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Corso di Laurea Magistrale

Biologia marina

Caratterizzazione morfologica e macromolecolare degli spermatozoi dello squalo bamboo (*Chiloscyllium punctatum*)

Morphological and macromolecular characterization of bamboo shark sperm (*Chiloscyllium punctatum*)

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“When everything seems to be going against you,

remember that the airplane takes off against the wind, not with it”

Henry Ford

To my family, for the sacrifices and support

To the patience and perseverance, that never abandon me,

To myself, because despite everything, I did it!

RIASSUNTO

Nonostante gli squali popolino da più di 400 mila anni il nostro pianeta, la corretta e completa conoscenza dei cicli riproduttivi è ancora scarsa. Negli oceani e mari di tutto il mondo, i condroitti stanno affrontando un rapido declino, in particolare nelle zone dove è presente la barriera corallina a causa dell'overfishing e dello shark finning, ovvero l'uccisione degli squali per prelevare le pinne dorsali e poi venderle. A questo, si aggiungono i cambiamenti climatici, l'inquinamento e la distruzione degli habitat. Inoltre, gli squali sono suscettibili all'estinzione in quanto crescono e si riproducono molto lentamente; raggiungono la maturità sessuale in età avanzata, la gestazione dura per molti mesi e il numero di piccoli per parto è limitato sia per le specie ovipare che vivipare. Conoscere quindi il ciclo riproduttivo è molto importante per la conservazione della specie. Si sa dalla letteratura che molte femmine di squalo possono conservare lo sperma all'interno del loro corpo anche per lunghi periodi.

Lo scopo della tesi è quello di caratterizzare a livello citologico e macromolecolare il liquido seminale e gli spermatozoi di maschi di squalo bamboo (*Chiloscyllium punctatum*) a differenti stadi di maturità (immaturo, in via di maturazione e maturo).

Per fare ciò è stato sviluppato e applicato un protocollo di estrazione dello sperma assolutamente non invasivo per determinare lo stadio di maturità sessuale degli individui. Sono state utilizzate tecniche di colorazione citologica per osservare la morfologia degli spermatozoi, mentre per la caratterizzazione macromolecolare sono state utilizzate la spettroscopia Raman per gli spermatozoi e la spettroscopia FT-IR per il plasma seminale.

Dai risultati ottenuti, ciò che è stato osservato a livello morfologico ha trovato conferma in ciò che è stato osservato a livello macromolecolare: nel liquido seminale dei maschi immaturi sono presenti solo spermatidi, mentre negli animali maturi sono presenti spermatozoi. Nei maschi immaturi, gli spermatidi vengono rilasciati, ma non hanno completato il processo di differenziazione. L'acquisizione della maturità sessuale o della pubertà avviene quando il maschio è in grado di produrre per la prima volta gameti maturi e funzionanti.
Il protocollo applicato quindi potrebbe aprire molte strade per studi futuri. Con una semplice tecnica non invasiva e senza sedazione dell’animale si può prelevare un campione di liquido spermatico e analizzarlo con i suddetti strumenti. In questo modo si può determinare velocemente lo stadio di maturità sessuale di un individuo, evitando così di ricorrere alle analisi citologiche o istologiche che sarebbero più dispendiose a livello di tempo.

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1. INTRODUCTION

1.1 Chondrichthyes systematic

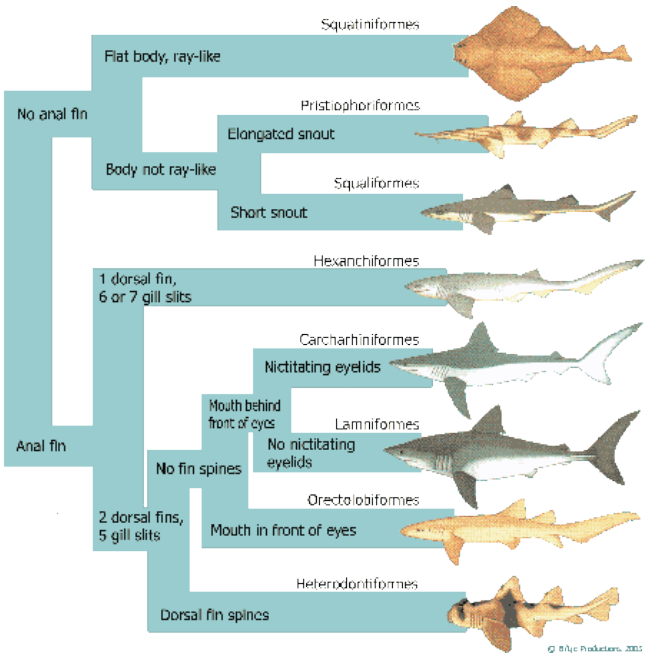

Figure 1. Chondroichthyes classification

Sharks include a variety of cylindrical, elongated, or depressed jawed fishes with paired pectoral and pelvic fins and relatively simple internal skeletons made of cartilage and lacking internal or external bones. Living sharks are members of the Class Chondrichthyes (the cartilaginous fishes), which includes the Subclass Elasmobranchii (including living sharks and rays), and the Subclass Holocephali (chimaeras). It is traditional to classify living elasmobranchs into 2 formal taxonomic groups, sharks (Selachii) and rays (Batoidea), but modern cladistic studies show that the rays comprise a single group of highly derived and extremely diverse 'flat' or 'winged' sharks that is closest to the small group of sawsharks (Pristiophoridae) and which nests within 1 of 2 superorders of living sharks, the Squalomorphii (Compagno, L. J., 2001).

1.2 General remarks

Traditional ‘sharks’, differ from the rays in having lateral gill openings and the pectoral fins not fused to the sides of the head over the gill. The greatly depressed angel sharks (Family Squatinidae) might be mistaken for rays at first sight and are the immediate relatives of the rays and sawsharks; they have large, broad, ray-like pectoral fins that extend as triangular lobes alongside the gill openings, but are not fused to the head above them. Sharks have eyes on the dorsal surface or sides of the head. There are usually 5 gill openings on each side of the head, rarely 6 or 7; spiracles (when present) are on the dorsal or dorsolateral surfaces of the head between the mouth and first gill openings. The mouth is usually ventral or subterminal on the head, but terminal or nearly so in a few species. The teeth on the jaws are set in numerous transverse rows and are constantly replaced from inside the mouth. Most species of sharks are more or less covered by small (occasionally enlarged) tooth-like placoid scales or dermal denticles. The tail and caudal fin are always well developed and propel the animal by lateral undulations; the pectoral fins are mostly not used for propulsion through the water but aid in stabilizing and steering the shark. Most sharks have 2 (rarely 1) dorsal fins, sometimes with spines on their front edges; an anal fin is usually present, but missing in several families (Compagno, L. J., 2001).

Mature sharks vary in total length from about 15 to 19 cm (dwarf species of Etmopteridae, Dalatiidae, and Proscylliidae) to 18 m more (whale shark, Family Rhincodontidae) and range in weight from between 10 and 20 kg to at least 30 t. Most sharks are of small or moderate size; about 50% are small, between 15 cm and 1 m; 32% between 1 and 2 m; 14% between 2 and 4 m; and only 4% are over 4 m in total length (Compagno, L. J., 2001).

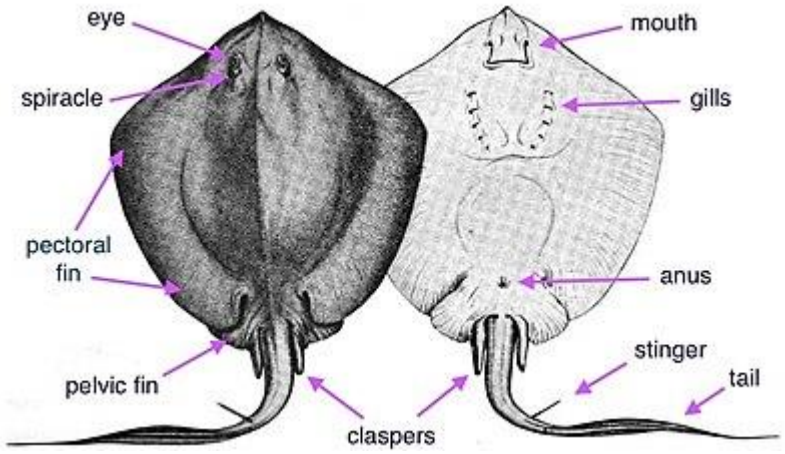

Figure 2. Stingray external anatomy

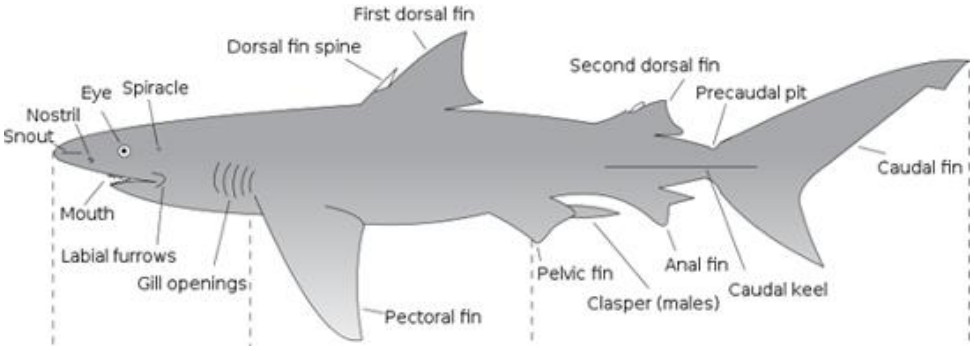

Figure 3. Shark external anatomy

1.3 Elasmobranchs ecology

Sharks are primarily marine, but a few requiem sharks (Carcharhinidae) have broad salinity tolerances, and one species (bull shark, *Carcharhinus leucas*) is wide-ranging in tropical lakes and rivers with sea access as well as shallow inshore waters. No sharks are known to be confined to fresh water. Sharks are widely distributed in all oceans, from the Arctic to subantarctic islands, and from close inshore on reefs, off beaches, and in shallow, enclosed bays to the lower continental slopes, the abyssal plains, sea mounts and ridges, and the high seas. They are most diverse in continental waters of tropical and warm-temperate seas, from inshore waters down to upper continental slopes, but are less so in colder waters, at great depths (below 1 500 to 2 000 m), in the open ocean and off oceanic islands. The richest shark faunas occur in the Indo-West Pacific from South Africa and the Red Sea to Australia and Japan.

The Western Central Atlantic has a moderately diverse shark fauna compared to other parts of the world, but includes at least 23 families, 42 genera, and 100 species of sharks. Worldwide there are 34 families, 104 genera, and between 397 and 488 species of sharks (estimate as of 23 January 2001). Many species of sharks are endemic to the area and have restricted ranges within it. More new species and many records of described species will be discovered with further collecting in poorly known parts of the area (Compagno, L. J., 2001).

2. REPRODUCTION

Chondrichthyan reproduction is characterized by internal fertilization, diverse reproductive modes, complex reproductive cycles, late sexual maturity, iteroparity (several litters per lifetime), and small brood size.

Reproductive anatomy is the same for each sex across elasmobranch taxa, although there are some specializations in each sex and asymmetries, particularly with respect to the female reproductive tract.

The principal components of the reproductive tract in female elasmobranchs include the: ovaries; ostia (ostium); oviduct; shell gland; uterus; and cervix.

Ovaries are located in a dorsal retroperitoneal position, supported by mesenteries, mesovaria. Both left and right ovaries and oviducts are functional in some groups (e.g., skates). The right ovary and both oviducts are functional in other groups (e.g., lamniform and carcharhiniform sharks), whereas only the left ovary and oviduct are functional in others (e.g., many myliobatiform rays).

The main specializations in the female reproductive tract occur in the shell gland and the uteri. The shell gland is reduced in viviparous forms.

Uterine specializations include infoldings, uterine villi or trophonemata (in myliobatiform rays), and compartmentalization (in placental viviparous sharks).

Additional specializations have occurred in the reproductive tracts for the storage and packaging of sperm in the seminal vesicles, prior to copulation, in males of *Alopias superciliosus, Odontaspis taurus, Carcharodon carcharias, Isurus oxyrinchus, Lamna nasus, Squalus acanthias, Hydrolagus colliei, Carcharhinus limbatus, Carcharhinus plumbeus, Carcharhinus falciformis Carcharhinus obscurus, Carcharhinus porosus, Prionace gluaca, Rhizoprionodon terraenovae, and Sphyrna lewini* (Pratt and Tanaka, 1994), and prior to ovulation and fertilization, in the shell gland, in females of some species such as *Alopias vulpinus, Lamna nasus, Carcharhinus obscurus, Carcharhinus plumbeus, Galeocerdo cuvieri, Prionace glauca, Rhizoprionodon terraenovae, Sphyrna lewini* and *Sphyrna tiburo* (Pratt, 1993), in those species that have been investigated.

Spermatozoa may be stored in the female reproductive tract from the short-term (*Rhizoprionodon terraenovae*) to periods exceeding two years (*Prionace glauca* and *Carcharhinus obscurus*) (Dodd,1983; Castro et al., 1988; Pratt, 1993). In other species, such as the Atlantic stingray (*Dasyatis sabina*), there was no evidence for sperm storage by females in a distinct study area (Maruska et al., 1996; Tricas et al., 2000).

Elasmobranch reproductive strategies include oviparity, aplacental viviparity (ovoviviparity) and placental viviparity (Wourms, 1977).

Oviparous species (1/3 of the species) enclose eggs in an egg case and deposit them into the environment, where embryos develop external to the body of the mother. Embryos remain in the egg case to develop for a period ranging from less than two months to over one year (Compagno, 1990).

Viviparous species retain eggs within the uteri where the embryos develop. The yolk sac of placental viviparous species interdigitates with the uterine wall to form a placenta in which nutrients from the mother are transferred to the embryo. Gestation for viviparous species ranges from less than six months to greater than two years (Compagno, 1990).

Ovoviviparous lamnoid sharks of the families Odontaspididae, Alopiidae, and Lamnidae practice uterine cannibalism, in which one or more foetuses in each uterus resorb their yolk sacs and then devour eggs passed down the oviducts for nutriment (oophagy) and grow to considerable size with massive yolk stomachs before birth.

In the Odontaspididae (*Carcharias taurus*) the largest foetus kills and eats its siblings (adelphophagy) and only 1 foetus survives in utero, while several young may cohabit the uterus in the other families (Compagno, L. J., 2001).

2.1 Male reproductive anatomy

Principal components of the reproductive tract in male elasmobranchs are: testes; epidymis; Leydig's gland; vas deferens; seminal vesicle; siphon sac, clasper gland or alkaline gland; and the claspers.

The testes are paired structures located in a dorsal retroperitoneal position and supported by a mesorchium and in all male elasmobranchs are embedded in the epigonal organ (Engel and Callard, 2005), an important tissue related to the production of leukocytes, granulocytes and lymphocytes. They are the location in which spermatogenesis occurs and they also play a role in producing and secreting steroid hormones.

The location of the germinal zone within the testis and the direction of spermatocysts maturation vary between elasmobranch species.

Pratt (1988) identified three types of testes in elasmobranchs: radial, diametric and compound, defined by their pattern of seminiferous follicle origin and propagation.

In radial testes, the germinal zone is located at the centre of multiple lobular structures in the testis that are separated by a duct system.

Developing spermatocysts progress in a radial pattern toward the outer margin of the lobe and are collected by the duct system after spermiation.

Species with a radial testis type include the lamnoid sharks such as the white shark (*Carcharodon carcharias*) (Pratt, 1988).

In diametric testes, the germ cells are located in a strip on one side of the testis, and developing spermatocysts progress across the width of the testis to the efferent ductules located on the opposite side.

This testis type is the most common among elasmobranchs. It is found in the carcharhinid sharks and is also observed in the Port Jackson shark (*Heterodontus portusjacksoni*) (Jones and Jones 1982), piked dogfish (*Squalus acanthias*) (Dobson and Dodd 1977b; Callard *et al.* 1985; Dubois and Callard 1989) and draughtboard shark (*Cephaloscyllium laticeps*) (Awruch *et al.* 2008b).

Compound testes are a combination of both radial and diametric types. Spermatocysts develop within multiple lobular structures that are not separated by a duct system.

The germinal zone is located at the centre of each lobule, with spermatocysts developing first towards the outer edge and then across the diameter of the testis to the efferent ductules located on the opposite surface.

The compound testis is presumed to be the testis type common to all skate species (Pratt, 1988; Hamlett, 1999; Engel and Callard, 2005).

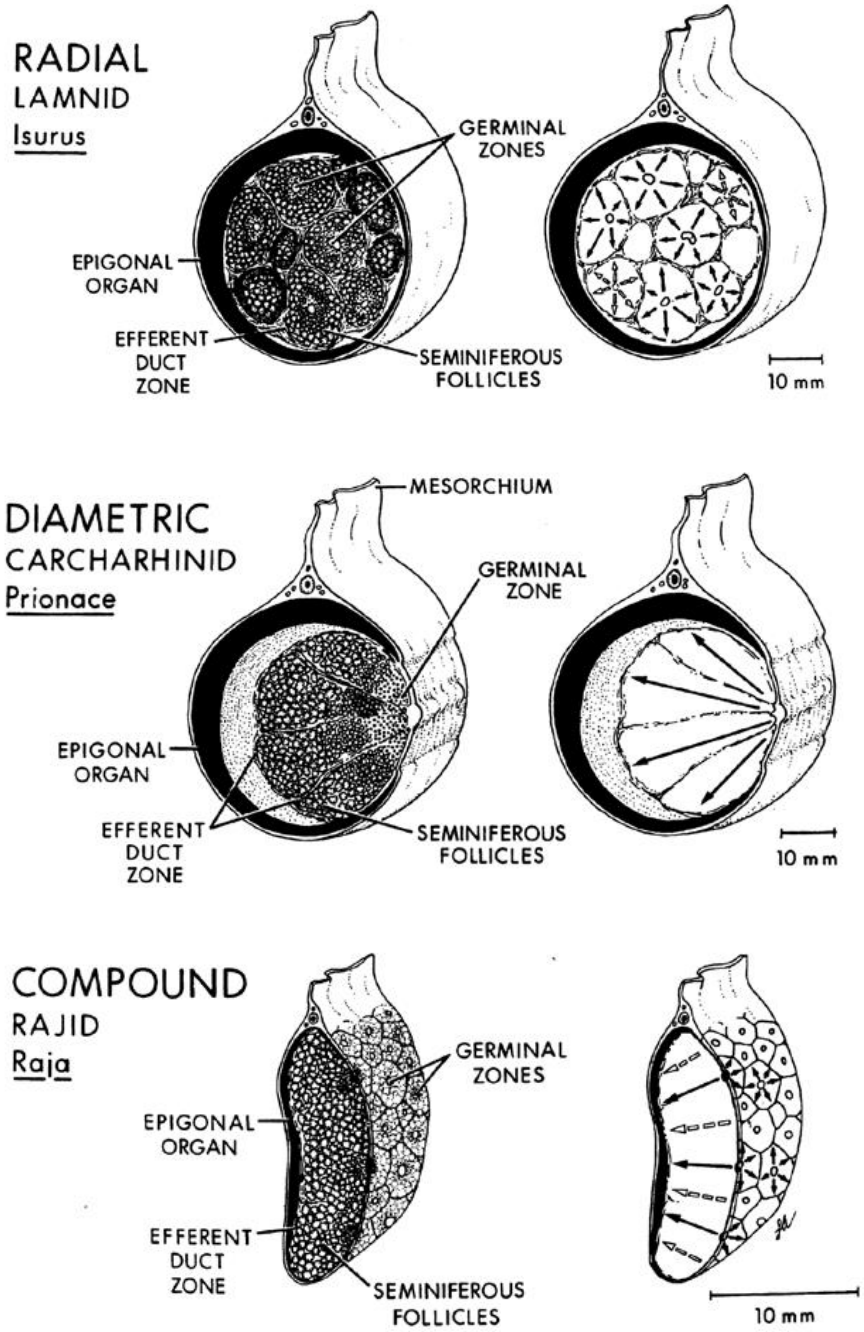

Figure 4. Types of testes in male elasmobranch

Both testes are active in all species. The siphon sac in male sharks is replaced by the clasper gland and alkaline gland in batoids.

The epididymis is connected to the testis via the ductus efferens, which are fine tubules which cross the mesorchium at the anterior edge of the testis.

Mature sperm are discharged from the testis through the ductus efferens (Wourms, 1977).

The efferent ducts join the epididymis, which expands to form a long tube with complex convolutions.

The epididymis is continuous with the next section of the genital duct, the ductus deferens, also known as the vas deferens or Wolffian duct.

The ductus deferens is continuous with the seminal vesicle or ampulla ductus deferens. The ductus deferens and seminal vesicle function as storage areas for seminal products and in some species sperm is packaged into either spermatozeugmata or spermatophores here (Wourms, 1977).

The ureter becomes entwined with the terminal portion of the seminal vesicle and both end in the anterior wall of the urogenital sinus.

The urogenital sinus vents into a common cloaca by means of a single large papilla. Two accessory glands are present, Leydig glands and the alkaline gland. Leydig glands are a series of branched tubular glands that secrete

seminal fluids into the epididymis and ductus deferens.

The alkaline gland of batoids may be involved in sperm protection (Hamlett, 1999).

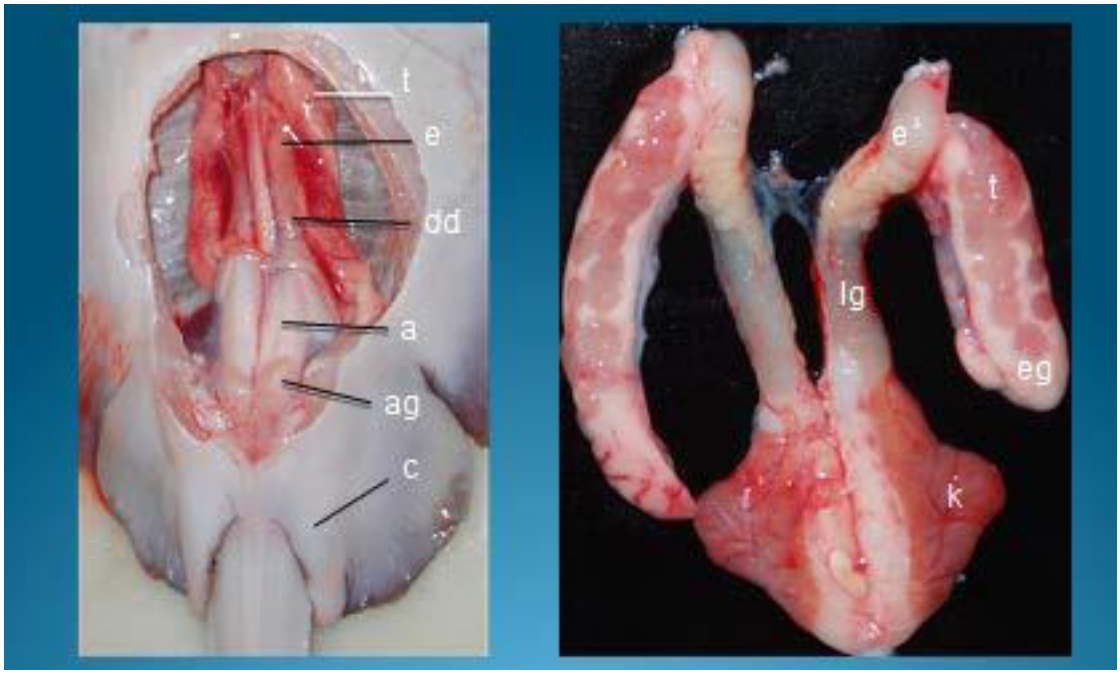

Figure 5. Male reproductive anatomy of a sparsely spotted stingaree (Urolophus paucimaculatus) (courtesy of Jon Daly).

KEY: ampulla of ductus deferens (a), alkaline gland (ag), clasper (c), ductus deferens (dd), epididymis (e), epigonal organ (eg), kidney (k), leydig gland (lg), testis (t).

Male elasmobranch copulatory appendages consist of the paired claspers, which extend posteriorly from the pelvic fins (Gilbert and Heath 1972; Hamlett 1999).

The claspers in immature males are relatively small and the cartilaginous supports soft, but at puberty, the claspers undergo a rapid increase in size and the cartilage becomes calcified (Gilbert and Heath 1972; Walker 2005). Calcification of the claspers has been used as an indicator of male maturity in several shark species (Pratt 1979; Castro *et al.* 1988; Heupel *et al.* 1999; Walker 2005).

During copulation, the male inserts one clasper into the females' cloaca and flares the terminal cartilages to ensure that the clasper stays in position so sperm can be delivered (Gilbert and Heath 1972; Jones *et al.* 2005; Pratt and Carrier 2005); the siphon sac, placed under the skin of the animal, is filled with water, to facilitate the transfer of sperm to the female (Gilberth and heath, 1972) by inducing uterine contractions or assist sperm competition by washing female genital tract before ejaculation with water flux.

Claspers do not have a lumen like the mammalian penis, but instead have a groove running longitudinally on the dorsal side of the claspers to aid the passage of semen (Hamlett 1999; Jones *et al.* 2005).

The groove is either completely or partially enclosed by the axial and marginal cartilages making up the clasper shaft (Compagno 1999a).

Figure 6. Claspers on a male shark.

2.1.1 Spermatogenesis

Spermatogenesis is the developmental process of mature male gametes.

During this process, a small number of diploid spermatogonial stem cells produce a large number of highly differentiated spermatozoa carrying an haploid, recombined genome.

Those cells are extremely specialized and adapted to motility.

Comparing them with the female gametes, are smaller in terms of dimension and, in terms of metabolic effort, are largely less costly.

Indeed, the cellular basis of sexual reproduction in animals are haploid gametes, one sex producing a relatively small number of large gametes, eggs that are rich in reserves, the other sex producing a high number of small gametes, spermatozoa.

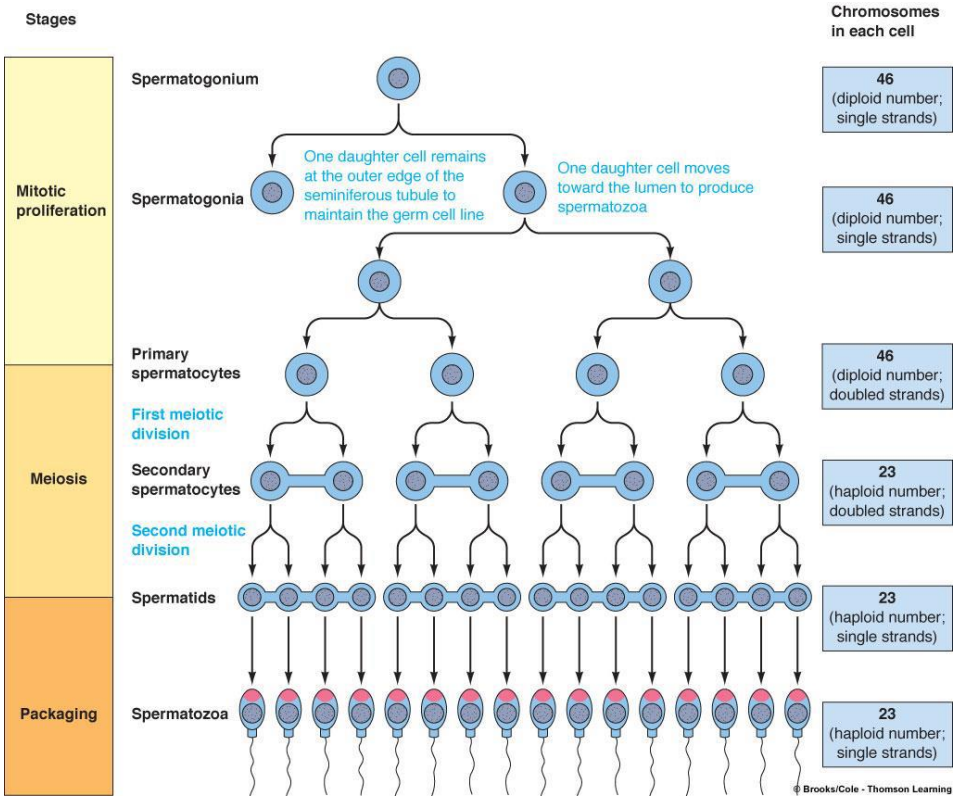

Figure 7. Schematic representation of spermatogenesis

Spermatogenesis can be defined as a sequence of complex cellular events:

1. commitment to spermatogenesis proper
2. mitotic proliferation
3. apoptosis (in some situation)
4. meiosis (the central event of spermatogenesis)
5. spermiogenesis (metamorphosis of an immotile round cell to a free-swimming spermatozoon) (Roosen-Runge 1977).

Spermiogenesis involves condensation and elongation of nuclear chromatin, reduction of the cytoplasm, and formation of the acrosome, midpiece and flagellum (Stanley 1966; Callard *et al.* 1988; Sourdaine and Jegou 1989; Parsons and Grier 1992; Maruska *et al.* 1996; Hamlett 1999; Engel and Callard 2005; Awruch *et al.* 2008b).

Elongated spermatids gradually become laterally aligned at the head to form bundles, which are closely associated anteriorly with the Sertoli cells (Stanley 1966; Callard et al. 1988; Sourdaine and Jegou 1989; Parsons and Grier 1992; Maruska et al. 1996; Engel and Callard 2005).

Spermatids in mature spermatocysts have the helical nucleus characteristic of elasmobranch spermatozoa and form tightly packed bundles embedded in the Sertoli cell cytoplasm (Sourdaine and Jegou 1989; Parsons and Grier 1992; Maruska *et al.* 1996; Hamlett 1999).

At spermiation, the Sertoli cells rupture to release the spermatids, which flow out of the spermatocyst into the collecting duct system (Stanley 1966; Callard *et al.* 1988; Maruska *et al.* 1996).

The remains of the spermatocyst and Sertoli cells left behind gradually break down and are resorbed (Stanley 1966).

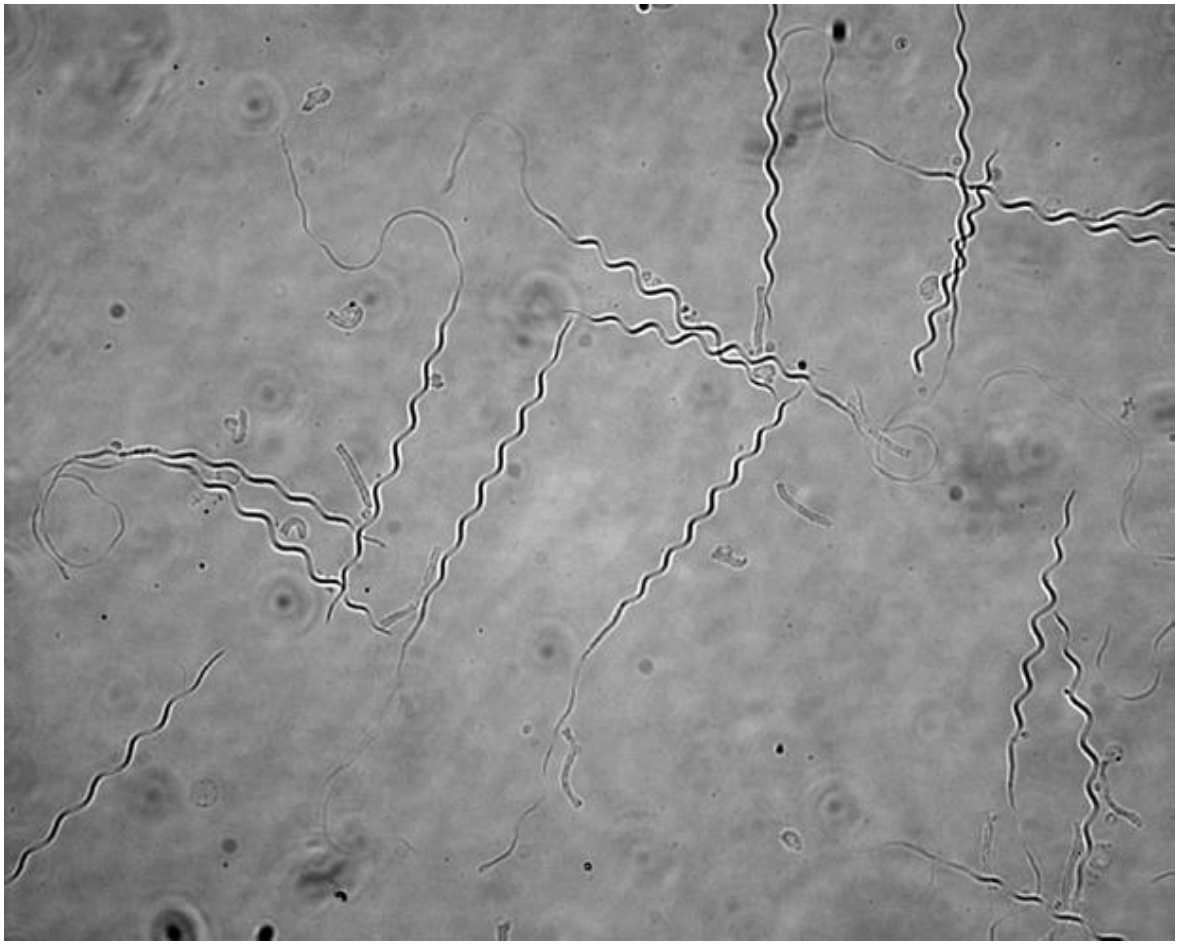

Figure 7. - Semen sample from a sparsely spotted stingaree (Urolophus paucimaculatus) (courtesy of Jon Daly).

Generally, mature spermatozoa are considered such when they are still inside the cyst ready to reach the epididymis through the efferent ductules.

From the epididymis, they go to the ductus deferens and seminal vesicles, along with secretions from the genital ducts and from the Leydig gland.

Secretions from the Leydig gland facilitate maturation of spermatozoa and matrical material form in the lumena of the seminal vesicles and associate with individual and previously bundled sperm.

The sperm are held together by a sticky matrix as either spermatophores (sperm encapsulated) or spermatozeugmata (sperm not encapsulated but tails of peripheral sperm protruding).

Ciliated epithelial columnar cells lining the lumen convey the spermatozoa through the genital ducts; only the seminal vesicles have a muscular wall.

At copulation and ejaculation, sperm transfer from the seminal vesicle through the urogenital papilla to the dorsal groove of each clasper.

Spermatozoa acquire the potential for modest motility while in the terminal regions of the genital ducts but acquire active, robust motility at ejaculation (Hamlett,1999).

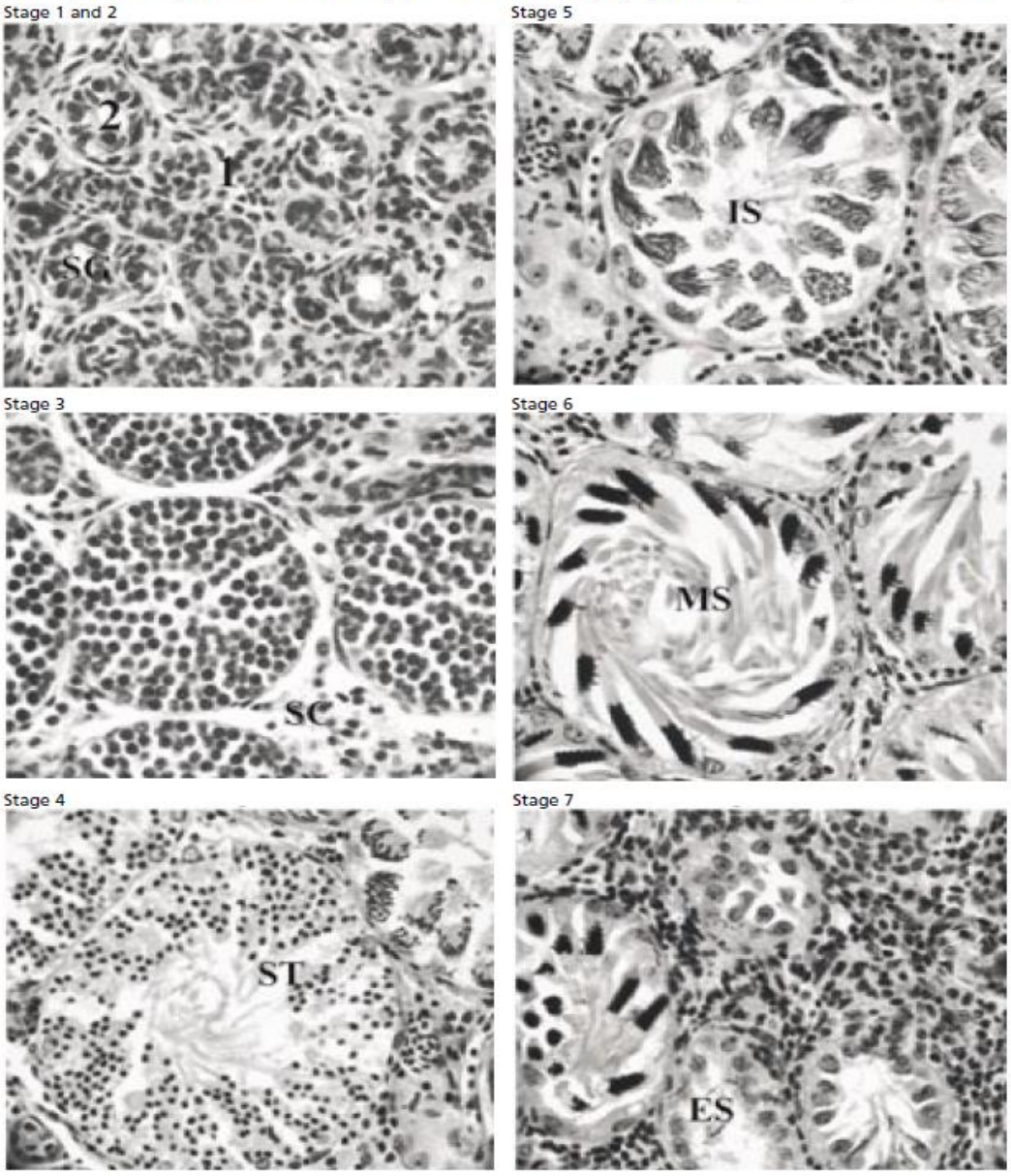

Figure 8. Sperm stages of the testes: stages 1-7, SG = spermatogonia, SC = spermatocytes, ST = spermatids, IS = immature sperm, MS = mature spermatozoa, ES = empty spermatocyst, SG = spermatogonia

2.1.2 Sperm morphology

As in mammals, the sperm of elasmobranchs is made up of a head, midpiece, and tail. The head of all chondrichthyan sperm is long (> 30 μm) and helical in structure, with the nucleus following the same course (Jamieson 2005).

The midpiece of elasmobranch sperm consists of an axial midpiece rod around which the mitochondria are arranged (Stanley 1971a; 1971b), and these in turn are surrounded by a fibrous sheath (Jamieson 2005).

The axial midpiece rod is a structure unique to the class Chondrichthyes, and takes the place of the nine coarse fibres that make up the centre of the mammalian sperm midpiece (Stanley 1971a; Jamieson 2005).

The flagellum (or tail) is comprised of two key structures, the central axoneme and the longitudinal columns. As the central axoneme rotates on its longitudinal axis along the length of the flagellum, the longitudinal columns remain fixed at doublet positions 3 and 8 to create a double helix structure (Stanley 1971b; 1983; Jamieson 1991).

Sperm morphology is variable across sharks, with head length ranging from 26.81 to 63.90 μm, midpiece length from 5.40 to 16.77 μm and flagellum length from 67.88 to 146.13 μm. In sharks, flagellum length is significantly positively associated with relative testes mass.

However, flagellum length is negatively associated with body mass in sharks, suggesting that larger-bodied shark species produce sperm with smaller flagella. Similarly, sperm total length is positively associated with testes mass and negatively associated with body mass. Neither sperm head nor midpiece length is associated with relative testes mass (Rowley, Amy, et al., 2019).

Longer flagella can be advantageous during competitive fertilizations under three possible mechanistic scenarios, none of which is mutually exclusive. First, longer flagella may provide greater thrusting force to propel sperm more quickly as they swim towards the egg (Fitzpatrick et al., 2009; Lüpold, Calhim, Immler, & Birkhead, 2009), particularly as sperm swimming speed is an important predictor of competitive fertilization success in a wide range of taxa (Simmons & Fitzpatrick, 2012).

Second, sperm with longer flagella may be better able to displace rival sperm from advantageous positions within the female's reproductive tract, for example by being better positioned to fertilize the egg or enter sperm storage organs (Lüpold et al., 2012).

Finally, longer sperm may be selected for if cryptic female choice favours the use and storage of sperm with longer flagella (Baer, Schmid-Hempel, Høeg, & Boomsma, 2003; Miller & Pitnick, 2002).

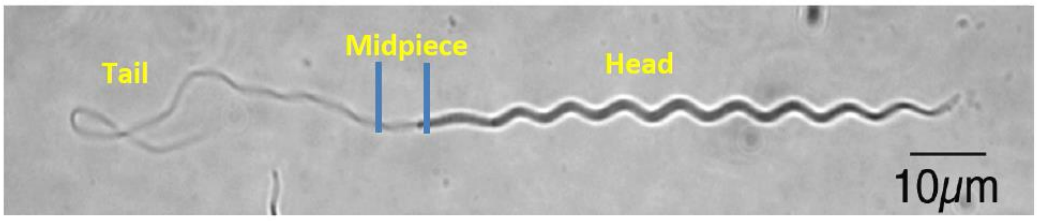

Figure 9. Sperm from a sparsely spotted stingaree (Urolophus paucimaculatus) (courtesy of Jon Daly)

2.1.3 Sperm membrane lipid composition

The lipid composition of sperm membranes is crucial for fertilization and differs among species. Reasons for a diverse lipid composition could be the habitat specificity, the diet, the mating frequency, the capability of sperm storage and the mode of fertilization (Engel, Kathrin M., et al. (2020)).

Dzyuba and collaborators, studied lipid composition of spermatozoa from ocellate river stingray *Potamotrygon motoro*, a representative of cartilaginous fishes possessing internal fertilization.

Analysis of lipid class composition revealed the presence of phospholipids (PL), cholesterol (Chol), free fatty acids (FFA), triacylglycerols (TAG), and cholesterol esters (CholEst) in spermatozoa.

Phospholipids showed the highest percentage (64.2 ± 1.5%) among all detected lipid classes while proportion of TAGs was the lowest (2.8 ± 1.2%).

The main PL classes identified were: phosphatidylethanolamine (PEA; had the highest content), phosphatidylcholine (PC), cardiolipin (CL), sphingomyelin (SM), phosphatidylserine (PS) and phosphatidylinositol (PI).

Higher content of PEA in membrane could increase its viscosity.

High level of CL can be reated to the presence of numerous mitochondria in midpiece of spermatozoa in representatives of Chondrychthyes.

It is well accepted that general lipid composition and particularly cholesterol content determine the ability of spermatozoa to resist osmotic shock and mechanical properties of the cell in general (Dzyuba, Viktoriya, et al., 2019). The cholesterol/SM ratio could be crucial for the rigidity of the cell membrane and, thus, for cell function (Engel, Kathrin M., et al., 2020).

Analysis of FA composition showed that the content of saturated FAs (SFAs) was two times lower than that of monounsaturated FAs (MUFAs) and polyunsaturated FAs (PUFAs) (Table 1).

From the SFAs, palmitic (16:0) and stearic (18:0) acid were present in significantly higher proportions compared to the other SFAs.

Fatty acid	Mean ± Std	Fatty acid	Mean ± Std
16:0 (palmitic acid)	9.4 ± 0.4	unknown2	0.5 ± 0.1
16:1 (palmitoleic acid)	1.8 ± 0.2	20:1 n-9 (gondoic acid)	7.4 ± 0.4
16:1 trans n-9 (palmitelaidatic acid)	0.7 ± 0.1	20:2 n-6 (eicosadienoic acid)	0.4 ± 0.1
16:1 trans2 n-7	0.6 ± 0.1	unknown3	3.7 ± 0.3
17:0 (margaric acid)	2.1 ± 0.2	20:4 n-3 (eicosatetraenoic acid)	0.7 ± 0.1
18:0 (stearic acid)	10.1 ± 0.5	22:4 n-6 (docosatetraenoic acid)	14.4 ± 0.8
unknown1	3.5 ± 0.5	22:3 n-3	2.1 ± 0.5
18:1 n-9 (oleic acid)	7.1 ± 0.2	22:5 n-6 (osbond acid)	2.1 ± 0.5
18:1 n-7 (vaccenic acid)	6.6 ± 0.6	22:5 n-3 (docosapentaenoic acid)	2.5 ± 0.4
18:1 trans hydroxy (ricinelaidic acid)	2.0 ± 0.2	22:6 n-3 (docosahexaenoic acid)	3.0 ± 1.7
18:1 n-5	2.1 ± 0.2	SFA	32.7 ± 0.8
18:2 trans (linolelaidic acid)	0.4 ± 0.2	MUFA	28.2 ± 0.9
18:2 n-6 (linoleic acid)	0.5 ± 0.1	PUFA	41.4 ± 1.4
20:3 n-6 (dihomo-γ-linolenic acid)	1.0 ± 0.2	n-3	8.2 ± 1.1
20:4 n-6 (arachidonic acid)	13.4 ± 1.4	n-6	32.3 ± 1.9
22:0 (behenic acid)	1.0 ± 0.2	n-3/n-6	0.3 ± 0.0

SFA – saturated fatty acid; MUFA – monounsaturated fatty acid; PUFA polyunsaturated fatty acid. Data are presented as mean and standard deviation; n = 6.

Table 1. Fatty acid composition in P. motoro sperm (% of total identified FA from total lipid fraction)

From MUFAs, the most abundant were oleic (18:1 n-9), vaccenic (18:1 n-7) and gondoic (20:1 n-9) acid.

Docosatetraenoic (22:4 n-6) and arachidonic (20:4 n-6) acids were the major representatives of PUFAs in stingray sperm. The high level of arachidonic acid was found when diets were rich in linoleic acid, which is known to be possible precursor of arachidonic acid. It is known to be among the metabolic regulators of spermatogenesis.

Low level of docosahexaenoic acid was observed in stingray sperm. It is usually present in high amounts in the spermatozoon membrane and providing high membrane fluidity; it is necessary for sperm motility and fertility.

2.1.4 Sperm activation and motility

There is relatively little information available on the activation and motility of elasmobranch sperm. Jones et al. (1984) reported that mature sperm from *H. portusjacksoni* were activated by a phosphate-buffered elasmobranch ringer solution based on the ionic composition of elasmobranch blood.

Similarly, work by Minamikawa and Morisawa (1996) showed that sperm from the banded houndshark, *Triakis scyllia*, could maintain activity in solutions that replicate blood or uterine conditions. Glucose was found to have a positive effect on the duration of motility, and the investigators concluded that hexoses were likely to be important for maintaining sperm motility in elasmobranchs.

This is supported by observations in a recent study by Luer et al. (2007), who reported that sperm from the clearnose skate (*Raja eglanteria*) maintained high motility in an elasmobranch-modified poultry semen extender containing fructose. Although hexoses appear to have an influence on maintaining motility, the specific factors controlling activation and immobilization (sperm storage) of elasmobranch sperm are still not clear.

2.1.5 Sperm storage in female shark

Observations of mating in the wild suggest that female sharks commonly mate with more than one male within a reproductive cycle (e.g. Carrier, Pratt, & Martin, 1994; Whitney, Pratt, & Carrier, 2004).

In addition, female sharks retain sperm in specialized storage organs (i.e. oviducal glands). Although the length of sperm storage may vary (Pratt, 1993), in some species offspring can be produced using sperm stored for up to four years after mating (Bernal et al., 2015). The potential for long-term sperm storage uncouples mating from fertilization, increasing competition between rival ejaculates, and imposes selection on sperm morphology to enter and remain viable in sperm storage organs (Fitzpatrick et al., 2012; Orr & Brennan, 2015; Orr & Zuk, 2014).

It was hypothesized that post-copulatory sexual selection influences sperm morphology and variance. Sperm competition arising from female polyandry influences the evolution of sperm flagellum length and variance in sharks (Rowley, Amy, et al., 2019).

2.2 Reproductive cycles

Reproductive cycles have been classified by several authors (Wourms, 1977; Dodd and Sumpter, 1984; Koob and Callard, 1999; Hamlett and Koob, 1999). The cycles as defined by Koob and Callard (1999) are:

1. continuous for those species that reproduce throughout the year,

2. seasonal for those species that are reproductively active for only a part of the year, and

3. punctuated for those species that are pregnant for about a year and the next pregnancy is at least a year later.

3. FEEDING REQUIREMENTS

3.1 Diet composition in the wild

All sharks are top predators, with a wide prey range from planktonic crustaceans and benthic invertebrates to pelagic cephalopods, small to large bony fishes, other cartilaginous fishes, marine mammals, and other marine and terrestrial vertebrates. (Compagno, 2002)

Examining the stomach contents of wild elasmobranchs (e.g., Cortés and Gruber, 1990, Stevens and McLoughlin, 1991; Stillwell and Kohler, 1993; Castro, 1996) has provided an indication of food preferences, food availability, and nutritional requirements in natural habitats.

Table 2 gives an overview of the diet composition of different species of wild elasmobranchs.

Stillwell and Kohler (1993) conducted a feeding habit survey on sandbar sharks (Carcharhinus plumbeus) off the northeastern coast of the United States. The stomach content of sub-adult and adult sharks consisted of 43% teleosts, 16% elasmobranchs, and 3% cephalo-pods. The size of the prey ingested appeared to be an important factor and the majority of prey items observed were small enough to be swallowed in one piece. Generally, it can be said that the main food item for sandbar sharks is teleosts for adult sharks and crustaceans for

juvenile sharks. Other adult carcharhinids include teleosts as the largest part of their diets.

Family	Common name	Diet composition
Callorhinchidae	elephantfishes	Echinoids, bivalves, crustaceans, and small teleosts[2]
Carcharhinidae	requiem sharks	Large and small teleosts, crustaceans, cephalopods, and elasmobranchs[1]
Dasyatidae	whiptail stingrays	Crustaceans, teleosts, bivalves, polychaetes, and cephalopods[2]
Ginglymostomatidae	nurse sharks	Small teleosts (88%[5]), cephalopods (14%[5]), crustaceans (8%[5]), bivalves, and echinoderms [1]
Gymnuridae	butterflyrays	Crustaceans, teleosts, cephalopods, gastropods, and polychaetes[2, 7]
Hemiscylliidae	bamboo sharks	Bivalves, crustaceans, small teleosts, cephalopods, and gastropods[1]
Heterodontidae	horn sharks	Echinoids, crustaceans, mollusks, polychaetes, and small teleosts[1]
Mobulidae	devilrays	Plankton and small teleosts[2]
Myliobatidae	eaglerays	Bivalves (40%[6]), crustaceans (40%[6]), polychaetes (10%[6]), tunicates, cephalopods, and small teleosts[2]
Odontaspididae	ragged-tooth sharks	Large and small teleosts, small sharks, crustaceans, and cephalopods[1]
Orectolobidae	wobbegongs	Bivalves, echinoids, crustaceans, cephalopods, and small teleosts[1]
Pristidae	sawfishes	Small teleosts, shellfish, and crabs[2]
Pristiophoridae	saw sharks	Small teleosts, crustaceans, and squid[1]
Rajidae	skates	Teleosts, small sharks, crustaceans, bivalves, cephalopods, and polychaetes[2]
Rhincodontidae	whale sharks	Plankton and small teleosts[1]
Rhinidae	sharkfin guitarfish	Crabs, shrimp, bivalves, and small teleosts[2]
Rhinobatidae	guitarfish	Crustaceans, bivalves, polychaetes, and small teleosts[2]
Rhinopteridae	cownose rays	Bivalves[2]
Scyliorhinidae	catsharks	Bivalves, crustaceans, echinoderms, cephalopods, and small teleosts[1]
Sphyrnidae	hammerheads	Large and small teleosts, elasmobranchs, gastropods, echinoderms, crustaceans, and cephalopods[1]
Squalidae	dogfish sharks	Small teleosts (55%)[3], crustaceans (35%)[3], cephalopods (5%)[3], polychaetes, sea cucumbers, and jellyfish[1]
Squatinidae	angelsharks	Small teleosts, crustaceans, cephalopods, gastropods, and bivalves[1]
Stegastomatidae	zebra sharks	Bivalves, crustaceans, and small teleosts[1]
Torpedinidae	electric rays	Teleosts, cuttlefish, and small sharks[2]
Triakidae	houndsharks	Small teleosts, crustaceans, and cephalopods[1]

Table 2 Overview of diet composition for elasmobranchs in the wild

Stevens and McLoughlin (1991) found large quantities of fishes (>76% for n>35) in the stomachs of the graceful (Carcharhinus amblyrhynchoides), pigeye (Carcharhinus amboinensis), spinner (Carcharhinus brevipinna), whitecheek (Carcharhinus dussumieri), and hardnose (Carcharhinus macloti) sharks. Castro (1996) found that the stomachs of blacktip sharks (Carcharhinus limbatus) taken from the southeastern U.S. coastline contained 76% fish and

9% crustacean remains. In 96% of the stomachs only one type of prey was found.

Even within a species there are dietary differences between distinct populations. A survey done on 116 specimens of Australian sandbar sharks showed that 88% of the animals had recently eaten teleosts, 22% had eaten cephalopods, 8% had eaten crustaceans, 1% had eaten mollusks (other than cephalopods), and 2% had eaten miscellaneous material (Stevens and McLoughlin, 1991).

Diet can differ according to the age of a shark. Stillwell and Kohler (1993) found crustaceans (82%) and teleosts (14%) in the stomach of pup and juvenile sandbar sharks. Crustaceans were represented primarily (75%) by soft blue crabs (*Callinectes sapidus*). Fish prey consisted of small flounder, anchovy, Atlantic silversides, and mullet. Castro (1989) found similar results for the smalleye hammerhead (*Sphyrna tudes*), whereby 90% of the juveniles had eaten shrimp and 18% had eaten fish remains, while 89% of adults had eaten teleosts and 18% of adults had eaten shrimp.

Seasonal changes in diet may be important. Jones and Geen (1977) found that spiny dogfish (*Squalus acanthias*) predominantly ate teleosts in the winter and invertebrates in the summer.

3.2 Diet composition in captivity

Different studies on the diet of wild elasmobranchs demonstrate that a large variety of prey is consumed. This variety should be the basis of a dietary composition for elasmobranchs in captivity. In addition, it is important to consider changes in diet that may occur during the life history of a species. For example, juvenile sandbar sharks should be fed mainly on crustaceans, while adults should be fed on teleost. An overview of food items regularly fed to elasmobranchs in captivity has been provided in Table 3. Some of the ranges described are due to seasonal variations. For example, a study of Atlantic mackerel (Scomber scomber) revealed a fat content of 10 g 100 g-1 during the fall and 17 g 100 g-1 during the winter (Karakoltsidis et al., 1995). Both lean (i.e., <2 g 100 g fat-1) and fatty fish, as well as crustaceans, cephalopods, and possibly some bivalves or gastropods, should be included in a diet for elasmobranchs. Feeding different items with varying nutritive values will increase the probability that most of the essential elements are ingested. Variation in the diet is easy to coordinate when multiple feedings occur each week. Sharks may prefer certain food items over others.

By feeding one type of food per feeding session, animals will be encouraged to eat fewer appealing items and thus vary their diet. The choice of food types depends on availability, quality, supply consistency, and price.

Diets should be selected to reflect the natural diet components as closely as possible.

Food type (Species name)	Food type (Common name)	Water (g 100 g^{-1})	Protein (g 100 g^{-1})	Fat (g 100 g^{-1})	Energy (kcal 100 g^{-1})
Invertebrates					
Crangon crangon	shrimp	78[d]	19[d,h]	1[d]	87[d]
Euphausia superba	krill	78[d]	15[d]	3[d]	91[d]
Mytilus edulis	mussel	80[c]	8[h]-10[d]-12[c]-13[h]	1[d,h]-2[h,i]	51[d]-80[c]
Loligo sp.	squid	79[c,h]	13[h]-15[a,b,h]-16[f]-17[c]	0.2[h]-1[a,b,c,f,h]	84[f]-85[c]
Octopus vulgaris	octopus	84[h]	11[h]-15[f,h]	0.2[h]-1[e,f]	73[f]
Peaneus duorarum	gamba	78[c]	20[c]	1[c]	87[c]
Sepia sp.	sepia	80[h]-81[d]	16[d,f]-17[h]	1[d,f]	73[d]-81[f]
Callinectes sapidus	blue crab	80[c]	16[c]	1[c]	78[c]
Cerastoderma edule	cockle	-	17[f]	1[f]	81[f]
Fishes					
Caranyx chrysos	blue runner	75[c]	22[c]	2[c]	96[c]-163[g]
Clupea harengus	Atlantic herring	65[d]-74[c]	18[c,d]	10[c,d]-19[d]	160[c]-223[g]-233[d]
Engraulis encrasicholus	anchovy	74[h]-75[d]	18-21[h]	0.6-4[h]	-
Gadus morhua	Atlantic cod	81[c]	18[c,d]	1[d]	73[c]-77[d]
Merluccius merluccius	European hake	80[d]-81[h]	16[h]-17[d]-18[h]	0.4[h]-2[h]-3[d]	92[d]
Micropogonias undulatus	Atlantic croaker	78[h]	18[f]-20[h]	1[f,h]	84[f]
Pleuronectus flesus	flounder	80[d]	17[d]	1[d]	86[d]
Pollachius virens	saithe	79[c]-80[d]	18[d]-19[c]	1[c,d]	81[d]-84[c]
Pomatomus saltatrix	bluefish	75[c]	21[c]	4[c]	96[c]
Salmo trutta	trout	65[d]	20[d]	3[d]	103[d]
Sarda sarda	Atlantic bonito	70[h]	19-25[h]	3-8[h]	97[g]
Scomber scombrus	Atlantic mackerel	62[c]-68[d]-70[h]	18[h]-19[d]-20[c]	10[h]-12[d]-17[h]	182[d]-198[c]
Scomber japonicus	Pacific mackerel	71[c]	21[c]-25[h]	4[h]-7[c]	142[c]-152[g]
Sprattus sprattus	sprat	66[c]	17[d]	17[d]	217[d]
Trachurus trachurus	scat	75[d]-79[h]	18[h]-20[d]	1[h]-4[d]	114[d]

Table 3 Protein, fat and energy content of the edible portion of some food items used to feed elasmobranch in captivity

3.4 Feeding frequency

Feeding frequency depends on many factors (e.g., metabolism, age class, hormonal status, food availability, etc.). Of course, feeding frequency in captivity depends on the number of feeding sessions per week and the amount of food given during each session, and there is a demonstrated variety of opinions as to what is considered appropriate. There is a need to use biological parameters (e.g., gastric evacuation rates) to help determine feeding frequencies for captive populations of elasmobranchs.

Several models have been used for measuring food consumption in teleosts (Gerking, 1994) and elasmobranchs (Medved et al., 1988; Cortés and Gruber, 1990). Linear and exponential evacuation models examine the mean weight of food in the stomach, over a 24-hour period, and the hours required for complete gastric digestion. For elasmobranchs, a number of studies have measured stomach weights (Stillwell and Kohler, 1982; Medved et al., 1988; Cortés and Gruber, 1990). The bioenergetics model of estimating food consumption employs a balanced energy equation (Winberg, 1956; Gerking, 1994), where consumption is expressed as the sum of energy used for growth, reproduction, metabolism, and fecal and other excretory wastes. The food values of prey items are expressed as energy, in kcal (Table 3). Ultimately, comparisons between the weights of similar-sized wild and captive sharks will give a good

indication of whether feeding rations are correct (Mohan, 1996). Even within a species large differences for feeding ration are observed, making it difficult to predict precisely the ration for a particular species. Feeding ration will depend on the type of food available, the age of the animal, health and hormone cycle, size and shape of the tank, and water temperature and quality. When using daily feeding rate to calculate weekly ration, the number of days a shark is fed must be taken into consideration. For example, sharks fed at a daily rate of 1% BW day-1 three times a week are receiving 3% BW week-1 and not 7% BW week-1. Since sharks held at different institutions may be fed at different frequencies, useful comparisons of feeding ration can only be made if intake is expressed as percent body weight per week (i.e., % BW week-1).

To ensure meaningful adjustments of the feeding ration, it is important to observe the collection closely. If possible, monitor gut distension and evacuation to determine how rapidly food is moving through an animal. These observations will give a better understanding of the effect of captive conditions and dietary regime on metabolism. In addition, monitor overall growth, body width behind the head, and un-gorged gut size. These parameters will give an indication of the need to adjust feeding rations. Food type, and therefore energy content, will of course influence feeding ration. Atlantic herring (*Clupea*

harengus) have a much higher food energy, of 223 kcal 100 g-1, than the leaner flounder (*Pleuronectus flesus*), with a food energy of 86 kcal 100 g-1 (Table 3).

In general, juvenile sharks require a higher feeding ration than adults, using the excess energy for growth. Since feeding ration is dependent on many factors, it is impossible to give an exhaustive list of rations for all elasmobranchs. However, as a starting point, the following feeding rations may be applied to adult animals of the following taxa: (1) bottom dwelling sharks (e.g., Hemiscylliidae and Stegostomatidae) 4-6% BW week-1; (2) slow-swimming ram ventilating sharks (e.g., Odontaspididae) 1-2.5% BW week-1; (3) fast-swimming ram ventilating sharks (e.g., Carcharhinidae) 3-4% BW week-1; (4) bottom dwelling rays (e.g., Dasyatidae) 4-6% BW week-1; and, (5) ram ventilating rays (e.g., Myliobatidae) 4-6% BW week-1.

4. THE BROWN-BANDED BAMBOO SHARK (*Chiloscyllium punctatum*, Müller and Henle, 1838)

The brown-banded bamboo shark (*Chiloscyllium punctatum*) is a member of the Chondrichthyes class, jawed fish with paired fins, paired nares, a 2-chambered heart and cartilaginous skeletons. They are further classified as sharks within the Elasmobranch subclass.

The brown-banded bamboo shark is a small and benthic shark; it is a particularly hardy species and bodes well in captivity. What makes the species even more popular is its ability to be successfully housed with a wide variety of aquatic animals from larger rays to an array of small tropical fish. Furthermore, housing *C. punctatum* allows for further research into the not only such an unknown species, but also into little known areas in the group such as parthenogenesis with the publication of potential 'virgin births' in a number of species of sharks, including a close relative of the brown-banded bamboo shark, the white-spotted bamboo shark, *Chiloscyllium plagiosum*.

4.1 Nomenclature

Class: Chondrichthyes

Subclass: Elasmobranchii

Order: Orectolobiformes

Family: Hemiscyllidae (Gill, 1862)

Genus: *Chiloscyllium*

Species: *C. punctatum*

The brown-banded bamboo shark is a member of the family Hemiscylliidae (the bamboo sharks) which divides into two genuses, Hemiscyllium and Chiloscyllium. These genus' represent 11 species of typically smaller sharks, all species averaging less than 150 cm in length.

There are a number of other common names that refer to *Chiloscyllium punctatum* other than the brown-banded bamboo shark. These include the brown-spotted catshark, grey carpet shark and the spotted catshark. The suffix catshark is a common addition due to the small 'whiskers' that develop on the snout. Grey carpet shark is perhaps the most suited common name, especially when discussing adults, as only juveniles possess the banding pattern referred in the common name brown-banded bamboo shark.

4.2 Morphology

Brown-banded bamboo sharks possess slender bodies with concave posterior margined dorsal fins and an elongated and thick precaudal tail. Mouth well in front of eyes. The body lacks a lateral dermal ridge. No colour patterns are observed in the adult form, just a solid variation of grey-brown. The juveniles are distinct with few dark spots and dark transverse banding across their pale form which fades to the adult form.

Figure 10. Juvenile brownbanded bamboo shark

Prepectoral length 16.2 to 18.4% of total length. Snout rounded anteriorly. Eyes moderately large. Body and tail moderately slender. No lateral ridges on trunk, and predorsal and interdorsal ridges not prominent. Interdorsal space fairly short, slightly greater than first dorsal-fin base.

Dorsal fins large and angular, larger than pelvic fins, and with concave posterior margins and prominently projecting free rear tips. Total vertebral count between 136 and 170 (mean = 154.7, n = 6). Intestinal valve count 20 (n = 4) (Compagno, J. V. L., 1984).

Wild specimens of *C. punctatum* have been known to reach lengths of 117cm (Bennett and Kyne, 2003). In captivity, the brown-banded bamboo shark can grow up to 144cm.

Figure 11. Adult male brownbanded bamboo shark

Sexual dimorphism is not clearly evident based on size. However, as with other Chondrichthyes, sexual dimorphism is evident as males possess claspers. Claspers are formed from the posterior portion of the pelvic fins which serve as an intromittent organ for semen direction into the female's cloaca.

4.3 Distribution

The brown- banded bamboo shark is a widely distributed tropical specie found on Indo-West Pacific: India (east coast, Andaman Islands), Malaysia, Singapore, Thailand, Indonesia (Java, Sumatra, Sulawesi, Komodo), Vietnam, China, Taiwan (Province of China), Japan, Philippines, south coast of New Guinea (Papua-New Guinea and Irian Jaya, Indonesia), north coast of Australia (Northern Territory, Western Australia, Queensland). (Compagno, J. V. L., 1984)

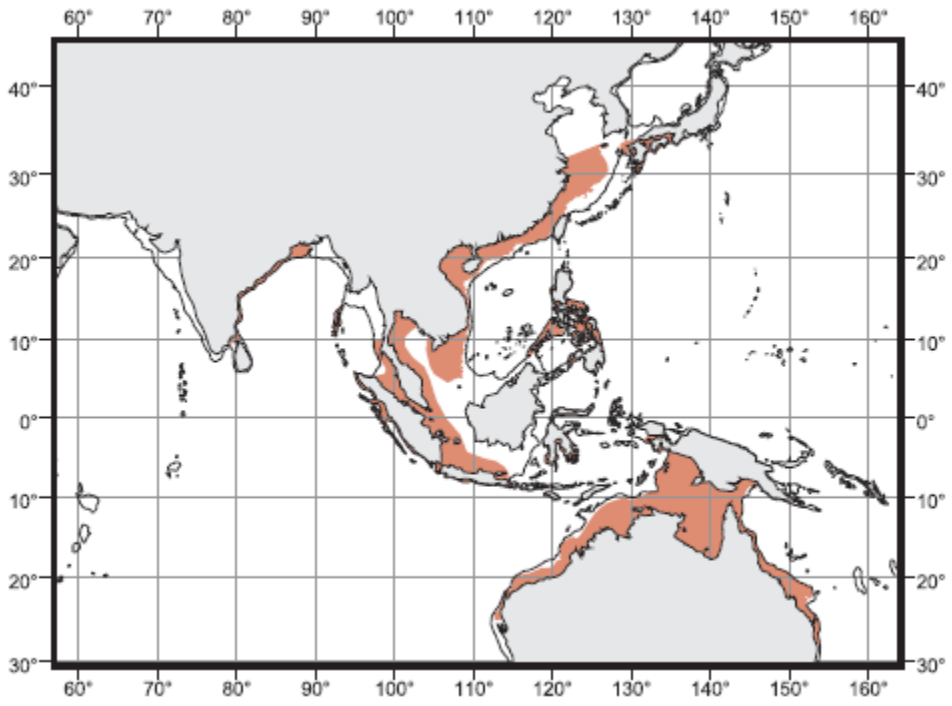

Figure 12. Map of brownbanded bamboo shark distrubution

4.4 Ecology

C. punctatum is found on coral reefs, often in tidepools sand and sand/mud substrates throughout its range. Found in the intertidal down to at least 85 m. (Compagno, J. V. L., 1984). It can tolerate severe environmental hypoxia (low oxygen levels), one reason this species is favoured in captivity. It prefers to hide in crevices during the day being a nocturnal feeder. The banding pattern found on juvenile's further helps it camouflage with the coral based surroundings.

4.5 Conservation status

The species is listed as near threatened on the IUCN red list. There are insufficient data for upgrading it to vulnerable. Research into C. punctatum populations is necessary as the species is common in by-catch, threatened by overfishing for human consumption or the display-aquarium trade, and also habitat degradation and loss.

4.6 Feeding requirements

The brown banded bamboo shark is a carnivorous species, more specifically though, a portion of its diet deems it a piscivore. In the wild, populations of these sharks will typically eat mostly invertebrates (such as squid), crustaceans (shrimp) and other benthic organisms. Small fish may also be preyed upon by larger juveniles and adults.

The species is a demersal one and tends to inhabit coastal areas and tidal pools, including the continental shelf in tropical regions and thus, there is slight seasonal variability depending on prey populations (Daley et al. 2002). Though the diet primarily remains the same, the portion that each group makes up of the diet can vary. Due to this species being commonly kept in aquaria, more is known on this species diet or prey portions percentages in captivity.

The brown-banded bamboo shark's captive diet similarly reflects that of its wild diet. A variety of squid, prawns, mussels and small marine fish such as whitebait can be offered. The size of the individual will determine the size of the food able to be offered. Being a nocturnal species, the brown-banded bamboo shark has limited day time activity. The day usually involves resting in dark places such as caves, crevices, under rocks and other shaded areas. Juveniles are also known to hide among corals as due to their colour banding they camouflage well. At night, these sharks will hunt for food.

4.7 Reproduction

No known courtship displays between males and females has been observed in the brown-banded bamboo shark. As with most species of shark however, a sign that a female will possibly soon lay eggs is bite marks on her body (N. Weller, pers. comm.). The brown banded bamboo shark is an oviparous species. During mating, the male is required to insert one of his claspers, located on the inner side of his pelvic fin, into the female's genital opening (cloaca). In order to achieve this, the male uses his mouth to hold on to areas such as her gill slit or pectoral fin; these once mated with, the female lays a group of elongated, flattened eggs. These then attach to rocks, inside crevices, etc with filamentous material. The developing embryo feeds exclusively on the yolk sac until they hatch. In captivity, the eggs take between 4-6 months to hatch, with the time varying with temperature. The young are around 13-17cm when they emerge from the pod-like eggs and possess a distinct banding pattern, giving the name brown-banded bamboo shark. Once hatched, the juveniles are on their own, with no parental care exhibited.

Figure 13. C. punctatum egg

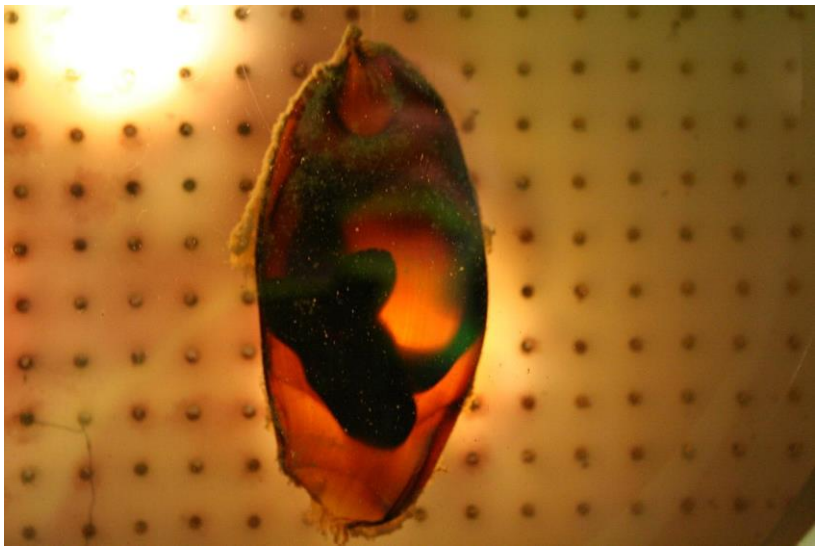

Figure 14. C. punctatum embryo

4.7.1 Reproductive condition

- Females

To reproduce females must first reach maturity which is age and size related at approximately 63 cm total length (Bennett and Kyne, 2003). The female must also have a healthy body condition to be able to provide the nutrients and energy to develop healthy eggs and offspring. In captivity, this is dependent on aquarists maintaining a high standard of care and holding animals in their optimal conditions especially breeding individuals.

- Males

Males too must also reach maturity before breeding which is age and size related also (68-76 cm total length) (Bennett and Kyne, 2003). Seeing as though males are required to hold on to the female during mating, males must also have a peak body condition to have the opportunity to mate every season. In captivity, this is dependent on aquarists maintaining a high standard of care and holding animals in their optimal conditions especially breeding individuals.

The brown banded bamboo shark is an oviparous species that lays brown rectangular egg cases. Fine tendrils covering the outside of the egg case are used to attach it to rocks and coral. (Harahush, 2007). Eggs are generally laid between late July and February with mating occurring between July and September (Harahush et al. 2007); however, if temperature and photoperiod are

monitored, the species is capable of breeding year round. There is also the potential for parthenogenesis to occur. Furthermore, females in the related epaulette shark (*Hemiscyllium ocellatum*) have the ability to store sperm to fertilise the ova for up to several months after mating and it is possible that the same scenario can occur in brown-banded bamboo sharks.

4.8 Sperm storage in female

C. punctatum is the leading actor of the longest documented case of sperm storage (45 months) for any species of shark. What makes this event unusual is that one female of *C. punctatum* laid eggs after at least 45 months of complete isolation from males (Bernal, M. A., et al., 2015).

Previous studies on sharks have reported sperm storage periods of 13 (Storrie *et al.*, 2008) to 28 months (Castro *et al.*, 1988).

Considering that the females had not been in contact with males for an extended period, there are two hypotheses that could explain the origin of the pup:

(1) long-term sperm storage by one of the female *C. punctatum* or

(2) facultative parthenogenesis, which has been previously demonstrated for the closely related whitespotted bamboo shark *Chiloscyllium plagiosum* (Bennett 1830) (Feldheim *et al.*, 2010).

Genetic analyses suggest that is the longest documented case of sperm storage (Bernal, M. A., et al., 2015).

5. CATTOLICA ACQUARIUM: “LE NAVI”

The buildings and the history

1934 - 1999: from the colony to the restoration project

In the early 1930’s, the “Figli del Littorio” foundation, in collaboration with the directorate general of “Figli degli Italiani all’Estero”, an organization working with the children of Italians living abroad, commissioned the Rome-born architect and engineer Clemente Busiri Vici to design a marine colony to be built northeast of Cattolica, in an area between the rivers Ventena and Conca. The plans for the nucleus of the project were ready by 1933, with a rigidly symmetrical layout comprising five buildings inspired by the world of ships, aircraft, flying boats, trains and submarines. The complex was built in nine months, and was inaugurated on 28 June 1934, in the presence of the head of government, Benito Mussolini.

Great attention was dedicated to this new complex in the Italian and international press of the period, with comments ranging from enthusiasm to amazement. In the context of the cultural and architectural debate of the time, which contrasted classical and modernist styles, meaning architects who preferring the model of the late 1800’s and those who proclaimed instead a new architecture more suited to the times, it is easy to see innovative forms in the “Le Navi” complex that bring it close to Italian Futurism.

It very evidently offers in fact intensely symbolic contents linked with the modernist theme of machines and machinery, and strong emotional and psychological atmospheres deriving also from the Expressionist use of reinforced concrete. In late 1934 Busiri Vici developed a project for the extension and alteration of the colony, maintaining the naval references, even though in a more moderate form, in the two torpedoes on the landward side, which in plan and elevation are "Rationalist" citations of the four ships closer to the beach, in the restructured semi-underground chapel, and in the guard building. Between 1935 and 1943, the "XXVIII Ottobre Marine Colony" was a self-sufficient structure, with its own farm, still existing today, capable of accommodating about 2000 young naval cadets, living under almost military discipline. In 1944, after the passage of the front, it became a military hospital.

After the war the complex was restored to its former function as a holiday centre, becoming the "G. De Michelis Marine Colony". During the years of the economic boom, the company Maraldi of Cesena planned to divide the entire complex up into construction lots. Cattolica's new town planning regulations were approved in 1963, but excluded the area occupied by "Le Navi" from its development plans, initially protected but now left free for building as proposed by government authorities.

This allowed Maraldi to obtain permission for its planned subdivision of the area, demolishing some of the buildings and building hotels and apartments in the area, almost halving the surface area occupied by buildings on which the colony once stood. In the mid-1970's the activities of "Le Navi" as a summer holiday centre and ownership of its buildings were taken over by the Emilia-Romagna Region, which assigned its management to the Municipality of Cattolica. In the mid-1980's a start was made on an initial restoration of the entire complex, now destined for use as the "Le Navi International Youth Centre", a student holiday centre for young people from the whole of Europe, making joint agreements with the municipalities of Bologna, Modena and Cattolica, and managed from 1993 to 1997 to the LE NAVI Cooperative of Cattolica.

In 1993, the Urbanistics Office of the Municipality of Cattolica prepared a series of preliminary studies and projects for the conversion of the complex into a multifunctional centre with specific educational, cultural and recreational characteristics, by restoring or regenerating the existing buildings, at the same time requesting once again the management of the entire complex from the Emilia - Romagna Region. In 1997, the municipality obtained the transfer of the complex from the region for the creation of a themed marine park.

Simultaneously, the municipality become the promoter for the constitution of a company with mixed public and private capital, to be called “Parconavi SpA”, the aim of which was to create the marine park. Work on the park started in 1999, and on 10 June 2000, after only eleven months of work, it was inaugurated and opened to the public.

Figure 15. Cattolica Acquarium

From 2000 to today: preservation and restoration

Today, the “Le Navi” complex, restructured and restored to use, is an important touristic and cultural attraction for Cattolica, also representing significant architectural values brought once again to life. The recovery and reutilization programme for the complex as a marine theme park has made a successful start, developing in four major directions identified as being fundamental.

The first direction was that of the development of a conceptually unified structure, respecting the original nature of the buildings as devised and designed in 1932 by Clemente Busiri Vici. The second direction was that of creating a park with green areas and public spaces freely available for use by the community, constituting nothing less than an important public park integrated into the urban fabric, also given its specific geographical location. A third aspect was that of display themes, characterized by an ample and articulated variety of contents.

The final point regarded the conscious attempt to present a sophisticated interactive educational and cultural product, destined nevertheless for use by the general public. The first of these issues was respected with great sensitivity by the designers of the Acca C Studio of Rome.

In changing the use of the complex, all elements of the new project of a certain impact, such as the system of paths linking the individual buildings and the imposing seawater and aquaculture filtration systems, were installed underground, and more precisely at a depth of -4.7 metres below sea level. The only new additions that have been introduced, with great discretion, rest on the ground surfaces of the park, and are modular metallic structures of limited dimensions. Three of these, light and empty, serve as information points. The other three, with their different colours of light blue, yellow and pink, give external indications of the points where the three main underground itineraries meet the four ship buildings used for the marine park's themed exhibits, destined to the discovery of the underwater habitat and its evolution from its origins to the present day. The spaces used for the other activities of the park, including hospitality, catering, leisure and shopping, were all obtained using structures already existing, by simply changing their utilization, and accommodating them in the Torpedoes, the Admiral ship, the former Chapel and the former Infirmary, which are accessed directly at sea level.

The only new element can be seen in the tiered arena that leads visitors into the large covered square called “Templa Serena”, at a level of -4.7 metres, which is the starting point for the long itinerary that leads through the buildings “West Ship”, “East Ship”, “Mistral Ship” and “Admiral Ship”, now “Geopolis” and “Aquapolis”.

Figure 16. Map of the park

6. AIM OF THE THESIS

The correct and complete knowledge of the reproductive cycle of a species is very important for its conservation. Although there are fossil records dating back 400 million years, little is still known about sharks reproduction. To date, sharks are highly susceptible to extinction because they grow and reproduce slowly. Many reef shark populations have been threatened in recent decades by overfishing and shark finning, the practice of killing sharks to sell their valuable dorsal fins.

Since the information present in the literature is still scarce, the aim of the thesis is to characterize at cytological and macromolecular level the semen of brown-banded bamboo shark males (*Chiloscyllium punctatum)* at different maturity stages (immature, maturing and mature).

The brown-banded bamboo shark is easy to breed in captivity and has suitable size to take samples.

Establishing a non-invasive protocol to determine the sexual maturity of an individual could be very useful, especially in captive reproduction.

The protocol developed and applied in this thesis for the collection of semen is absolutely non-invasive and could avoid and or replace histological analysis or GSI evaluation as methods to assess sexual maturity.

To reach the goal, a protocol for the collection of semen without use of anaesthetics was established. In addition, different approaches such as cytological analysis, and the FT-IR (Fourier Transform Infrared Spectroscopy) analysis and Raman analysis were used to characterize and to assess the presence and the quality of spermatozoa and the macromolecular composition of seminal plasma.

Cytological analysis and Raman spectroscopy were used for the analysis and characterization of spermatozoa.

FT-IR spectroscopy have been applied to characterize the macromolecular composition of seminal plasma.

7. MATERIAL AND METHODS

The experimentation was carried out at the Cattolica Acquarium. An experimental group of five males of brown-banded bamboo shark (*Chiloscyllium punctatum*) has been defined. They are siblings but not of the same age: some were born in 2013 – 2014, other in 2015. Before the experimentation they have been maintained under the same conditions of T, pH, salinity and nutrition. All specimens were kept in a circular tank of the dimensions of 200 cm diameter, 110 cm high, carrying 3454 l of water with the following values: T 23.6° C, pH 7.43 - 8.08, salinity 33.4 - 33.8 %, photoperiod 12 h of light and 12 h of darkness.

Each individual has a microchip for recognition: 39466, 39424, 39660, 39783, 39861.

Sperm samples were taken at the Cattolica Aquarium. All subsequent analyses were carried out at the Development and reproduction biology laboratory of the Università Politecnica delle Marche.

The sharks were taken from the tank where they were kept and placed in a smaller one. Each specimen was identified, turned on its back and, without being anesthetized, the sample was taken. It was done by inserting a cat urinary catheter 1x130 mm in one of the two claspers and by aspiration with syringe,

the sperm material was taken. The contents were then placed in a Falcon tube, diluted with 0.9% NaCl saline and kept cool for transport.

Figure 17. Semen collection

For all samples two washes have been done. In each tube 1 ml of saline was added, vortexed and then the contents were transferred into a 2 ml tube. Centrifugation was performed at 700 xg for 7 minutes at + 4°C.

The supernatant was removed, while the pellet obtained was resuspended with 1 ml of saline, vortexed and centrifuged at 1000 xg for 10 minutes at + 4°C. The supernatant was removed; about 2 ml of Formol was added to the pellet obtained to fix the sample. Everything was stored in the refrigerator at +4°C.

7.1 Citology analysis

A sub-sample of each sample was taken with a pipette and placed on a microscope slide. The slides were dried under a hood and transferred at a temperature of 37° C to facilitate drying (about 3-4 hours). Then they were stained by Mayer's Hematoxylin and Eosin technique. The first dye utilized is the Hematoxylin, and the second one is the Eosin. For a proper staining, we choose to retain the samples, 50 seconds for the Hematoxylin dye, and 20 seconds for the Eosin dye. After the staining, the slides are placed in a crescent graded ethanol bath (70%, 80%, 95%, 100%). The protocol requires a last bath in Xylene, to better facilitate the mounting of the slides under the hood. Some drops of glue are needed to allow the mount of the slides using the coverslip. Samples were then observed and evaluated under a light microscope with the aim of detecting the presence of spermatozoa by Zeiss Axioscop (Oberkochen, Germany) and using the software Zen (Carl Zeiss, Oberkochen, Germany) to take pictures.

The slides were initially observed with a 20x magnification lens to make a general analysis and check the presence of spermatozoa. The slides were then moved to a 40x magnification lens to observe in more detail the areas where the presence of sperm was detected. In those cases where it was possible, an observation was made with a 100x magnification lens in oil immersion. An area of 0.25 cm^2 was taken as a reference for the count, chosen on the basis of the greater coverage of the sample, as it is assumed that there is a greater probability of finding spermatozoa. Once identified, they were photographed and then the head and tail measurements were taken using a reference scale in μm.

7.2 Raman Microspectroscopy (RMS) measurements and analysis

Spermatozoa were washed by a series of centrifugations in physiological solution (NaCl 0.9%); then, a 10-μm aliquot was seeded onto glass windows and let dry at room temperature for 30 minutes.

RMS measurements were performed in duplicate at the Laboratory of Advanced Research Instrumentation (ARI), Department of Life and Environmental Sciences, Università Politecnica delle Marche (Ancona, Italy). A Horiba Jobin-Yvon XploRA Raman microspectrometer, equipped with a 785-nm diode laser was used as source. All measurements were acquired by

using a ×100 objective (Olympus, N.A. 1). The spectrometer was calibrated to the 520.7 cm^{-1} line of silicon prior to spectral acquisition. A 600 lines per mm grating was chosen. A 100 μm confocal pinhole was used for all measurements. The spectra were dispersed onto a 16-bit dynamic range Peltier cooled CCD detector.

For each sample, bidimensional Raman maps were acquired from 5 single spermatozoa seeded on the glass slide, on the tail, middle piece and head regions. The spectral range from 400 to 1800 cm^{-1} was chosen and spectra were acquired for 3×10 seconds at each spot. Raman spectra displayed homogeneous profiles; no contribution of glass to the spectra was observed. Raman maps were corrected by smoothing procedure (9 smoothing points), baseline-corrected with the polynomial method (2 iterations) (OPUS 7.5 software, Bruker Optics GmbH, Ettlingen, Germany).

For the rough evaluation of the spatial distribution of unsaturated lipids, glycogen, ATP (adenosine triphosphate), and DNA, within the mapped regions, false color images were generated by integrating the Raman images under the following peaks: 1450 cm^{-1} (vibration of CH_2 groups, *Unsaturated lipids*), 935 cm^{-1} (vibration of CCH, *Glycogen*), 752 cm^{-1} (ring vibrations of adenine moieties, *ATP*), 1340 cm^{-1} (ring vibrations of adenine and guanine, *DNA*).

7.3 FT-IR measurements and analysis

A Bruker VERTEX 70 interferometer coupled with a Hyperion 3000 Vis-IR microscope was used. The spectrometer was equipped with a liquid nitrogen cooled bidimensional Focal Plane Array (FPA) detector that allows to perform the imaging analysis of non-homogeneous biological samples by simultaneously acquiring 4096 spectra on an area of 164x164 μm2. The visible image of seminal plasma was obtained with a 15X condenser/objective and used to select areas containing spermatozoa at different development stages (immature, maturing and mature). On these selected areas, IR maps were collected in transmission mode in the 4000-900 cm-1 MIR range with a spatial resolution of ~2.56 μm. Each spectrum was the result of 256 scans with a spectral resolution of 4 cm-1. Background spectra were acquired on clean regions of CaF2 optical windows.

Raw IR maps were corrected by applying the Atmospheric Compensation routine, to remove the contribution of atmospheric carbon dioxide and water vapour, and then vector normalized in the 4000-900 cm-1 spectral range, to avoid artefacts due to differences in thickness (OPUS 7.1 software, Bruker Optics, Ettlingen, Germany).

These preprocessed IR maps were integrated under the following spectral regions, to obtain false colour images representing the topographical

distribution and relative amount of the most relevant biochemical features: 2995–2825 cm-1 54 (containing the vibrational modes of lipids, named LIPIDS); 1718–1481 cm-1 (containing the vibrational modes of proteins, named PROTEINS); 1274–1181 cm-1 (containing the vibrational modes of phosphates groups inside nucleic acids, named PHI DNA), and 1130–1013 cm-1 (containing above all the vibrational modes of pentos, named PHII DNA).

8. RESULTS AND DISCUSSION

Elasmobranchs are often considered ancestral in their design compared to extant vertebrate species (Helfman et al. 1997), and are considered virtually unchanged since their development over 400 million years ago. Nevertheless, they have evolved diverse, highly complex mechanisms for assuring reproduction success. Elasmobranchs are considered K-strategists, exhibiting slow growth, high maternal investment, and production of only a few, often well developed, offspring. Examples of their adaptation are the internal fertilization for all species, viviparity, with peculiar mechanisms of nutrition transmission from the mothers to the embryos, and finally the oviparity. Another interesting characteristic is the sperm storage in males and/or females has evolved in different elasmobranch species (Pratt and Tanaka1994).

At last, the production of enough numbers of functionally competent spermatozoa is considered one of the best successes of all the strategies.

Sexual maturity stages can be estimated using several methods, from microscopic gonadal inspection to macroscopic observation of reproductive characteristics. In male elasmobranchs, non-invasive macroscopic reproductive variables commonly used for classifying maturity stages include the clasper calcification degree and a measure of the clasper length.

By using clasper calcification alone, the determination of maturity stages is to some extent subjective (Walker 2005).

In elasmobranch species the differentiation of testes usually anticipates the full development of claspers (asynchronous development), which rigidity is a condition necessary but not enough for the determination of the maturity stage. In general, the complex life cycle of oviparous elasmobranchs such as *Chiloscyllium punctatum* is translated into an extended reproductive cycle and it is associated with high energy requirements.

Microscopically, spermatogenesis starts both from the germinal zone or germinal papilla located in the centre of the lobe and dorsally in the testis. Spermatocysts are spherical units composed of germ cells and Sertoli cells surrounded by an acellular basal lamina. In all elasmobranchs, the germ cells of only a single developmental stage are associated with a Sertoli cell at any given time and then degenerate after the development is complete (Stanley 1966). Spermatocysts go through several stages leading to the production of mature spermatozoa: stages I (primordial germ cells or gonocytes), stages II (spermatogonia), stages III (primary spermatocytes), stage IV spermatocysts are formed from a division of primary spermatocytes into secondary spermatocytes, containing small, round nuclei and condensed chromosomes.

Stage V consists of spermatids, which are produced after the second meiotic division of secondary spermatocytes; the spermatids show elliptical nuclei and emerging flagella and are separated and unorganized inside the spermatocyst. In stage VI (early spermatozoa), spermatids that have undergone spermiogenesis are transformed into more elongated immature spermatozoa, which form loose bundles with heads facing the basement membrane and tails projecting toward the lumen. Stage VII consists of mature spermatozoa that are organized in tight bundles associated with Sertoli cells arranged in the periphery. The testes are completely formed and are filled with lobules containing all the spermatocyst stages and there are a greater proportion of stages V–VII (spermatids to mature spermatozoa).

The mature spermatozoa exit the testes via efferent ducts. The sperm is then transported through the epididymis and vas deferens to the seminal vesicles and the claspers.

In this work, analysing the semen manually collected, of males at different gonadal maturation stages we observed the presence of germinal cells at different maturation stage; in particular it was possible to retrieve spermatids in the immature males respectively.

Observing the morphology of spermatids we can define them as human-like (Figure 18) because they have a morphology similar to human spermatozoa, with a small rounded head, an evident mid-peace and a thick tail. The dimensions detected are 5 µm head and 55 µm tail. To confirm the immature male condition, the morphology was compared with the sperm found in the *Mustelus mustelus* testis (Figure 19), a work carried out by Dott. Lorenzo De Santis.

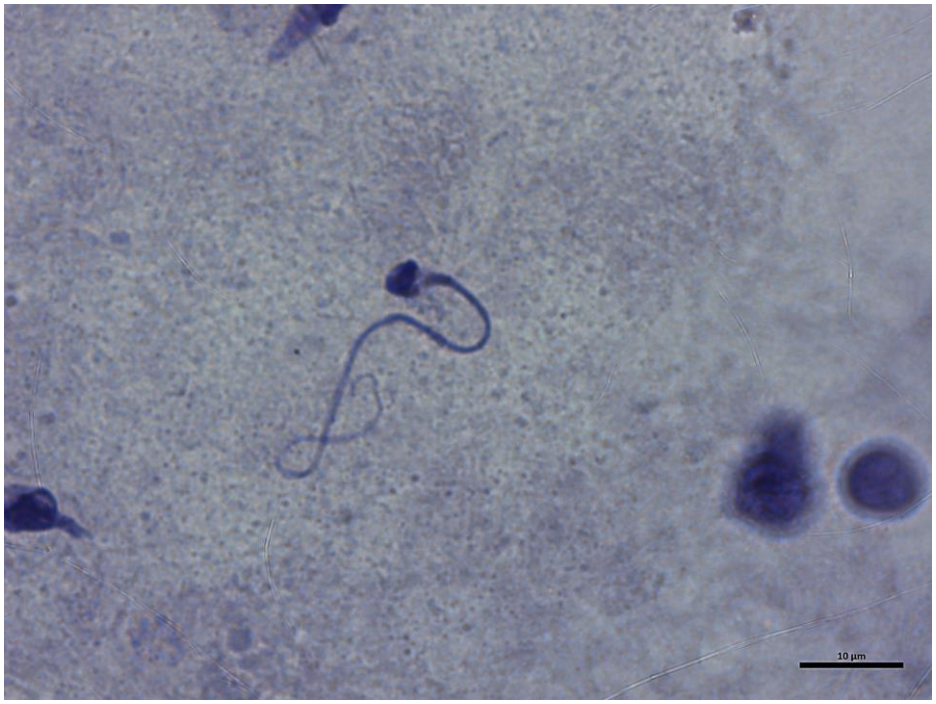

Figure 18. Spermatids of Chiloscyllum punctatum immature male (enlargement 100x)

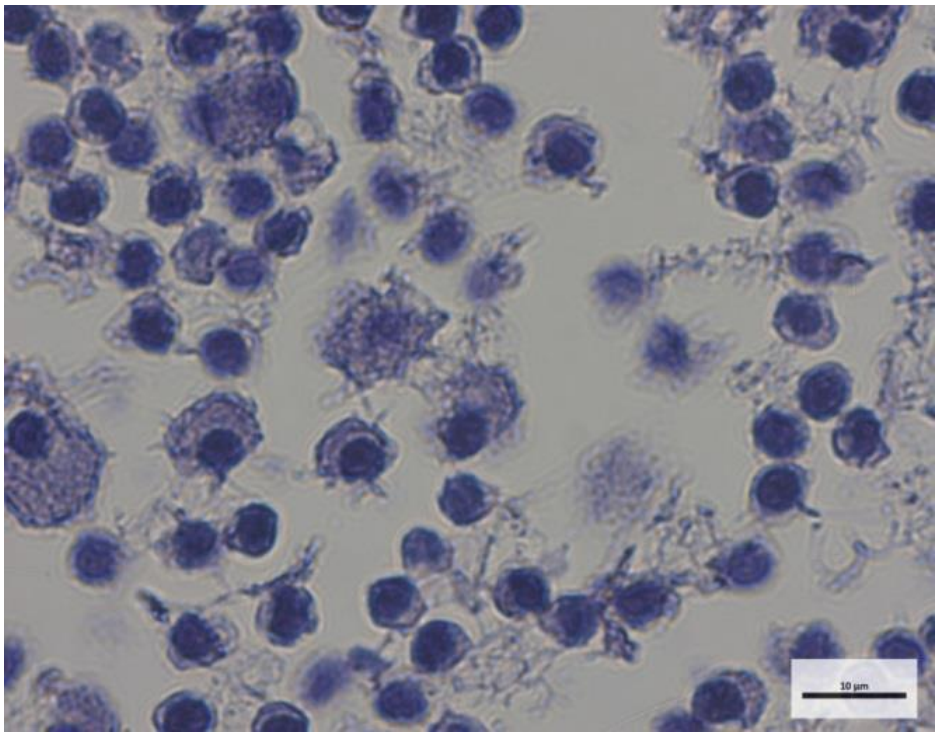

Figure 19. Spermatids in Mustelus mustelus testis (enlargement 100x)

In the early spermatozoa phase they are “maturing” as shown in the Figure 20 and Figure 21. They have an elongated elliptical head and a thinner tail. The dimensions detected are 20 µm head and 32 µm tail (Figure 20) and 40 µm head and 70 µm tail (Figure 21). Also in this case from the observations made in the *Mustelus mustelus* testis (Figure 22) we can see a compatibility with the maturation stage.

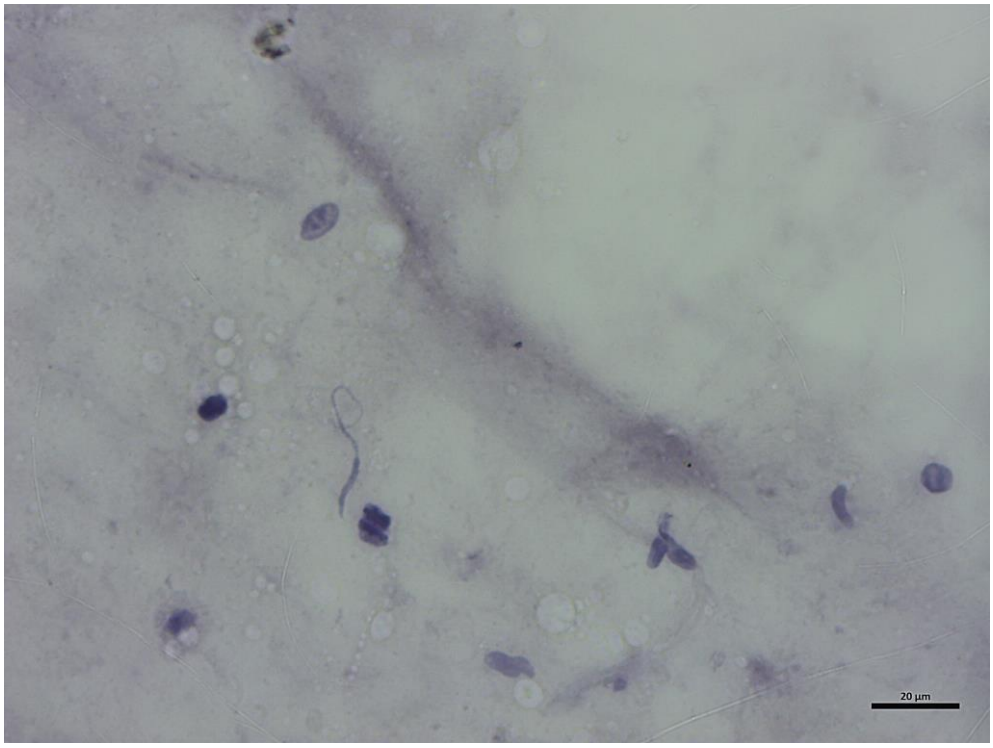

Figure 20. Early spermatozoa in Chiloscyllum punctatum maturing male (enlargement 40x)

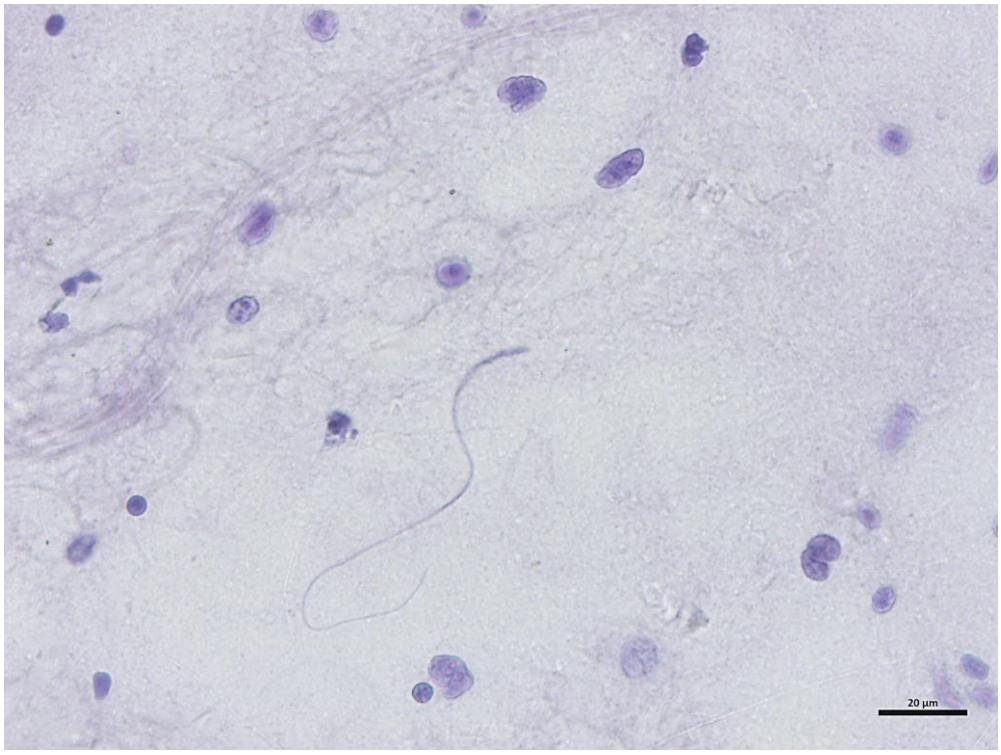

Figure 21. Early spermatozoa in Chiloscyllum punctatum maturing male (enlargement 40x)

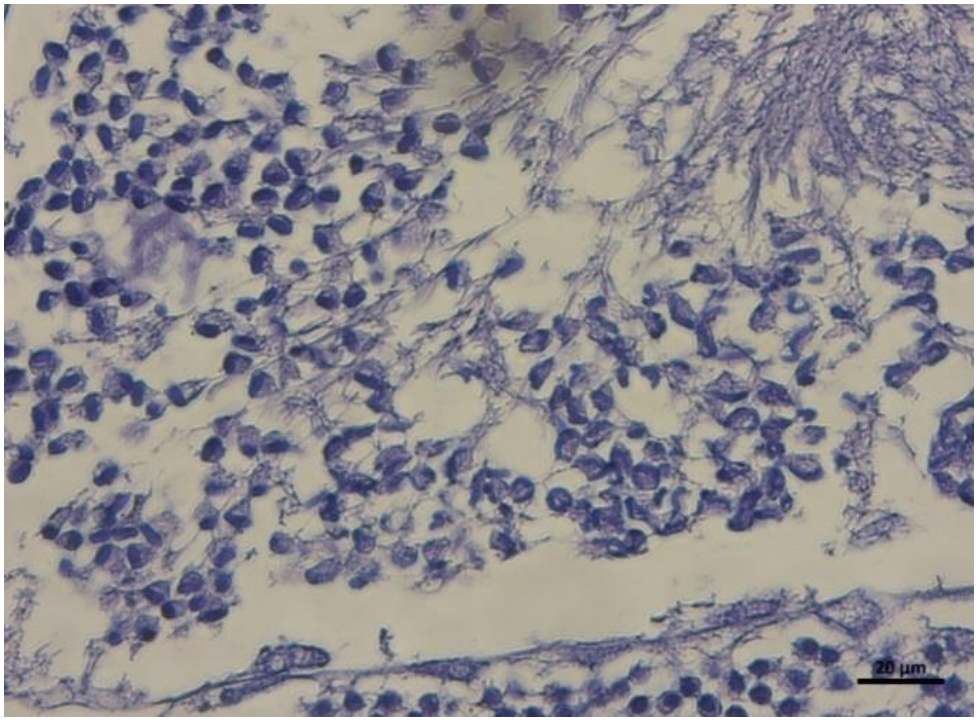

Figure 22. Early spermatozoa in Mustelus mustelus testis (enlargement 40x)

In the last stage we observed the real mature spermatozoa that were called shark-like spermatozoa. They present development of the helical head, a characteristic feature of chondrichthyan spermatozoa and a very long and thin tail. The dimensions detected are 40 µm head and 66 µm tail (Figure 23). Also in the *Mustelus mustelus* testis was found this morphology (Figure 24), so in this case we have a mature male.

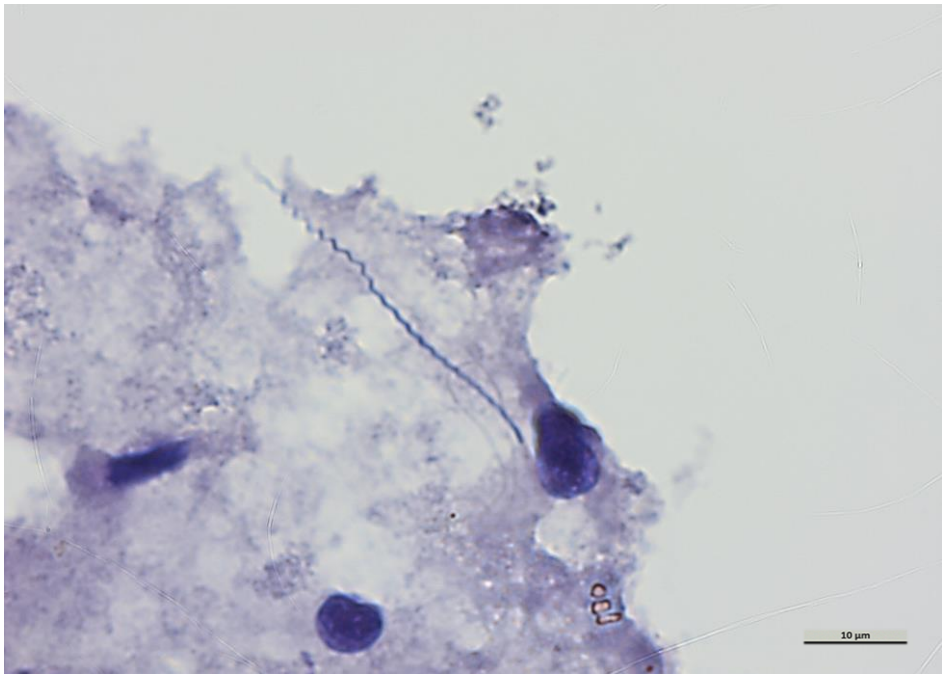

Figure 23. Spermatozoa in Chiloscyllum punctatum mature male (enlargement 100x)

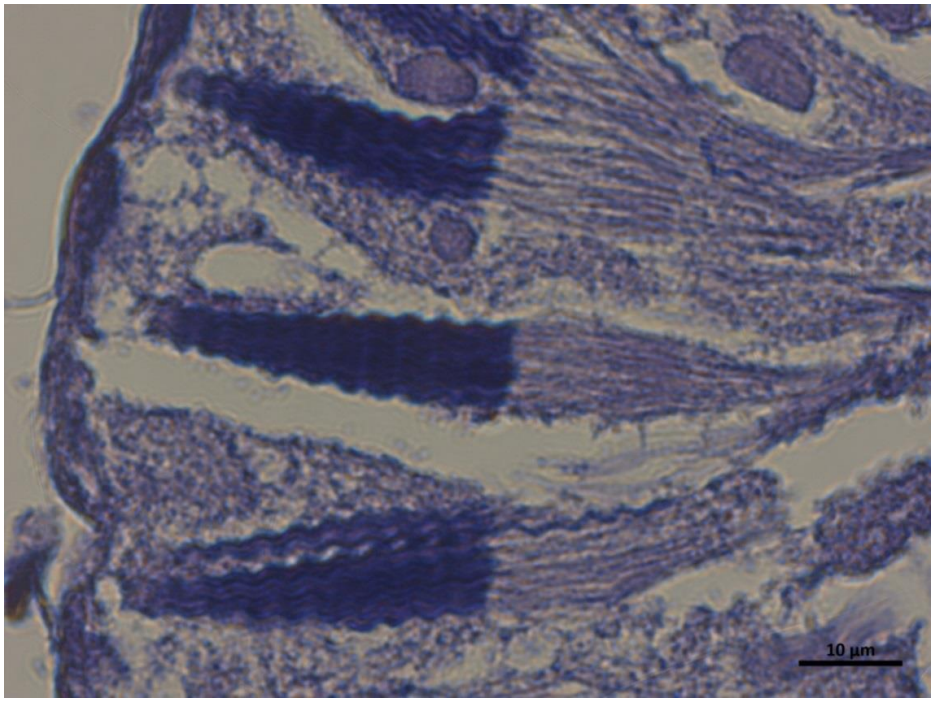

Figure 24. Mature spermatozoa in Mustelus mustelus testis (enlargement 100x)

In addition to the individual spermatids/spermatozoa were also found aggregation for both the spermatids and the early spermatozoa, as can be seen in the Figure 25 and Figure 26. Supporting the hypothesis that sharks ejaculate multiple spermatozoa “packaged” into spermatophores.

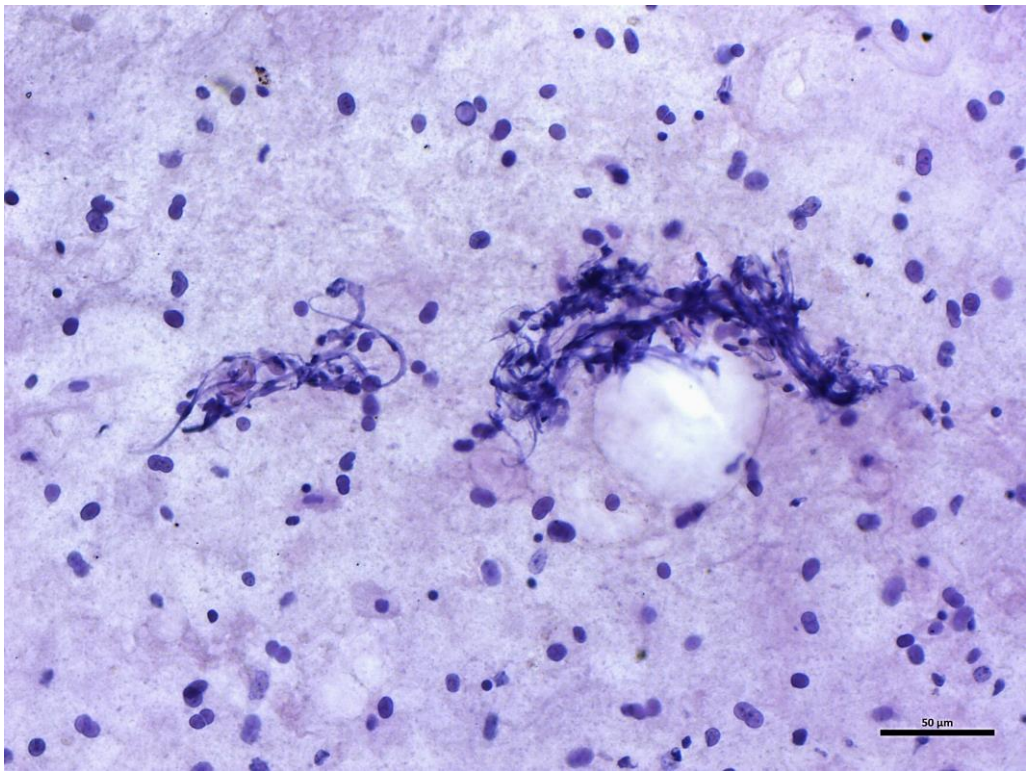

Figure 25. Spermatids aggregation (enlargement 20x)

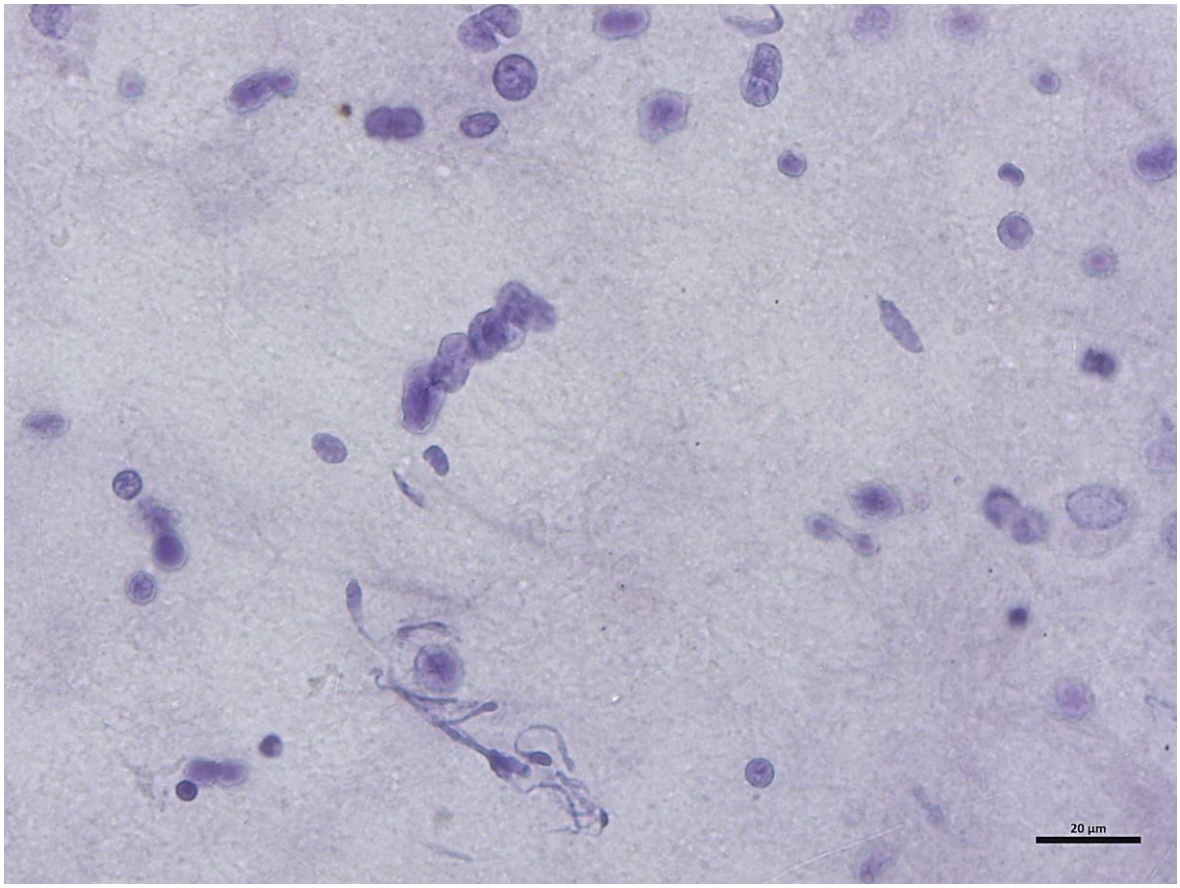

Figure 26. Early spermatozoa aggregation (enlargement 40x)

From what has been observed we can say that in the seminal fluid of immature males only spermatids are present, while mature spermatozoa are present in mature animals. In immature males, spermatids are released but they have not completed the differentiation process and therefore have not been differentiated into mature spermatozoa. The acquisition of sexual maturity or puberty takes place when the male is able to produce mature and functioning gametes for the first time.

By analysing the spermatozoa at the various stages of maturation with Raman Microspectroscopy (RMS) we obtained the following results.

Figures 27-29 report the microscopy images of representative spermatozoa from immature (Fig. 27), maturing (Fig. 28) and mature (Fig. 29) samples (H&E stained), together with the false color images representing the spatial distribution of *Unsaturated lipids* and *Glycogen* in the tail region, of *ATP* in the mid-piece region, and of *DNA* in the head region.

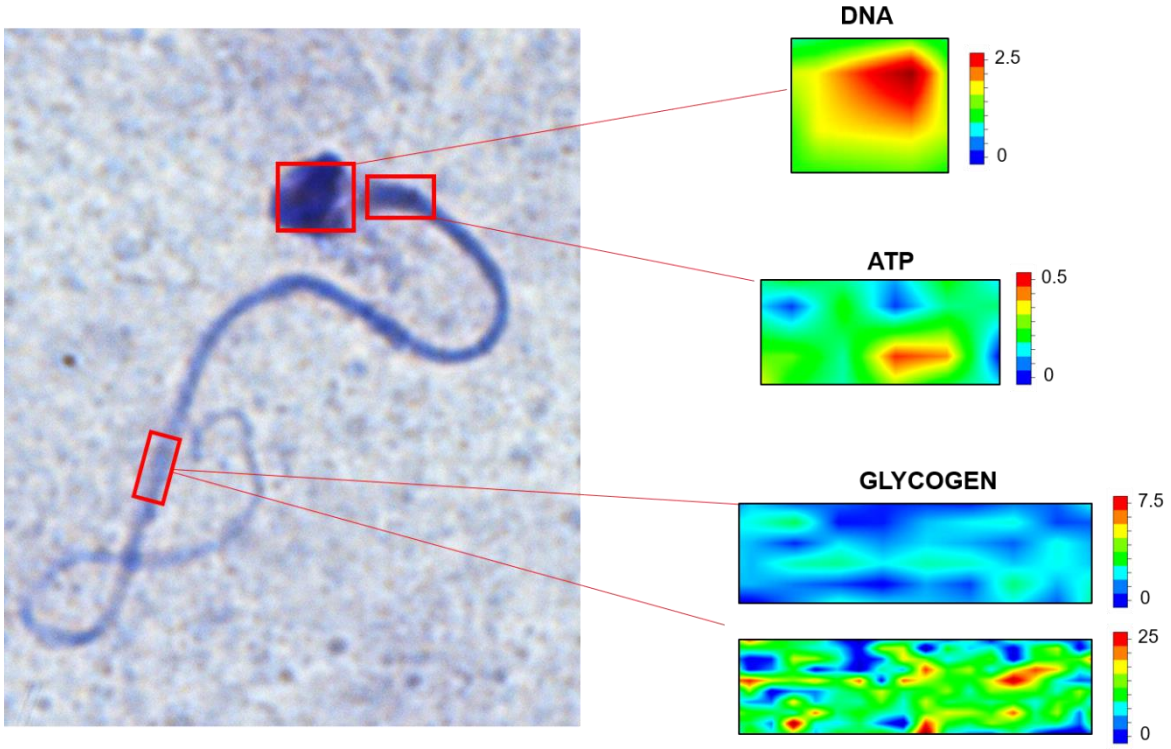

Figure 27. Spermatids from immature male

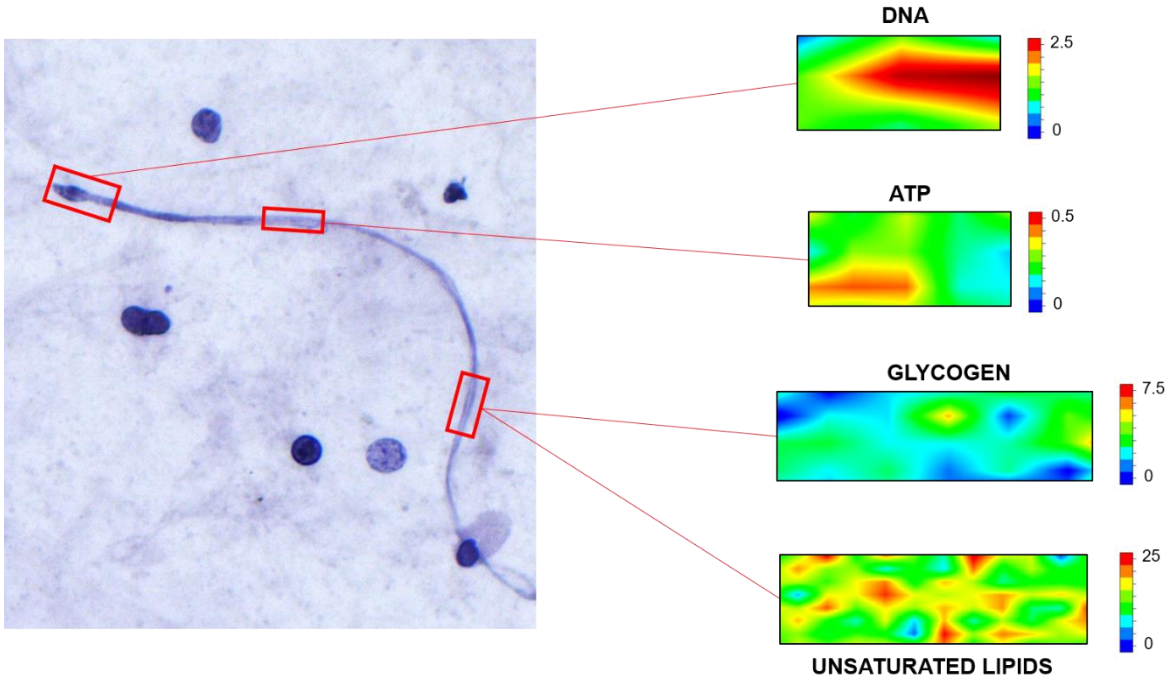

Figure 28. Early spermatozoa from maturing male

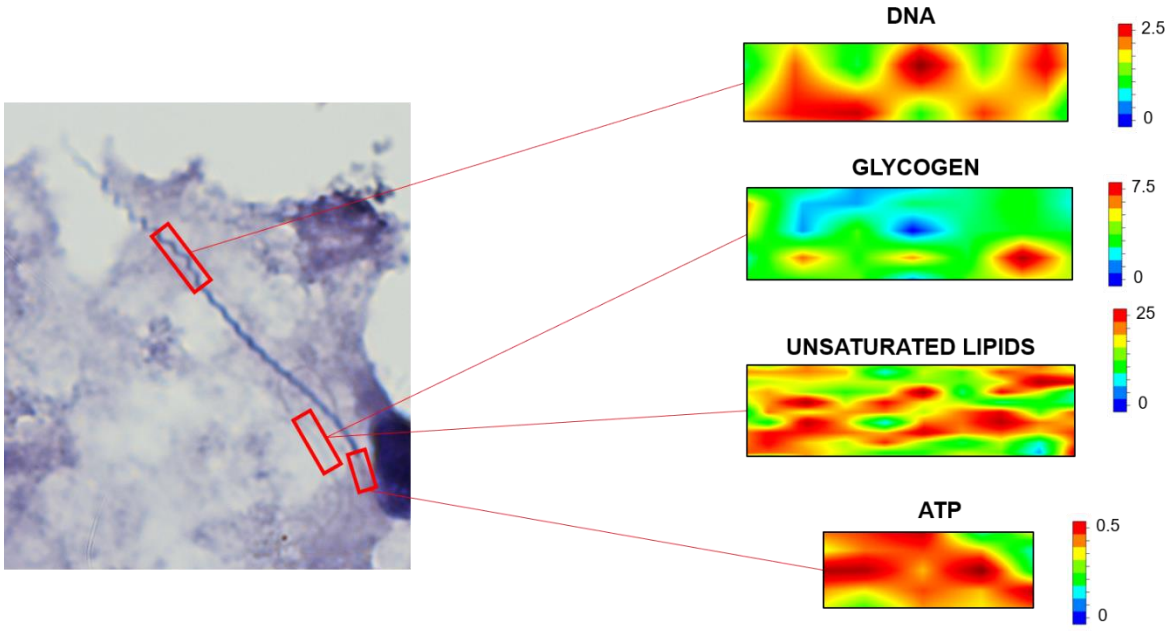

Figure 29. Spermatozoa from mature male

As summary, Figure 4 displays all the Raman maps, to compare the distribution of unsaturated lipids, glycogen, ATP and DNA among the three experimental groups.

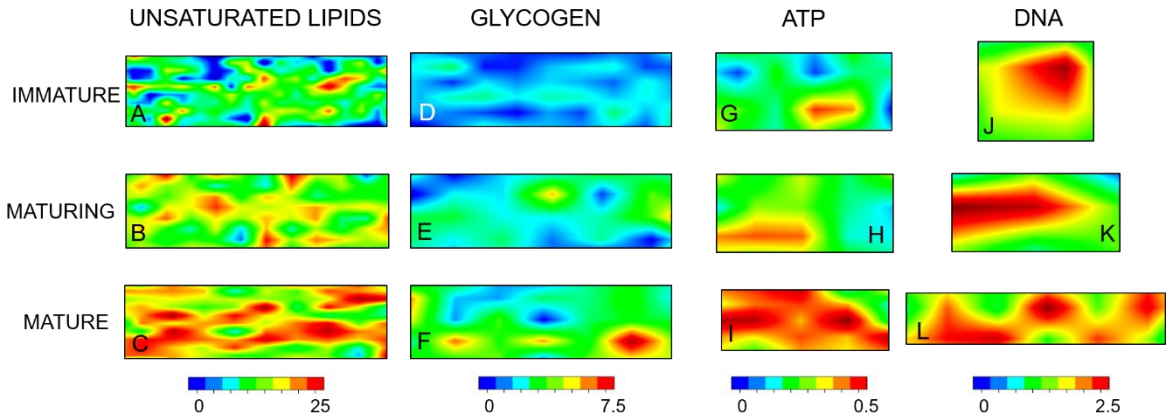

Figure 30. Representative Raman maps showing the distribution of: unsaturated lipids (A-C) and glycogen (D-F) in the tail; ATP (G-I) in the mid-piece, and DNA (J-L) in the head, of spermatozoa from immature, maturing and mature C. punctatum males. Different color scales were used for a better interpretation of the data.

Raman maps show an increasing concentration of unsaturated lipids along the tail, in spermatids and spermatozoa from immature to mature males, with the same spotted distribution (Fig. 4A-C); similarly, tails of spermatozoa from immature males show a lower concentration of glycogen, with respect to maturing and mature males (Fig. 4D-F).

As regards the mid-pieces, the ATP concentration is significantly higher in spermatozoa from mature males, with respect to immature and maturing ones (Fig. 4G-I). Finally, the DNA concentration in spermatozoa heads is comparable among the three experimental groups, with a localization that reflect the particular morphology of heads in spermatozoa from immature, maturing and mature males (Fig. 4J-L).

The higher concentration of unsaturated lipids in the tail gives the cell membrane greater fluidity and therefore sperm motility. At the same time, the higher concentration of ATP in the mid-piece indicates that there is a lot of energy that will be used by the spermatozoa to move and fertilize the egg. Therefore we can say that it is a mature and functioning spermatozoa.

In addition to the characterization by Raman spectroscopy and cytological staining of spermatozoa from mature, maturing and immature males, the use of FTIR microspectroscopy enabled us to characterize also the macromolecular composition of their seminal plasma. In Figure 31 are reported the average spectra representative of seminal plasma of each maturation stage of *C. punctatum* male. Comparing all the representative spectra it was possible to evidence how the maturation process is detectable also by evaluating the composition of the seminal plasma. Seminal plasma of immature males is composed mainly on proteins, with a very low amount of carbohydrates and phosphates and completely lacking lipids. In contrast, in the seminal plasma of maturing male, an increase of carbohydrates and phosphates amount have been showed.

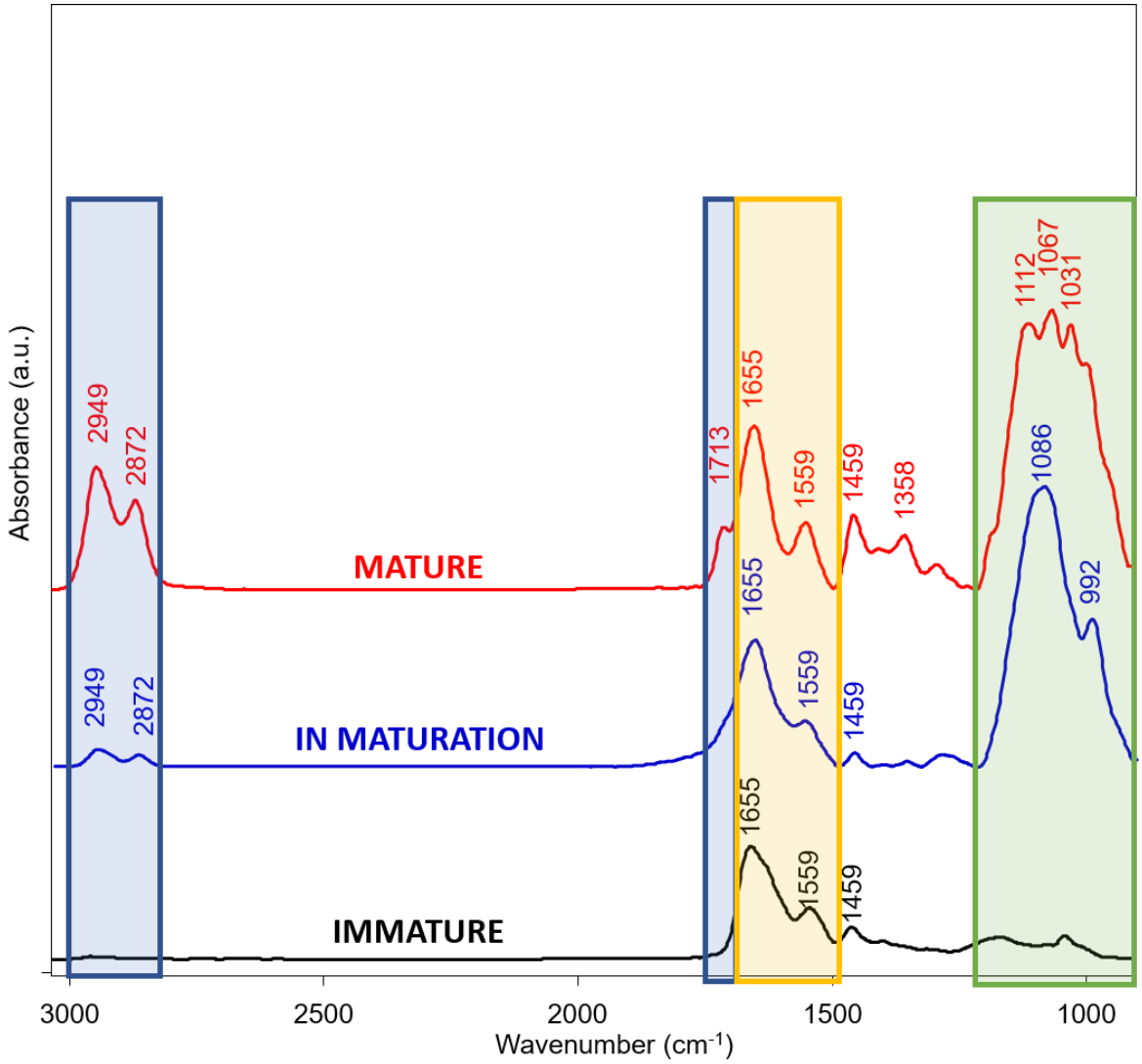

Figure 31. Average spectra representative of seminal plasma of each maturation stage of C. punctatum male. In blue square the bands representatives of lipids, in yellow square the bands representatives of proteins and in green square the bands representatives of carbohydrates and phosphates

9. CONCLUSION

From the results obtained we can conclude that the new protocol developed and applied for the study of sperm in *Chiloscillyum punctatum* shark has shown excellent results. Through cytological analyses the different morphology of germ cells at different stages of maturation, in particular spermatides, early spermatozoa and mature spermatozoa, was observed. Further confirmation of the maturity stage of males comes from the macromolecular analyses carried out with Raman spectroscopy for spermatozoa and FT-IR spectroscopy for seminal plasma. Furthermore, being animals of different ages, we should expect differences in the maturity stage (immature, maturing and mature).

The protocol applied could therefore open up many pathways for future studies. With a simple non-invasive technique, without animal sedation, the sperm can be collect and analyzed with the above instruments. In this way we can quickly determine the sexual maturity of an individual avoiding and or replace histological and cytological analysis which would take longer.

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