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ASSOCIATION BETWEEN THE PRESENCE OF A 38 kDa FACTOR IN THE SEMINAL PLASMA AND INHIBITION OF SPERM MOTILITY IN JUNDIÁ FISH *Rhamdia quelen*

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ABSTRACT

Protein factors have been identified in the seminal plasma of fish and mammal species and, in some situations, associated to sperm quality indicators. However, for jundiá fish (*Rhamdia quelen*), such factors and those potential associations remain unknown. In the present study, we aimed to identify some protein factors present in the seminal plasma of jundiá fish and to evaluate their association to sperm motility. SDS-PAGE was used to identify 14 bands, with molecular weight ranging from 217.1 to 7.1 kDa. Sperm motility was evaluated for 21 males. Four protein bands (81.5; 60.4; 33.6; and 25.5 kDa) were present in all seminal plasma samples. One protein band with molecular weight of 38.3 kDa was associated to reduced sperm motility of jundiá ($P<0.01$), since it was detected in 91.4% of the samples having motility lower than 80%. These results suggest that this seminal protein band associated to lower sperm motility may be considered a potential biochemical marker for sperm quality.

KEYWORDS: Protein, *Rhamdia quelen*, SDS-PAGE, seminal plasma, sperm motility.

RESUMO

ASSOCIAÇÃO ENTRE A PRESENÇA DE UM FATOR DE 38 kDa NO PLASMA SEMINAL E A INIBIÇÃO DA MOTILIDADE ESPERMÁTICA NO JUNDIÁ *Rhamdia quelen*

Fatores proteicos tem sido indentificados no plasma seminal de peixes e mamíferos e, em algumas situações, associados com indicadores de qualidade espermática. Entretanto, para o jundiá (*Rhamdia quelen*), tais fatores como aqueles com potenciais associações ainda não foram descritos. Os objetivos deste estudo foram de identificar alguns fatores proteicos presentes no plasma seminal do jundiá e avaliar suas associações com a motilidade espermática. Através de eletroforese do tipo SDS-PAGE foram identificadas 14 bandas proteicas com peso molecular entre 217.1 e 7.1 kDa. A motilidade espermática foi avaliada em 21 machos. Quatro bandas proteicas (81.5; 60.4; 33.6 e 25.5 kDa) foram detectadas em todas as amostras de plasma seminal analisadas. Uma banda proteica com peso molecular de 38.3 kDa foi associada com a baixa motilidade espermática no jundiá ($P< 0,01$), uma vez que foi detectada em 91.4% das amostras com motilidade menor que 80%. Estes resultados sugerem que esta banda proteica seminal associada com a baixa motilidade espermática poderá ser considerada como um potencial marcador bioquímico de qualidade seminal.

PALAVRAS-CHAVES: Motilidade espermática, plasma seminal, proteínas, *Rhamdia quelen*, SDS-PAGE.

INTRODUCTION

The jundiá fish (*Rhamdia quelen*) is endemic in South America. It is a teleost specie from the Pimelodidae family, commonly known as "Silver catfish", and has great relevance for aquaculture systems in temperate and subtropical climates. Although its habitat extends from Southern México to Central Argentina, it is spreading in Southern Brazil. Fish farmers are interested in its culture because it presents omnivorous feeding habits, good growth rate, high fertilization and hatching rates, and good acceptance by the consumers (BARCELLOS et al., 2001; GOLOMBIESKI et al., 2003).

In teleost fish species, the efferent duct system (testicular main ducts and spermatic ducts) synthesizes the seminal plasma, besides acting in sperm storage and having phagocytotic activity (LAHNSTEINER et al., 1993a, b; LAHNSTEINER et al., 1994). The seminal plasma provides the fluid media for spermatozoa transport, but, in all the studied fish species, spermatozoa present in the seminal plasma have no motility. The acquisition of motility is mainly controlled by variation in the osmotic pressure, which, in the environment, is caused by the contact of the sperm cells with water (ALAVI & COSSON, 2005).

The composition of the seminal plasma in mammals and fish is similar, including monosaccharides, lipids, proteins, ions, sugars, and other molecules, which give support to the spermatozoa, maintaining their motility, viability and fertilizing ability (WOJTCZAK et al., 2005). According to LOIR et al. (1990), proteins represent the largest organic component in the seminal plasma of teleost fish, with concentrations of approximately 1-3 mg/ml. Sperm protein losses may compromise sperm motility, fertilization ability and the early post-fertilization events in fish (ZILLI et al., 2005).

Proteins present in the seminal plasma are described as having an important role for the maintenance of sperm motility in bulls (BAAS et al., 1983; JOBIM et al., 2004; MOURA, 2005; MOURA et al., 2007), rams (GRAHAM, 1994; CARDOZO et al., 2006) and boars (STRZEZEK et al., 1992; KORDAN et al., 1998). Additionally, such proteins would be associated to increased spermatozoa resistance to cold shock, in boars (BERGER et al., 1985) and rams (BARRIOS et al., 2000) and thus could be considered as cryomarkers for the mentioned species (RONCOLETTA et al., 1999; JOBIM et al., 2004; BARRIER-BATTUT et al., 2005; MOURA, 2005; BIANCHI et al., 2008). In fish, the acquisition of sperm motility is associated to the presence of some glycoproteins in the seminal plasma, which act as sperm immobilizing factor, as described for the Nile tilapia (*Oreochromis niloticus*) (MOCHIDA et al., 1999). For the rainbow trout (*Oncorhynchus mykiss*), there are studies showing associations between sperm viability and the presence of some protein factors (LAHNSTEINER et al., 2004; LAHNSTEINER, 2006). However, there are no studies investigating such associations in jundiá fish (*Rhamdia quelen*).

The aims of this study were to characterize protein factors present in the seminal plasma of jundiá fish seminal plasma and to investigate their potential association to sperm motility.

MATERIALS AND METHODS

The study was carried out on a sexually mature brood stock of reared *Rhamdia quelen* males (2 years old, length 25-30 cm, weight 300-400 g). The experiments were conducted during the reproductive period, from September to March, in Pelotas, Rio Grande do Sul State, in South of Brazil. Fish were kept in an indoor tank at density of 0.9 kg/m^3. The semen samples were collected from 21 males of the Aquaculture Station of the Catholic University of Pelotas. Only one semen sample was collected from each male because there was no individual identification for the males in the tank, so they were all captured and collected at once. Semen collection was conducted through a gentle pressure applied to the testes and sperm ducts, to obtain a drop of milt on the previously cleaned gonophore's area. Urine and potentially urine-polluted semen were discarded carefully. Sperm was collected in 5 ml syringes and stored at 4°C on conical tubes (TPP®, Switzerland).

Due to the high sperm concentration in milt, the assessment of sperm motility required a high dilution rate (BILLARD & COSSON, 1992). An aliquot of sperm used to measure motility were diluted 1:200 in a solution of NaCl (75 mOsm) to activate the spermatozoa. Motility was evaluated within the first 10 seconds after activation, by putting 20 μL of the diluted sample

over microscope slides, for observation in an optical microscope (100x) connected to a video monitor. Motility was evaluated by two independent observers, in three replicates for each sample. Motility was defined as the percentage of motile sperm, considering only forward-moving spermatozoa, whereas those simply vibrating or turning on their axes were considered to be immotile.

An aliquot of sperm used on the SDS-PAGE was centrifuged at 1000 g for 10 minutes at 4°C and the supernatant was stored in liquid nitrogen. Seminal plasma was centrifuged a second time (3000 g, 10 min., 4°C) to eliminate possible contamination with spermatozoa. Each sample was prepared with 50 µL of seminal plasma and 25 µL of sample buffer (Glycerol; Tris-Hcl 0,6173M - pH 6,8; β-mercaptoethanol; 10% SDS; bromophenolblue; H2O) at 100°C for 10 minutes.

Electrophoresis was performed for each seminal plasma sample in a BIO-RAD Mini-Protean 3 Cell® system using 15% bis-acrylamide gels. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted in a Tris buffer system (LAEMMLI, 1970), with 10 µL of sample loaded in gels. Samples were concentrated at 60 V for 20 minutes, and the separation was performed at 120 V for 70-80 minutes. The BenchMark Protein Ladder™ (Invitrogen®, Carlsbad, USA) was used as the standard molecular weight. Gels were stained with Coomassie Brilliant Blue and were scanned and analyzed through the TotalLab TL 100 analysis software (Nonlinear Dynamics, UK).

Descriptive statistics were generated for the molecular weight of the protein factors identified in the seminal plasma samples. The sperm motility observed in the semen sample was categorized in 80% or higher, and lower than 80%. The presence or absence of each identified protein factor was compared across the two categories of sperm motility using either the chi-square test or the Fischer´s exact test, depending on the number of observations in each combination of categories. The analysis was conducted using the STATISTIX® program (2003).

RESULTS AND DISCUSSION

The computer imaging analysis identified a total of 14 protein bands. Its frequency distribution and the number of males in which they were detected are demonstrated in Table 1. No ejaculate contained all 14 protein bands. The proteins with molecular weights equal to 81.5 ± 2.6 kDa; 60.4 ± 1.6 kDa; 33.6 ± 0.4 kDa; 25.5 ± 0.2 kDa were identified in all samples. The proteins with molecular weights equal to 97.3 ± 3.64 and 7.1 ± 0.5 kDa occurred in 37% of the samples.

TABLE 1. Molecular weights (kDa) of jundiá fish seminal plasma protein bands detected by SDS-PAGE and the number of males in which those bands were present

Bands	Molecular weight (kDa) Mean ± SD	Number of semen samples (%)*
1	217.1 ± 3.31	9 (42)
2	147.5 ± 2.18	17 (79)
3	97.3 ± 3.64	8 (37)
4	81.5 ± 2.61	21 (100)
5	60.4 ± 1.60	21 (100)
6	52.1 ± 0.36	19 (89)
7	45.3 ± 0.86	20 (93)
8	38.3 ± 0.46	13 (61)
9	33.6 ± 0.47	21 (100)
10	25.5 ± 0.20	21 (100)
11	21.8 ± 0.43	15 (70)
12	16.3 ± 0.61	11 (51)
13	14.2 ± 0.93	10 (47)
14	7.1 ± 0.59	8 (37)

*n = 21

One protein band with molecular weight equal to 38.3 ± 0.46 kDa (Figures 1 and 2) was identified in 13 males (61% of the samples). This is the only one associated to sperm motility ($P < 0.01$), since such band was detected in 91.7% of the samples having motility <80%, but it was not detected in 77.8% of the samples having ≥80%. Figure 1 shows the electrophoretic profile of the proteins present in the seminal plasma samples from nine analyzed males, and the band having 38.3 kDa is present in five males. The band with 81.5 ± 2.61 kDa presented larger concentrations than the other bands (Figure 2).

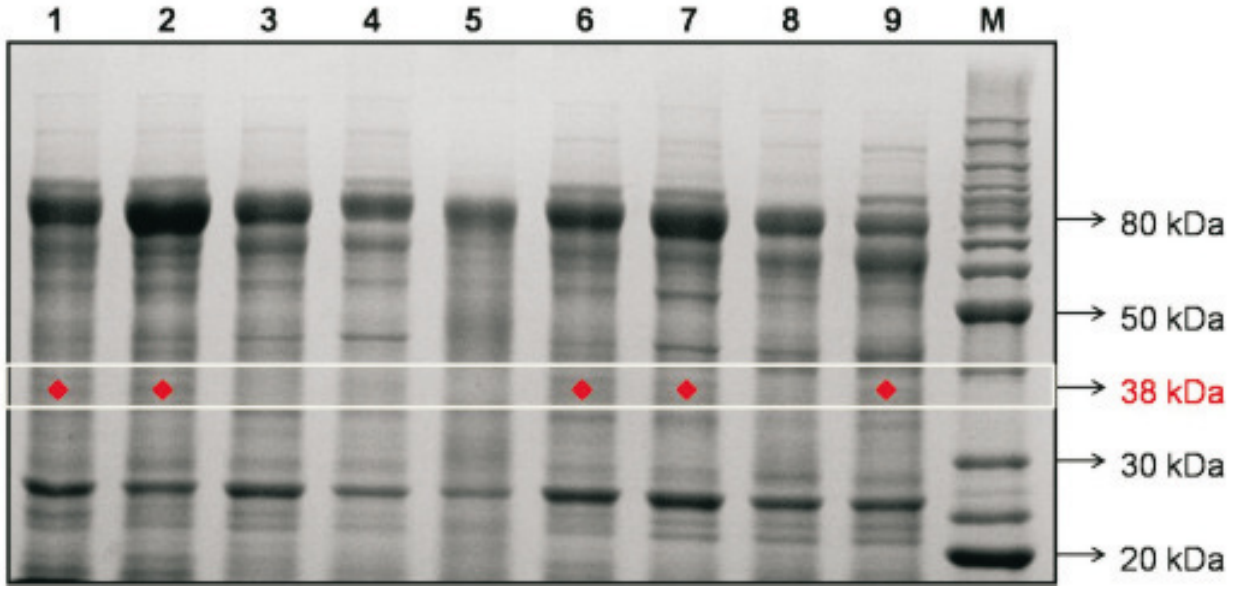

FIGURE 1. Protein profile of seminal plasma of 9 jundiá fish as revealed by SDS–PAGE gel electrophoresis. Lane M indicates the molecular weight BenchMark Protein Ladder™ (Invitrogen®, Carlsbad, USA) of the standard in kDa. Gels were stained with Coomassie Brilliant Blue.

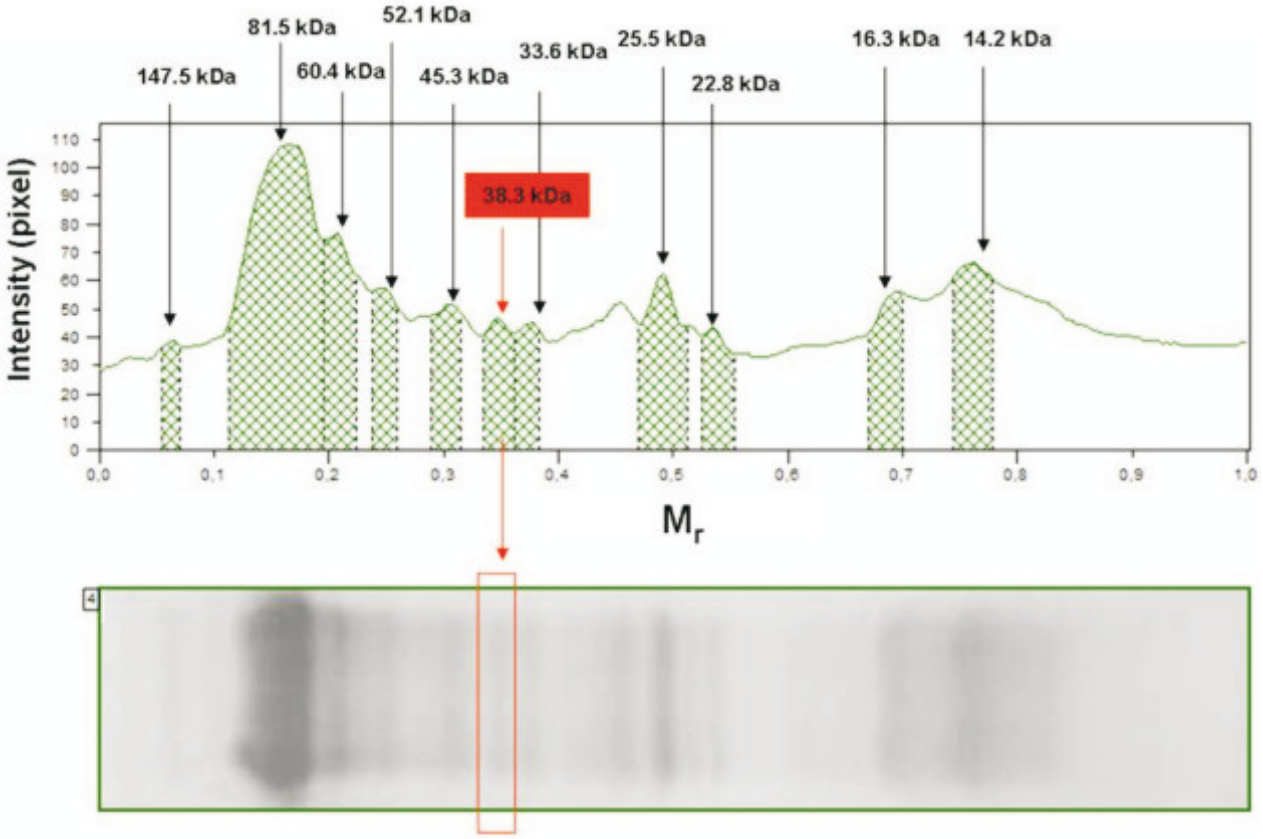

FIGURE 2. Quantitative protein bands (based on pixel intensity) and M_r (migration rate) of the seminal plasma of jundiá fish. Analyzed in TotalLab TL 100 analysis software (Nonlinear Dynamics, UK).

The present study was the first one to detect a group of 14 protein factors in the seminal plasma of jundiá fish (*Rhamdia quelen*). Most importantly, for the first time, a protein band associated with sperm motility was detected in this species. Depending on future studies that would investigate such association in more detail, this protein band may be a potential biochemical marker for sperm quality. Considering that such wild species has a very good marketing acceptance (BARCELLOS et al., 2001; GOLOMBIESKI et al., 2003), that finding represents an opportunity to increment assisted reproduction programs based on semen cryopreservation of selected males, what would render feasible raising this species in aquaculture systems.

The presence of protein factors in the seminal plasma have been described for many fish species, such as Nile tilapia – *Oreochromis niloticus* (MOCHIDA et al., 1999; MOCHIDA et al., 2002); rainbow trout – *Oncorynchus mykiss* – (LOIR et al., 1990; KOWALSKI et al., 2003; LAHNSTEINER et al., 2004; MAK et al., 2004; LAHNSTEINER, 2006); common carp *Cyprinus carpio*; bream Abramis brama; ide *Leuciscus idus*; chub *Leuciscus cephalus*; grayling *Thymallus thymallus;* perch *Perca fluviatilis*; pike *Esox lucius*; goldfish *Carassius carassius*; and pikeperch *Stizostendion lucioperca* (KOWALSKI et al., 2003); different marine teleost fish species - *Diplodus sargus, Mullus barbatus, Thalassoma pavo, Trachinus draco, Uranuscopus scaber, Sparisoma cretense, Synodon saurus* – (LAHNSTEINER, 2003). In some of the studies mentioned above (MOCHIDA et al., 1999; LAHNSTEINER et al., 2004; LAHNSTEINER, 2006), the identified protein factors were associated to parameters of sperm quality.

Such associations between seminal plasma protein factors and sperm quality parameters were also described for other animal species. In humans, an 18-22 kDa sperm motility inhibiting factor (SMIF) has been purified from the seminal plasma and associated to the inhibition of dynein ATPase (IWAMOTO & GAGNON, 1988). In bulls, two types of protein factors have been described by BAAS et al. (1983): the first type included low molecular weight factors that restore motility, whereas the second type included high molecular factors that caused permanent spermatozoa inactivation. Other protein factors associated to sperm motility in bulls were reported elsewhere (JOBIM et al., 2004; MOURA, 2005; MOURA et al., 2007). Furthermore, a 78 kDa SMIF having antibacterial property has been identified in rooster seminal plasma (MOHAN et al., 1995). The potential of using seminal plasma protein factors as biochemical markers for resistance to cold chock in semen cryopreservation protocols has been considered in different mammal species (BERGER et al., 1985; RONCOLETTA et al., 1999; BARRIOS et al., 2000; JOBIM et al., 2004; BARRIER-BATTUT et al., 2005).

Although not associated to sperm motility, the other protein bands detected in the seminal plasma may play important roles for sperm physiology. Some enzymes leaking into seminal plasma have been described for many fish species (MAK et al., 2004; KOTLOWSKA et al., 2005; WOJTCZAK et al., 2005). Most of those enzymes are proteolytic, proteinases and proteinase inhibitors. Most of the proteinases found in seminal plasma are serine proteinases, although proteinases from other classes are present in the semen of humans and domestic mammals (MÉTAYER et al., 2002). The activities of these proteinases are controlled by their inhibitors. The potential role of proteinase inhibitors is to protect the sperm duct epithelial cells, seminal plasma proteins, or viable spermatozoa from the potentially detrimental proteolytic action of acrosin released from the acrosomes of dead and damaged spermatozoa (SUOMINEN & SETCHELL, 1972; KOTLOWSKA et al., 2005). But in fish the biological role of those enzymes is not clear (MAK et al., 2004). The presence of non-proteinous compounds (such as monosaccharides and triglycerides) in seminal plasma may be associated to the presence of seminal plasma proteins (CIERESZKO & DABROWSKI, 1994; LAHNSTEINER et al., 2004). In jundiá fish, the biochemical composition of the seminal plasma was described (BORGES et al., 2005) and compounds such as triglycerides and creatinine were identified.

Similarities among some blood and seminal proteins have been reported in mammals (SKINNER et al., 1987), chicken (THURSTON et al., 1982), and fish (LOIR et al., 1990). Such proteins may have functions related to reproduction, whereas proteins found exclusively in the reproductive tract may have more specific functions (KOTLOWSKA et al., 2005). LAHNSTAINER (2003) concluded that the testicular main ducts and spermatic ducts of some marine tele-

ost fish species act on the synthesis of some seminal plasma proteins, similar to what occurs in fresh water fish. Thus, some seminal plasma proteins detected in our study may be synthesized in these spermatic ducts. In teleosts, spermatozoa already having potential for motility remain immotile in the seminal plasma due to motility immobilizing factors such as potassium ions, in salmonid fish, and osmolality, in cyprinid fish (MORISAWA et al., 1983). On the other hand, the motility immobilizing factor present in seminal plasma characterized by MOCHIDA et al. (1999) was a glycoprotein. However, the protein factor detected in the present study, although associated to decreased sperm motility, cannot be characterized as a motility immobilizing factor, unless further studies can be conducted in the future.

The SDS-PAGE technique used in this study has limitations because it did not allow the characterization of the 38 kDa factor as a single protein or a protein group. On the other hand, the results of the present study represent the first description of an association between sperm motility and a seminal plasma protein factor in jundiá fish.

CONCLUSIONS

We concluded that seminal protein factor with 38 kDa have an association to lower sperm motility. When this band is detected in seminal plasma, the low motility is found on the same sample. Such discovery would be very helpful for future studies in this field, especially considering the possibility of rearing this wild specie in aquaculture systems, which would require assisted reproduction programs.

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