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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

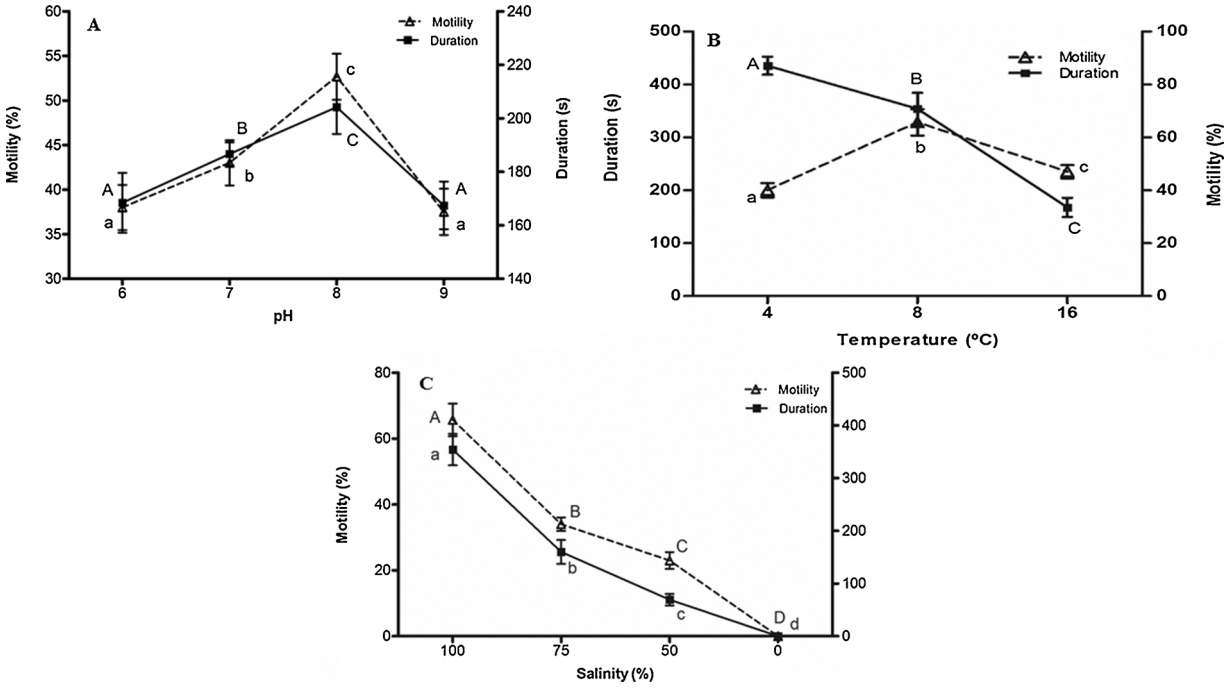

Fig. 1. A) Duration of motility (s) and percentage of motility of pink cusk-eel spermatozoa at different pH (6, 7, 8 and 9), osmolality = 1010 mOsm/kg and temperature = 16 ºC. B) Duration of motility (s) and percentage of motility of pink cusk-eel spermatozoa at different temperatures (4, 8 and 16 ºC),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