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STUDY OF SPERM QUALITY-MARKERS IN SEMEN OF pink cusk-eel (*Genypterus blacodes*, Schneider 1801) GROWN UNDER EXPERIMENTAL FARMING CONDITIONS

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"STUDY OF SPERM QUALITY-MARKERS IN SEMEN OF pink cusk-eel (*Genypterus blacodes*, Schneider 1801) GROWN UNDER EXPERIMENTAL FARMING CONDITIONS"

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THESIS OUTLINE

Chile is a country with abundant aquatic resources that has allowed fishery and aquaculture to develop in the last decades and *Genypterus blacodes* has become important specie for the Chilean aquaculture industry. *G. blacodes* is one of the most important species and commercialized in Chile, this species constitutes very important resources in artisanal and industrial fishing. In recent decades, the harvest of this species has been decreasing due to overexploitation, but also because it is strongly regulated by catch quotas that are low compared to other species. Their catches have remained under 50 thousand ton per year in the period 2000-2014. It is a species with potential for Chilean aquaculture due to meat quality and its high commercial value. The characteristics project *G. blacodes* as candidate for the development of their farming technology. Currently, the Chilean fishery has developed new technology and knowledge for handling this species; this includes conditions for live storage, management, catch and processing. The specimens of *G. blacodes* captured from the natural environment has had an active response to live, fresh and inert food, in addition to a rapid growth, and good health status. This potential to farm *G. blacodes* will open interesting business prospects for Chile in the coming years. Despite the importance of this species, very little is known about its reproductive biology. Reports on the breeding of this fish in captivity are lacking and hatchery production of this species is yet to be developed for large-scale farming. Considering the importance of protecting this fish, it is essential to understand its reproductive biology.

Therefore, the following hypothesis were proposed: "The parameters of sperm functions (motility, mitochondrial membrane potential, plasma membrane integrity and DNA fragmentation) morphology and ultrastructure of pink cusk-eel (*Genypterus blacodes*) are within ranges similar to those described for other teleosts". "Sperm motility depends on physicochemical characteristics of the activation media, such as: pH, osmolality, and temperature".

In **Chapter I**, general introduction, hypotheses, general, and specific objectives are presented. The general objective of this Doctoral Thesis was to assess the spermatic functions (motility, mitochondrial membrane potential, cytoplasm membrane integrity and viability, DNA integrity and fertility) and the effects of pH, osmolality and temperature on pink cusk-eel sperm motility (*Genypterus blacodes*).

Chapter II corresponds to the manuscript entitled ‘Effect of pH, osmolality and temperature on sperm motility of pink cusk-eel (*Genypterus blacodes*)’’. In this chapter, we evaluate the effects of pH, temperature and osmolality at different values on the sperm motility of *G. blacodes* intratesticular spermatozoa. In addition, we determined the fertilization rate. Findings revealed that the sperm motility is initiated on contact with a hyperosmotic swimming medium under normal conditions. The longest motility duration was recorded at 4°C. The maximum percentage of motile cells was recorded at 8°C at osmolality 1010 mOsm/kg, whereas an optimum was observed at pH 8 and a high fertility rate. In conclusion, the results of this chapter permit a baseline to be established for further research and protocols for artificial reproduction of this species to be developed and optimized. In addition, the information gathered in this research will be useful for developing the biotechnology of *G. blacodes*.

Chapter III corresponds to the manuscript entitled ‘’Study of spermatic function pink cusk eel (*Genypterus blacodes*, Schneider 1801)’’. In this chapter, we determined the percentage of mitochondrial membrane potential (JC-1 staining), cytoplasm membrane integrity (SYBR-14/PI) and DNA integrity [transferase dUTP (deoxyuridine triphosphate) nick-end labelling (TUNEL) by flow cytometry, whereas the motility was evaluated subjectively by optical microscope and Computer Assisted Sperm Analyzer (CASA) and the fertility was evaluated. Sperm motility was initiated in contact with a hyperosmotic swimming medium with a percentage subjective and CASA >80% and >20%, respectively. Additionally, the motility showed a positive correlation with mitochondrial integrity and citoplasmatic membrane integrity. Additionally, the velocity straight line and velocity curved-line correlated very well with the mitochondrial integrity and fertility, respectively.

Chapter IV corresponds to the manuscript entitled ‘’Morphology and ultrastructure of pink cusk-eel (*Genypterus blacodes*) spermatozoa by scanning and transmission electron microscopy’’. In this chapter, morphological and ultrastructure of *Genypterus blacodes* spermatozoa were studied using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). *G. blacodes* spermatozoon have a primitive type and is uniflagellated, lacks an acrosome, and is differentiated into a head, midpiece, and flagellum. The short mid-piece contains 4 or 5 mitochondria. The axoneme composed the typical 9+2 microtubular doublet structure.

Finally, **Chapter V** corresponds to general discussion, conclusions, and future directions.

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CHAPTER I

General introduction, hypothesis and objectives

1. GENERAL INTRODUCTION

Aquaculture is an industry that generates revenue based on the application of different technologies for the potential development of high demand aquatic species for human consumption (Ovando-Solis, 2013). The sustainability of world fisheries has been an important especially on the directions of current trends in fish abundance and the prospects for stock, food security, and biodiversity conservation (Worm *et al.*, 2009; Youn *et al.*, 2014; McClanahan *et al.*, 2015). In 2014, aquaculture production increased 11% compared to 2012, and it currently provides half of all the fish destined for human consumption, with the foreseeable future growth of the fisheries sector deriving mainly from aquaculture (FAO, 2016). During the past years, the overexploitation of fisheries resulted in an increase of aquaculture production to fulfil the market demands on marine products of a global exponentially growing human population (Lahnsteiner *et al.*, 2009; Aksnes & Browman, 2016).

Chile is the largest fisheries producer nation in the world in terms of production (FAO, 2014, 2010). It is among the top ten most important aquaculture producers in the world with abundant aquatic resources that has allowed aquaculture to develop in the last decades especially for Atlantic salmon. *Genypterus blacodes* is a species that distribute both in the Pacific and Atlantic seawaters of South America (Díaz *et al.*, 2012). It is an opportunistic benthic predator and their populations can be found in Uruguay, Argentina, New Zealand, southern Australia, Brazil and Chile (Francis *et al.*, 2002; Nyegaard *et al.*, 2004).

According to the compiled logbooks, the pink cusk-eel fishery is developed in Chilean waters between Talcahuano (36º 44'S) and south of Cabo de Hornos (57º00'S) from Coquimbo to austral zone (41º28'-57º00'S) (Ward *et al.,* 2001; Wiff *et al.,* 2007). The reproductive activity of the species occurs mainly during the summer in Patagonian coastal waters, from 41ºS to 45ºS (Louge *et al.,* 1992). It is one of the two species of the *genus Genypterus* traditionally exploited in the Argentine Sea, and in Chile (Cohen *et al.,* 2015). Considering the commercial importance of *G. blacodes* for Chilean aquaculture, it is essential to understand the reproductive biology of this species. Commercial capture of *G. blacodes* represents a significant source of income for Chilean fishery. However, its population has declined to such a degree that major concerns have been raised for its long-term survival (Wiff *et al.,* 2006; Chong *et al.,* 2014).

It is a species with the greatest farming potential in Chile, due the exceptional quality of its flesh and high commercial value (Vega *et al.,* 2012). Studies about the genus *G. blacodes* in other parts of the world, have suggested the existence of multiple stocks in comparatively smaller areas than the distribution presented by this species in Chile (Wiff *et al.,* 2011). In Chile *G. blacodes* is a specie sexually immature, sexual maturation can be influenced by a number factors including age, size, geographic distribution, season, nutritional status, and fishing pressure (Bromley, 2000; Smith *et al.,* 2005). There are numerous publications regarding the fisheries studies of *G. blacodes* such as its taxonomy, stomach contents, parameters of age and growth (Chong & Aguayo, 1990), macroscopic and microscopic structure of the ovary in samples from its Atlantic range, description of spawning stages from Argentinean waters (Machinandiarena *et al.,* 1998), regional morphometric variations in New Zealand (Colman, 1995), instantaneous rate of natural mortality (Ojeda *et al.,* 1986) and

population structure (Canales-Aguirre *et al.*, 2010). Annual catches of *G. blacodes* in Chile ranged from 45-50 thousand tons (Chong *et al.*, 2014). During the last decades, fish farmers of the world have become attracted to *G. blacodes* farming, especially in Chile, Australia, Argentina and New Zealand. For the time being, there is management plan to repopulate the environment of this species. There are not international databases recording the production and sales of aquacultured *G. blacodes*. Studies on the reproductive status of *G. blacodes* in the coast of Chile are relatively sparse and consider the austral zone as a single closed population. These include studies on fecundity and maturity (Chong, 1993; Paredes & Bravo, 2005). Key aspects of the reproduction of this species, and how these may differ between management zones and across time, remain largely unknown (Baker *et al.*, 2013). *G. blacodes* is a spawner with low fertility and a high frequency of spawning events (Cordo *et al.*, 2002).

It has been established that this species has a partial spawning type, with a pattern of indeterminate oocyte development. Since *G. blacodes* has relatively low levels of fecundity and matures at a larger size than, for example, the two Chilean hake species such as *Merluccius gayi* and *Merluccius australis*, with fecundities of 500 000 oocytes approximately, it may be more susceptible to overexploitation than the hake (Paredes & Bravo, 2005). Reproduction of this fish takes place in the deepest of its habitat. During the spawning season, *G. blacodes* moves to the deepest depths low layers, even as deep as a thousand meters (Freijo *et al.*, 2009). The eggs of *Genypterus*, ranges between ten million, resemble tiny balloons of very transparent glass and fluctuate between the surface and 500 meters deep, the tiny organisms are born in sheet form, called leptocephali. While studies of the reproduction of this species are improving, questions about many aspects of the life history and stages of reproduction of this fish remain (George-Nascimento & Muño, 1997).

The main export market of *G. blacodes* for Chile is Spain with 72% of frozen concentrated shipments, equivalent to 563 t, and 100% of shipping fresh cooled, followed by the United States, Brazil, Russia, Brazil and Portugal (SUBPESCA, 2013). The export price level of this fish in Chile vary between US $ 3.5 per kilo for fresh produce refrigerated and US $ 4.2 per kilo for frozen format, positioning it as a very attractive business. Artisanal sector catches are processed mainly in fresh cooled format, while the industrial sector is essentially intended for frozen (SUBPESCA, 2013). In Chile, the marketing chain craft sector *G. blacodes* is quite simple, it is distributed wholesale nationwide retailers that distribute in their regions or to processing plants that are responsible for processing mainly in fresh chilled for export.

After capture, *G. blacodes* is either processed immediately or kept alive. Live storage includes storage in water tanks near processing facilities and dry transport to the destination market. Live storage enables the industry to control the processing time or transport of live *G. blacodes*. The development of live-storage technology requires a reliable, consistent supply of crabs. It is through live storage that *G. blacodes* can be transported to overseas markets in good condition (SUBPESCA, 2013). Processing of *G. blacodes* includes a series of operations, from slaughtering to the final product. To increase the market share and position of Chilean *G. blacodes*, marketing and promotion efforts are required. Nevertheless, it will be of the utmost importance that the product is handled optimally from catch to the destination market in order to secure the supply of consistent, quality products. In Chile, the *G. blacodes* is a sexually immature species and the overexploitation reduces growth rates to reach a marketable size. The successful intensive production of *G. blacodes* is dependent on high quality sexually mature males and females.

Sexual maturation and reproductive behavior are controlled by an interaction of endogenous and exogenous forces (Baggerman, 1980; Baroiller & Jalabert, 1989). To carry out the culture of *G. blacodes*, previously it is required to control the reproductive cycle and broodstock management systems. An alternative is the rearing of broodstock in recirculating aquaculture systems with the maturation cycles (Migaud, 2006; Philipsen, 2008). Many studies have found broodstock nutrition to have an effect on the quality of reproduction of farmed fish (Henrotte *et al.*, 2010). In *G. blacodes*, as in most commercially important farmed finfish, hormone stimulation is frequently applied to synchronize ovulation of females and/or stimulate release of semen in males.

One of the strategies to protect the reproductive potential of this species is to evaluate the quality of the gametes, broodstock management and the larval culture. The main challenge to repopulate the environment of *G. blacodes* will be to optimize and improve the captive production to reduce the mortality rate and maximize the survival of the larvae, also, improve the in vivo storage conditions. To produce *G. blacodes* larvae with good yields, it is important to know the nutritional requirements of the species, particularly in the first larval stages. In addition, it is known that the nutrition of the broods directly influences the quality of the larvae. Studies of methods of artificial reproduction, with emphasis on broodstock reproductive physiology, to define those optimal for achievement of stable mass production of *G. blacodes* larvae and fry are still needed.

The study of sperm quality is essential to understand the overall dynamic of fertilization process in fish. In fish, the spermatozoon is immobile while it remains in the seminal fluid and its flagellar activity is only triggered when it comes into contact with water (Alavi & Cosson, 2005, 2006). Sperm quality has been a focus of research since it can be used as a biomarker of the male fish status (Chauvaud *et al.,* 1995; Cabrita *et al.,* 2009). The knowledge of the motility patterns of spermatozoa in this species is a key tool to determine the quality of the semen during the procedures of artificial fertilization (Alavi & Cosson, 2005; Hu *et al.,* 2009; Valdebenito *et al.,* 2009). According to Alavi & Cosson (2006), the parameters such as: temperature, pH and osmolality affect the capacity for and duration of mobility in fish spermatozoa, but pH has been reported as having little effect on the motility activation of fish spermatozoa. The motility duration, fertilizing ability and velocity of spermatozoa depend on temperature of the activation medium (Billard *et al.,* 1995b).

Efforts have been made to understand the life cycle and reproductive biology of this species. Sperm quality has been a focus of research, since it can be used as a biomarker of the male status (Chauvaud *et al.,* 1995; Cabrita *et al.,* 2009). Study of sperm function is essential to understand the overall dynamics of fertilization process in fish. A quality assessment must be reliable and fast to be useful in commercial aquaculture (Cabrita *et al.,* 2009). The parameters of spermatic function, such as motility, mitochondrial membrane potential (JC-1/rhodamine), cytoplasm membrane integrity (SYBR-14/PI), and DNA integrity [transferase dUTP (deox-yuridine triphosphate) nickend labelling (TUNEL)] single-cell electrophoresis (COMET)], have been determined in several species using flow cytometry or electrophoresis (Lahnsteiner *et al.,* 1996, 1998; Fauvel *et al.,* 1998; Geffen & Evans, 2000; Chowdhury & Joy, 2001; Rurangwa *et al.,* 2004; Figueroa *et al.,* 2016).

The evaluation of sperm motility and other kinetic parameters, like curvilinear, straight line and average path velocities, is an essential tool in the examination of sperm quality in many fish species, including the *G. blacodes* (Marco-Jiménez *et al.,* 2006; Asturiano *et al.,* 2007; Gallego *et al.,* 2012). However, a few studies have used flow cytometry to evaluate the sperm function in intratesticular spermatozoa of *G. blacodes*. Various studies have demonstrated that the majority of the characteristics of sperm function analyzed contribute to the general quality of the spermatozoa (Alavi *et al.,* 2008; Bobe & Labbé 2010).

Motility, one of the most frequently used parameters to assess semen quality, generally presents a positive correlation with fertilizing capacity (Figueroa *et al.,* 2016). Traditionally, motility was assessed subjectively by determining the percentage of motile spermatozoa in a scale such as the one of Sanchez-Rodriguez & Billard (1977) and Cosson *et al.* (2008b) with a subjective classification, which assigns a level of zero when no spermatozoa are moving and five when all of them are moving vigorously, additionally by Computer Assisted Sperm Analyzer (CASA). In the teleost species studied to date, the spermatozoa structure has revealed a high diversity, predominately between systematic families. This diversity is reflected in the head shape, in the number, shape and location of mitochondria, and in the number, length and structure of the flagellum (Guo *et al.,* 2016). Teleost spermatozoa exhibit a diverse range of structural features that makes it difficult to depict a common sperm type (Mattei, 1991). The structure of fish spermatozoa varies between families: from aflagellate to biflagellate, while shape, size, and structure can vary significantly according to whether a species adopts internal or external fertilization (Jones & Butler, 1988).

Spermiogenesis in teleosts shows a wide variety of patterns which is broadly categorized as two types (I and II) of spermiogenesis (Mattei, 1970). With Type I, rotation of the nucleus occurs and the diplosome enters the nuclear fossa and the flagellum is symmetrically located, while with Type II, there is no nuclear rotation, the diplosome remains outside the fossa and the flagellum is asymmetrically located. Mattei (1970) have reported that the teleost sperm exhibit a broad range of varying structural features that makes it difficult to depict a common sperm type. The characterization and the study of spermatic function of *G. blacodes* would allow establish a base line of sperm quality-markers and also improve the management *in vitro* of the gamete species. The aim of this work was to assess the spermatic functions (motility, mitochondrial membrane potential, cytoplasm membrane integrity and viability, and DNA integrity) and the effects of pH, osmolality and temperature on pink cusk-eel sperm motility (*Genypterus blacodes*, Schneider 1801). This thesis was realized with intratesticular spermatozoa because for this species it is difficult to find wild fully-sexually mature male individuals; and sexual maturation in captivity has not been yet reported. The use of intratesticular spermatozoa for *in vitro* fertilization is a key tool for breeding this species in captivity and hence would allow its introduction in aquaculture industry.

1.1. HYPOTHESES

Currently, there are not publications regarding study on sperm quality-markers in semen of *Genypterus blacodes*. The evaluation of the effect pH, osmolality and temperature on sperm motility, spermatic function, morphology and ultrastructure are essential to understand the reproductive biology of this species with the potential to contribute to the protection of this endangered species.

Therefore, the following hypothesis is proposed:

- The parameters of sperm functions (motility, mitochondrial membrane potential, plasma membrane integrity and DNA fragmentation), morphology and ultrastructure of pink cusk-eel spermatozoa (*Genypterus blacodes*, Schneider 1801) are within ranges similar to those described for other teleosts.

- Pink cusk-eel sperm motility (*Genypterus blacodes*, Schneider 1801) depends on physicochemical characteristics of the activation media, such as pH, osmolality, and temperature.

1.2. GENERAL OBJECTIVE

To assess the spermatic functions (motility, mitochondrial membrane potential, cytoplasm membrane integrity and viability, DNA integrity and fertility) and the effects of pH, osmolality and temperature on pink cusk-eel sperm motility (*Genypterus blacodes*, Schneider 1801).

1.3. SPECIFIC OBJECTIVES

1. To determine the effects of pH, osmolality and temperature on pink cusk-eel sperm motility (*Genypterus blacodes*, Schneider 1801).

2. To assess the spermatic functions (motility, mitochondrial membrane potential, Cytoplasm membrane integrity and viability, DNA integrity and fertility) of pink cusk-eel sperm (*Genypterus blacodes*, Schneider 1801).

3. To determine the morphology and ultrastructure of spermatozoa pink cusk-eel (*Genypterus blacodes*, Schneider 1801).

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CHAPTER II

Effect of pH, osmolality and temperature on sperm motility of pink cusk-eel (*Genypterus blacodes*, (Forster, 1801))

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Effect of pH, osmolality and temperature on sperm motility of pink cusk-eel (*Genypterus blacodes*, (Forster, 1801))

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Abstract

In this research we evaluated simple aspects of the sperm biology of *Genypterus blacodes*, in particular assessing the effects of pH (6, 7, 8 and 9), temperature (4, 8 and 16° C) and osmolalities 100% sea water (1010 mOsm/kg, Control), 75% sea water (774 mOsm/kg, T_1), 50% sea water (488 mOsm/kg, T_2) and distilled water (0 mOsm/kg, T_3)) on the motility of *Genypterus blacodes* intratesticular spermatozoa. In addition, we determined the fertilization rate. Our results show that *G. blacodes* spermatozoa have a sperm density of 5.35 ± 0.16 x 10^9 spermatozoa/mL. Sperm motility is initiated on contact with a hyperosmotic swimming medium under normal conditions (1010 mOsm/kg, pH 8 and 8°C). The longest motility duration (432.48 ± 8.89 s) was recorded at 4°C. The maximum percentage of motile cells was recorded at 8°C (65.66 ± 4.95) at osmolality 1010 mOsm/kg, whereas an optimum was observed at pH 8. This is the first report on sperm motility of *G. blacodes* spermatozoa. In conclusion, the results of this study permit a baseline to be established for further research and protocols for artificial reproduction of this species to be developed and optimized. In addition, the information gathered in this research will be useful for developing the biotechnology of *Genypterus blacodes*.

Keywords: *Genypterus blacodes*; sperm motility; osmolality; pH; temperature

2. Introduction

In recent years, the overexploitation of fisheries has resulted in increased aquaculture production. The optimization of the reproductive performance of broodstock has been essential to obtaining high quality fry, which is imperative if the aquaculture industry is to produce high quality fish (Lahnsteiner *et al.*, 2009). Within the order Ophidiiformes, genus *Genypterus* (*Genypterus Philippi*, 1857) contains the most economically important species. Three of these are found in Chilean waters: red cusk-eel (*Genypterus chilensis*, (Guichenot, 1848)), black cusk-eel (*Genypterus maculatus*, (Tschudi, 1846)) and pink cusk-eel (*Genypterus blacodes*, (Forster, 1801)), with the last being the most economically important (Canales-Aguirre *et al.*, 2010). *G. blacodes* is a benthic-demersal species found in the oceans around southern Australia, Chilean Patagonia, Brazil, Argentina and New Zealand in depths from 22 to 1000 m (Young *et al.*, 1984; Francis *et al.*, 2002; Nyegaard *et al.*, 2004). Adults exhibit a demersal behavior and they are usually found at depths between 45 and 350 m (Cousseau & Perrota, 2000; Nyegaard *et al.*, 2004). The pink cusk-eel fishery is developed in Chilean waters between Coquimbo (41º and 28'S) and south of Cape Horn (57º and 00'S) (Ward *et al.*, 2001; Wiff *et al.*, 2007). The global market for *G. blacodes* is around 45 thousand tons per year. Spain is the main destination of *G. blacodes* exports, accounting for 72% of frozen shipments, equivalent to 563 t, and 100% of shipping fresh chilled in 2013. Spain is followed by the United States, Brazil, Russia and Portugal (Chong *et al.*, 2014).

Biological studies of this fish are scarce and they only mention its taxonomy, stomach contents (Bahamonde & Zavala, 1981; Renzi, 1986;), age and growth parameters (Chong & Aguayo, 1990; Wiff *et al.,* 2007), macroscopic and microscopic structure of the ovary in samples from its Atlantic range, description of spawning stages from Argentinean waters (Machinandiarena *et al.,* 1998), regional morphometric variations in New Zealand (Colman, 1995), and instantaneous rate of natural mortality (Ojeda *et al.,* 1986; Wiff *et al.,* 2011). There is limited information about the morphological and functional aspects of the reproductive biology of *G. blacodes* (Chong, 1993; Paredes & Bravo, 2005; Freijo *et al.,* 2009; Diaz *et al.,* 2012). The reproductive activity of this species occurs in Patagonian coastal waters south of 42° S (Province of Chubut, Argentina), and the spawning area goes from 41° S to 45° S, mainly during summer (Cousseau & Perrotta, 2000). In Chile, the fecundity of *G. blacodes* in captivity is estimated to be between 66,167 and 706,658 oocytes per female (Paredes & Bravo, 2005).

In the fish, the spermatozoon is immotile in the seminal fluid and its flagellar activity is only triggered when it comes into contact with water (Alavi & Cosson, 2005, 2006). Sperm quality has been a focus of research given that it can be used as a biomarker of the status of the male fish (Chauvaud *et al.,* 1995; Cabrita *et al.,* 2009). Knowledge of sperm motility is a key tool to determine semen quality during artificial fertilization procedures (Alavi & Cosson, 2005; Hu *et al.,* 2009; Valdebenito *et al.,* 2009). According to Valdebenito *et al.* (2016), the parameters such as temperature, pH and osmolality affect the capacity and duration of motility in fish spermatozoa. The objective of this study was to determine the effects of osmolality, temperature and pH on sperm motility of pink cusk-eel spermatozoa; and fertilization tests were conducted.

In this study, intratesticular spermatozoa were used because for this species it is difficult to find wild fully-sexually mature male individuals; and sexual maturation in captivity has not been yet reported. The use of intratesticular spermatozoa for *in vitro* fertilization is a key tool for breeding this species in captivity and hence would allow its introduction in aquaculture industry.

2.1 Materials and Methods

2.1.1 Broodstock males

This study was conducted at the Engineering Biotechnology and Applied Biochemistry Laboratory (LIBBA) and at the Center for Biotechnology in Reproduction (CEBIOR), Universidad de la Frontera, Chile, as well as at the Aquaculture Biotechnology Laboratory, Catholic University of Temuco, Chile. The specimens of *G. blacodes* were caught between April and May 2017 in Puerto Montt, Region de Los Lagos, Chile with average weight of 1.96 ± 1.06 kg and a total length of 62.5 ± 4.68 cm, respectively.

2.1.2 Collection of gametes

This study was carried out with intratesticular spermatozoa, which were collected according to the procedure described by Cabrita *et al.* (2005). During transport, the specimens were kept alive. The specimens of *G. blacodes* were anesthetized by immersion with AQUI-S® (BAYER S.A. Animal Health-Chile) for a few minutes and then decapitated. Their testicles were surgically extracted and carefully cleaned with distilled water, dried and blood remnants were removed. The testis were transferred individually into an Eppendorf tube on ice and were transported using oxygenated containers with a constant temperature of 4°C. The testes were sectioned directly in the Eppendorf tube (on ice) using a scalpel and collecting the sperm by dripping directly into a graduated, sterile, dry, disposable plastic container maintained at 4 °C.

In addition, the intratesticular spermatozoa were diluted in StorFish® (Imv, Technologies, France) medium (dilution 1:1) and centrifuged twice at 1800 rpm for 5 minutes. Immediately after collection, sperm motility and concentration were determined using a phase contrast microscope (Carl Zeiss, Jena, Germany).

2.1.3. Sperm density

Sperm density (number of spermatozoa/mL) was determined in six males using a Neubauer hemocytometer according to the methodology described by Merino *et al.* (2011) and Figueroa *et al.* (2015) for blood cells and spermatozoa at a dilution of 1 µL of sperm in 1200 µL of StorFish® medium (Imv, Technologies, France) using a phase contrast microscope (Carl Zeiss, Jena, Germany).

2.1.4. Activation solutions

For sperm motility activation, three solutions with different proportions of seawater (35g/L, pH 8) and distilled water were used: control, seawater 100% (1010 mOsm/kg); T_1, seawater 75% (774 mOsm/kg); T_2, seawater 50% (488 mOsm/kg); and T_3, distilled water (0 mOsm/kg), as suggested by Cosson et al. (2008b). These activation solutions were assessed at three different temperatures (4°, 8° and 16 °C). The osmolalities used in each activation solution were determined by a Fiske® Micro-Osmometer, 2010 model (Germany). The pH of the control (seawater) was adjusted to three pH units above and below the normal value (8) for seawater (6, 7 and 9) by adding HCl [1%] and NaOH [1%] respectively, using a pH-meter model pH 21. The effect on motility was assessed at ambient temperature of 4, 8 and 16 °C.

2.1.5. Assessment of sperm motility

Sperm motility was assessed in a cold-room, where temperatures of 4, 8 and 16 °C were used for the experiment. All treatments were assessed by the same person using a Nikon Eclipse E400 microscope (Japan) at 40× magnification. Approximately 4 h after the semen were obtained, the flagellar activity periods were recorded using a chronometer from the initiation of movement (progressive motility) until the start of local circular movement (vibratory or stationary movement) (Groison *et al.*, 2010). To assess the percentage of motile cells, values from 0 to 100% were used as suggested by Cosson *et al.* (2008b), on a scale from 1 to 5: 1 = 0-5%; 2 = 5-25%; 3 = 25-50%; 4 = 50-75%; 5 = 75-100%. The percentage and duration of motility were assessed in 15 aliquots of 1 μL of semen activated in 10 μL of each of the activating solutions (Control, T_1, T_2, and T_3) at temperatures of 4, 8 and 16 °C as per Cosson *et al.*, 2010 and Valdebenito *et al.* (2016) using a Nikon Eclipse E400 (Tokyo, Japan) microscope at 10x magnification and with 15 repetitions per treatment for osmolality and temperature.

2.1.6. Statistical analysis

The motility results were analyzed with the statistical software GraphPad Prisma® version 5.0 (GraphPad Software, San Diego CA). A one-way ANOVA was used for nonparametric samples to analyze the percentage and duration of motility. Additionally, the analysis of differences between the average values of the variables of treatment groups were compared by applying the Tukey test. The level of significance was set at $p < 0.05$, $n = 15$ replicate. The results of the level of motility are presented as a sample mode.

2.2. Results

2.2.1. Spermatological parameters

Table 1 shows the morphometric and spermatological parameters of pink cusk-eel (*Genypterus blacodes*) samples, while Table 2 shows the levels sperm motility.

Table 1. Morphometric and spermatological parameters of pink cusk-eel samples (n = 9 males).

Parameters	Minimum	Maximum	Mean	SD
Weight (kg)	0.98	4.08	1.96	± 1.06
Length (cm)	56	70	62.5	± 4.68
Testicle volume (mL)	1.2	2.5	1.47	± 0.56

Table 2. Levels of sperm motility of the pink cusk-eel (*Genypterus blacodes*) activated with solutions at different temperatures and osmolalities: control (100% seawater), T_1 (75% seawater), T_2 (50% seawater) and T_3 (distilled water) *n* =15.

Activator (mOSm/kg)	**Temperature**		
	4ºC	8ºC	16ºC
Control (1010)	**Level**		
	3	4	3

Treatment	**Temperature (16ºC)**
	Level
T_1 (774)	3
T_2 (488)	2
T_3 (0)	0

2.2.2. Sperm density

The sperm density in *G. blacodes* intratesticular spermatozoa was of 5.35 ± 0.16 x 10^9 spermatozoa/mL

2.2.3. Fertility and effects of pH on the duration and level of sperm motility

The rate of sperm motility of *G. blacodes* intratesticular spermatozoa and fertilization were expressed as mean ± SD. Statistically significant differences ($p < 0.05$) were observed in both duration of motility (s) and percentage of motility between pH 6, 7, 8 and 9. The highest level of motility was recorded at pH 8 (204. 14 ± 10.00 s and 52.66 ± 2.58 % of motility) compared to other treatments: pH 6 (168.4 ± 11.18 s and 38 ± 2.35 % of motility), pH 7 (186.64 ± 4.31 s and 43 ± 2.53 % of motility) and pH 9 (167.41 ± 8.89 s and 37.5 ± 2.59 % of motility), while there was no significant difference between pH 6 and 9 (Fig 1A). The determining rate for fertilization was 73.9 ± 17%.

2.2.4. Effects of temperature on the duration and level of sperm motility

The flagellar activity was recorded at 4°C (Control: 432.48 ± 8.89 s and 40 ± 2.67% of motility) comparing to 8ºC (Control: 354.12 ± 29.92 s and 65.66 ± 4.95% of motility) and 16ºC (Control: 167.52 ± 18.08 s and 47 ± 2.53% of motility), while there was significant difference in flagellar activity at 16 °C (control: 167.52 ± 18.08 s; T_1: 160.06 ± 22.57 s; T_2: 70.14 ± 11.16 s) and T_3: distilled water, the motility of which was not observed (Fig 1B).

2.2.5. Effects of osmolality on the duration and level of sperm motility

In terms of osmolality, significant differences were found for the control (354.12. 14 ± 29.92 s) compared to T_1 (160.06 ± 22.57 s) and T_2 (70.14 ± 11.16 s) at 16 °C (Fig 1C). *G. blacodes* intratesticular spermatozoa were only activated on contact with seawater (1010 mOsm/kg), including seawater at lower levels of osmolality (772 mOsm/kg) and (448 mOsm/kg). The *G. blacodes* spermatozoa were immotile in the testicle and only began intense flagellar movement on contact with a hyperosmotic activation medium including seawater at lower levels of osmolality.

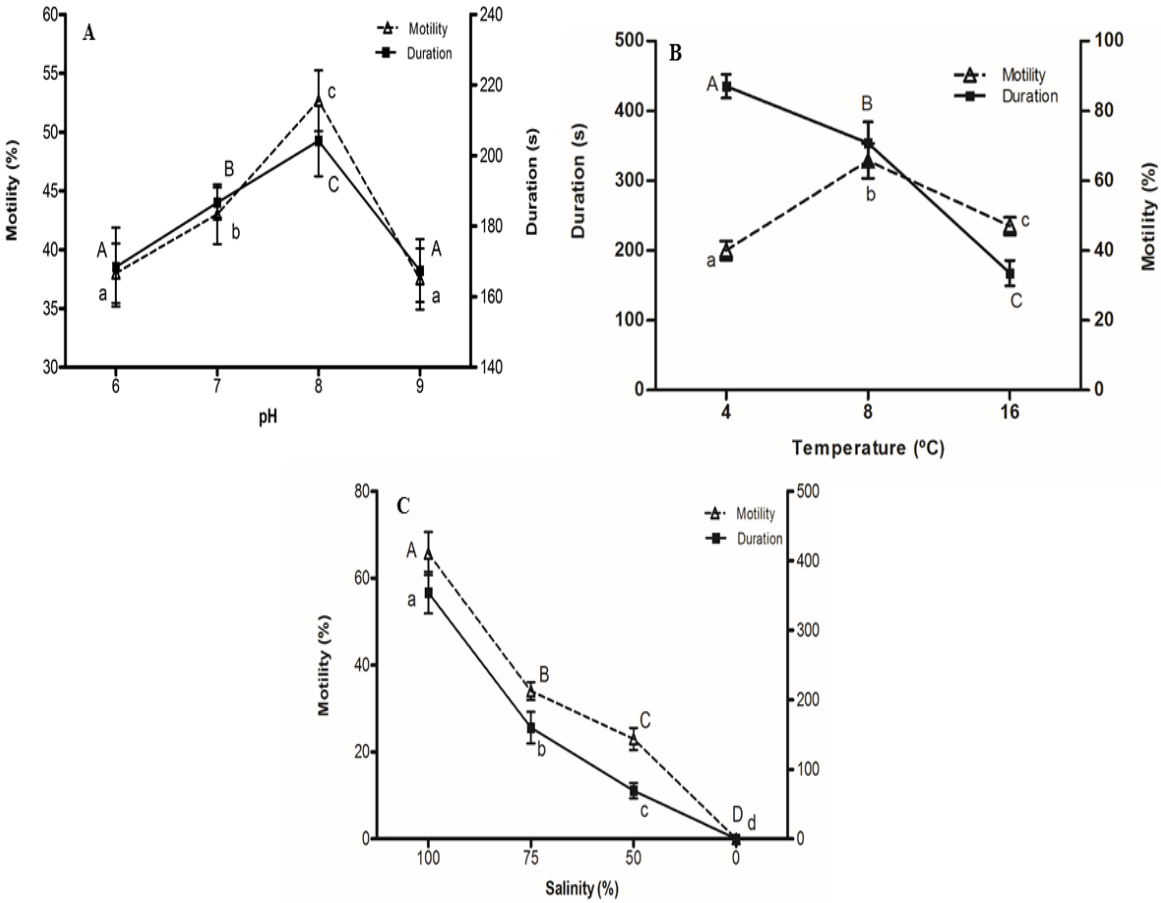

Figure 1. A) Duration of motility (s) and percentage of motility of pink cusk-eel spermatozoa under different pH (6, 7, 8 and 9) conditions, osmolality = 1010 mOsm/kg and temperature =16ºC; B) Duration of motility (s) and percentage of motility of pink cusk-eel spermatozoa under different temperatures (4, 8 and 16ºC), pH 8 and osmolality

= 1010 mOsm/kg; C) Duration of motility (s) (mean ± SD) and percentage of motility of pink cusk-eel spermatozoa activated in different salinity conditions, 100% sea water (1010 mOsm/kg); 75% sea water (774 mOsm/kg); 50% sea water (488 mOsm/kg) and distilled water (0 mOsm/kg), pH 8 and temperature 16ºC. The values are shown as mean ± SD, capital and small letters indicate significant differences in motility (%) and duration of motility, respectively with $p < 0.05$ and $n = 15$ replicates.

2.3. Discussion

2.3.1. Sperm density and motility

Cosson *et al.* (2008a, b) have reported in marine teleosts with external fertilization the osmolality is the main factor controlling sperm motility. However, the sperm motility rapidly decrease after activation therefore progressive movement needed by the sperm to effectively reach the egg surface is limited. According to sperm duration of motility, the spermatozoa of *G. blacodes* can move for up to 354.12 s as shown in this study; sperm cells commence any flagellar activity only when they come into contact with a hyperosmotic medium. Also, the motility observed in *G. blacodes* are similar to what has been described for other marine fish species (Cosson, 2004; Cosson *et al.*, 2008a; Groison *et al.*, 2008; Valdebenito *et al.*, 2009; Groison *et al.*, 2010; Effer *et al.*, 2013). Additionally, duration of motility of other marine species of commercial importance can be mentioned, including halibut (110-120 s), turbot (600 s), sea bass (50-60 s), cod (7-800 s) and tuna (140 s) (Cosson *et al.*, 2008a, b). Although the motilities presented for each of the above species were not determined under the same experimental conditions, these data allow us to indicate differences and similarities between the main marine species of commercial interest.

In all these species, the spermatozoa are immotile in seminal fluid (Cierezko, 2008; Cosson *et al.*, 2008b,c; Cosson, 2004) and only commence flagellar activity when they come into contact with a hyperosmotic medium (Cosson, 2004; Cosson *et al.*, 2008a, 2008b, 2008c; Morisawa, 2008; Cosson *et al.*, 2010). However, the total motility time will depend on fluctuations in the parameters of the microenvironment (pH, temperature and osmolality) in which the reproduction of these species occurs (Cosson *et al.*, 2008b; Morisawa, 2008; Groison *et al.*, 2010).

2.3.2. Effect of temperature, pH and osmolality on the duration of sperm motility

In marine teleosts, sperm motility is activated by contact with seawater mainly through a positive increase in osmolality, and several factors are known to affect this process, including temperature (Alavi & Cosson 2005, 2006). The effect of temperature on sperm motility has scarcely been studied in marine and freshwater teleostei (Morisawa, 1994; Valdebenito *et al.*, 2009, 2016). Nevertheless, it has been reported that with a decrease in temperature, flagellar beating frequency is lower (Billard & Cosson, 1988) and the duration of motility is longer (Cosson *et al.*, 2008a, b). In this research, our data showed that the activation medium indicated an optimum at 8°C 354.12 s (5.9 min) compared to 4°C 432.48 s (7.20 min) and 16 ºC 167.52 s (2.79 min), when *G. blacodes* spermatozoa were activated with sea water. The spawning temperature for this species is 10-14°C. The duration of sperm motility in fish depends on the temperature of the activation medium (Billard & Cosson 1988). According to Alavi & Cosson (2005, 2006), low temperatures reduce the intensity of flagellar movement, significantly prolonging motility, whereas high temperatures increase the intensity of flagellar movement, reducing the motility time.

However, there have been thorough studies focusing on the specific ions that trigger sperm motility, such as K^+, Ca^{2+} and Mg^{2+} present in seawater and their influence on sperm motility activation (Cosson, 2004). *G. blacodes* spermatozoa were only activated on contact with seawater (1010 mOsm/kg), including seawater at lower levels of osmolality (772 mOsm/kg) and (448 mOsm/kg). However, motility was not found when the semen was transferred to distilled water (mOsm/kg). Valdebenito and Figueroa (unpublished data) point out that the maximum mean duration recorded for *G. chilensis* was 1346 s in a saline solution (928 mOSm/kg) and for the black conger eel it was 1470 s in saline solution (754 mOsm/kg). Nevertheless, motility in marine fish may occur in a wide range of osmolalities, for example in Atlantic halibut (*Hippoglossus hippoglossus*) it occurs between 900 and 1100 mOsm/kg (Billard *et al.*, 1995), in *Dicentrarchus labrax* over 300 mOsm/kg (Chauvaud et al., 1995; Dreanno *et al.*, 1999), and in (*Sarathoredon melanotheron*, (Rüppell, 1852)) between 300 and 970 mOsm/kg (Legendre *et al.*, 2008). A recent study conducted by Valdebenito *et al.* (2016) demonstrated that osmolality of the activation medium (Control = 815 mOsm/ kg; T_1 = 716 mOsm/kg; T_2 = 590 mOsm/kg) influenced the sperm motility of Patagonian blenny (*Eleginops maclovinus*), a species that lives in the same areas as *G. blacodes*. The highest flagellar activity (percentage of motile sperm and duration) was shown at pH = 8. Effer *et al.* (2013) described for *M. australis*, a fish that also lives in the same areas as *G. blacodes*. The pH of the activation solution affects sperm motility to a low extent (Cosson, 2004). It is generally accepted that the pH or ions present in the activation solution polarize the cell membrane and stimulate the motility of fish spermatozoa by changing the Na^+/K^+ permeability (Morisawa & Morisawa, 1988; Boitano & Omoto, 1991). The pH of the internal cytoplasm is one of the most important factors that affect sperm motility (Woolsey & Ingermann, 2003).

The alkaline nature (pH = 8) of the activation solution seems to be the most suitable for species such as turbot (Chauvaud *et al.*, 1995), halibut (*Hippoglossus stenolepis*) (Billard *et al.*, 1993). According to Cosson *et al.* (2008b, c), the pH may reduce or prolong motility, but it is not the principal parameter in motility initiation, despite intracellular pH playing a key role in sperm maturation in vivo (Cierezko, 2008).

In conclusion, in the current study, we showed that the motility of *G. blacodes* intratesticular spermatozoa is initiated by hyperosmotic medium. Varying temperature and osmolality of the activation medium demonstrated an optimal effect on sperm motility, whereas the pH of the activation solution affected sperm motility to a lesser extent. The fertilization rate was high at 73.9 %; however, more tests need to be done to improve these results. Finally, considering the importance of fish reproduction in captivity and in the wild, it is advisable to deepen research in cellular aspects, thus providing useful information for the elaboration of means of cultivation and other inputs used in the management of gametes of this species.

Conflict of interest

The authors have declared that they have no conflicts of interest.

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CHAPTER III

Study of spermatic function pink cusk eel (*Genypterus blacodes*, Schneider 1801).

Associated to specific objectives 2
Paper in preparation to be submitted to Aquaculture Research

Study of spermatic function pink cusk eel (*Genypterus blacodes*, Schneider 1801).

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Abstract

The objective of this study was to evaluate the spermatic function such as mitochondrial membrane potential, cytoplasmic membrane integrity, DNA integrity, motility and fertility in *Genypterus blacodes* intratesticular spermatozoa. The intratesticular spermatozoa were diluted in StorFish® in a ratio of 1:1 (testicle volume: extender) and activated in seawater. The percentage of mitochondrial membrane potential (ΔΨMMit, JC-1 staining), cytoplasm membrane integrity (SYBR-14/PI) and DNA integrity [transferase dUTP (deoxyuridine triphosphate) nick-end labelling (TUNEL) were determined and evaluated by flow cytometry, whereas the motility was evaluated subjectively by optical microscope and Computer Assisted Sperm Analyzer and the fertility was evaluated using 4.5 ml sperm + 1.5L mass + 500 ml of sea water in a ratio of 1:1 (testicle volume: extender). The results of this study showed that *G. blacodes* intratescular spermatozoa had sperm density of 5.6 ± 0.12 x 10^9 spermatozoa/mL. Sperm motility was initiated in contact with a hyperosmotic swimming medium. The mean ± SD of DNA fragmentation was 1.175 ± 1.14%; plasma membrane integrity was 83.87 ± 6.06%; mitochondrial membrane potential was 46.26 ± 14.34%; motility (subjective) was 88.75 ± 8.56%; 23.125 ± 2.58% Computer Assisted Sperm Analyzer and fertility was 68.9 ± 25.91%. The mean ± SD rate of motility variables were velocity curved-line (67.3 ± 22.7 µm/s); velocity straight line (55.4 ± 24.29 µm/s); velocity average path (60.0 ± 23.6 µm/s); linearity (84.8 ± 7.62%); wobble (93.0 ± 4.18%). The motility showed a positive correlation with mitochondrial membrane potential (r= 0.94) and citoplasmatic membrane integrity (r= 0.92). Additionally, the velocity straight line and velocity curved-line correlated very well with the mitochondrial membrane potential (r= 0.97; r= 0.97) and fertility (r= 0.84; r= 0.86, respectively).

Moreover, fertility was positively correlated with motility (r= 0.89) and mitochondrial membrane potential (*r*= 0.87). The results provide new data on *G. blacodes* sperm quality evaluated by flow cytometry and fertility.

Keywords: *Genypterus blacodes*; sperm motility; sperm function; flow cytometry

3. Introduction

During the past years, the overexploitation of fisheries resulted in an increase of aquaculture production to fulfil the market demands on marine products of a global exponentially growing human population. The optimization of reproductive performance on husbandry broodstock has been essential to obtain high quality fry, which is imperative to allow the aquaculture industry produce high quality fish. Fish aquaculture production, as fish recruitment in the wild, depends directly on the quality and quantity of eggs available during the reproductive season (Lahnsteiner *et al.,* 2009). The economic importance and high commercial demand of the *Genypterus blacodes* primarily from Chile and world markets is well known. However, the population of the *G. blacodes* has declined to such a degree that major concerns have been raised for its long-term survival (Chong *et al.,* 2014). Efforts have been made to understand the life cycle and reproductive biology of this species. Sperm quality has been a focus of research, since it can be used as a biomarker of the male status (Cabrita *et al.,* 2009; Chauvaud *et al.,* 1995). Study of sperm function is essential to understand the overall dynamics of fertilization process in fish. A quality assessment must be reliable and fast to be useful in commercial aquaculture (Cabrita *et al.,* 2009). The parameters of spermatic function, such as motility, mitochondrial membrane potential (JC-1/rhodamine), cytoplasm membrane integrity (SYBR-14/PI), and DNA integrity [transferase dUTP (deoxyuridine triphosphate) nickend labelling (TUNEL)] single-cell electrophoresis (COMET)], have been determined in several species using flow cytometry or electrophoresis (Lahnsteiner *et al.,* 1996, 1998; Fauvel *et al.,* 1998; Geffen & Evans, 2000; Chowdhury & Joy, 2001; Rurangwa *et al.,* 2004; Figueroa et al., 2016).

In addition, the evaluation of sperm motility and other kinetic parameters, like curvilinear, straight line and average path velocities, is an essential tool in the examination of sperm quality in many fish species, including the *G. blacodes* (Marco-Jiménez *et al.,* 2006; Asturiano *et al.,* 2007; Gallego *et al.,* 2012). Various studies have demonstrated that the majority of the characteristics of sperm function analyzed contribute to the general quality of the spermatozoa (Alavi *et al.,* 2008; Bobe & Labbé 2010). Motility, one of the most frequently used parameters to assess semen quality, generally presents a positive correlation with fertilizing capacity (Figueroa *et al.,* 2016). Traditionally, motility was assessed subjectively by determining the percentage of motile spermatozoa in a scale such as the one of Sanchez-Rodriguez & Billard (1977) and Cosson *et al.* (2008b) with a subjective classification, which assigns a level of 0 when no spermatozoa are moving and 5 when all of them are moving vigorously, additionally, by Computer Assisted Sperm Analyzer (CASA). Nowadays, there is no research regarding characterization and the evaluation of sperm function *G. blacodes* in the literature. The characterization and the study of spermatic function of *G. blacodes* would allow establish a base line of sperm quality-markers and also improve the management in vitro of the gamete species. The present study is the first report on the evaluation of the sperm function in intratesticular spermatozoa of *G. blacodes*. Thus, the aim of this study was to evaluate the sperm function such as motility, mitochondrial membrane potential, cytoplasm membrane integrity and DNA integrity and fertility in intratesticular spermatozoa of *G. blacodes* through flow cytometry analysis.

3.1. Materials and Methods

All chemicals used in this study were purchased from Sigma (www.sigmaaldrich.com), unless otherwise indicated. All solutions were prepared using water from a Milli-Q Synthesis System. The live-dead sperm viability kit (SYBR-14/PI; Thermo-Fisher) the situ cell-death detection kit (TUNEL; Roche Diagnostics GmbH) and the mitochondrial permeability detection kit AK-116 (MiT-E-Ψ, JC-1; Biomol International LP) test kits were also used.

3.1.1. Broodstock males

This research was conducted at the Engineering, Biotechnology and Applied Biochemistry Laboratory (LIBBA) and at the Center for Biotechnology in Reproduction (CEBIOR), La Frontera University, Chile. Twelve (n = 12) males *G. blacodes* were caught in the wild with a trap in Piedra Azul (41º 56’40.86’’ S and 72º 73’46. 62’’ W), located near the city of Puerto Montt, Los Lagos Region, Chile with average weight 1.94 ± 1.06 kg and total length of 62.6 ± 4.45 cm.

3.1.2. Collection of gametes

This study was carried out with intratesticular spermatozoa, which were collected according to the procedure described by Cabrita *et al.* (2005). During transport, the specimens were kept alive. The specimens of *G. blacodes* were anesthetized by immersion with AQUI-S® (BAYER S.A. Animal Health-Chile) for a few minutes and then decapitated. Their testicles were surgically extracted and carefully cleaned with distilled water, dried and blood remnants were removed. The testis were transferred individually into an Eppendorf tube on ice and were transported using oxygenated containers with a constant temperature of 4°C. The testes were sectioned directly in the Eppendorf tube (on ice) using a scalpel and collecting the sperm by dripping directly into a graduated, sterile, dry, disposable plastic container maintained at 4 °C.

In addition, the intratesticular spermatozoa were diluted in StorFish® (Imv, Technologies, France) medium (dilution 1:1) and centrifuged twice at 1800 rpm for 5 minutes. Immediately after collection, sperm motility and concentration were determined using a phase contrast microscope (Carl Zeiss, Jena, Germany).

3.1.3. Sperm density

Sperm density (number of spermatozoa/mL) was determined in six males using a Neubauer hemocytometer according to the methodology described by Merino *et al.* (2011) and Figueroa *et al.* (2015) for blood cells and spermatozoa at a dilution of 1 µL of sperm in 1200 µL of StorFish® medium (Imv, Technologies, France) using a phase contrast microscope (Carl Zeiss, Jena, Germany).

3.1.4. Sperm evaluation

3.1.4.1. Motility by Computer Assisted Sperm Analyzer (CASA)

Motility (percentage of motile spermatozoa) was performed using a phase contrast microscope (Carl Zeiss Jena). In addition, the percentage of sperm motility was assessed using a modified protocol of Cosson (2004) and Li *et al.* (2012) for optical microscope with Exposure Scope stroboscopic light (FROV, Vodnany, Czech Republic) by CASA software. The percentage of motile spermatozoa and the spermatozoa average velocity (μms^{-1}) were determined at ×200 magnification in a phase contrast Olympus BX 41 microscope after activation of motility. To prevent the spermatozoa from adhering to the slide, 0. 25% (w/v) of Pluronic was added to the activator (seawater). The spermatozoa were recorded with a SSC-G818digital video camera mounted on the microscope, filming at 25 frames s^{-1} at 50 Hz. They were analyzed using ImageJ CASA software for processing images and videos.

The following sperm motility variables were evaluated: curved line velocity (VCL, μms^{-1}), average path velocity (VAP, μms^{-1}), straight-line velocity (VSL, μms^{-1}), linearity (LIN, %), wobble (WOB, %). The analysis was replicated three times in each trial.

3.1.4.2. Motility Subjective

Immediately after collection, spermatozoa motility and concentration were determined using a phase contrast microscope (Carl Zeiss, Jena, Germany). Sperm motility (percentage of motile spermatozoa) was evaluated subjectively according to the protocol described by Cosson *et al.* (2008b), values from 0 to 100% were attributed to the samples, i.e. from 0% of spermatozoa with progressive motility to 100% of spermatozoa with progressive motility according to the 1 to 5 scale: 1 = 0-5%; 2 = 5-25%; 3 = 25-50%; 4 = 50-75%; 5 = 75-100% by the same evaluator . Three observations were done per sample.

3.1.4.3. DNA fragmentation

To assess DNA fragmentation, the TUNEL (In Situ Cell Death Detection Kit, Fluorescein, Roche Diagnostics GmbH, Mannheim, Germany) procedure was used. The 500 µL sperm suspension at concentration 3 x 10^6 /mL was centrifuged for 5 min at 1300 rpm. The pellet was fixed at 500 µL with 4% formaldehyde in PBS for 1 h at room temperature. Next, the suspension was washed in PBS by centrifuging at 1300 rpm for 5 min; the pellet was re-suspended in 300 µL of 0.5% Triton X-100 prepared in 0.1% sodium citrate for 15 min at 4°C, and then washed in PBS for 5 min at 1300 rpm. The pellet was then re-suspended in 50 µL TUNEL reaction mixture (TdT and fluorescein-dUTP) and incubated for 60 min at 37ºC in a humidified atmosphere in the dark. Then the spermatozoa were stained with 5 µL propidium iodide (1.5 mM), re-suspended in 200 µL PBS and washed for 5 min at 1300 rpm.

Afterwards, the pellet was re-suspended in 200 µL PBS. After washing, the label at the damaged DNA sites was analyzed directly by flow cytometry and confocal microscopy. The TUNEL positive spermatozoa, with fragmented DNA, appeared stained green. The analysis in each trial was replicated three times.

3.1.4.4. Cytoplasm membrane integrity

The viability of the spermatozoa and the integrity of the cytoplasm membrane were assessed using the LIVE/DEAD Sperm Viability Kit (SYBR-14/PI dye) (L7011-Invitrogen Molecular Probes, USA). For this test, 3 x 10^6 spermatozoa/mL were resuspended in 200 µL PBS + 1 µL SYBR-14 (0.025 mM) + 1 µL propidium iodide (PI) (2.4 mM). After the spermatozoa had been exposed to this solution for 10 min at 4°C, the sample was centrifuged at 1.300 rpm for 5 minutes, a further 200 µL of PBS were added prior to sperm analysis by flow cytometry and confocal microscopy. The SYBR-14 positive spermatozoa, with cytoplasm membrane integrity, appeared stained green. The analysis in each trial was replicated three times.

3.1.4.5. Mitochondrial membrane potential (ΔΨM)

To evaluate mitochondrial activity, changes in the mitochondrial membrane potential (ΔΨM) were determined using JC-1 (MitoProbe™ JC-1 Assay Kit, M34152, Life technologies, Molecular Probes) a fluorescent cation dye, 5,5´,6,6´-tetrachloro-1,1´,3,3´ tetraethylbenzymidazolyl carbocyanine iodide. The JC-1 is a lipophilic dye that is internalized by all functioning mitochondria, where it fluoresces green. In highly functional mitochondria, the concentration of JC-1 inside the mitochondria increases and the stain forms aggregates that fluoresce red. This test was performed according to the manufacturer's instructions for the Mitochondrial Permeability Detection Kit AK-116 (MīT-E-Ψ™, BIOMOL International LP, Plymouth Meeting, PA, USA).

Briefly, 1 µL JC-1 was added to the 250 µL Sperm suspension and incubated for 10 min at 4°C in the dark. The cell suspension was then centrifuged for 5 min at 1300 rpm, the supernatant was discarded and the sperm pellet re-suspended in 200 µL PBS and immediately analyzed by flow cytometry and confocal microscopy. The percentage of red-stained cells was recorded as a cell population showing high ΔΨM, and the percentage of green-stained cells as a cell population with low or reduced ΔΨM. The analysis in each trial was replicated thrice.

3.1.4.6. Fertility

The fertility was evaluated with 4.5 ml sperm + 1.5 L mass + 500ml of seawater. It was mixed well and allowed to keep for 5 min, after was added 1.5 L of seawater at 12ºC. Fertilization was evaluated by observation of the first cleavages (segmentation), those with segmented blastodiscs were considered as fertilized.

3.1.5. Flow cytometry

The FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) was used to determine the following variables: sperm membrane integrity (with SYBR-14/PI), mitochondrial membrane potential (with JC-1) and DNA fragmentation (by TUNEL). A minimum of 10,000 spermatozoa were examined in each assay at a flow rate of 100 cells/s. The spermatozoon probe was gated using 90-degree and forward-angle light scatter to exclude debris and aggregates. The excitation wavelength was 488 nm, supplied by an argon laser at 15 mW. Green fluorescence (480-530 nm) was measured in the FL-1 channel and red fluorescence in the FL-2 channel (580-630 nm) and FL-3 channel (610 nm).

3.1.6. Statistical analysis

The percentage data were analyzed using the statistics program Prisma® version 6.0. Mean ± SD was used to assess rates of DNA fragmentation, viability/plasma membrane integrity, mitochondrial membrane potential, motility and fertility. Pearson correlation was used to relate the motility with membrane integrity, mitochondrial membrane potential, and fertilization rates. The level of significance was set at $p<0.05$.

3.2. RESULTS

3.2.1. Spermatological parameters

The testicle volume for each males of *G. blacodes* was between 1.2 and 2.5 mL, the mean was 1.47 ± 0.56 mL (n = 10).

3.2.2. Sperm density

Sperm density (number of spermatozoa/mL) was an average 5.6 ± 0.12 x 10^9 spermatozoa/mL

3.2.3. Evaluation of membrane integrity, mitochondrial membrane potential, DNA integrity, motility and fertility

The rate of sperm motility of intratesticular spermatozoa *G. blacodes* and its variables were expressed by mean ± SD. The motility was assessed subjectively and by CASA with a level of 88.75 ± 8.56% and 23.12 ± 2.58%, respectively. The rate of plasma membrane integrity of intratesticular spermatozoa registered by flow cytometer was 83.87 ± 6.06%, mitochondrial membrane potential was 45.26 ± 14.34%; DNA fragmentation was 1.175 ± 1.14%, whereas the fertility showed a rate of 68.9 ± 25.9% (Table 1).

Figure 1 shows the mean ± SD rate of motility variables a) VCL (67.3 ± 22.79 µm/s); VSL (55.4 ± 24.29 µm/s); VAP (60.0 ± 23.66 µm/s, b) LIN (84.8 ± 7.62%); WOB (93.0 ± 4.18%) and motility by CASA.

Table 1. Mean ± S.D rate of sperm functionality variables: citoplasmatic membrane integrity; mitochondrial membrane potential; DNA fragmentation, motility and fertility of intratesticular spermatozoa *G. blacodes* (*P*<0. 05, n=12).

Sperm function	Minimum	Maximum	Mean	SD
Motility (%)	75	100	88.75	± 8.56
Plasma membrane integrity (%)	77.9	93.5	83.87	± 6.06
Mitochondrial membrane potential (%)	16.9	71.8	45.26	± 14.34
DNA fragmentation (%)	0.2	3.8	1.175	± 1.14
Fertility (%)	36.3	100	68.9	± 25.9

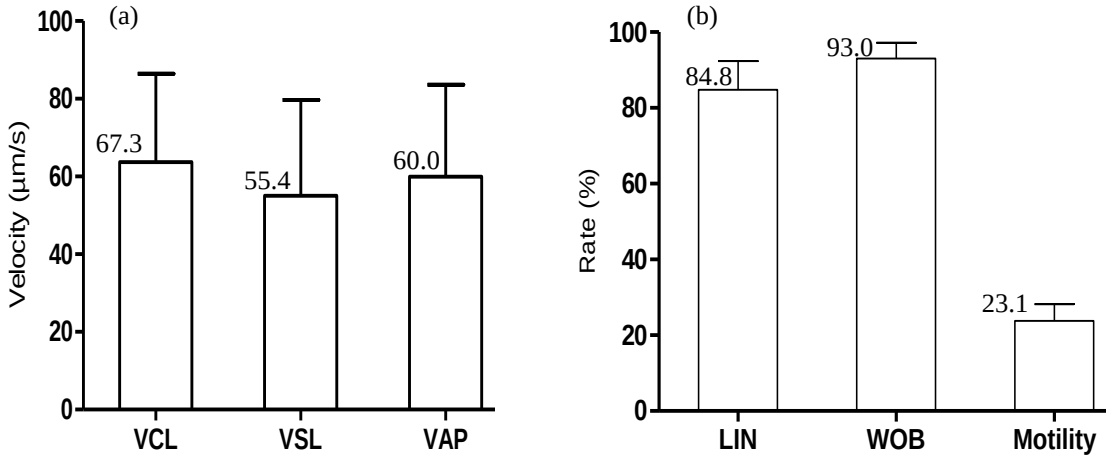


Figure 1. (a) Mean ± S.D velocity: VCL, VSL and VAP; (b) Mean ± S.D rate of motility variables (Motility, LIN and WOB) of intratesticular spermatozoa *G. blacodes* that were activated in sea water (P<0.05, n=10).

3.2.4. Relationships between motility and plasma membrane integrity, mitochondrial membrane integrity, DNA fragmentation and fertility

The motility calculated subjectively shows positive correlation with mitochondrial membrane potential, citoplasmatic membrane integrity, (Figure 2). Additionally, the motility variables such as VCL and VSL show positive correlation with mitochondrial membrane potential (Figure 3). In addition, the fertility shows positive correlation with the motility and mitochondrial membrane potential, also shows a good correlation with VCL and VSL (Figure. 4).

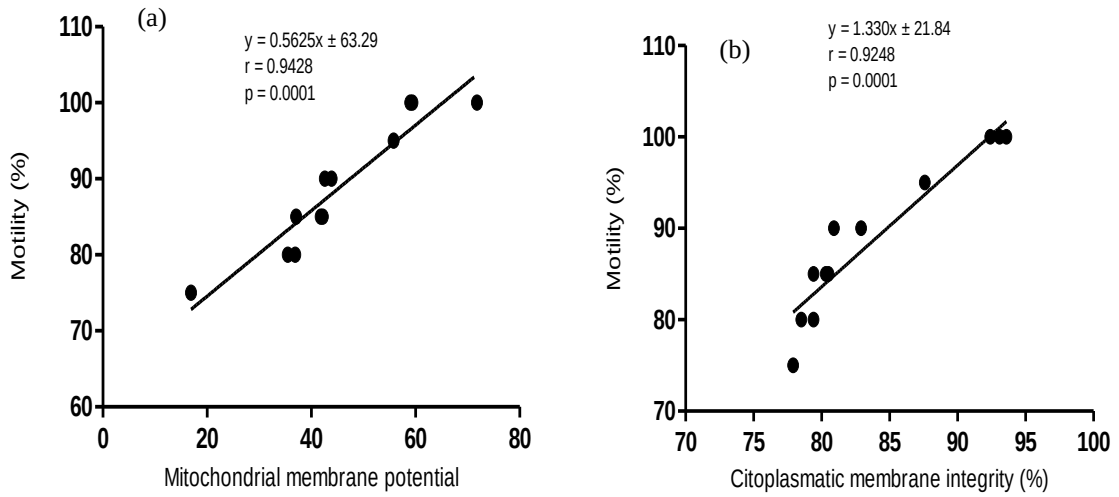


Figure 2. Relationship between motility, mitochondrial membrane potential and citoplasmatic membrane integrity of intratesticular spermatozoa *G. blacodes* that were activated in seawater: a) Positive correlation between motility and mitochondrial membrane potential (r= 0.94); b) citoplasmatic membrane integrity (r =0.92) (n= 10).

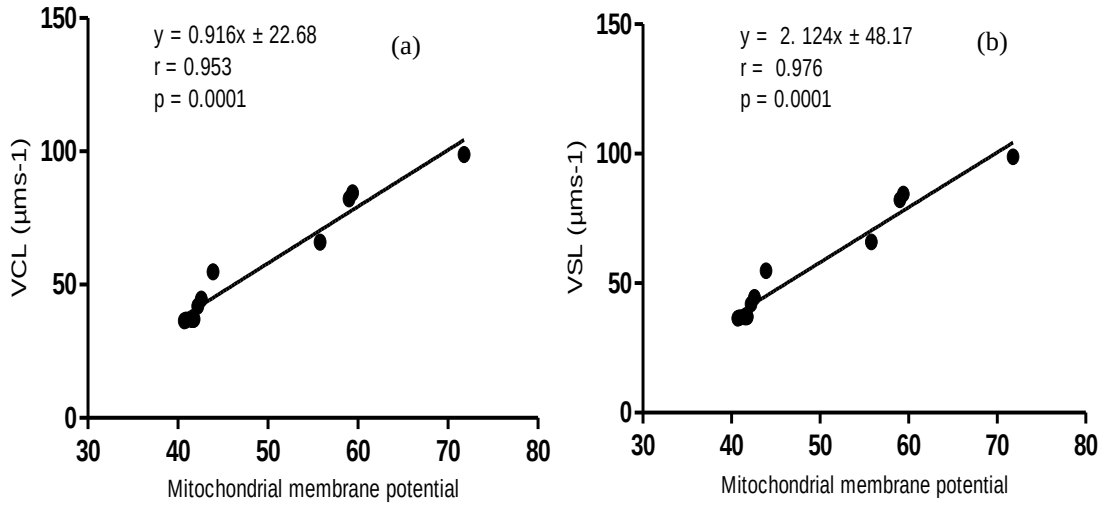
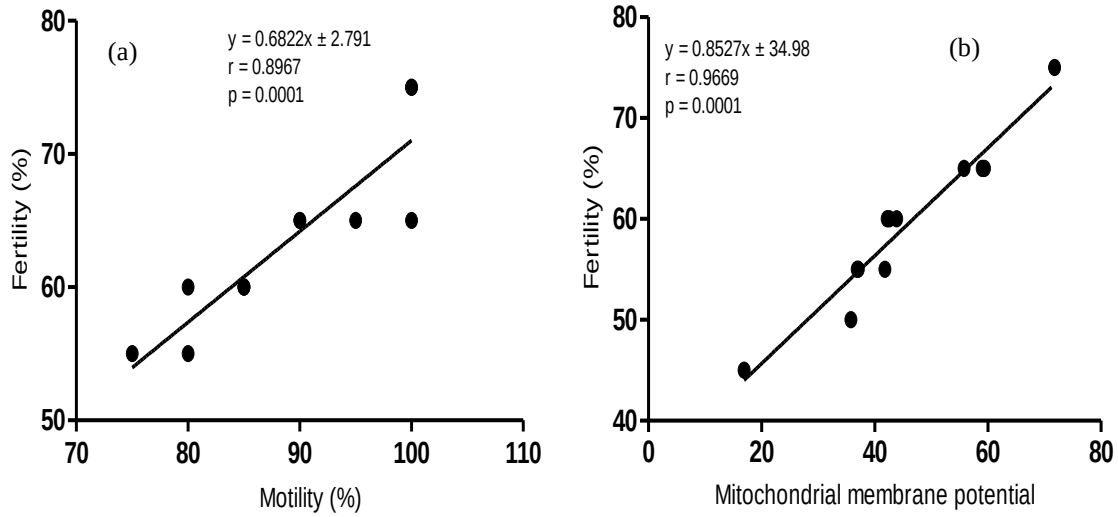


Figure 3. Relationship between VCL, VSL and mitochondrial membrane potential of intratesticular spermatozoa *G. blacodes* that were activated in seawater. (a) Positive correlation between VSL and mitochondrial membrane potential (r = 0.97); (b) Positive correlation between VCL and mitochondrial membrane potential (r = 0.91) (n= 10).



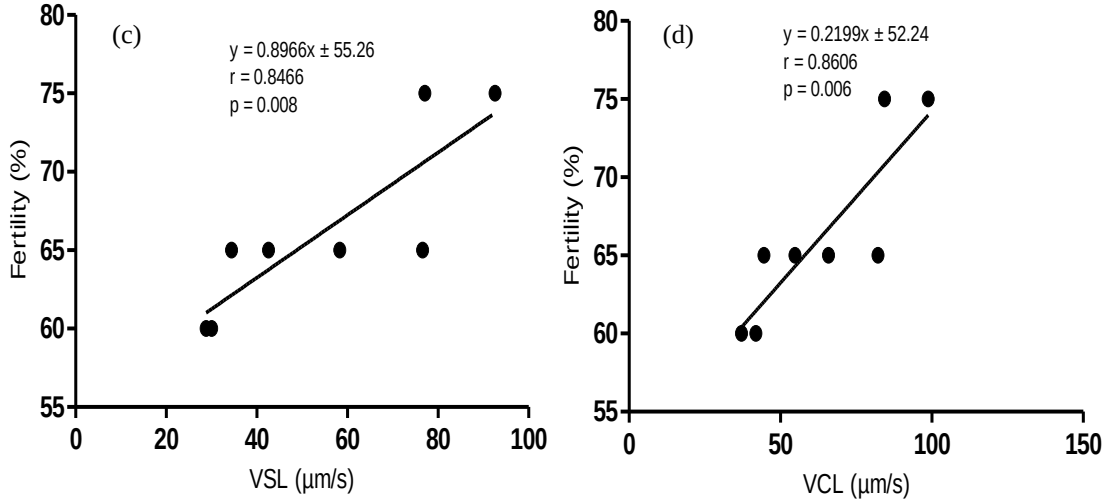


Figure 4. Relationship between fertility, motility, mitochondrial membrane potential VSL and VCL of intratesticular spermatozoa *G. blacodes*: a) positive correlation between fertility and motility (r = 0.89); b) correlation between fertility and mitochondrial membrane potential (r = 0.87); c) positive correlation between fertility VSL (r = 84) and d) VCL (r = 0.86) (n= 10).

3.3. Discussion

The evaluation of spermatic function has been reported for various marine species (Li *et al.*, 2009). Motility is regarded as one of the most essential parameters related to sperm quality, and decrease in spermatozoa movement performance under *in vivo* or *in vitro* conditions is a common reason for observation of reduced sperm fertility (Trigo *et al.*, 2014). The results of this study showed that the rate of sperm motility was gradually reduced subjectively to CASA due to the evaluation interval 88.75 ± 8.56% (subjective) to 23.12 ± 2.58% (CASA) (Table 1), motility subjective was evaluated after four hours of the sample collection, whereas sperm motility by CASA was evaluated five hours after sample collection. Additional parameters, such as DNA integrity, citoplasmatic membrane integrity and mitochondrial membrane potential, are also important indicators of sperm fertilizing potential (Anna *et al.*, 2012).

Sperm viability and mitochondrial membrane potential are two of several properties that can be determined using SYBR-14/PI dual-staining technique and JC-1/PI, respectively, these fluorescent dye combinations have been used to estimate cell quality and viability with sperm of *G. blacodes* spermatozoa. The SYBR-14 is able to penetrate the cell membrane of the sperm head and stain the nucleic acids of viable cells. Propidium iodide is not able to pass through the membrane of living cells, but it is able to penetrate and stain the nuclear DNA of degenerated or dead sperm. The lipophilic compound JC-1 has been used to evaluate the depolarization of the mitochondrial membrane in the spermatozoa of several species of mammals and fishes (Figueroa *et al.,* 2014). The use of fluorescent dyes to evaluate viability and mitochondrial function should be compared with fertilization rates to further evaluate the utility of these flow cytometry procedures assessing sperm quality of fish. The importance of the sperm plasma membrane lies mainly in the ion transport functions, motility, water balance, signal receivers involved with fertilization (gametes fusion), and regulation of fluidity and permeability (Lahnsteiner *et al.,* 2009; Berrios *et al.,* 2010). The importance of physiological parameters such as the integrity of the cytoplasm and mitochondrial membranes in fertilization is known. The DNA quality of spermatozoa is an important indicator of the correct transmission of genetic material from one generation to the next. The DNA damage correlates strongly with the appearance of mutagenic alterations; however, spermatozoa with genetic material damaged by freezing are capable of fertilization (Twigg *et al.,* 1998). The low rate of the parameter such as mitochondrial membrane integrity may be associated with stress oxidative (Figueroa *et al.,* 2014. Nevertheless, the motility correlated with mitochondrial membrane potential, and citoplasmatic membrane integrity (r= 0.94; r= 0.92, respectively) (Figure 2).

This may result from the high integrity of the cytoplasm membranes (88.75 ± 8.56%) and the low mitochondrial potential (45.26 ± 14.34%) due to the possible formation to osmotic changes. The advantage of diluting semen is a reduction in sperm density, thus, oxidation of plasma and mitochondrial membranes are prevented, that is, the dilution produces a buffering capacity and delivers energy substrate that allows the maintenance of spermatozoa with a stable pH and does not allow cells to dehydrate (Bobe & Labbe, 2009; Dziewulska *et al.*, 2010). The role of the mitochondria has been considered one of the key factors in sperm functionality and fertilizing capacity in all species. Changes in mitochondrial membrane potential are a good indicator of functional impairment (Merino *et al.*, 2011a, b).

Very few studies have correlated motility with mitochondrial membrane integrity. Additionally, studies performed by Figueroa *et al.* (2014, 2015b) in *Salmo salar* showed a positive correlation between VCL, VSL and mitochondrial membrane potential. The results of this study showed positive correlation between VCL, VSL and mitochondrial membrane potential (r= 0.97; r= 0.97, respectively). The activation and duration of motility require a large amount of ATP, which is supplied by the mitochondrion in most fish. For each examined sampling, we observed considerable variation in the percentages of spermatozoa regarding motility, plasma membrane integrity, mitochondrial membrane potential and DNA fragmentation. Additionally, the results of this study indicate a positive correlation of the fertility with motility, mitochondrial membrane potential, plasma membrane integrity, VSL and VCL (r= 0.89; r= 0.87; r= 0.92; r= 0.84 and r= 0. 86, respectively) (Figure 3 and 4).

Studies of embryonic development are important in terms of understanding basic biology and potential applications (Ishigaki *et al.*, 2016). Teleost fertilization exhibits special distinct characteristics from those of other vertebrates and even other fish groups. In economically productive aquaculture, one very important aspect is the availability of an adequate supply of fertile eggs. The assessment of the fecundity of fishes is of especial importance in biological studies, particularly as far as the management of heavily fished commercial species is concerned. The average rate of fertility was 68.9 % using *G. blacodes* intratesticular spermatozoa of wild males with eggs of females in captivity. This result is very important for artificial reproduction of this specie. It is the first report on fertility of *G. blacodes.* The use of intratesticular spermatozoa for *in vitro* fertilization is a key tool for breeding this species in captivity and hence would allow its introduction in aquaculture industry. Gage *et al.* (2004) concluded that fertilization was more successful for spermatozoa capable of faster curvilinear swimming, although high motility often results in high fertility rates. Good fertilization rates were also obtained from Atlantic salmon (*Salmo salar*) and rainbow trout (*O. mykiss*) spermatozoa that showed little or no motility (Levanduski *et al.*, 1988; Figueroa *et al.*, 2016).

In conclusion, in this study we observed a high percentage of membrane integrity and motility (subjective), low mitochondrial membrane potential, DNA fragmentation and motility (CASA) and high percentage of fertility. Finally, considering the importance of the fish reproduction in captivity and wild, it is advisable to deepen research in cellular aspects, hence, providing useful information for the elaboration of means of cultivation and other inputs used in the management of gametes of this species. Moreover, this study shows great prospects for development in biotechnology and production for the world *G. blacodes* industry.

Conflict of interest

The authors have declared that they have no conflicts of interest.

Acknowledgements

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CHAPTER IV

Morphology and ultrastructure of pink cusk-eel (*Genypterus blacodes*, Schneider 1801) spermatozoa by scanning and transmission electron microscopy

Associated to specific objectives 3
Paper under review in Tissue and Cell

Morphology and ultrastructure of pink cusk-eel (*Genypterus blacodes*, Schneider 1801) spermatozoa by scanning and transmission electron microscopy

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Abstract

In this study, scanning electron microscopy and transmission were used as techniques to describe the ultrastructure and morphology the *Genypterus blacodes* spermatozoa. Findings revealed that the *G. blacodes* spermatozoa can be differentiated into three major parts: an ovoid head without an acrosome, a short mid-piece, and a long flagellum. The mean length of the spermatozoa was 57.6 ± 6.08 µm, with flagella length of 56.2 ± 7.2 µm, a head length of 1.47 ± 0.2 µm, and head width of 0.89 ± 0.06 µm. The mid-piece measure 0.72 ± 0.16 µm total, with 0.31 ± 0.02 µm length, 0.6 ± 0.05 µm wide. The short mid-piece contains 4 or 5 mitochondria. The main piece of the flagellum had short irregular side-fins. The axoneme composed the typical 9+2 microtubular doublet structure and the mitochondria were separated from the axoneme by a cytoplasmic canal. The present study reveals that *G. blacodes* sperm can be categorized as being of a primitive type. This study for the first time provides a comprehensive detail on the ultrastructure and morphology in *G. blacodes*.

Keywords: Morphology, ultrastructure, *Genypterus blacodes*, spermatozoa, scanning electron microscopy, transmission electron microscopy

4. Introduction

Descriptions and knowledge on morphology and ultrastructural of fish sperm provide information for understanding the taxonomic classifications, relationships at family, subfamily and species and establish phylogenetic relationships among fish species (Mattei, 1991; Lahnsteiner & Patzner, 2008), also, allow a better *in vitro* management of the species. In the teleost species studied to date, the spermatozoa structure has revealed a high diversity specially, in the head shape, in the number shape and location of mitochondria, and length and from aflagellate to biflagellae according to whether a species adopts internal or external fertilization (Guo *et al.,* 2016). The application of scanning electron microscopy (SEM) and transmission (TEM) in fish taxonomy, however, is a relatively recent development (Kaur & Dua, 2004; Johal *et al.,* 2006; Liu *et al.,* 2008). There are difficulties for comparing the results of different studies (Psenicka *et al.,* 2007) because; (i) each study may not describe an identical set of fine structural parameters, ii) fixation and staining procedures may also play an important role in affecting results comparability, and (iii) the number of samples varies greatly between studies.

Currently, research and development of *G. blacodes* aquaculture has been initiated in over six countries including Chile. The *G. blacodes* fishery is developed in Chilean waters between Talcahuano (36º 44'S) and south of Cabo de Hornos (57º00'S) from Coquimbo to austral zone (41º28'-57º00'S) (Wiff *et al.,* 2011). It is a species with the greatest farming potential in Chile, due the exceptional quality of its flesh and high commercial value (Vega *et al.,* 2012). Despite the importance of this species, very little is known about its reproductive biology. Currently, in Chile, breeding of this fish in captivity is found in the initial stage and hatchery production of this species is yet to be developed for large-scale farming.

Considering the importance of protecting this fish, it is essential to understand its reproductive biology. Therefore, the present study aimed to investigate the ultrastructure and morphology of *Genypterus blacodes* spermatozoa using SEM and TEM.

4.1. Materials and methods

The specimens were caught in the wild with a trap at Piedra Azul (41º 56'40.86'' S and 72º 73'46. 62'' W), located near the city of Puerto Montt, Los Lagos Region, Chile with an average (body weight = 1 960 ±1.06 g, Total body length 62.6 ± 4.45 cm, *n* = 10). This study was carried out with intratesticular spermatozoa and they were collected according to the procedure described by Cabrita *et al.* (2005). *G. blacodes* males were anaesthetized and decapitated, the testicles were surgically extracted and carefully cleaned. The testes were cut into pieces directly in the Eppendorf tube (on ice) using scalpel and collecting sperm by directly dripping it into graduated, sterile, dry, disposable plastic containers, kept at 4 °C. Sperm samples were diluted at a ratio of 1:1 (testicle volume: extender medium nonactivating (Storfish® 1x = 300 mOsm/kg and pH 8.1) and centrifuged twice at 1800 rpm for 5 minutes.

Morphology and ultrastructure were assessed by means of scanning electron microscopy (SEM) and transmission (TEM), respectively following the methodology of Luo *et al.* (2011), modified, fixing for 48 h in glutaraldehyde 2.5% in a buffer of sodium cacodylate 0.1 M (pH 7.5) at 20-25 °C. For SEM, spermatozoa were dehydrated in a graded series of acetone and dried to the critical point (Polaron E 3000), changing acetone for CO_2 (four times, 10 min each). After fixation, the sperm pellets were processed for TEM. For TEM, the samples were dehydrated as described above, and embedded in epoxide resin. Then, ultra-thin sections of 60-100 nm thickness were collected using glass knives.

Later, the sections were placed on copper grids, stained with uranyl acetate and lead citrate, and screened under a TEM (Hitachi H700 TE) for screening the ultra-cellular structure of sperm. Spermatozoa were coated with gold/palladium at approx. 20 nm, observed and photographed using SEM (ZEISS 409 DMS, Germany). All SEM and TEM measurements were evaluated using Prisma® software version 6.0. (Version 4.0.1 for Windows, Olympus Optical Co., Hamburg, Germany). Spermatozoa morphological characteristics were assessed and expressed as mean ± standard deviation, number, and range. The experiment of SEM and TEM has been repeated four times.

4.2. Results and discussion

Morphologically, *G. blacodes* spermatozoa consists of three major parts: an ovoid head without acrosome, short midpiece and a single uniflagellate and can be categorized as a primitive type (Fig. 1A, B and E). Spermatozoa without acrosome are common characteristics in many teleost species such as *Eleginops maclovinus* (Valdebenito *et al.,* 2016), *Merluccius australis* (Effer *et al.,* 2013) *Brachymystax lenok tsinlingensis* (Guo *et al.,* 2016), *Scatophagus argus* (Madhavi *et al.,* 2015) except, Atlantic eel sperm (Gibbons *et al.,* 1983) and chondrostean fishes (sturgeons) containing acrosome (Ginsburg, 1968). According to the Kim *et al.,* 2011, the sperm with an acrosome-less head is closely associated with the egg micropyle diameter of the species and it is the likely result of coevolution with possessing a micropyle, an opening in the zona pellucid through which sperm enter to fertilize the egg. *G. blacodes* spermatozoa have total length of 57.6 ± 6.08 µm and flagellum 56.2 ± 7.2 µm (n =20). The head of the spermatozoa was small, spherical 1.47 ± 0.2 µm long and 0.89 ± 0.06 µm wide (n = 20) (Table 1). *G. blacodes* sperm head has ovoid shape and contains an ovoid nucleus.

The small ovoid head found in *G. blacodes* is the result of a simple spermiogenesis process. However, Psenicka *et al.* (2007) concluded that an elongated head present in Siberian sturgeon (*Acipenser baerii*) indicated a more complex spermiogenic process that is considered as an advanced morphological sperm feature. The hydrodynamic shape of the head can moderate swimming ability and velocity (Malo *et al.*, 2006) and is considered to be a primitive or ect-aquasperm. *G. blacodes* morphology described in this research using SEM agrees with these patterns and the characteristics described by Cosson *et al.* (2008b) in turbot 50 µm (0.6 µm in diameter) and halibut; 40 µm (0.4 µm in diameter) in sea bass; 50 µm in hake; 60-70 µm in cod; and 45 µm in tuna. Additionally, Ginzburg (1972) reports that herring (*Clupea harengus pallasis*) shows a total length of 43 µm and for Atlantic salmon (*Salmo salar*) a total length of 63 µm. Comparing the morphological features of a *G. blacodes* spermatozoon with those of other fish species, showed them to be closely similar in overall shape to *T. bifasciatum*, *Coris. julis* (Mattei, 1991; Lahnsteiner & Patzner, 2008;). In the latter species, spermatozoa head length varied from about 1 to 3 µm as compared with *G. blacodes* spermatozoa, of length 1.47 ± 0.2 µm. Similar to *E. masquinongy*, *E. lucius* spermatozoon have a spherical head, 1.40 mm in diameter (Alavi *et al.*, 2008). The midpiece is elongated in *G. blacodes* spermatozoa, different of those that have been demonstrated in salmonid spermatozoa or sturgeons (Lahnsteiner & Patzner, 2008; Psenicka *et al.*, 2008).

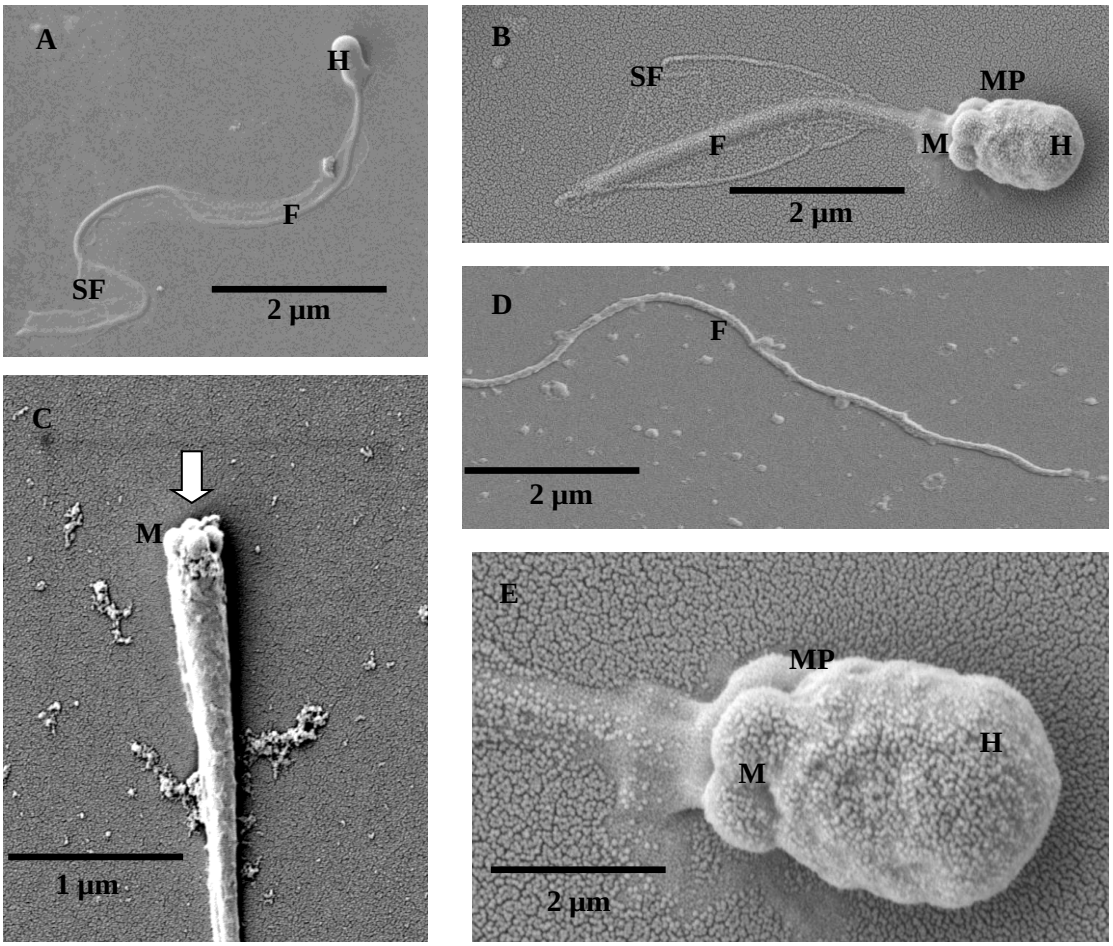

Figure 1. Scanning electron microscopy (SEM) micrographs of *Genypterus blacodes*. A) Spermatozoa with their characteristic: H, head; F, flagella; SF, side-fins: B) General views of different rotations of the sperm with mitochondrial regions. H, head; MP, mid-piece; F, flagellum; M, mitochondria; SF, side-fins; C) The mid-piece with mitochondria between 4 or 5; D) Flagellum without head and mid-piece; E) Magnified view of a spermatozoon with prominent H, head; F, flagellum; M, mitochondria, . Scale bars: 1µm (A, B, C and E), 2 µm (E).

Table 1. Ultrastructural and morphological variables of *Genypterus blacodes* spermatozoa.

Spermatozoa	Variables			
Sperm size (µm)	Total length 57.6 ± 6.08			
Head (µm)	Length 1.47 ± 0.2	Width 0.89 ± 0.06	Nucleus length 0.52 ± 0.08	Nucleus width 1.0 ± 0.06
Mid-piece (µm)	Length 0.31 ± 0.02	Width 0.6 ± 0.05	Mitochondria diameter 0.42 ± 0.04	Mitochondria number 4 or 5
Flagellum (µm)	Length 56.2 ± 7.2	Width 0.14 ± 0.02	Flagellum diameter 0.14 ± 0.02	
Axoneme (nm)	PDM 31.35 ± 5.13	CDM 38 .42 ± 3.21	Microtubule diameter 15.25±1.05	Axoneme pattern 9+2

Data are mean ± SD (n = 20), PDM: Peripheral doublets of microtubules width; CDM Central doublets of microtubules width

The present investigation confirmed that *G. blacodes* sperm possess the configuration of the Uniflagellate acrosome-less aquasperm which is consistent with this species having external fertilization. It is reported that the shape of the head is highly variable among teleosts sperm. The shape of the nucleus in fish sperm is species specific and it varies from species to species (Maricchiolo *et al.,* 2010). According to Baccetti *et al.* (1984), swimming speed and duration of motility of sperm are influenced by the size of the mid-piece. The midpiece has an extension of 0.72 ± 0.16 µm with 0.31 ± 0.02 µm on length, and 0.6 ± 0.05 µm wide (*n* =20). In *G. blacodes,* the mid-piece is short which is a common feature in teleost where fertilization is external (Vergílio *et al.,* 2013).

The small mid-piece length and a long flagellum in *G. blacodes* sperm clearly suggests the sperm cell can move quickly through the water column to fertilize a free floating egg which is released by the female brooder. It is believed that swimming speed of sperm help to reduce the distance between the dispersed gametes within the water column. The short mid-piece of *G. blacodes* spermatozoa shows 4 or 5 mitochondria as a circle like structure (Fig. 1C). The role of mitochondria in the mid-piece of the sperm is an energy source for generating adenosine triphosphate (ATP) for sperm motility as previously documented (Billard *et al.,* 2000). The ATP is also used by the dynein arms, which helps in a self-oscillatory bending behavior of the flagellar axoneme (Maricchiolo *et al.,* 2004). The number of mitochondria present in the mid-piece of sperm of various species have been reported for the Common two-banded seabream *Diplodus cervinus cervinus* which have 1 mitochondrium (Mahmoud, 2010); and there are 4-6 in Common barbel *Barbus barbus* (Alavi *et al.,* 2008); 5-6 in Leopard coralgrouper *Plectropomus leopardus* (Gwo *et al.,* 1994); 6 in Atlantic bluefin tuna *Thynnus thynnus* (Abascal *et al.,* 2002) and 6-9 in Longtooth grouper *Epinephelus bruneus* (Kim *et al.,* 2013). The presence of 4 or 5 mitochondrion in *G. blacodes* indicate a greater energy delivering capacity for the sperm compared with those species possessing a fewer number of mitochondria. The number of mitochondria is an important factor for sperm motility as a source of energy and thereby has a significant role in fertilizing eggs, also, plays an important role in reproductive activity of the fish (Lahnsteiner & Patzner, 1995). Movement of the flagellum depends on how it is attached to the mid-piece and the structural support provided by the associated membranes.

The mid-piece, has several foldings of the cytoplasmic membrane and a shallow collar-like structure providing structural support for the movement of flagellum during swimming (Markovina, 2008).

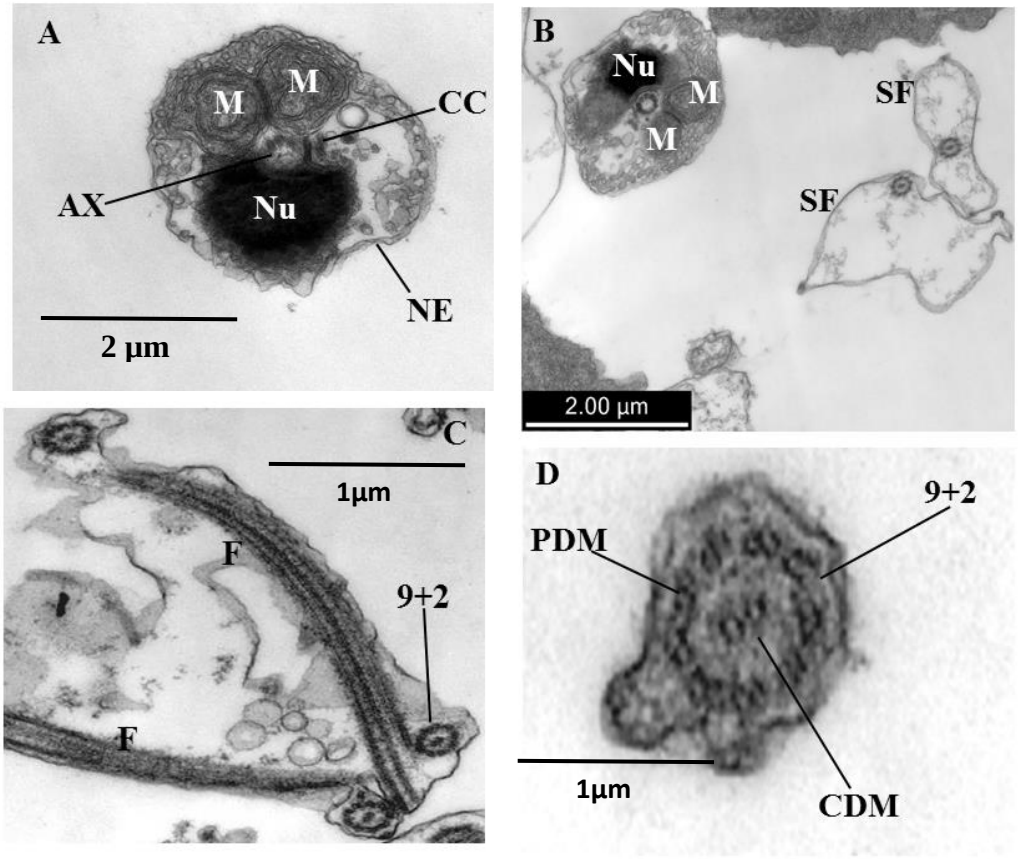

Figure 2. TEM micrographs of *Genypterus blacodes*. A) Nu, nucleus; M, mitochondria; Ax: axenoma; CC, cytoplasmic channel; NE, nuclear envelope: B) Nu, nucleus; M, mitochondria; SF, side-fins; C) axoneme pattern (9+2): E) PDM, peripheral doublets of microtubules; CDM, central doublets of microtubules; axoneme pattern (9+2).

Transmission electron microscopic observations indicated the nucleus occupied a part of the head and was composed of electron-dense, granular materials (chromatin) surrounded by a nuclear envelop (Fig. 2A and 2B). The axoneme composed the typical 9+2 microtubular doublet structure with two central and nine peripheral doublet microtubules. The mitochondria were separated from the axoneme by a cytoplasmic canal (Fig. 2 A, B, C and D). The flagellum has surrounded by a cell membrane that projected to form two side-fins located on the both sides of the flagellum (Fig 1A and B). Even though the functions of side-fins are still unknown (Maricchiolo *et al.,* 2004), the presence could be accelerate the flagellar forward motion and also increase the friction with the surrounding medium which results in an increased probability of fertilization (Zhang *et al.,* 1993; Cosson *et al.,* 2000; Psenicka *et al.,* 2007). The presence of side-fins is not order or family-specific, as shown by Maricchiolo *et al.* (2004) in Sparidae; Kristan *et al.* (2014) Percidae; and Hatef *et al.* (2011) Acipenseridae. The mean width of the outer doublet and the central pair is 31.35 ± 5.13 and 38 .42 ± 3.21 nm, respectively (Table 1) (n = 20). Based on ultrastructure, the spermatozoon of *G. blacodes* can be classified into Type I spermatozoa. Mattei (1991) has reported that the spermatozoa of Type I contain an axoneme with a 9+2.

4.3. Conclusions

The present study provides important background on morphology and ultrastructure of sperm *G. blacodes*. Detailed spermatozoa characteristics have been revealed in this study including an ovoid shaped head without acrosome, a short midpiece, and a long uniflagellum that attribute to the potential swimming ability for *G. blacodes* sperm and primitive type. The information on the ultrastructure and morphology of *G. blacodes* spermatozoa improves the understanding of its reproductive biology, with the potential to contribute to the protection of this endangered species.

In addition, this knowledge can assist in the development of artificial reproduction for enhanced production of this species.

Author contributions

All authors reviewed and contributed to the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interest to any of the internal or external funding sources.

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CHAPTER V

General discussion and conclusions

5. GENERAL DISCUSSIONS

As mentioned in the introduction, *G. blacodes*, is one of the most important species and commercialized in Chile, this species constitutes very important resources in artisanal and industrial fishing. During the last years, they have been increasing their importance in the artisanal fishing of internal waters. The characteristics *G. blacodes* profile it as a species to diversify and generate competitiveness in Chilean aquaculture. However, in recent decades landings of this species has been decreasing due to overexploitation, but also because it is strongly regulated by catch quotas that are low comparatively to other species. Currently, in Chile the culture of *G. blacodes* as a native fish is found in the initial stages, is mainly oriented to the capture of wild juveniles and larvae production, and requires a great research effort to obtain essential information. Sperm characterization of *G. blacodes* during this Doctoral Thesis allows establishing a baseline for further research. During this Doctoral Thesis, we have used intratesticular spermatozoa because for this species it is difficult to find wild fully-sexually mature male individuals; and sexual maturation in captivity has not been yet reported. The use of intratesticular spermatozoa for *in vitro* fertilization is a key tool for breeding this species in captivity and hence would allow its introduction in aquaculture industry.

Studies based on morphometric analysis of *G. blacodes* populations were realized in Australian waters (Ward & Reilly, 2001) and New Zealand waters (Smith & Francis, 1982; Colman, 1995). Canales-Aguirre *et al.* (2010), used microsatellites to determine genetic variability and population zones where *G. blacodes* is being fully exploited in Chilean waters. During the eighties the fleet began directly targeting the pink cusk-eel and since the early nineties, were recording high levels of capture.

Population attributes of *G. blacodes* such as its low resilience to exploitation, slow growth, medium longevity, and a sedentary life-style facilitate the study of life history tradeoffs (Ward *et al.,* 2001; Wiff *et al.,* 2007). According to indicators studied by Céspedes *et al.* (2011), in Chile, the size structures of industrial capture of *G. blacodes* between 2009 and 2010 showed unimodal distributions, with a greater presence of juveniles. New Zealand has determined the existence of at least three *G. blacodes* stocks through several techniques including allozymes (Smith & Francis, 1982), morphometry (Colman, 1995), vital parameters and size structure (Horn, 1993). The maximum length in Chile of *G. blacodes* is 160 cm, whereas the average length of capture is 80-90 cm (Céspedes *et al.,* 2014). Importantly, the *G. blacodes* fishery is characterized by infringing a significant proportion of juveniles (under 90 cm height) with age at first maturity 6 years (82 cm) (Wiff *et al.,* 2011).

Studies using allozyme and microsatellite techniques to look at the stock structure of *G. blacodes* inhabiting the Australian coast have found no statistical evidence to reject or accept the hypothesis of a single stock in distribution. It is therefore difficult to determine, whether the existence of genetic variations is local (Canales-Aguirre *et al.,* 2010). Landings of *G. blacodes* have been decreasing considerably in Chile. However, from 2014, fishery has been on the increase with a steep increase during the last two years. In Chile, according to Contreras *et al.* (2018) the stock of *G. blacodes* is at 20% spawning biomass. The artisanal fishery contributed to 60% of the national landings in 2013, followed the industrial fleet with 40%. In recent years, given the high levels of recorded catches, the stock is in a state of overexploitation and, due to high fishing mortalities, is still at risk of overfishing (Wiff *et al.,* 2011).

Currently, stock depletion of *G. blacodes*, double increasing of the fish demand and further upward movement of fish prices are observed. With increased marketing efforts and population growth, the demand for fish product could be tremendously increased (Janko, 2014). Types of fishing, fishing methods, socio-economic factors, lack of facilities and infrastructure, ineffective administration setup, lack of expertise lack of scientific data are the major fishery management and conservation challenges of *G. blacodes*. Thus, effective management setup, regular stakeholders follow-up, and encouraging the development of aquaculture are very important to sustain the resources and meet the demand.

Semen analysis is the most important diagnostic tool used to assess fertility (Hwang *et al.,* 2011). Sperm motility constitutes the basis for evaluating milt and controlling the ability of sperm to fertilize eggs (Cejko *et al.,* 2013). Sperm motility is a key factor to determine semen quality and clearly is related to osmolality which having important relevance in fertilizing capacity and the duration of sperm motility (Sadeghi *et al.,* 2017; Ingermann *et al.,* 2011). Sperm motility and duration are influenced by various determinants providing activation of axonemal movement such as pH, temperature, ions, salinity, and osmolality (Islam & Akhter, 2011; İnanan & Ögretmen, 2015). Marine fish display higher velocity and duration compared to freshwater species (Cosson *et al.,* 2008a; Browne *et al.,* 2015). Sperm motility parameters in marine fish are actually influenced or sensitized to pH, temperature, osmolality, and ions as well as to dilution (Alavi *et al.,* 2007; Le *et al.,* 2011; Browne *et al.,* 2015). Therefore, determination of good diluent with optimal sperm motility parameters is very important to increase the fertilizing ability of artificial propagation (Cosson *et al.,* 2008a, 2008b; Browne *et al.,* 2015).

Sperm dilution is a main factor in the activation of spermatozoa motility (Alavi *et al.*, 2007; Le *et al.*, 2011) and the ability to fertilize eggs (Cosson *et al.*, 2008a, 2008b). It was shown that sperm dilution ratio in activation medium influenced fertilizing ability throughout changes in sperm motility parameters, such as sperm velocity and sperm motility percentage (Alavi *et al.*, 2007; Le *et al.*, 2011; Browne *et al.*, 2015). In this Doctoral Thesis, sperm motility of *G. blacodes* is initiated on contact with a hyperosmotic swimming medium under normal conditions (1010 mOsm/kg, pH 8 and 8°C). The longest motility duration (432.48 ± 8.89 s) was recorded at 4°C (Chapter II; Fig. 1, A, B and C). Cosson *et al*. (2008a) have been reported that pH is one of the key factors indirectly or directly influencing sperm motility parameters in marine Conversely, the sperm motility parameters in *G. blacodes* were activated at pH from 6.0 to 9.0, but the optimal sperm motility parameters were observed at pH 8.0. Sperm motility parameters also depend on temperature of activation medium (Alavi *et al.*, 2007; Le *et al.*, 2011; Browne *et al.*, 2015) which tends to be similar to optimal temperature for growth.

Alavi & Cosson (2005) and Browne *et al.*, (2015) reported optimal sperm motility temperatures of less than 21 °C in species that are distributed in cold waters. The results of this study showed that sperm motility parameters were near maximum in an activation medium of 16°C. Fish sperm motility parameters were also affected by different osmolalities in the surrounding media (Alavi *et al.*, 2007; Le *et al.*, 2011; Browne *et al.*, 2015). According to these authors, sperm motility parameters are stimulated by hypotonic and hypertonicity osmolality in freshwater and marine fish species, respectively. In *G. blacodes*, a gradual reduction in motility time was observed as the osmolality of the swimming medium diminished (Chapter II; Fig. 1C).

The optimal osmolality for sperm motility parameters has been reported for various fish species: 900-1100 in halibut, 300-1100 in turbot, 333-645 in tilapia (Cosson *et al.*, 2008a; Alavi & Cosson 2006). In the case of ions, the sperm motility parameters in marine fish were also affected and controlled through their sensitivity to ionic concentrations (Alavi *et al.*, 2007; Le *et al.*, 2011; Browne *et al.*, 2015). It is clear that sperm motility parameters play an important role in evaluating the fertilizing ability of fish sperm. . It is possible to increase the fertilizing capacity of the fish sperm by using suitable activated solutions that can retain sperm motility for a long time. In summary, spermatozoids activated with medium with a high osmolality medium showed higher values in motility in comparison with low osmolality medium and the low salinity levels in the swimming medium affect both motility time and the percentage of motile cells.

Study of sperm function is essential to understand the overall dynamics of fertilization process in fish (Cabrita *et al.*, 2009). The parameters such as motility, mitochondrial membrane potential, cytoplasm membrane integrity, and DNA integrity play an important role in the sperm quality and fertilization capacity (Figueroa *et al.*, 2016; Rurangwa *et al.*, 2004). According to our results presented in Chapter III, *G. blacodes* intratesticular spermatozoa shows a low rate of DNA fragmentation 1.175%); high rate of plasma membrane integrity (83.87%) mitochondrial membrane potential 46.26%) motility (subjective) (88.75%) CASA (23.125%) (Chapter III, Table 1). Additionally, sperm motility showed a positive correlation with mitochondrial membrane potential and citoplasmatic membrane integrity, whereas, the velocity straight line and velocity curved-line correlated very well with the mitochondrial membrane potential and fertility. Moreover, fertility was positively related to sperm motility and mitochondrial membrane potential.

Very few studies have correlated sperm motility with fertility. Studies realized by Figueroa *et al.* (2014, 2016) in *Salmo salar* have demonstrated a relationship between sperm motility and fertlity.

Understanding the morphology and ultrastructure of sperm cells is a prerequisite to evaluating and establishing methods for analyzing sperm motility, fertilizing ability and cryopreservation conditions (Psenicka *et al.*, 2007). In addition, allow to determine the taxonomic and phylogenetic relationships at either inter- or intra-specific levels (Afzelius, 1978; Jamieson, 1991; Mattei, 1991). Fish spermatozoa are widely divergent in both morphology and ultrastructure (Ginsburg, 1968; Jamieson, 1991). In this Doctoral Thesis, morphology and ultrastructure of *G. blacodes* spermatozoa were describing through scanning and transmission electron microscopy. Our results showed that the sperm cell differentiated into a head without acrosome, a midpiece and a simple flagellar.

The head morphology of *G. blacodes* sperm is ovoid. In the mid-piece, the axoneme is separated from the plasma membrane by a cytoplasmic canal. The cytoplasmic canal is located between the axoneme and mitochondria. In the mid-piece, is possible to observe 4 or 5 mitochondria arranged in circle form. The axoneme composed the typical 9+2 microtubular doublet structure with two central and nine peripheral doublet microtubules. The flagellum of *G. blacodes* has surrounded by a cell membrane that projected to form two side-fins located on the both sides of the flagellumn. One possible role of these fin-like flagella could be to contribute to the efficiency of the thrust generated by waves, by increasing the flagellar surface used for friction on the surrounding medium. A second role could also be to contribute to the large increase in the membrane surface, due to the presence of such fins, compared with that of a simple cylindrical axoneme.

As the sperm flagellar membrane in fishes has these unusual fin-shaped folds (Cosson *et al.*, 2000), these significantly increase the membrane surface area, thus contributing to an apparent membrane excess favoring water exchange, but also can be easily distorted, eventually leading to blebs on exposure to extreme osmotic situations (Perchec *et al.*, 1996; Cosson *et al.*, 2000). In addition, comparison of the present results with the current literature on other teleosts species reveals clear differences, which could be used to determine phylogenetic and taxonomic. Briefly, the results obtained in this Doctoral Thesis allow: i) characterizing sperm *G. blacodes*; ii) controlling some factors that influence in the sperm quality iii) established a base line for further research and protocols for artificial reproduction of this species to be developed and optimized; iv) contributing to productive diversification plans through the development of biotechnological tools. Finally, we can indicate that the hypothesis of this thesis were validated according to the main results in this study.

5.1. CONCLUDING REMARKS

According to our results, we can conclude that sperm motility of *G. blacodes* intratesticular spermatozoa is initiated by hyperosmotic medium (1010 mOsm/kg, pH 8 and 8°C). Parameters such as pH, temperature and osmolality affect the sperm motility. Additionally, Sperm motility it correlates positively with the fertility, plasma membrane integrity, and mitochondrial membrane potential. In addition, our results showed that fertility it correlates positively plasma membrane integrity, mitochondrial membrane potential, VCL and VSL. Regarding morphology and ultrastructure, *G. blacodes* spermatozoa can be differentiated into three major parts: a ovoid head without an acrosome, a short mid-piece, and a long flagellum that attribute to the potential swimming ability.

The mid-piece of *G. blacodes* spermatozoa contains 4 or 5 mitochondria. The axoneme composed the typical 9+2 microtubular doublet. *G. blacodes* sperm can be categorized as being of a primitive type.

5.2. FUTURE DIRECTIONS

Aquaculture is continuing to grow and expand worldwide, in Chile, the main development prospects for aquaculture up to 2030 is the production developments connected with the diversification process with native species. Aquaculture in Chile is a promising field with a great capability for expansion and development. Recent investments in new technologies, fishing vessels, processing plants and skilled human resources have made the Chilean fishing and aquaculture industries highly competitive in a global context. Chile is making concerted efforts to manage its fisheries in a sustainable and appropriate manner. However, future fisheries and aquaculture developments will require an increased emphasis on sustainability. *G. blacodes* occupies a good position in the aquaculture industry and market. *G. blacodes* fischery can become an important tool for promoting sustainable development. Nevertheless, its culture is in the initial stages and requires large research effort to obtain essential information. Currently, *G. blacodes* fisheries sector is in an expansion phase in Chile. However, its overexploitation has meant a significant impact on levels of biomass in Chile. As a solution to overcome overexploitation, the development of crop technology to culture is considered a good solution. *G. blacodes* is a species with an unsatisfied demand in Chile. One of the strategies to protect the reproductive potential of this species is to assess the size selectivity of first capture and the size average of sexual maturity.

Undoubtedly, future growth for this species should be raised on the basis of incorporating added value to the extracted species, whose physical production does not exceed catch volumes in recent years. *G. blacodes* is a species of very easy upbringing, it has a low metabolism compared to other fishes. The main challenge for *G. blacodes* species will be to optimize and improve the production in captivity of this specie to reduce the mortality rate by improving live storage conditions from harvest to destination markets. The culture and catch *G. blacodes* resources require more attention in Chile, due to this specie is dwindling in the landings. The egg collection and larval rearing under the present technology, it allows improving the culture of *G. blacodes* resources and the replacement of wild seed stock by artificial fingerling to *G. blacodes* farms have firmly been improving in Chile. The development and implementation of a particular fishery or aquaculture management system has important repercussions in terms of environmental, economic and social outcomes. Consequently, the Chilean fishery industry needs to have important modifications in the different stages of its productive practice, including extraction and processing, as well as in the development and expansion of aquaculture. The diversification of native species and culture systems could provide an add economic, social and ecological insurance to aquaculture systems in Chile.

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Annexes

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Effect of pH, osmolality and temperature on sperm motility of pink cusk-eel (*Genypterus blacodes*, (Forster, 1801))

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ABSTRACT

In this research we evaluated simple aspects of the sperm biology of *Genypterus blacodes*, in particular assessing the effects of pH (6, 7, 8 and 9), temperature (4, 8 and 16 °C) and osmolalities 100% sea water (1010 mOsm/kg, Control), 75% sea water (774 mOsm/kg, T_1), 50% sea water (488 mOsm/kg, T_2) and distilled water (0 mOsm/kg, T_3)) on the motility of *Genypterus blacodes* intratesticular spermatozoa.

In addition, we determined the fertilization rate. Our results show that *G. blacodes* spermatozoa have a sperm density of 5.35 ± 0.16 × 10^9 spermatozoa/mL. Sperm motility is initiated on contact with a hyperosmotic swimming medium under normal conditions (1010 mOsm/kg, pH 8 and 8 °C). The longest motility duration (432.48 ± 8.89 s) was recorded at 4 °C. The maximum percentage of motile cells was recorded at 8 °C (65.66 ± 4.95) at osmolality 1010 mOsm/kg, whereas an optimum was observed at pH 8. The fertility rate was 73.9 ± 17%. This is the first report on sperm motility of *G. blacodes* spermatozoa. In conclusion, the results of this study permit a baseline to be established for further research and protocols for artificial reproduction of this species to be developed and optimized. In addition, the information gathered in this research will be useful for developing the biotechnology of *Genypterus blacodes*.

1. Introduction

In recent years, the overexploitation of fisheries has resulted in increased aquaculture production. The optimization of the reproductive performance of broodstock has been essential to obtaining high quality fry, which is imperative if the aquaculture industry is to produce high quality fish (Lahnsteiner et al., 2009). Within the order Ophidiiformes, genus *Genypterus* (*Genypterus Philippi*, 1857) contains the most economically important species. Three of these are found in Chilean waters: red cusk-eel (*Genypterus chilensis*, (Guichenot, 1848)), black cusk-eel (*Genypterus maculatus*, (Tschudi, 1846)) and pink cusk-eel (*Genypterus blacodes*, (Forster, 1801)), with the last being the most economically important (Canales-Aguirre et al., 2010).

G. blacodes is a benthic-demersal species found in the oceans around southern Australia, Chilean Patagonia, Brazil, Argentina and New Zealand in depths from 22 to 1000 m (Young et al., 1984; Francis et al., 2002; Nyegaard et al., 2004). Adults exhibit a demersal behavior and they are usually found at depths between 45 and 350 m (Cousseau and Perrotta, 2000; Nyegaard et al., 2004). The pink cusk-eel fishery is developed in Chilean waters between Coquimbo (41° and 28′S) and south of Cape Horn (57° and 00′S) (Ward et al., 2001; Wiff et al., 2007). The global market for *G. blacodes* is around 45 thousand tons per year. Spain is the main destination of *G. blacodes* exports, accounting for 72% of frozen shipments, equivalent to 563 t, and 100% of shipping fresh chilled in 2013. Spain is followed by the United States, Brazil, Russia and Portugal (Chong et al., 2014). Biological studies of this fish are scarce and they only mention its taxonomy, stomach contents (Bahamonde and Zavala, 1981; Renzi, 1986), age and growth parameters (Chong and Aguayo, 1990; Wiff et al., 2007), macroscopic and microscopic structure of the ovary in samples from its Atlantic range, description of spawning stages from Argentinean waters (Machinandiarena et al., 1998), regional morphometric variations in New Zealand (Colman, 1995), and instantaneous rate of natural mortality (Ojeda et al., 1986; Wiff et al., 2007). There is limited information about the morphological and functional aspects of the reproductive biology of *G. blacodes* (Chong, 1993; Paredes and Bravo, 2005; Freijo

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et al., 2009). The reproductive activity of this species occurs in Patagonian coastal waters south of 42 °S (Province of Chubut, Argentina), and the spawning area goes from 41 °S to 45 °S, mainly during summer (Cousseau and Perrotta, 2000). In Chile, the fecundity of *G. blacodes* in captivity is estimated to be between 66,167 and 706,658 oocytes per female (Paredes and Bravo, 2005).

In the fish, the spermatozoon is immotile in the seminal fluid and its flagellar activity is only triggered when it comes into contact with water (Alavi and Cosson, 2005, 2006). Sperm quality has been a focus of research given that it can be used as a biomarker of the status of the male fish (Chauvaud et al., 1995; Cabrita et al., 2009). Knowledge of sperm motility is a key tool to determine semen quality during artificial fertilization procedures (Alavi and Cosson, 2005; Hu et al., 2009; Valdebenito et al., 2009). According to Valdebenito et al. (2016), the parameters such as temperature, pH and osmolality affect the capacity and duration of motility in fish spermatozoa. The objective of this study was to determine the effects of osmolality, temperature and pH on sperm motility of pink cusk-eel spermatozoa; and also fertilization tests were conducted. In this study, intratesticular spermatozoa were used because for this species it is difficult to find wild fully-sexually mature male individuals; and sexual maturation in captivity has not been yet reported. The use of intratesticular spermatozoa for *in vitro* fertilization is a key tool for breeding this species in captivity and hence would allow its introduction in aquaculture industry.

2. Materials and methods

2.1. Broodstock

This study was conducted at the Engineering Biotechnology and Applied Biochemistry Laboratory (LIBBA) and at the Center for Biotechnology in Reproduction (CEBIOR), Universidad de la Frontera, Chile, as well as at the Aquaculture Biotechnology Laboratory, Catholic University of Temuco, Chile. The specimens of *G. blacodes* were caught between April and May 2017 in Puerto Montt, Region de Los Lagos, Chile with average weight of 1.96 ± 1.06 kg and a total length of 62.5 ± 4.68 cm, respectively.

2.2. Collection of gametes

This study was carried out with intratesticular spermatozoa, which were collected according to the procedure described by Cabrita et al. (2005). During transport, the specimens were kept alive. The specimens of *G. blacodes* were anesthetized by immersion with AQUI-S® (BAYER S.A. Animal Health-Chile) for a few minutes and then decapitated. Their testicles were surgically extracted and carefully cleaned with distilled water, dried and blood remnants were removed. The testis were transferred individually into an Eppendorf tube on ice and were transported using oxygenated containers with a constant temperature of 4 °C. The testes were sectioned directly in the Eppendorf tube (on ice) using a scalpel and collecting the sperm by dripping directly into a graduated, sterile, dry, disposable plastic container maintained at 4 °C. In addition, the intratesticular spermatozoa were diluted in StorFish® (Imv, Technologies, France) medium (dilution 1:1) and centrifuged twice at 1800 rpm for 5 min. Immediately after collection, sperm motility and concentration were determined using a phase contrast microscope (Carl Zeiss, Jena, Germany). Sperm density (number of spermatozoa/mL) was determined in six males using a Neubauer hemocytometer according to the methodology described by Figueroa et al. (2015) and Merino et al. (2011) for blood cells and spermatozoa at a dilution of 1 µL of sperm in 1200 µL of StorFish® medium (Imv, Technologies, France).

2.3. Activation solutions

For sperm motility activation, three solutions with different proportions of seawater (35 g/L, pH 8) and distilled water were used: control, seawater 100% (1010 mOsm/kg); T_1, seawater 75% (774 mOsm/kg); T_2, seawater 50% (488 mOsm/kg); and T_3, distilled water (0 mOsm/kg), as suggested by Cosson et al. (2008b).

These activation solutions were assessed at three different temperatures (4°, 8° and 16 °C). The osmolalities used in each activation solution were determined by a Fiske® Micro-Osmometer, 2010 model (Germany). The pH of the control (seawater) was adjusted to three pH units above and below the normal value (8) for seawater (6, 7 and 9) by adding HCl [1%] and NaOH [1%] respectively, using a pH-meter model pH 21. The effect on motility was assessed at ambient temperature of 4, 8 and 16 °C.

2.4. Assessment of sperm motility

Sperm motility was assessed in a cold-room, where temperatures of 4, 8 and 16 °C were used for the experiment. All treatments were assessed by the same person using a Nikon Eclipse E400 microscope (Japan) at 40× magnification. Approximately 4 h after the sperm were obtained, the flagellar activity periods were recorded using a chronometer from the initiation of movement (progressive motility) until the start of local circular movement (vibratory or stationary movement) (Groison et al., 2010). To assess the percentage of motile cells, values from 0 to 100% were used as suggested by Cosson et al. (2008b), on a scale from 1 to 5: 1 = 0–5%; 2 = 5–25%; 3 = 25–50%; 4 = 50–75%; 5 = 75–100%. The percentage and duration of motility were assessed in 15 aliquots of 1 µL of semen activated in 10 µL of each of the activating solutions (Control, T_1, T_2, and T_3) at temperatures of 4, 8 and 16 °C as per Cosson et al., 2010 and Valdebenito et al. (2016) using a Nikon Eclipse E400 (Tokyo, Japan) microscope at 10x magnification and with 15 repetitions per treatment for osmolality and temperature.

2.5. Fertility

Fertility was evaluated with 4.5 mL sperm + 1.5 L mass + 500 mL of seawater. It was mixed well and allowed to keep for 5 min, after which time 1.5 L of seawater was added at 12 °C. All the fertility tests were carried out five times with 200 oocytes per replication. The eggs were incubated in open flow at 10 °C. Fertilization was evaluated by observation of the first cleavages (segmentation) after 16 h incubation at 10 °C. Those with segmented blastodiscs were considered fertilized.

2.6. Statistical analysis

The motility results were analyzed with the statistical software GraphPad Prisma® version 5.0 (GraphPad Software, San Diego CA). A one-way ANOVA was used for nonparametric samples to analyze the percentage and duration of motility. Additionally, the analysis of differences between the average values of the variables of treatment groups were compared by applying the Tukey test. The level of significance was set at $p < 0.05$, n = 15 replicate. The results of the level of motility are presented as a sample mode.

3. Results

3.1. Spermatological parameters

Table 1 shows the morphometric and spermatological parameters of pink cusk-eel samples, while Table 2 shows the levels sperm motility.

3.2. Fertility and effects of pH on the duration and level of sperm motility

The rate of sperm motility of *G. blacodes* intratesticular spermatozoa and fertilization were expressed as mean ± SD. Statistically significant differences ($p < 0.05$) were observed in both duration of motility (s) and percentage of motility between pH 6, 7, 8 and 9. The highest level

Table 1
Morphometric and spermatological parameters of pink cusk-eel samples (n = 9 males).

Parameters	Minimum	Maximum	Mean	SD
Weight (kg)	0.98	4.08	1.96	± 1.06
Length (cm)	56	70	62.5	± 4.68
Sperm volume (mL)	1.2	2.5	1.47	± 0.56
Density (x 10^9/mL)	5.1	5.6	5.35	± 0.16

SD = Standard Deviation.

Table 2
Levels of sperm motility of the pink cusk-eel activated with solutions at different temperatures and osmolalities: control (100% seawater), T_1 (75% seawater), T_2 (50% seawater) and T_3 (distilled water).

Activator (mOSm/kg)	Temperature		
	4 °C	8 °C	16 °C
Control (1010)		Level	
	3	4	3

Treatment	Temperature (16 °C) Level
T_1 (774)	3
T_2 (488)	2
T_3 (0)	0

of motility was recorded at pH 8 (204. 14 ± 10.00 s and 52.66 ± 2.58% of motility) compared to other treatments: pH 6 (168.4 ± 11.18 s and 38 ± 2.35% of motility), pH 7 (186.64 ± 4.31 s and 43 ± 2.53% of motility) and pH 9 (167.41 ± 8.89 s and 37.5 ± 2.59% of motility), while there was no significant difference between pH 6 and 9 (Fig. 1 A). The determining rate for fertilization was 73.9 ± 17%.

3.3. Effects of temperature on the duration and level of sperm motility

The flagellar activity was recorded at 4 °C (Control: 432.48 ± 8.89 s and 40 ± 2.67% of motility) comparing to 8 °C (Control: 354.12 ± 29.92 s and 65.66 ± 4.95% of motility) and 16 °C (Control: 167.52 ± 18.08 s and 47 ± 2.53% of motility), while there was significant difference in flagellar activity at 16 °C (control: 167.52 ± 18.08 s; T_1: 160.06 ± 22.57 s; T_2: 70.14 ± 11.16 s) and T_3: distilled water, the motility of which was not observed (Fig. 1B).

3.4. Effects of osmolality on the duration and level of sperm motility

In terms of osmolality, significant differences were found for the control (354.12. 14 ± 29.92 s) compared to T_1 (160.06 ± 22.57 s) and T_2 (70.14 ± 11.16 s) at 16 °C (Fig. 1C). *G. blacodes* intratesticular spermatozoa were only activated on contact with seawater (1010 mOsm/kg), including seawater at lower levels of osmolality (772 mOsm/kg) and (448 mOsm/kg). The *G. blacodes* spermatozoa were immotile in the testicle and only began intense flagellar movement on contact with a hyperosmotic activation medium including seawater at lower levels of osmolality.

4. Discussion

4.1. Sperm density and motility

Cosson et al. (2008a, b) have reported in marine teleosts with external fertilization the osmolality is the main factor controlling sperm motility. However, the sperm motility rapidly decrease after activation therefore progressive movement needed by the sperm to effectively reach the egg surface is limited. According to sperm duration of motility, the spermatozoa of *G. blacodes* can move for up to 354.12 s as

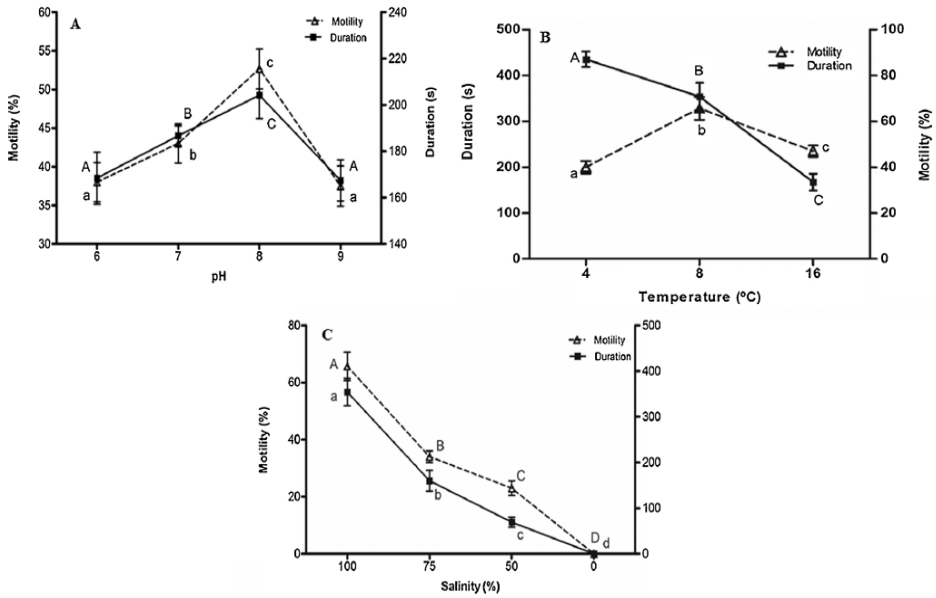

Fig. 1. A) Duration of motility (s) and percentage of motility of pink cusk-eel spermatozoa at different pH (6, 7, 8 and 9), osmolality = 1010 mOsm/kg and temperature = 16 °C. B) Duration of motility (s) and percentage of motility of pink cusk-eel spermatozoa at different temperatures (4, 8 and 16 °C), pH = 8 and osmolality = 1010 mOsm/kg. C) Duration of motility (s) and percentage of motility of pink cusk-eel spermatozoa activated in different salinity conditions, 100% sea water (1010 mOsm/kg), 75% sea water (774 mOsm/kg), 50% sea water (488mOsm/kg) and distilled water (0 mOsm/kg), pH = 8 and temperature = 16°C. The values are mean ± SD, capital and small letters indicate significant differences in motility (%) and duration of motility, respectively with $P < 0.05$ and N = 15 replicates.

shown in this study; sperm cells commence any flagellar activity only when they come into contact with a hyperosmotic medium. Also, the motility observed in *G. blacodes* are similar to what has been described for other marine fish species (Cosson, 2004; Cosson et al., 2008a, 2010; Valdebenito et al., 2009; Groison et al., 2008, 2010; Effer et al., 2013). Additionally, duration of motility of other marine species of commercial importance can be mentioned, including halibut (110–120 s), turbot (600 s), sea bass (50–60 s), cod (7–800 s) and tuna (140 s) (Cosson et al., 2008a,b).

Although the motilities presented for each of the above species were not determined under the same experimental conditions, these data allow us to indicate differences and similarities between the main marine species of commercial interest. In all these species, the spermatozoa are immotile in seminal fluid (Cierezko, 2008; Cosson, 2004; Cosson et al., 2008b,c) and only commence flagellar activity when they come into contact with a hyperosmotic medium (Cosson, 2004; Cosson et al., 2008a,b,c, 2010; Morisawa, 2008). However, the total motility time will depend on fluctuations in the parameters of the microenvironment (pH, temperature and osmolality) in which the reproduction of these species occurs (Cosson et al., 2008b; Groison et al., 2010; Morisawa, 2008).

4.2. Effect of temperature, pH and osmolality on the duration of sperm motility

In marine teleosts, sperm motility is activated by contact with seawater mainly through a positive increase in osmolality, and several factors are known to affect this process, including temperature (Alavi and Cosson, 2005, 2006). The effect of temperature on sperm motility has scarcely been studied in marine and freshwater teleostei (Morisawa, 1994; Valdebenito et al., 2009, 2016). Nevertheless, it has been reported that with a decrease in temperature, flagellar beating frequency is lower (Billard and Cosson, 1988) and the duration of motility is longer (Cosson et al., 2008a,b). In this research, our data showed that the activation medium indicated an optimum at 8 °C 354.12 s (5.9 min) compared to 4 °C 432.48 s (7.20 min) and 16 °C 167.52 s (2.79 min), when *G. blacodes* spermatozoa were activated with sea water. The spawning temperature for this species is 10–14 °C. The duration of sperm motility in fish depends on the temperature of the activation medium (Billard and Cosson, 1988).

According to Alavi and Cosson (2005, 2006), low temperatures reduce the intensity of flagellar movement, significantly prolonging motility, whereas high temperatures increase the intensity of flagellar movement, reducing the motility time. However, there have been thorough studies focusing on the specific ions that trigger sperm motility, such as K^+, Ca^{2+} and Mg^{2+} present in seawater and their influence on sperm motility activation (Cosson, 2004). *G. blacodes* spermatozoa were only activated on contact with seawater (1010 mOsm/kg), including seawater at lower levels of osmolality (772 mOsm/kg) and (448 mOsm/kg). However, motility was not found when the semen was transferred to distilled water (mOsm/kg). Valdebenito and Figueroa (unpublished data) point out that the maximum mean duration recorded for *G. chilensis* was 1346 s in a saline solution (928 mOSm/kg) and for the black conger eel it was 1470 s in saline solution (754 mOsm/kg). Nevertheless, motility in marine fish may occur in a wide range of osmolalities, for example in Atlantic halibut (*Hippoglossus hippoglossus*) it occurs between 900 and 1100 mOsm/kg (Billard et al., 1995), in *Dicentrarchus labrax* over 300 mOsm/kg (Chauvaud et al., 1995; Dreanno et al., 1999), and in (*Sarathoredon melanotheron*, (Rüppell, 1852)) between 300 and 970 mOsm/kg (Legendre et al., 2008). A recent study conducted by Valdebenito et al. (2016) demonstrated that osmolality of the activation medium (Control = 815 mOsm/ kg; T_1 = 716 mOsm/kg; T_2 = 590 mOsm/kg) influenced the sperm motility of Patagonian blenny (*Eleginops maclovinus*), a species that lives in the same areas as *G. blacodes*. The highest flagellar activity (percentage of motile sperm and duration) was shown at pH = 8. This is similar to what Effer et al. (2013) described for *M. australis*, a fish that also lives in the same areas as *G. blacodes*. The pH of the activation solution affects sperm motility to a low extent (Cosson, 2004).

It is generally accepted that the pH or ions present in the activation solution polarize the cell membrane and stimulate the motility of fish spermatozoa by changing the Na^+/K^+ permeability (Morisawa and Morisawa, 1988; Boitano and Omoto, 1991). The pH of the internal cytoplasm is one of the most important factors that affect sperm motility (Woolsey and Ingermann, 2003). The alkaline nature (pH = 8) of the activation solution seems to be the most suitable for species such as turbot (Chauvaud et al., 1995), halibut (*Hippoglossus stenolepis*) (Billard et al., 1993). According to Cosson et al. (2008b,c), the pH may reduce or prolong motility, but it is not the principal parameter in motility initiation, despite intracellular pH playing a key role in sperm maturation *in vivo* (Cierezko, 2008).

In conclusion, in the current study, we showed that the motility of *G. blacodes* intratesticular spermatozoa is initiated by hyperosmotic medium. Varying temperature and osmolality of the activation medium demonstrated an optimal effect on sperm motility, whereas the pH of the activation solution affected sperm motility to a lesser extent. The fertilization rate was high at 73.9%; however, more tests need to be done to improve these results. Finally, considering the importance of fish reproduction in captivity and in the wild, it is advisable to deepen research in cellular aspects, thus providing useful information for the elaboration of means of cultivation and other inputs used in the management of gametes of this species.

Conflict of interest

The authors have declared that they have no conflicts of interest.

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Protein phosphorylation and ions effects on salmonid sperm motility activation

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Abstract

Sperm motility is considered as a key factor allowing determination of semen quality and predicts fertilizing capacity. In many fish species, the spermatozoa are immotile in the testes and seminal plasma, and motility is induced when they are released in the aqueous environment. Initiation and activation of sperm motility are prerequisite processes for the contact and fusion of male and female gametes at fertilization. Many proteins are involved in the activation of sperm motility in many species. Cell signalling for the initiation of sperm motility in the salmonid fish has drawn much attention during the last two decades. In some species, protein phosphorylation process was shown to be involved in flagellar motility regulation. Hyperpolarization of the sperm membrane induces synthesis of cAMP (cyclic AMP), which triggers further cell signalling processes, such as cAMP-dependent protein phosphorylation that finally initiates sperm motility in salmonid fish. Ions such as Na^+, K^+ and Ca^{2+} play also an important role in the activation of sperm motility in many species, more specifically in salmonids. Salmonid fish sperm motility can be suppressed by millimolar concentrations of extracellular K^+, and dilution of K^+ upon spawning is enough to trigger the cAMP-dependent signalling cascade leading to motility initiation. This review aims to update the present knowledge about the roles of ions and protein phosphorylation process in the sperm motility activation in salmonids.

Key words: cell signalling, fish spermatozoa, motility initiation, protein phosphorylation, sperm motility.

Introduction

Sperm motility is a key prerequisite determining the quality and fertilizing ability of semen. Motility is a minimal condition that enables the spermatozoon to reach the oocyte to fertilize it successfully and has been considered to be one of the principal variables of sperm quality in fish (Rurangwa *et al.* 2004) as it is an integrated quality variable, combining various cell components responsible for the activation and sustainability of the motility and progressive movement of the spermatozoon (Bobe & Labbé 2010). The osmolality and ion content (K^+, Na^+, Ca^{2+}, Mg^{2+}) of the aquatic medium are central factors in activating motility (Figueroa *et al.* 2014). Extracellular factors such as temperature, osmotic pressure, pH, dilution and gaseous components such as nitric oxide or CO_2 affect the percentage and duration of fish sperm motility (Inaba 2003; Cosson 2004; Wilson-Leedy & Ingermann 2011; Barman *et al.* 2013; Dzyuba & Cosson 2014). The mechanisms involved in activating sperm motility are considered of vital importance in regulating processes such as artificial fertilization and cryopreservation. In fish with external fertilization, the activation of sperm motility is triggered by various factors such as ionic changes (K^+ and Ca^{2+}), decrease in osmolality in freshwater species, increase in osmolality in saltwater species (Cosson 2010; Dzyuba & Cosson 2014) or a combination of some of these factors. Hypotonic exposure after dilution into freshwater is the triggering signal in non-salmonid freshwater fish such as carp (Krasznai *et al.* 2003; Morita *et al.* 2003). These changes occur when the

spermatozoa leave the seminal fluid (osmolarity ranging 300 mOsmol kg^{-1}) and come into contact with external water (osmolarity ranging 0 mOsmol kg^{-1}), following their ejaculation during the reproduction process (Cosson 2012). In salmonids, the inhibition of motility in semen is mainly controlled by the concentration of K^+ ion, the most important factor for initiation of sperm motility (Alavi *et al.* 2004). In the signalling cascade that regulates sperm motility, the best characterized factors are cAMP and Ca^{2+} (Walczak & Nelson 1994): cAMP-dependent phosphorylations of axonemal proteins have been reported to regulate the motility of sperm in salmonid fish (Morisawa & Okuno 1982). In teleost fish, the phosphorylation and dephosphorylation processes play an important role in the mechanism of spermatozoa motility activation (Morita *et al.* 2006; Zilli *et al.* 2008). For sperm initiation, motility activation and hyperactivation, cAMP-dependent protein phosphorylation plays a role important triggering in many organisms including sea urchin, salmonid fish and mammals (Zilli *et al.* 2016). In salmonids, a cAMP-dependent protein phosphorylation is induced by membrane hyperpolarization as a result of K^+ efflux and Ca^{2+} influx that triggers cell signalling for initiation of sperm motility (Morita *et al.* 2005). The results of several studies suggest that protein phosphorylation/dephosphorylation events are involved in sperm motility activation in many fish species having either internal or external mode of fertilization (Zilli *et al.* 2016).

Some membrane proteins are also involved in the sperm motility initiation in fish, in relation to osmoregulation (Cerdá & Finn 2010; Chauvigné *et al.* 2013). The analysis of protein phosphorylation within intact cells may represent with best accuracy the status of specific signalling networks. Regulation of sperm motility has been linked to cyclic adenosine monophosphate (cAMP) signalling pathways in several animal species, including mammals (Tash & Bracho 1994), salmonid fish (Morisawa & Okuno 1982), sparid seawater teleosts (Zilli *et al.* 2008), tilapia (Morita *et al.* 2003) and even invertebrates such as sea urchins (Bracho *et al.* 1998) or ascidians (Nomura *et al.* 2000). In salmonid fish sperm, the cAMP-dependent phosphorylation (by protein kinase A) of axonemal proteins is essential for the initiation of sperm motility (Ikegami *et al.* 2010).

Inhibition and activation of sperm motility in Salmonidae

Spermatozoa are immobile in fish testis and also in seminal plasma in many species (Muller *et al.* 2014). The seminal plasma analysis includes inorganic constituents (Na^+, K^+, Ca^{2+} and Mg^{2+}) involved in the process of inhibition or activation of sperm motility in most species. In salmonid fish, transmembrane cell signalling for the initiation of sperm motility is controlled by the changes under environmental ionic conditions at spawning from the male reproductive tract to the spawning ground, which is freshwater in the external fertilization species (Kho *et al.* 2005). In fish with external fertilization, the activation of sperm motility is triggered by ionic changes (K^+ and Ca^{2+}) in synergy with reduced osmolality in freshwater species and augmented osmolality in saltwater species (Cosson 2010; Dzyuba & Cosson 2014). In salmonids, a decrease in environmental K^+ concentration causes K^+ efflux through specific membrane channels, leading to membrane hyperpolarization, which, in turn, determines the Ca^{2+} influx by calcium channels (Fig. 1) (Alavi & Cosson 2006; Figueroa *et al.* 2015 modified). The response of sperm cells of different fish species to activation solutions may be different because of seminal plasma Ca^{2+} and K^+ concentration changes among species. However, the decrease in extracellular K^+ in Salmonidae is the sperm motility activator (Morisawa & Suzuki 1980). Generally, fish spermatozoa present a simplified structure as compared to mammal sperm (Cosson 2008). In freshwater spawning fish, spermatozoa experience a hypo-osmotic change, that is a transfer into a medium with lower osmolality, as they are released from the seminal plasma into freshwater, a medium where their motility may last less than two minutes after activation (Alavi & Cosson 2005; Gasparini *et al.* 2010). Again, the link between cAMP increase and motility initiation at the axoneme level was mainly investigated in Salmonidae (Jin *et al.* 1994). The cAMP and Ca^{2+}, as second messengers, play key roles in the initiation of sperm motility in fish (Zilli *et al.* 2008). The Ca^{2+} ion concentration regulates sperm motility apparently by acting as a cofactor of protein kinases or phosphatases (Tash *et al.* 1988). In some fish species, protein phosphorylation/dephosphorylation is involved in flagellar motility regulation (Zilli *et al.* 2009). Dilution of external K^+ induces an intracellular K^+ efflux and an intracellular Ca^{2+} ion concentration increases (Cosson *et al.* 1989).

Effects of ions on sperm motility in Salmonidae (K^+ and Ca^{2+})

K^+ ion

The intracellular signalling for the activation of sperm motility in teleosts has been well investigated in salmonid fish (Kho *et al.* 2005). Motility is initiated by a decrease in potassium ions (K^+) concentration in salmonid fish surrounding the spawned spermatozoa, when they are released into freshwater, due to dilution of semen in solutions containing low K^+ concentration. Dilution media containing K^+ concentrations that are elevated in relation to the ionic composition and osmotic pressure of the seminal plasma eliminate the initiation of sperm motility in salmonids (Billard *et al.* 1995; Kho *et al.* 2001; He & Woods 2003).

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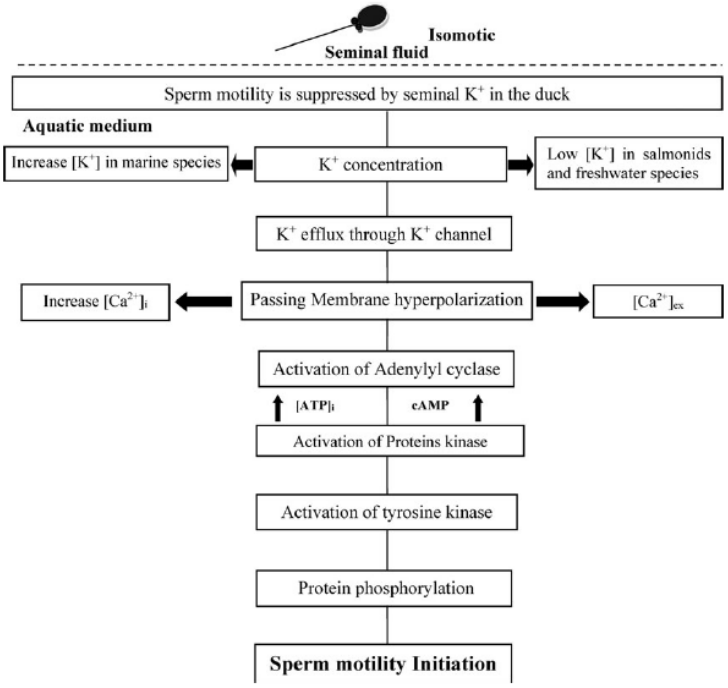

Figure 1 Model of sperm motility activation in salmonids. The spermatozoa are immobile in the seminal fluid (testicle) because SF is an iso-osmotic medium and due to the presence of specific proteins (SPP). Motility activation occurs (aquatic medium) at a low potassium concentration in the case of salmonids and in other species in response to a reduction in osmolarity (most other freshwater species). Change in osmolality of the external milieu would lead to membrane polarization changes mainly because of variation in the internal concentration of K^+, Ca^{2+} and a cyclic AMP (cAMP)-independent mechanism for activation of sperm motility. In salmonid fish, sperm motility is suppressed by high seminal K^+. A decrease in K^+ concentration surrounding spawned sperm in freshwater causes K^+ efflux, through the K^+ channel, resulting in hyperpolarization of the plasma membrane of the sperm flagellum. This increase in membrane potential could directly activate adenylyl cyclase, which provokes an increase in intracellular cAMP concentration. Increased intracellular Ca^{2+} may also activate this enzyme in cooperation with membrane hyperpolarization. The ATP content becomes lower because the renewal by mitochondrial phosphorylation is too slow. Following these stages in the process, cAMP activates protein kinase A (PKA), resulting in activation of tyrosine kinase and phosphorylation that triggers a final step leading to the initiation of sperm motility (Alavi & Cosson 2006; Figueroa *et al.* 2015 modified).

Woolsey *et al.* (2006) report the important influence of the ionic composition of the external environment on the sperm motility and how ion channels are essential elements for this cellular process. The effect of K^+ on sperm motility in other teleost fish is less clear, but it has been determined that it does not inhibit flagellar motility in some species (Morisawa 1994). Cosson *et al.* (1999) reported that, in trout, monovalent ions such as Na^+ and bivalent as Ca^+ Sr^{2+}, Ba^{2+} and Mg^{2+} reduce the inhibitory effect of K^+ and bivalent cations are more effective than monovalent. In addition, it has been reported that the inhibition by K^+ ions is mainly regulated by Ca^{2+} ions, possibly due to simultaneous flow of Ca^{2+} and K^+ (Cosson *et al.* 1999); the output of K^+ favours the opening of Ca^{2+} channels and the income of the same towards the inside of the cell; the entering Ca^{2+} favours the release of stored intracellular Ca^{2+} and modifies the pH_i (intracellular pH) that represents the first signal, independent of the cAMP concentration for the start of the mobility in carp (Krasznai *et al.* 2000, 2003). Currently, it is known that the presence of Ca^{2+} and cAMP is important for sperm motility initiation in salmonids to counteract the inhibitory effect of K^+ (Cosson 2008). The initiation of sperm motility is inhibited in media containing 20–40 mM of K^+ ions, concentrations that are normally present in the seminal plasma (Scheuring 1924; Turdakova 1970; Morisawa & Suzuki 1980; Baynes *et al.* 1981; Cosson *et al.* 1989). The inhibitory K^+ concentration for sperm activation has been reported between 0.1 and 2 mM in salmonids and between 10 and 40 mM in different carp species (Billard *et al.* 1987).

Potassium ions also inhibit motility at very low concentrations (in the range of 0.01 mM) in paddlefish and

shovelnose sturgeon spermatozoa (Cosson & Linhart 1996; Cosson *et al.* 2000). A K^+ concentration at 20–40 mM (Billard *et al.* 1987; Cosson *et al.* 1989) completely suppresses sperm motility in the rainbow trout. The inhibition of motility in salmonids is mainly due to K^+ ions. In other words, membrane hyperpolarization caused directly by transmembrane K^+ efflux is the first trigger for initiating sperm motility in salmonid fish. A similar role of potassium ions is also occurring in chondrostean fish (sturgeons); in those species, K^+ ion inhibition of motility can be bypassed by a hyperosmotic shock (Prokopchuk *et al.* 2016).

Ion Ca^{2+} as second messenger

The Ca^{2+} ions play important roles in the control of the ciliary/flagellar activity, and it is a ubiquitous intracellular messenger, which encodes information by temporal and spatial patterns of concentration. In spermatozoa, Ca^{2+} plays a key role in the initiation of sperm motility. Experimental evidence suggests that spermatozoa possess sophisticated mechanisms for the regulation of cytoplasmic Ca^{2+} concentration and the generation of complex Ca^{2+} signals (Cosson *et al.* 1989; Krasznai *et al.* 2000). An increase of Ca^{2+} probably activates the adenylyl cyclase (AC) leading to a cAMP-dependent protein phosphorylation that in turn activates axoneme. The AC/cAMP pathway plays an important role in sperm motility initiation in mammals (Nolan *et al.* 2004), amphibians (O'Briena *et al.* 2011) and fish (Zilli *et al.* 2008). The Ca^{2+} apparently regulates sperm motility by acting as a cofactor of protein kinases or phosphatases (Tash *et al.* 1988).

Calcium ions interact with an axoneme-located calmodulin (CaM) determining the activation of a Ca^{2+}/CaM-dependent protein phosphorylation that in turn triggers sperm motility (Dymek & Smith 2007). Intracellular cAMP concentration controls the net level of phosphorylation of certain specific proteins, especially protein kinase A (PKA) (Leclerc *et al.* 1996) that directly leads to initiation of axoneme movement in mammals. Calcium increased cAMP through activation of adenylyl cyclase in the spermatozoa of sea urchins and salmonid fish (Cook *et al.* 1994). Both adenylate cyclase activity and the cAMP concentration increase at motility initiation in intact trout sperm (Morisawa & Ishida 1987). The addition of a minimal concentration of cAMP is necessary for reactivating the demembranated trout spermatozoa (Morisawa & Okuno 1982), and cAMP must be permanently present to sustain axonemal motility (Cosson *et al.* 1995); the same authors also show in this paper that cAMP and ATP act synergestically. In salmonid fish, a transient increase in $[Ca^{2+}]_i$ occurs due to the release of Ca^{2+} from intracellular stores (Boitano & Omoto 1992). Three different mechanisms of action have been proposed for physiological roles of Ca^{2+}: (i) Ca^{2+} would act directly on the axonemal structures in case of sea bass and tuna (Cosson *et al.* 2008b); (ii) Ca^{2+} regulates Ca^{2+}/calmodulin-dependent protein phosphorylation that, in turn, activates the axoneme (e.g. in puffer fish or seawater-acclimated euryhaline tilapia *Oreochromis mossambicus*) (Krasznai *et al.* 2003; Morita *et al.* 2004); (iii) Ca^{2+} leads to a cAMP-dependent protein phosphorylation that activates axoneme in gilthead sea bream and striped sea bream (Zilli *et al.* 2008). Calcium ions have different effects on the flagellum: (i) it determines its activation to start motility; (ii) it changes the flagellar beating pattern; (iii) it modifies spermatozoa velocity (Dzyuba *et al.* 2013).

Both pathways (cAMP/PKA and calcium/calmodulin/CaMK) are upstream regulators of AMP-activated kinase (AMPK) that play a role in mammal sperm motility regulation (Hurtado de Llera *et al.* 2014). In carp and sturgeon spermatozoa, recent results show that PKA and PKC are involved in the phosphorylation of several sperm proteins that are implicated in the regulation of the motility period (Gazo *et al.* 2015). Furthermore, in the euryhaline tilapia *Sarotherodon melanotheron heudelotii* the sensitivity to external Ca^{2+} ions concentration is adapted to the environmental (freshwater, marine or hypersaline) conditions of the fish (Legendre *et al.* 2016).

Motility signalling pathways

In spermatozoa of externally fertilizing fish species, sperm motility is one of the most important viability parameters and the major ATP-utilizing process (Cosson 2004, 2008). Numerous studies suggest that the characteristics of motility of fish spermatozoa are related to their fertilizing capacity (Rhemrev *et al.* 2001). Motility of spermatozoa is sustained by hydrolysis of ATP catalysed by dynein ATPase, which is coupled with sliding of adjacent microtubules leading to the generation of flagella beating (Gibbons 1968, 1981). Motility activation of fish spermatozoa is a fraction of a second lasting process, making studies biochemical processes are necessary to understand that occur in sperm motility and during fertilization (Kowalski *et al.* 2003; Wojtczak *et al.* 2003; Cosson 2004). Spermatozoa motility signalling is a complex and highly orchestrated process that has not been only partially studied in fish (Alavi *et al.* 2008; Morisawa 2008; Cosson 2010). The extracellular factors controlling sperm motility (osmolality, ions, sperm-activating peptides and chemoattractants) act on the flagellar motile apparatus, the axoneme, through signal transduction across the plasma membrane (Alavi & Cosson 2006; Cosson 2010, 2016; Dzyuba & Cosson 2014). In salmonid fish spermatozoa, the cAMP-dependent phosphorylation (by protein kinase A) of axonemal proteins is essential for the initiation of sperm motility (Inaba *et al.* 1998). The cAMP-independent initiation of flagellar motility in sperm

has also been observed in puffer fish (Morisawa 1994), striped bass (Shuyang *et al.* 2004) and carp (Cosson & Gagnon 1988). Second messengers (cAMP and Ca^{2+}) determine the sperm motility initiation modifying dynein-mediated sliding of the axonemal outer-doublet microtubules through protein phosphorylation in different species, such as mammals (Lindemann & Kanous 1989), rainbow trout, chum salmon, sea urchin (Inaba *et al.* 1999), sea bass (Zilli *et al.* 2012) and tunicate (Nomura *et al.* 2000). In some freshwater teleosts, the flagellar axoneme is regulated by calcium/calmodulin-dependent protein phosphorylation (Krasznai *et al.* 2000; Morita *et al.* 2006).

Protein phosphorylation in the activation of sperm motility

The final event in the mechanism of sperm motility initiation is the transmission of the exogenous signals to the axoneme (Dzyuba & Cosson 2014; Zilli *et al.* 2016). In some fish species, protein phosphorylation/dephosphorylation is involved in flagellar motility regulation (Zilli *et al.* 2012). The cAMP-dependent phosphorylation of flagellar proteins is required for the initiation and maintenance of sperm motility. The major targets of the protein phosphorylation/dephosphorylation, which cause the activation of sperm motility, are structural components of inner and outer dynein arms, kinases and phosphatases anchored in the axoneme and the radial spoke proteins (Porter & Sale 2000; Yanga & Tierscha 2009). Kinases and phosphatases are required for local control of motor activity (Aparicio *et al.* 2007), and radial spoke proteins regulate inner arm dynein by phosphorylation/dephosphorylation.

Recent studies demonstrated that different factors could affect phosphorylation of sperm proteins after motility activation in fish (Zilli *et al.* 2011; Li *et al.* 2013; Gazo *et al.* 2015). In puffer fish and tilapia sperm, the activity of the flagellar axoneme is regulated by Ca^{2+}/calmodulin-dependent protein phosphorylation, whereas in gilthead sea bream and striped sea bream, it is regulated by cAMP-dependent protein phosphorylation (Zilli *et al.* 2009; Dzyuba *et al.* 2010). Protein phosphorylation occurs during initiation and activation of sperm motility in salmonid fish, echinoderms and mammals (Nomura *et al.* 2000). The motility of spermatozoa is initiated and maintained by the hydrolysis of ATP catalysed by dynein ATPase, which is coupled with sliding of adjacent microtubules, leading to the generation of flagellar beating (Gibbons 1981; Okamura *et al.* 1985; Bracho *et al.* 1998). Dyneins are ATPases capable of transducing chemical energy derived from the hydrolysis of ATP into the mechanical force necessary for cilia and flagella bending (King *et al.* 1986). Following phosphorylation, the dynein ATPase is activated and microtubule sliding occurs. A prerequisite of spermatozoa motility is that hydrolysis of ATP be catalysed by dynein ATPase, which liberates chemical energy and is coupled with mechanical sliding of adjacent microtubules (Tash 1989).

Proteins involved in sperm motility activation in different fish

Furthermore, various glycoproteins have been reported to be motility activators (Figueroa *et al.* 2015). Billard (1983) found that the sperm dilution rate could have an influence on the fertilizing ability of salmonid spermatozoa and suggested that possibly, the presence of some proteins in seminal plasma may play a role in the sperm protection mechanisms. Moreover, it was demonstrated that after fractionation of seminal plasma proteins, several fractions were found to be the most effective for saving sperm longevity (Lahnsteiner 2007).

While multiple forms of proteolytic enzymes exist in seminal plasma of teleosts and differ among fish families and species, the exact role of these enzymes remains poorly understood (Table 1). It was also suggested that transferrins and lipoproteins found in fish seminal plasma participate in the protection of spermatozoa during storage in the spermatic duct, together with proteinase inhibitors (Cierezko 2008).

Axoneme dynein ATPase activation

Phosphorylation of axonemal dynein appears to be a critical regulatory point in the initiation of flagellar motility. Following this phosphorylation step, the dynein ATPase is activated and microtubule sliding occurs. Right after, dephosphorylation of dynein by the calmodulin-dependent protein phosphatase calcineurin occurs and reverses this process. This requires that phosphorylation and dephosphorylation are acting in an asynchronous manner along the length of the axoneme (Luconi *et al.* 2011). In marine fish, the activation of axoneme is achieved by different mechanisms (Zilli *et al.* 2012). In sea bass and tuna spermatozoa, the key factor to start the beating of the flagella is the variation of intracellular ionic strength (Alavi & Cosson 2006; Cosson *et al.* 2008a). In herring sperm, increasing concentration of calcium ions is the main factor that determines the activation of the axoneme. In this case, a sperm motility initiation factor (SMIF) liberated by the egg induces calcium influx by opening the voltage-gated calcium channels and activating a reverse Na^{+}/Ca^{2+} exchange (Vines *et al.* 2002).

The presence of SMIF provokes an approximately fourfold increase in the sperm intracellular Ca^{2+} concentration that acts on the axoneme and induces its motility (Cherr *et al.* 2008). The major targets of protein phosphorylation/

Table 1 Proteins involved in sperm motility activation and in seminal plasma in different fish

Proteins	Species	Functions/roles	References
Metalloproteinase; serine proteases	Salmonid; Cyprinid; Percid	Can regulate spermatogenesis by activation of proenzymes and prohormones, stimulation of sperm motility and metabolism and removal of immature and damaged spermatozoa at the end of spawning	Kowalski *et al.* (2003)
Acid phosphatase; Alkaline phosphatase; β-D-glucuronidase	Salmonid; Cyprinid	Responsible for elimination of degenerating spermatozoa at the end of spawning	Lahnsteiner *et al.* (1998)
Inhibitor II α1-Antiproteinase	*Oncorhynchus mykiss*; *Cyprinus carpio*	May participate in protection of spermatozoa from proteolytic attack	Mak *et al.* (2004) and Wojtczak *et al.* (2007)
Transferrin	*C. carpio*	May protect spermatozoa against microbes, oxidative and heavy metal toxicity	Wojtczak *et al.* (2007)
Lipoproteins	*O. mykiss*	Interaction with sperm plasma membranes to maintain optimal lipid composition during storage in the spermatic duct	Loir *et al.* (1990)
Malate dehydrogenase; lactate dehydrogenase (LDH); aspartate aminotransferase; adenosine triphosphatase	Salmonid; Cyprinid; *Eleginus lucíś*, *Perca flavescens*	Enzymes involved in metabolism	Lahnsteiner *et al.* (1998)
Ca^{2+}-binding protein	*Oreochromis mossambicus*	Regulates the flagellar motility in a calcium-dependent manner by modifying both the sliding velocity and flagellar waveform	Morita *et al.* (2009)
Ca^{2+}/CaM-dependent protein kinase IV	*O. mossambicus*	Localized along the flagellum and sleeve structure, that is involved in the activation and regulation of sperm flagellar	Morita *et al.* (2006)
A-kinase anchor proteins	*Sparus aurata*	AKAP as a key regulator of sperm motility has been already established. Have the function of binding to the regulatory subunits (RI and RII)	Zilli *et al.* (2008)
Acetyl-CoA synthetase	*S. aurata*	This enzyme could be activated in motile sperm to increase the level of ATP, which is necessary for flagellar movement	Zilli *et al.* (2008)
Novel protein similar to phosphatase and actin regulator 3 of *Danio rerio*	*S. aurata*	May be a protein phosphatase inhibitor	Zilli *et al.* (2008)
Myotubularin-related protein 1	*Lithognathus mormyrus*	Belongs to the protein tyrosine phosphatase family, and DYRK3 is a protein kinase and regulated kinase family	Zilli *et al.* (2008)

dephosphorylation causing the activation of sperm motility are structural components of dynein arms (inner and outer), kinases and phosphatases anchored in the axoneme and in the radial spoke proteins (Yang *et al.* 2001). Kinases and phosphatases are required for local control of motor activity (Aparicio *et al.* 2007), and radial spoke proteins regulate inner arm dynein by phosphorylation/dephosphorylation. Dynein constitutes one of the major families of molecular motors that produce directed movement along axonemal microtubules (Dzyuba & Cosson 2014). The ATP is probably the main source of energy for sperm motility (Ingermann *et al.* 2003; Zilli *et al.* 2004; Cosson 2012). In fish species such as trout (Christen *et al.* 1987), carp (Perchec *et al.* 1995), catfish (Linhart *et al.* 2004), sea bass (Dreanno *et al.* 1999), sturgeon (Billard *et al.* 1999) or turbot (Dreanno *et al.* 1999), ATP stores showed shifting down to values reaching one-third to one-tenth of the initial content during intensive motility phase. Nevertheless, it is worth to remark that final ATP concentration at the end of a motility phase is usually high enough to fulfil the dynein ATPase requirements (Cosson 2004). Motility of spermatozoa is sustained by ATP hydrolysis catalysed by dynein ATPases, which are coupled with sliding of adjacent microtubules leading to the generation of flagellar beating. Intracellular ATP is the main energy source in spermatozoa, but it also acts as a substrate for the generation of the second messenger cAMP by adenylyl cyclases and serves as a phosphate donor for protein phosphorylation (Miki *et al.* 2004; Cao *et al.* 2006). The immediate source of mechanical energy for motility is hydrolysis of ATP catalysed by dynein ATPase.

The production of ATP is insured by the sperm mitochondria, in addition to a biochemical shuttle present in a flagellum that involves other molecules with high-energy

bonds and are in charge of distributing homogenously the ATP concentration along the flagellar compartment (Tombes *et al.* 1987; Saudrais *et al.* 1998). Regulative aspects of flagella activity, which are under control of ATP-related molecules such as cyclic AMP (cAMP) in sperm of many species, will also be reviewed. It has been shown for freshwater fish that during sperm movement under hypotonic conditions, a reduction in the ATP concentration and a rise in the sperm cell volume are observed due to water penetration into the spermatozoon due to the highly hypotonic environment (Dzyuba *et al.* 2010; Bondarenko *et al.* 2013). Dyneins are microtubule-dependent force-generating enzymes and constitute one of the major families of molecular motors producing directed movement along axonemal microtubules (Mocz & Gibbons 2001). Dyneins are ATPases capable of transducing chemical energy derived from the hydrolysis of ATP into the mechanical force necessary for bending of cilia and flagella (King *et al.* 1986). For motility activation, the activity of dynein, the molecular motor, has to be initiated and regulated to produce the coordinated sliding of microtubules in the axoneme (Cosson & Prokopchuk 2014; Zilli *et al.* 2016). The dynein ATPase of the axoneme utilizes ATP to bring about movement of the flagellum (Suarez & Ho 2003). Flagellar dynein activity is regulated by phosphorylation. The dynein ATPases belong to a family of molecular motors responsible for diverse cellular functions, not only axonemal beating but also includes retrograde microtubule-based transport of organelles, assembly and function of the Golgi and mitotic apparatus (Brokaw 2009). It has been revealed that dynein is one of the major targets of cAMP-dependent protein phosphorylation (Alavi *et al.* 2008; Lahnsteiner *et al.* 2010).

Conclusions

Motility activation of spermatozoa is a complex and highly organized process especially for salmonids. The ions such as Na^+, K^+ and Ca^+ play an important role in the sperm motility activation. Various studies have shown that phosphorylation of sperm proteins is an important aspect of capacitation and is associated with hyperactivated motility in mammals, and extensive research has also started to elucidate various pathways involved in protein phosphorylation during sperm capacitation. However, the role of protein phosphorylation regulation in sperm motility and the understanding of the links between the different phosphorylated proteins have not been studied in details in fish, yet. Some species, such as puffer fish, tilapia, gilthead sea bream and striped sea bream protein phosphorylation/dephosphorylation, have shown to be involved in flagellar motility regulation. The exact role of protein phosphorylations in the mechanisms that sustain sperm motility remains unclear, and the topic requires further comprehensive interspecific studies.

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Journal: Tissue and Cell

Dear Professor Farias,

I am pleased to inform you that your paper has been accepted for publication and will proceed to copy-editing and production.

Thank you for submitting your work to Tissue and Cell . We hope you consider us again for future submissions.

Kind regards,

Pietro Lupetti
Editor
Tissue and Cell

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