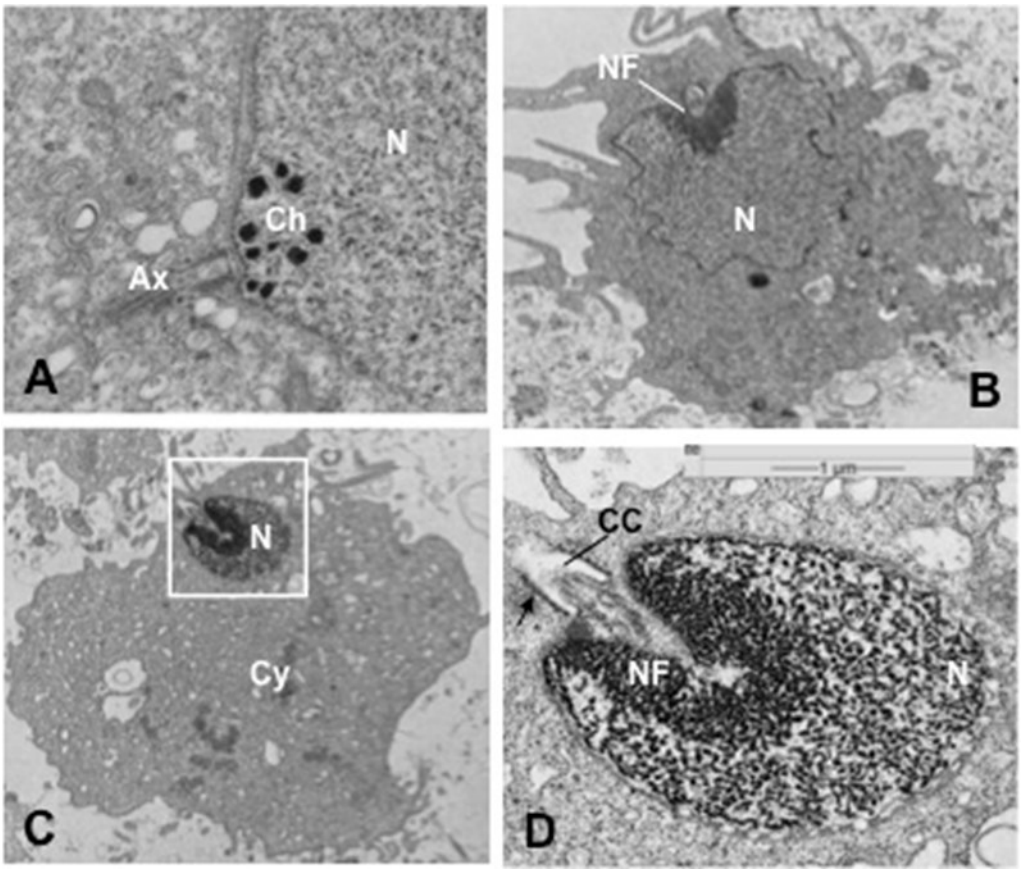

Figure 6. A. Transmission electron micrographs of young developing spermatid showing centrioles closed to the basal end of the nucleus inside which are several spots of condensed chromatin. **B**. More advanced spermatid with nucleus of irregular shape and basal nuclear fossa marked by condensed chromatin. Spermatid in a more advanced stage of development with nucleus of ovoid shape and chromatin more condensed. Developing midpiece is also recognizable. **D**. High magnification of the same spermatid showed in **C**. Spots of electron-dense material adhering to internal membrane of the future sperm collar (arrow); axoneme (AX); cytoplasmic canal (CC); nucleus (N); nuclear fossa (NF).

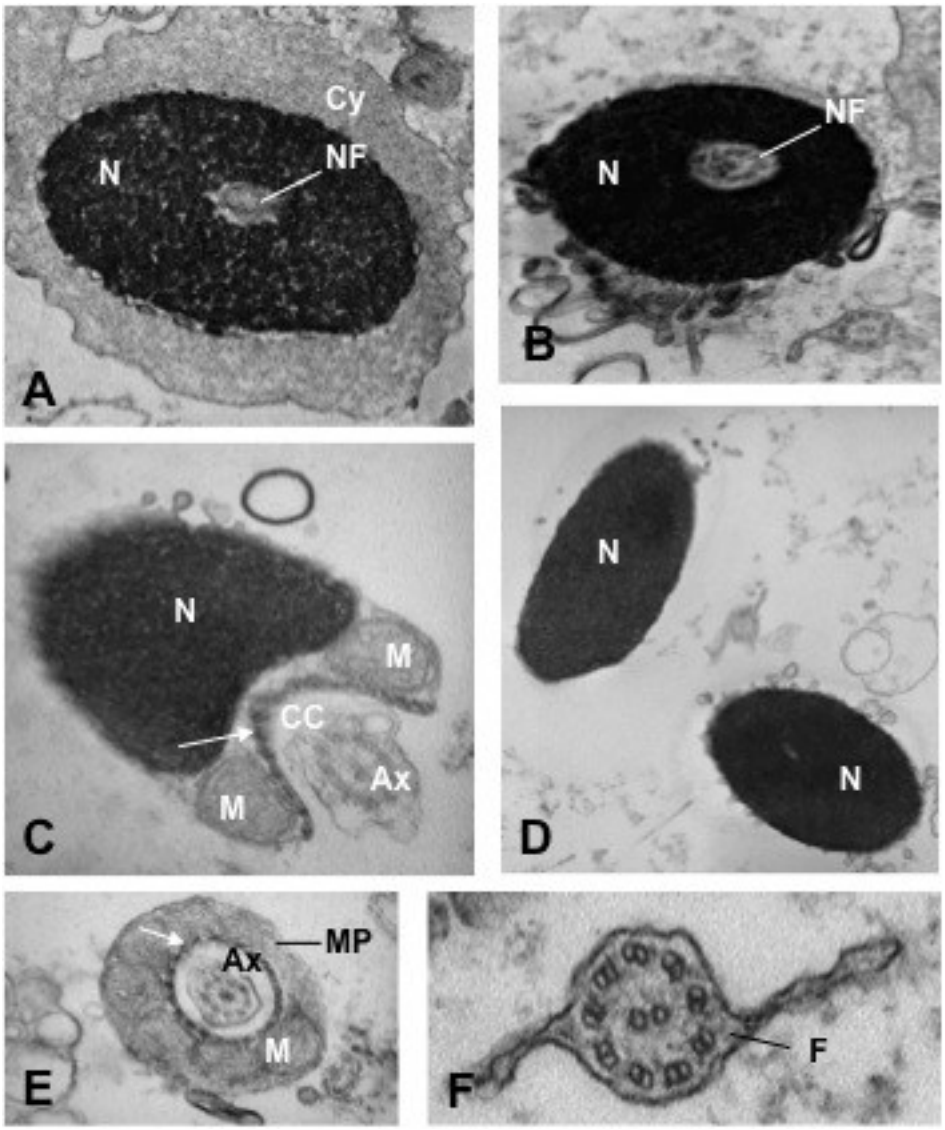

Figure 7. **A.** Late spermatid with a small amount of cytoplasm and chromatin almost completely condensed. **B**, **D**. Transverse section of mature sperm nuclei with chromatin completely condensed. **C.** Oblique section of mature sperm showing the basal end of the nucleus and the apica part of the midpiece. **E**. Transverse section of mature sperm midpiece. **F** Transverse section of mature sperm tail. Spots of electron-dense material adhering to internal membrane of the sperm collar (arrow); axoneme (AX); cytoplasmic canal (CC); cytoplasm (Cy) mitochondria (M); nucleus (N); nuclear fossa (NF).

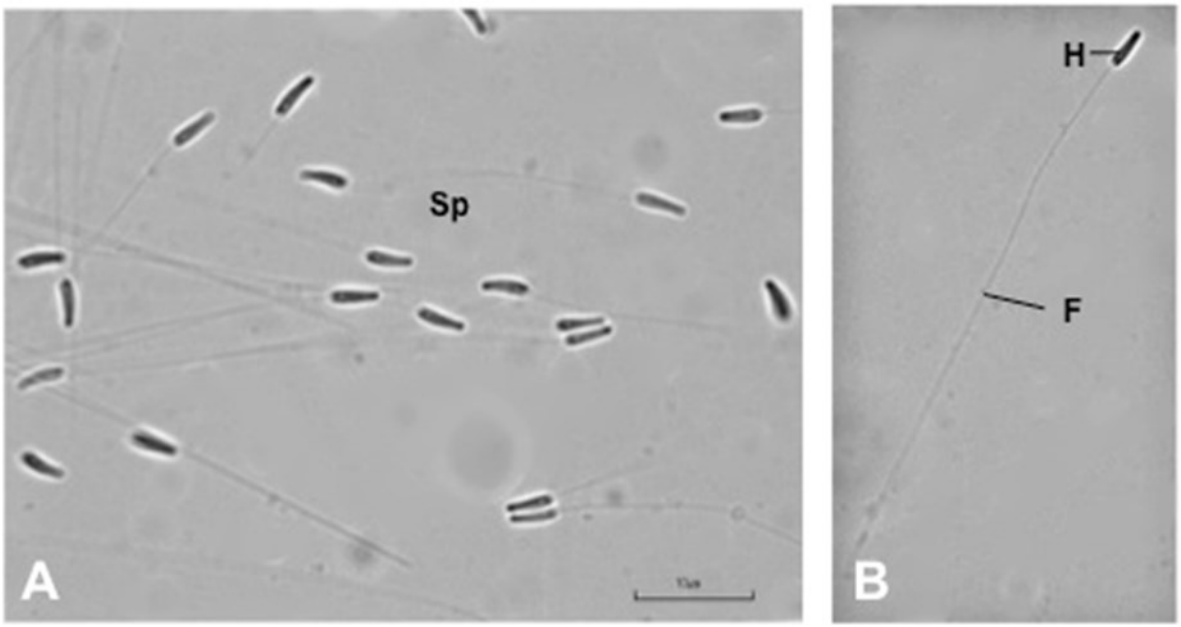

Figure 8. Light microscopic images of mature sperm showing sperm head of elongated and slightly curved shape followed by a thin and long flaglellum. Flagellum (F); sperm head (H) nucleus (N); sperm (Sp).

N.ophidion ***(N=20)***	Head width	Head length	Flagellum length	Tot. sperm length
Mean ± SD	1.34 ± 0.13	5.16 ± 0.31	52.16 ± 5.35	57.32 ± 5.45

Table 1. Values of the four morphometric traits analysed in *N. ophidiom* sperm. Data derived from 20 spermatozoa belonging to one male. All measurements are in μm, and are referred to mean ± Standard deviation

V

Characterization of the Dopaminergic system in adult and young males of the pipefish Syngnathus abaster (Syngnathidae)

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Abstract

In many but not all teleosts, the stimulatory control of hypothalamic-pituitary-gonadal axis by gonadotrophin-releasing hormones (GnRH) is regulated by the powerful inhibitory action of preoptic area (POA) dopaminergic (DA) neurons. In a previous work, on *Haplochromis burtoni,* it has been shown that soma size of the GnRH-containing neurons in the POA changes according to social status in male. Dominant fish have larger cells, in contrast to non territorial males. Interestingly, it has also been shown that the GnRH neurons are up to twice as larger in females that have never spawned or are in the act of spawning, than in females that are carrying broods. The primary factor controlling GnRH-immunoreactive (irGnRH) neurons size appears to be related to the reproductive state. Here we show that the cells body size of the DA neurons changes depending on the reproductive and maturational state in males *Syngnathus abaster*. In this study, we evaluated, in both young and adult reproductive males, the number and the size of Tyrosine-hydroxylase positive (presumably DAergic) neurons in the POA. In this area, we found that adult males have less but bigger cells than younger ones. The morphometric analisys of the cell body size, showing the phenotypic changes in neurons, which are implicated in the reproductive control, may be indicative of a high level of neuronal plasticity in the POA and may be important in the understanding of the events that regulate sexual maturation and/or gonadal developement. These results suggest that DA could be implicated in the regulation of sexual maturation in *Syngnathus abaster,* as in other teleosts.

Introduction

The endocrine control of reproduction in fishes is regulated, as in mammals, by complex neural networks, through the hypothalamic-pituitary-gonadal axis. The vertebrate nervous system integrates internal and external inputs in order to regulate sexual maturation and gonadal development. All the regulatory factors converge on gonadotropin-releasing hormone (GnRH)-containing neurons, which stimulates pituitary gland to synthetize and release gonadotropins: follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Kitahashi *et al.* 2013; Weltzien *et al.* 2004). Gonadotropins stimulate maturation of the gonads (ovaries and testes) by binding to their respective receptors.

The homeostasis of the reproductive system is regulated by sex steroid hormones (estrogens and androgens) secreted by mature gonads, which negatively regulate the hypothalamus and the pituitary gland (long- and short negative feedback loops). In most fishes, as the goldfish (Chang and Peter 1983; Chang *et al.* 1990), catfish (De Leeuw *et al.* 1986), Chinese loach (Lin *et al.* 1989), the tilapia (Levavi-Sivan *et al.* 1995) and the gray mullet (Aizen *et al.* 2005), the stimulatory signal by GnRH to pituitary gland may be antagonized by the inhibitory effects of DA-releasing neurons (Peter *et al.* 1986, 1991; Dufour *et al.*2010). In the goldfish, dopamine also acts on GnRH-releasing neurons to inhibit GnRH release (Trudeau 1997).

However, the inhibitory function of DA upon gonadotropins release was not found in some fish species, suggesting that physiological importance and role of the dopaminergic transmission is species-specific in teleosts. Anatomical studies in goldfish, trout and european eel (Lado *et al.* 2014; Linard *et al.* 1996; Sébert *et al.* 2008) revealed that DAergic neurones regulating the GnRH-releasing neurons, are located specifically in the nucleus preopticus anteroventralis and that project to the pituitary region (proximalis pars distalis,

PPD) (Fryer and Maler 1981; Hornby *et al.* 1987; Kah *et al.* 1987; Dufour *et al.* 2010). Moreover, in most teleost species, DAergic neurons are implicated in regulation of late oocyte maturation, of ovulation, of spermiation and sexual differentiation during the early steps of gametogenesis, and thus interact with GnRH-releasing neurones in the control of puberty (Dufour *et al.* 2010).

Pharmacological investigations in teleost showed that the inhibitory effects of DA on pituitary gonadotropin synthesis are mediated by DA-D2 type receptors. DA-D2 receptors have now been sequenced in several teleosts, and the coexistence of several DA-D2 subtypes has been demonstrated in few species (Nocillado *et al.* 2007). In teleosts, recent multiple microarrays analysis in *Carassius auratus* showed that DA was able to modulate the hypothalamic expression of genes related to neuroendocrine regulation of reproduction (Popesku *et al.* 2008).

Sexual steroids have been shown to regulate dopaminergic system in several teleost species, conditioning both DA synthesis and D2 receptors expression. This evidence demonstrates that sexual steroids feedback targets DAergic system, as well as the other components of the brain-pituitary gonadotropic axis, GnRH and gonadotropins (Dufour *et al.* 2010).

Recent studies showed that the African cichlid fish, *Haplochromis burtoni*, has a peculiar plasticity respect to soma size in a population of hypothalamic neurons, specifically to (GnRH)-containing neurons (Davis and Femald 1990; Francis *et al*. 1993). In this species, males are either territorial or not territorial, and the soma size is correlated to the two social states. In particular, the territorial males are dominant, aggressive, reproductively active, and have large GnRH-containing neurons in the POA. In contrast, non territorial males are not reproductively active, have smaller POA GnRH-containing neurons (Davis and Femald 1990).

In the family Syngnathidae (pipefish, seahorses, and seadragons), males provide parental cares by carrying developing embryos on the ventral surfaces of their boby (Hübner *et al*. 2013), in these species the role of DA in the neuroendocrine control of reproduction has never been studied. In this work, DAergic neurons of males of *Syngnathus abaster* were studied in order to determine if morphological changes occur in POA neurons in relation of sexual maturity.

Materials and Methods

In reproductive season (May-September 2014), six adult and six young males of *S.abaster* were sacrificed by decapitation during anaesthesia with MS-222 (ethyl 3-aminobenzoate, Sigma). Brains were rapidly (less than 1 min) removed and fixed by immersion in 4% paraformaldehyde (PFA, pH 7.4) in phosphate-buffered saline (PBS, pH 7.4) overnight at 4°C followed by an overnight incubation in 30% sucrose in phosphate-buffered saline (PBS) for cryoprotection. Coronal slices (25 μm thick) were obtained with a cryostat (Microm Cryo-Star HM 560, Walldorf, Germany) . Slices were washed for 30 min in PBS, then immerged in 5% normal goat serum (NGS), 1% BSA (bovine serum albumin) and 0.3% Triton X-100 in PBS and kept overnight at 4°C . After blocking, samples were incubated with primary antibody anti-Tyrosine Hydroxylase prediluited (Sigma-Aldrich Milano, Italy) at 4°C for 1day, then washed several times in PBS over a period of 30 min at room temperature. Subsequently, samples were incubated with secondary antibodies anti-mouse Alexa Fluor 594 (1:200) (Abcam) for 3 h. All slices were then washed three times (30 min) in PBS at room temperature and coverslipped with Vectashield (Vector Lab).

Analysis was performed 12 h later using a Leica 4D confocal laser scanning microscope with an argon–krypton laser. Confocal images were generated using PL Floutar 40X oil (na=1.00–

0.5) and 100X oil (na=1.3). Each frame was acquired eight times and then averaged to obtain noise-free images. For cell counts (i.e. cells per field), three-dimensional reconstructions were obtained with the 'maximum intensity algorithm which was used on 20 images scanned in a 10-µm Z-range with the PL Floutar 40 x. For morphometric analysis (i.e. area, perimeter and circularity), we used PL Floutar 100X.

All confocal images were white-labelled on a black background, in a grey scale ranging from 0 (black) to 255 (white) and processed with Scanware 4.2a Leica (Fig. 2).We then used ImageJ and bit plane Imaris softwares for cell counts and morphometric analysis. We evaluated the area of the cell's body obtained by marking its profile, excluding all dendritic trunks, perimeter and circularity, a real value of the ratio of the squared perimeter divided by the area (i.e. perimeter^2/area). Statistical analysis was performed by means of the Tukey t-test for post hoc comparisons with Microsoft Excel 2010. Statistical significance was set at $P<0.001$.

Results

In the present study, we evaluated morphometric parameters (area, perimeter and circularity) of TH-positive somata of the POA in two different sexual maturation stages: adults and young males of *Syngnathus abaster* (Fig. 1). Although most of the cells exhibited a pear-shaped appearance, considerable variability in cells shape was observed in both groups of males analized (Fig. 1). TH-positive neurons obtained from the young male group appear smaller (Fig. 2 A-C) than the adult ones, with a reduction in the mean calculated area and perimeter (Table 1). The analysis of 105 and 107 somata obtained and entirely reconstructed from the adult male and young males respectivelly revealed a statistically significant difference in both area and perimeter (Table 1). The size of the neurons was found to be 90.24 ± 8.7 µm^2 for the area and 36.94 ± 6.73 µm for the perimeter in the adults and 61.60 ± 5.4

μm^2 24.86 ± 4.8 μm in the young males.

Present results suggest that different developmental sexual stage profoundly affects the morphometrical features of TH+ presumably DA-containing neurons in the POA.
Post hoc analysis confirm that neurons in the POA of adult individuals have a larger cell body. Specifically, adults showed a statistically significant higher average of morphometric parameters (Area: t= 43.1, P <0.0001; Perimeter: t= 32.5, P <0.0001; Circularity: t= 24.7, P <0.0001) compared to the young individuals (Fig. 2 A-C). Irrespective of the difference in number of cells per field, these findings strongly suggests that the reductions of all morphometrics traits (area, perimeter and shape complexity) of preoptic region neurons are induced by the sexual maturity stage.

Discussion

According to previous reports (Kaslin and Panula, 2001), our results suggest that DA plays an important role in the neuroendocrine regulation of gonadotropins release in *S. abaster*. Celleular counts show that, in *S. abaster,* POA DA-containing neurons change significantly in size depending on male reproductive state. The adult males have significantly larger DAergic neurons compared to the young. This observed increased size is probably due to maturity sexual state and to the changes in the concentrations of sex steroid hormones during both the male reproductive development and reproductive cycle. However, further investigations based on a more frequent sampling design, throughout the entire annual cycle, could better support this hypothesis. Nonetheless, the present study demonstrates significant differences in cell size between adult and young males, suggesting that the trend toward larger cells during development is overlaid by a changing of the soma size, which is correlated to reproductive state. In *vitro* experiments, which use primary cell cultures of the

embryonic mouse midbrain, have demonstrated that estrogen is capable to stimulate neurite growth and plasticity of dopaminergic neurons (Beyer and Karolczak, 2000). Such a phenomenon has also been reported for hypothalamic dopaminergic cells in *vitro* and in *vivo* studies, where estrogen was found to be capable of increase the tyrosine hydroxylase (TH)-fiber density of periventricular dopaminergic neurons (Simerly *et al.* 1985) and dendritic expansion (Kawashima and Takagi, 1994) respectively. Previous studies in mammals demonstrated that, the sexually dimorphic population of dopaminergic neurons in the anteroventral periventricular nucleus of the hypothalamic (AVPV) preoptic region develops postnatally under the influence of testosterone. There are fewer dopaminergic neurons labeled with tyrosine hydroxylase (TH) in the male AVPV than the female, and sex steroids determine this sex difference (Waters and Simerly 2009). This mechanism could explain the difference in morphometric parameters and in the number of the cells per field observed between young and adult of *S. abaster* males. However in these teleosts, a positive correlation among DAergic neurons soma size, cells number and sex steroid levels remains to be demonstrated. The number and the size of neurons observed in this study seem to be coherent with the so-called 'size principle',which states that smaller motoneurons are more easily induced to fire action potentials than comparable units of larger size (Carpinelli 2008; Henneman *et al.* 1965). The smaller neurons react actively to low intensity stimuli; the larger need of greater amount of inputs to obtain an active reaction. Since the size of a motor neuron and the size of a motor unit are directly related, it is demonstrated that the participation of a motor unit in graded motor activity is dictated by its size (Carpinelli, 2008; Henneman, 1968).

We believe that in sexually immature males of *S. abaster* POA DAergic neurons are smaller in order to highly react to stimuli and maintain the inhibition upon GnRH-releasing neurons; in contrast, in mature males, the system recruits larger neurons (which have faster

and shorter action) in order to better modulate gonadotropin release during the different phases of reproductive cycle.

The phenotypic changes in both cells body size and number, seem to profoundly affect the physiological and biochemical properties of the DAergic system of *S.abaster*. Moreover, they suggest an involvement of dopaminergic transmission in orchestrating the events leading to changes in either sexual status and gonadal maturation. These changes represent important steps in the knowledge of reproductive biology of these fishes, including the sex-specific patterns of parental care.

Acknowledgements.

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TH+ Neurons Trait	Adult (N=105)	Young (N=107)
Area	90.24 ± 8.7	61.6 ± 5.4
Perimeter	36.94 ± 6.73	24.86 ± 4.8
Circularity	14.27 ± 2.7	9.44 ± 2.6
Cells per Field	9.48 ± 3.56	17.03 ± 5.21

Table 1. Numbers in parentheses indicate the number of neurons used for calculating area, perimeter and circularity. Area is given in μm^2, Perimeter in μm. Circularity is expressed as the ratio $perimeter^2$/area. Cells per field values are calculated from cell counts of 20 microscopic fields for each group. Data are expressed as means ± std dev.

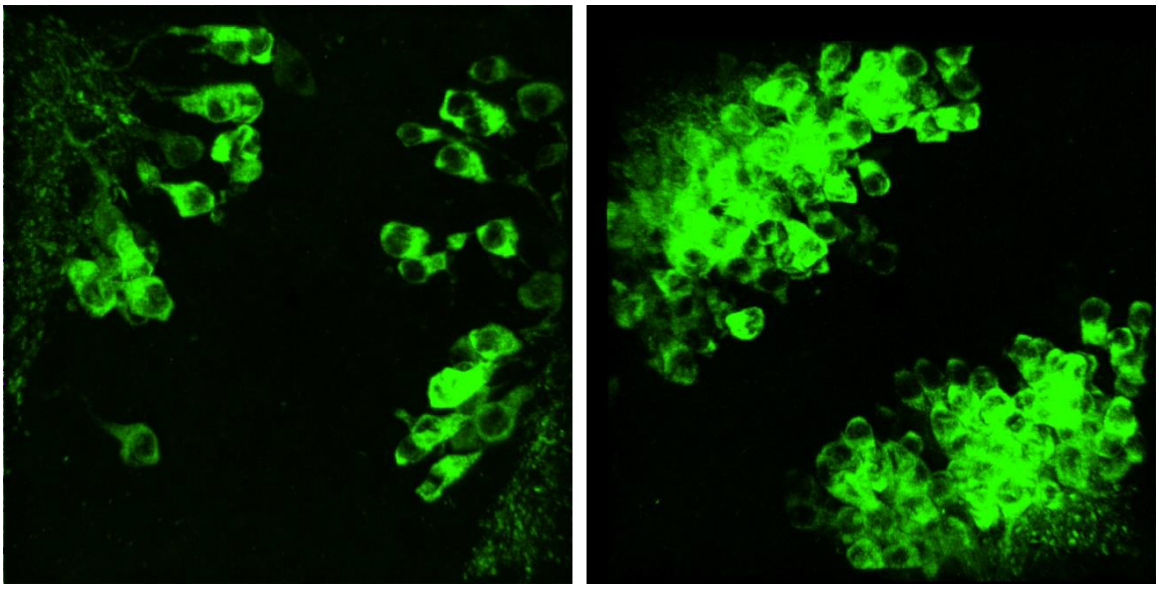
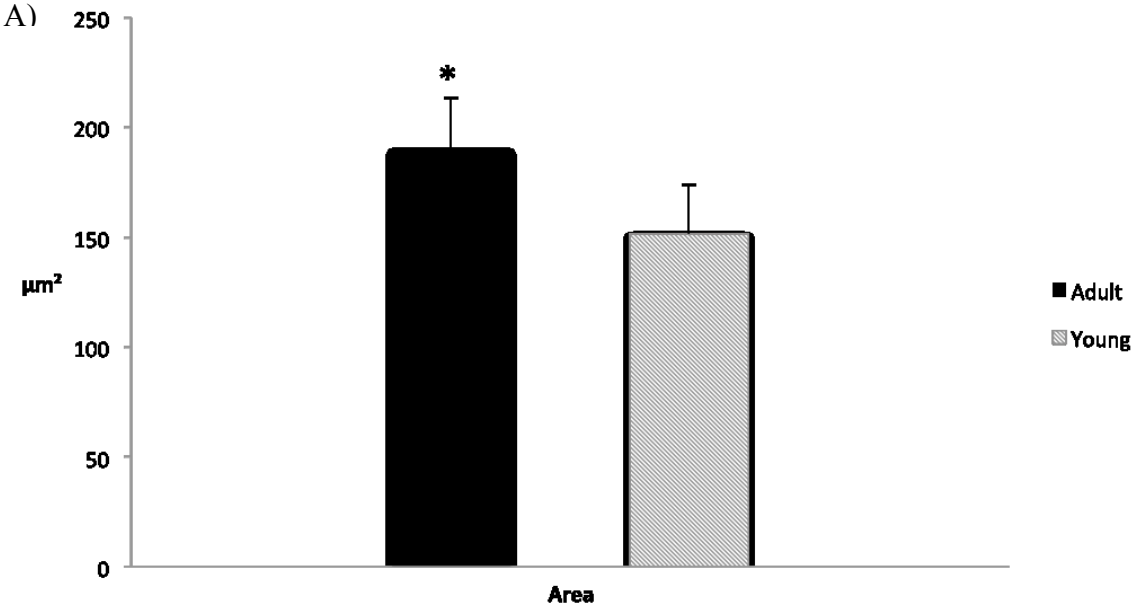
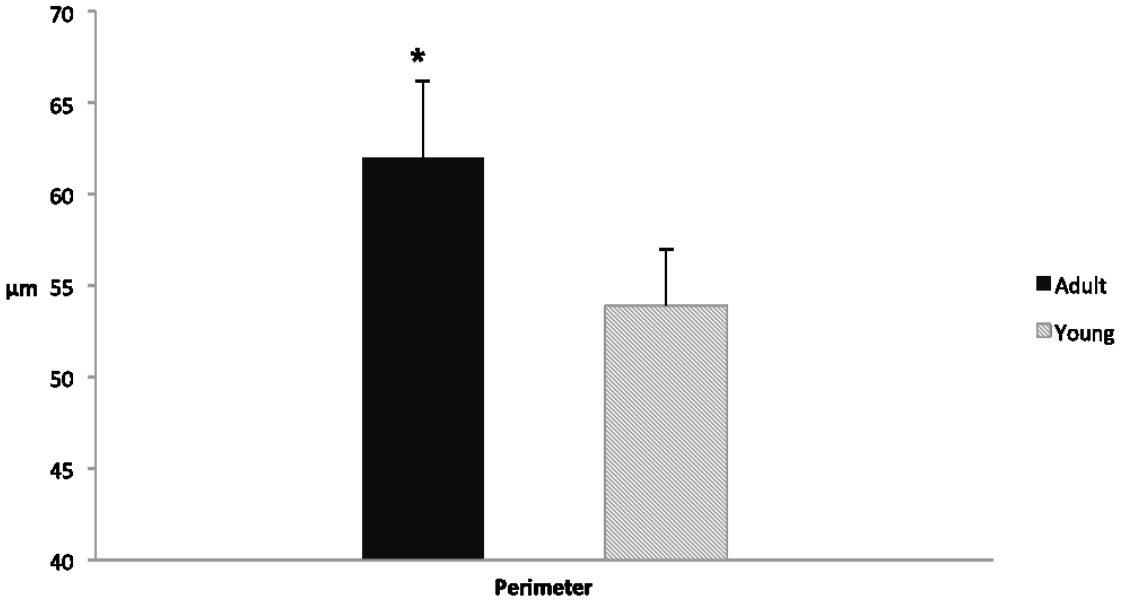


Figure 1. Confocal images of TH+ neurons of the POA. Each image is the projection of a 3-D reconstruction of 47 scans for a total of 23.5 µm in the z-axis, interscan distance is 0.5 µm. (**left**) Neurons from a *S. abaster* adult group. (**right**) Neurons from a *S. abaster* young group

A)


250
200
150
µm²
100
50
0
*
Area
Adult
Young

B)


70
65
60
µm 55
50
45
40
*
Perimeter
Adult
Young

C)

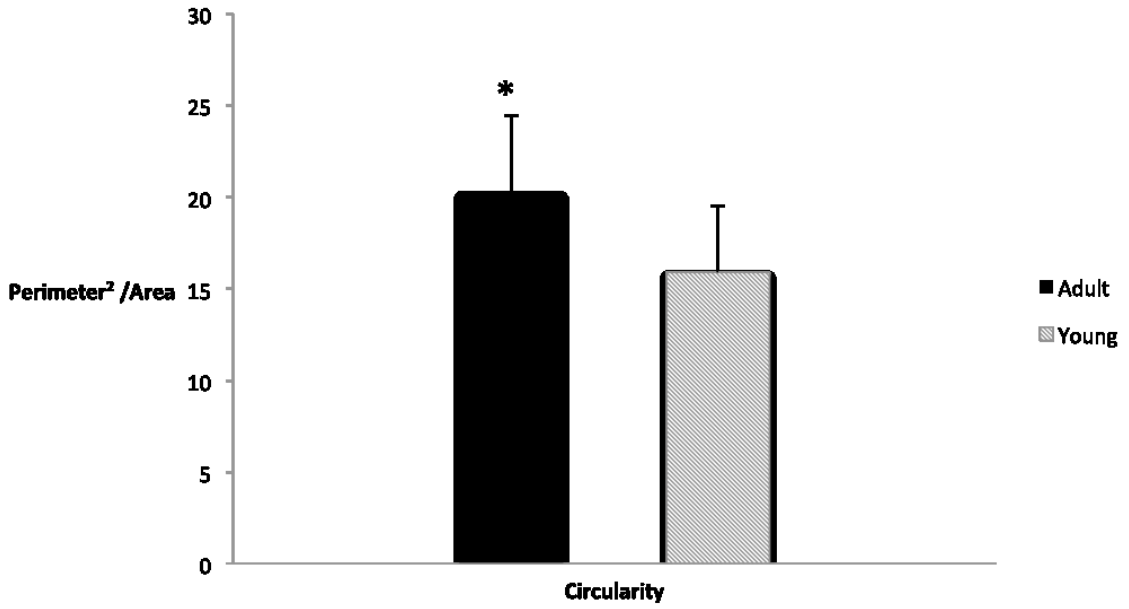


Figure. 2. Differences in morphometric parameters of POA TH+ neurons under different maturity state. **A)** soma area (μm^2, mean ± std dev), **B)** soma perimeter (μm, mean ± std dev) **C)** soma circularity (mean ± std dev) * = P < 0.0001.

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