GREAT LAKES FISHERY COMMISSION

2001 Project Completion Report[1]

Studies on the Role of Sperm-activating Proteins and the Mechanism of Protease-inhibitor(s) Reaction for Controlling Fertilization in Sea Lamprey

by:

Konrad Dabrowski[2] and Andrzej Ciereszko[3]

[2]School of Natural Resources
The Ohio State University
Columbus, Ohio
43210-1085

[3]Institute of Animal Reproduction
and Food Research
Polish Academy of Sciences
Olsztyn, Poland

February 2001

Final Report to the Great Lakes Fishery Commission

March 1, 2000 – Feb 28, 2001

Project title: Studies on the role of sperm-activating proteins and the mechanism of protease-inhibitor(s) reaction for controlling fertilization in sea lamprey

Principal Investigator: Konrad Dabrowski
School of Natural Resources
The Ohio State University
Columbus, OH 43210-1085

Co-Investigator: Andrzej Ciereszko
Institute of Animal Reproduction and Food Research
Polish Academy of Sciences
Olsztyn, Poland

1. Introduction

The invasion of the sea lamprey, *Petromyzon marinus*, in the Great Lakes had a devastating impact not only on the lake trout, *Salvelinus namaycush*, but also on all fish assemblages (Coble *et al.*, 1990). The recovery effort of the lake whitefish, *Coregonus clupeaformis*, in the Great Lakes might be impaired by lamprey attacks on the new host. With the opening of the St. Lawrence Seaway, the non-indigenous sea lamprey gained access to the Great Lakes in 1932. Current attempts to control are mainly 1) treatment of streams with lampricides to kill larvae and 2) production and release of sterile males to decrease spawning success.

Total reliance on a single control method is considered unwise. Therefore, a search for alternative lamprey control methods is necessary to create an integrated control program. Quality and quantity of lamprey sperm are important components of reproductive success in this species and may be potential targets for control of its reproduction (Kobayashi and Yamamoto, 1994). Undoubtedly, efficient reproduction of lampreys contributed to the colonization success of this parasite. Females of the landlocked sea lamprey produce about 60,000 eggs and males are able to produce milt for several spawning events (Manion and Hanson, 1980; Langille and Hall, 1988). However, because of the lack of knowledge on the biology of fertilization in the sea lamprey, we do not have a good understanding of gamete factors resulting in the enormous reproductive success of the sea lamprey. Understanding morphological changes in oocytes and sperm and the physiology of fertilization of lamprey might allow us to identify potential targets for preventing successful reproduction.

Acrosomal function related to fertilization

Unlike teleost fishes, which possess an anacrosomal spermatozoa, many species of primitive fishes have acrosomal sperm (Jamieson, 1991). It appears that the acrosome, a structure involved in sperm penetration through egg investments, was lost in teleosts, in concert with emergence of the micropyle in the eggs. However, in lampreys, there are no micropyle(s)

(Jamieson, 1991; Kobayashi *et al.*, 1994). Consequently, the presence of an acrosome was reported from spermatozoa of all examined lamprey species (Jamieson, 1991).

The acrosome is a structure involved in the spermatozoan's (1) species-specific egg recognition, (2) its further penetration through the egg investments, and (3) its fusion with the egg. The acrosome is characterized by heterogeneous contents of the acrosomal vesicle and, in some species of lower vertebrates including lamprey, a penetrating perforatorium. During the initial stages of fertilization, the acrosomal membrane fuses with the apical plasma membrane of the sperm causing the release of vesicular contents. This process is called the "acrosomal reaction" (AR). The acrosomal reaction has been described in *Lampetra japonica* (Kobayashi and Yamamoto, 1994), and is associated with an influx of calcium ions and Na^+ and K^+. These processes cause a membrane depolarization and intracellular alkalinization. In the sea urchin, the AR can be inhibited by: blockers of calcium or potassium channels, proteinase inhibitors, lowering pH below 8, or increasing the extracellular potassium concentration (Matsumara and Aketa, 1989). The two latter factors may also regulate sperm motility. Studies of the factors controlling the acrosomal reaction in lamprey may reveal potential targets for inactivation of spermatozoa.

Sperm proteolytic activity and anti-proteolytic activity of seminal plasma

The AR, usually triggered at the egg stage surface, leads to the release of a number of hydrolytic enzymes associated with the acrosome. The spermatozoan's penetration of egg membranes without distinguished micropyle(s) in lamprey is facilitated by acrosomal hydrolyzing enzymes. Acrosin is a trypsin-like enzyme found in the sperm acrosome and appears to be a widely distributed, conserved protein, present in spermatozoa of nearly all animals (Baccetti *et al.*, 1989). After comparative studies of amino acid sequences of rat proacrosin versus different serine proteases, Klemm *et al.* (1991) concluded that gene duplication, which resulted in the acrosin gene, had taken place a billion years ago during the period of early eukaryotic evolution. At that time, spermatozoa of Scyphozoa and Hydrozoa acquired a rudimentary acrosome. We documented, for the first time, the presence of acrosin in sturgeon sperm (Ciereszko *et al.*, 1994).

We found that trypsin-like activity in acinpenserides shares properties with mammalian acrosins, such as common inhibitors, substrate specificity and pH optimum (Ciereszko *et al.*, 1994).

There are no data on the acrosin activity in lamprey spermatozoa. We postulate that, due to the presence of an acrosome and lack of micropyle in eggs, acrosin might be present in spermatozoa of lampreys. Characterization of activity of this enzyme may lead to future attempts to inhibit acrosin activity. Inhibition of acrosin is one of the approaches currently studied to achieve male sterility in mammals (Kaminski *et al.*, 1985). Acrosin activity in spermatozoa is controlled by its inhibitors in acrosome and seminal plasma. Recently, we described antiproteinase activity in seminal plasma of teleost fishes (Dabrowski and Ciereszko, 1994; Ciereszko *et al.*, 1998). It seems that despite the loss of the sperm acrosome, anti-proteinase activity in teleosts was retained. Studies of the sperm acrosin/seminal plasma inhibitors system in lampreys are important for understanding mechanisms of fertilization in this species.

Sperm-activating peptides and proteins (SAF-1) and egg signals triggering the acrosomal reaction (AR) and/or acrosomal filament discharge (AFD) in spermatozoa.

To induce vigorous and prolonged swimming of sea lamprey spermatozoa, jelly coat substances (SAF-1) proved to be very effective (Dabrowski *et al.*, GLFC Report, 1998). However, upon encountering the jelly coat of an egg, lamprey spermatozoa do not undergo AFD and most likely do not undergo AR. The specificity of at least two factors need to be searched for, although in starfish spermatozoa, three jelly coat proteins act in concert to induce AR (Hoshi *et al.*, 1990).

Suzuki (1995) and his collaborators purified seventy-four peptides from the solubilized jelly coat layer of sea urchins. The biological effects and structures are specific at the ordinal level, although all are classified as sperm-activating peptides (SAP-1) and produce stimulation of the respiration and motility of spermatozoa. This includes an increase in cAMP and cGMP levels, activation of a NA/H exchange and increase in intracellular pH and Ca concentration at SAP-1 concentration as low as 10^{-10} M (Suzuki *et al.*, 1984; 1988).

In teleost fish, Pillai *et al.* (1994) and Morisawa et al. (1992) isolated a high molecular weight protein from the egg chorion in herring, which had similar sperm motility stimulatory action on this species' sperm. A purified glycoprotein from porcine follicular fluid of molecular weight 52 kDaltons possessed a potent ability to stimulate sperm motility (Lee *et al.*, 1992). To test this idea of SAP-1 presence, molecular characteristic and biological role as the chemoattractant during fertilization process, described as "gynogamone" by Scharatu and Montalenti (1941), we were prompted to characterize the sperm motility stimulator in lamprey.

2. Objectives

Sea lamprey males are currently sterilized using intra-peritoneal injections of bisazir. Because this is a potent mammalian mutagen, less hazardous substances for sterilization are desirable (Holmes, 1995). Protease inhibitors have proven *in vitro* as inhibitory to fertilization in lamprey. Understanding of the mechanism of reaction between protease(s), such as sperm-activating factor and their receptor(s) is critical to control efficiency of fertilization. Therefore, we proposed:

(1) to further characterize proteolytic activity of sperm and ovarian cells in order to increase substrate affinity and determine inhibitors specificity,

(2) to determine the effect of intraperitoneal injections of protease inhibitors, both in males and females, on gamete fertilizing ability, and fish viability as a means of understanding the sequence of events leading to release of proteases and their inhibitors (gender specificity).

(3) to continue evaluation of bisazir treated male lampreys and design a less labor-intensive method of estimating the efficiency of sterilization.

3. Methodology and Results

Animal handling and fertilization

Sea lampreys were obtained from the Cheboygan River, Michigan (Lake Huron Biological Station) and air-shipped overnight to Columbus, Ohio. They were kept in the aquaculture facility of the School of Natural Resources, The Ohio State University. Fish were kept in regular conditions (12L:12D) and anaesthetized with 0.1% tricaine (MS-222, Argent) in 0.3% sodium bicarbonate before sampling.

Gametes were obtained by stripping. They were used on the day of collection (sperm and eggs) or after one-day of storage in slightly refrigerated conditions (10°C)(eggs). Milt was stored on ice. Fertilization trials were performed in Petri dishes using 200 eggs and a known number of spermatozoa. Gametes were activated with water (10 mL) and incubated at room temperature (20°C) for 5 hours. After recording the fertilization rate (2-cell embryos), eggs were transferred to California-type hatching trays and kept in individual baskets

Fertilization rate in most experiments was estimated by calculations of percentage of fertilization at the 2-cell embryos state (5 hours after fertilization). Additionally, fertilization rate was assessed 3 minutes after fertilization (perivitelline space or cytoplasmic bleb test, CBT) or at hatching.

Proteolitic activity in seminal plasma

Experimental design

We evaluated the presence of bands of proteolytic activity using gelatin-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Siegel and Polakoski, 1985). We employed Zymogram Ready Gels supplied by Bio-Rad Laboratories (Hercules, CA, USA). These gels contained either copolimerized β-casein or gelatin. We used Ready Gel Cell apparatus (Bio-Rad) for electrophoresis. SDS-PAGE-gelatin was run at 25 mM for 1.5h. Gels were renaturated for 30 min at room temperature in 2.5% Triton X-100. Then gels were developed overnight in 50 mM Tris-

HCl buffer, pH 8.0 containing 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% Brij-35. Gels were stained with 0.5% Commassie Brilliant Blue R-250 in 40% methanol/10% acetic acid and destained in 40% methanol/10% acetic acid.

Results

Casein zymogram gels appeared not to be useful for detection of proteases in sea lamprey seminal plasma. However, when using SDS-page-gelatin, we identified for the first time the presence of proteolytic activity in sea lamprey seminal plasma (Fig. 1). Some proteolytic bands were common for all samples and seem to be universal for lamprey seminal plasma. In some samples, we found multiple bands of proteolytic activity. Origin of these bands is unclear. These samples were characterized by a high protein concentration and contained a large amount of cells of unknown origin. The role of proteolytic enzymes in sea lamprey seminal plasma is unknown at present.

Conclusion

A number of proteolytic enzymes are present in sea lamprey seminal plasma. Further studies are necessary to characterize their specificity and to elucidate if these enzymes are important for sperm physiology and if inhibition of these enzymes may be important for sperm fertilizing ability.

Anti-proteinase activity in seminal plasma

Experimental design

Anti-proteinase activity was measured by inhibition of cod trypsin amidase activity according to Dabrowski and Ciereszko (1994) and Ciereszko *et al.* (1998). Native PAGE gel electrophoresis was performed using Ready Gel 10% (Bio-Rad). After electrophoresis (50 mM, 1h) gels were stained for anti-trypsin activity according to Uriel and Berges (1968).

Results

We found for the first time anti-proteinase activity (measured as anti-trypsin activity) in sea lamprey seminal plasma. This activity did not differ between control and bisazir treated males (control: 6.9 ± 2.64 U/l, range 0-23.18 U/l vs. bisazir-treated 7.09 ± 3.22 U/l, range 0.95-25.01 U/l). Anti-protease activity correlated with protein concentration ($r=0.73$, $p<0.01$). Due to low anti-proteinase activity we were not able to detect any anti-trypsin bands after native PAGE.

Conclusion

The anti-proteinase activity found in sea lamprey seminal plasma is low as compared to teleost fish seminal plasma (177.6-1000.5 U/l; Ciereszko *et al.*, 1998) and rather resembles the activity of sturgeon seminal plasma (22.0 U/l; Glogowski *et al.*, unpublished data). This activity was also much lower than that of blood plasma (227.0 U/l, n=1). Due to low antiproteinase activity we were not able to identify this activity after PAGE. Low anti-proteinase activity in lamprey seminal plasma is puzzling in view of the presence of acrosome in sea lamprey spermatozoa. Further studies are required to test if seminal plasma of sea lamprey can inhibit chymotrypsin activity, because chymotrypsin-like activity has been identified in sea lamprey sperm (Dabrowski *et al.*, GLFC, 1998 annual Report).

Effect of TPCK (chymotrypsin-inhibitor) and TLCK (trypsin-inhibitor) injection on sea lamprey sperm quality

Experimental design:

Sea lamprey males were injected intraperitoneally (May 5) with 100 μM/kg of TPCK (n = 7) or TLCK (n = 6). Both proteinase inhibitors were dissolved in ethanol. Control fish (n = 7) received only ethanol.

Results

By July 8, all TLCK injected fish died. At the same time, 6 control and 7 TPCK injected fish were alive. These fish lived one more month. During this period, attempts to collect sperm

were unsuccessful or resulted in obtaining minute amounts of milt of low sperm density (color). This did not allow us to perform sperm quality analysis of TPCK injected and control fish.

Conclusion

TLCK at a dose of 100 μM/kg is lethal for sea lamprey. This suggests that trypsin-like activity is important for vital functions of sea lamprey during the spawning run. TPCK effect was not evaluated and should be tested in the future.

Evaluation of the fertilizing ability of eggs of bisazir-treated sea lamprey females

Results

We obtained eggs from three sterilized females.

Female #1:

Eggs of this female were fertilized with 20 μl of semen of 3 individual males and with combined semen (20 μl of each male, total 60 μl). The following fertilization rates were obtained:

Source of semen	2-cell embryos (%)	Pre-hatched larvae (%)
Combined semen	96.0	9.0
Male 1	96.0	9.5
Male 2	15.0	2.0
Male 3	91.0	13.5

Female #2

Eggs were of poor quality, and looked overripe. 30 μl of semen of 3 males were used for fertilization. The following fertilization rates were obtained:

Source of semen	2-cell embryos (%)	Pre-hatched larvae (%)
Male 1	21.5	0
Male 2	53.5	0
Male 3	62.0	0

Female #3

30 µl of semen obtained from 3 males was used. Only limited numbers of eggs were obtained. This suggested that we collected eggs at the beginning of ovulation. The following fertilization rates were obtained:

Source of semen	2-cell embryos (%)	Pre-hatched larvae (%)
Male 1	79.5	30.5
Male 2	30.5	11.0
Male 3	64.0	33.5

Conclusion

These data suggest that, opposite to males, injection of sea lamprey females does not result in complete sterilization. For this reason, more studies are necessary to optimize the bisazir dose to sterilize females.

Evaluation of the fertilizing ability of sperm of bisazir-treated sea lamprey males

Experimental design

Sperm of 13 non-treated and 11 bisazir-treated males was tested. Sperm motility was evaluated subjectively.

Results

Semen of bisazir-treated males did not differ in percentage of motility (Fig. 2A) but had a lower sperm concentration (Fig. 2B). Fertilizing ability of bisazir-treated males was impaired. It was seen already at the 2-cell stage (Fig. 3A). Very few larvae were observed at hatching (0.07%) due to 0.8% fertilization rate at hatching in 1 of 11 males (Fig. 3B).

Conclusion

These results confirm our earlier data concerning high efficiency of the male sterilization program. During the 2000 season we found for the first time a lower sperm concentration and fertilization success at the 2-cell stage of bisazir-treated males. This may suggest a variability in bisazir action on the male reproductive tract. The source of this variability needs to be identified.

4. References

Baccetti, B., Burrini, A.G., Collodel, G., Piomboni, P., Renieri, T. and Sensini, C. 1989. Localization of acrosomal enzymes in Arthropa, Echinodermata and Vertebrata. *J. Submicrosc. Cytol. Pathol.* 21:385-389.

Ciereszko, A., Piros, B., Dabrowski, K., Kucharczyk, D., Luczynski, M.J., Dobosz, S. and Glogowski, J. 1998. Serine proteinase inhibitors of seminal plasma of teleost fish: distribution of activity, electrophoretic profiles and relation to proteinase inhibitors of blood. *J. Fish Biol.* 53:1292-1305.

Ciereszko, A., Dabrowski, K., Lin, F. and Doroshov, S.I. 1994. Identification of trypsin-like activity in sturgeon spermatozoa. *J. Exp. Zool.* 268:486-491.

Coble, D.W., Brusewitz, R.E., Fratt, T.W., Scheirer, J.W. 1990. Lake trout, sea lampreys, and overfishing in the upper great lakes-A review and reanalysis. *Trans. Amer. Fish. Soc.* 119:985-995.

Dabrowski, K. and Ciereszko, A. 1994. Anti-proteinase inhibitor(s) in seminal plasma of teleost fish. *J. Fish Biol.* 45:801-809.

Holmes, J.A. 1995. Assessment of alternative sea lamprey control research priorities. Final Report to the Great Lakes Fishery Commision. JAH Envirinmental, pp. 1-97.

Hoshi, M., Amano, T., Okita, Y., Okinaga, T. and Matsui, T. 1990. Egg signals for triggering the acrosome reaction in starfish spermatozoa. *J. Reprod. Fertil.* 42:23-31.

Jamieson, B.G.M. 1991. Fish evolution and systematics: Evidence from spermatozoa. Cambridge University Press, Cambridge, New York, Port Chester, Melbourne, Sydney.

Kaminski, J.M., Nuzzo, N.A., Bauer, L., Waller, D.P. and Zaneveld, L.J.D. 1985. Vaginal contraceptive activity of aryl 4-guanidinobenzoates (acrosin inhibitors) in rabbits. *Contraception* 32:183-188.

Klemm, U., Maier, W-M., Tsaousidou, S., Adham, I.M., Willison, K. and Engel, W. 1990. Mouse preproacrosin: cDNA sequence, primary structure and postmeiotic expression in spermatogenesis. *Differentiation* 42:160-166.

Kobayashi, W. and Yamamoto, T.S. 1994. Fertilization in lamprey, *Lampetra japonica* eggs: implications of the presence of fast and permanent blocks against polyspermy. *J. Exp. Zool.* 269:166-167.

Kobayashi, W. Baba, Y., Shimozawa, T. and Yamamoto, T.S. 1994. The fertilization potential provides a fast block to polyspermy in lamprey eggs. *Dev. Biol.* 161:552-562.

Langille, R.M. and Hall, B.K. 1988. Artificial fertilization, rearing, and timing of stages of embryonic development of the andromonus sea lamprey, *Petromyzon marinus* L. *Can. J. Zool.* 66:549-554.

Lee, S-L., Kuo, Y-M., Kao, C-C., Hong, C-Y., and Wei, Y-H. 1992. Purification of a sperm motility stimulator from porcine follicular fluis. *Comp. Biochem. Physiol.* 101B:591-594.

Manion, P.J. and Hanson, L.H. 1980. Spawning behavior and fecundity of lampreys from the upper three Great Lakes. *Can. J. Fish. Aquat. Sci.* 37:1635-1640.

Matsumara, K. and Aketa, K. 1989. Activation of Ca^{2+} channels during the acrosome reaction of sea urchin sperm is inhibited by inhibitors of chymotrypsin-like proteases. *Gamete Res.* 23:255-266.

Morisawa, M., Tanimato, S. and Ohtake, H. 1992. Characterization and partial purification of sperm-activating substance from eggs of the herring. *J. Exp. Zool.* 264:225-230.

Pillai, M.C., Yanagimachi, R. and Cherr, G.N. 1994. *In vivo* and *in vitro* initiation of sperm motility using fresh and cryopreserved gametes from the pacific herring, *Clupea pallasi*. *J. Exp. Zool.* 269:62-68.

Schartau, V.O. and Montalenti, G. 1941. Untersuchungen uber die Befruchtung, insbesondere uber Gamone bei dem Flubneunauge (*Lampetra fluviatilis* L.). *Biologisches Zentralblatt* 61:473-478.

Siegel, M.S. and Polakoski, K.L. 1985. Evaluation of human sperm proacrosin-acrosin system using gelatin-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Biol. Reprod.* 32:713-720.

Suzuki, N. 1995. Structure, function and biosynthesis of sperm-activating peptides and fucose sulfate glycoconjugate in the extracellular coat of sea urchin eggs. *Zool. Sci.* 12:13-27.

Suzuki, N., Shimomura, H. Radany, E.W., Ramarao, C.S., Ward, G.E., Bentley, J.K. and Garbes, D.L. 1984. A peptide associated with eggs causes a mobility shift in a major plasma membrane protein of spermatozoa. *J. Biol. Chem.* 259:14874-14879.

Suzuki, N., Yoshino, K-I., Kurita, M., Nomura, K. and Yamaguchi, M. 1988. A novel group of sperm activating peptides from the sea urchin *Glyptocidaris crenularis*. *Comp. Biochem. Physiol.* Vol. 90C, 2:305-311.

Uriel, J. and Berges, J. 1968. Characterization of natural inhibitors of trypsin and chymotrypsin by electrophoresis in acrylamide-agarose gels. *Nature* 218:578-580.

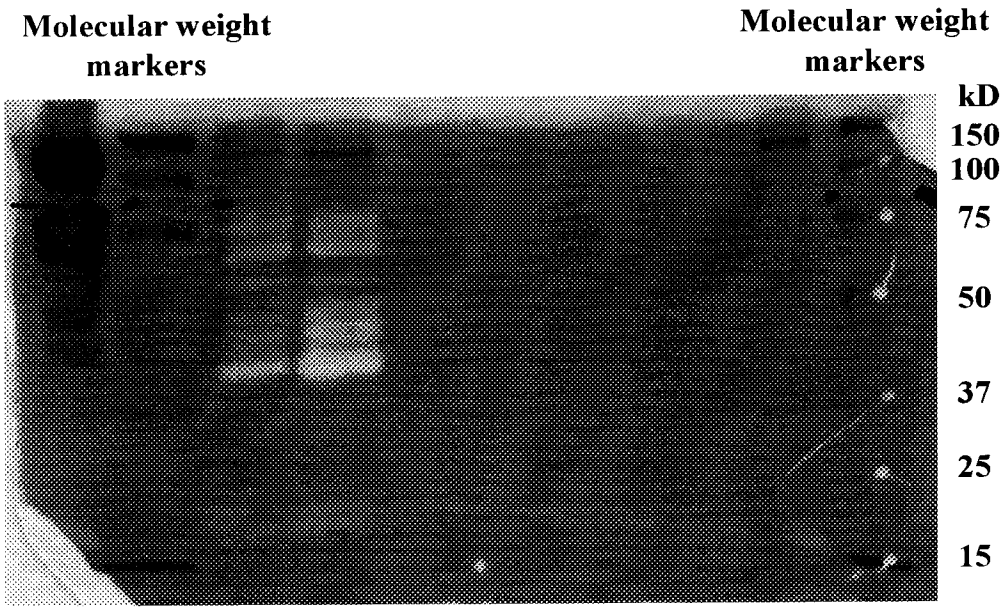

Figure 1: Identification proteolytic activity in sea lamprey seminal plasma using gelatin-sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

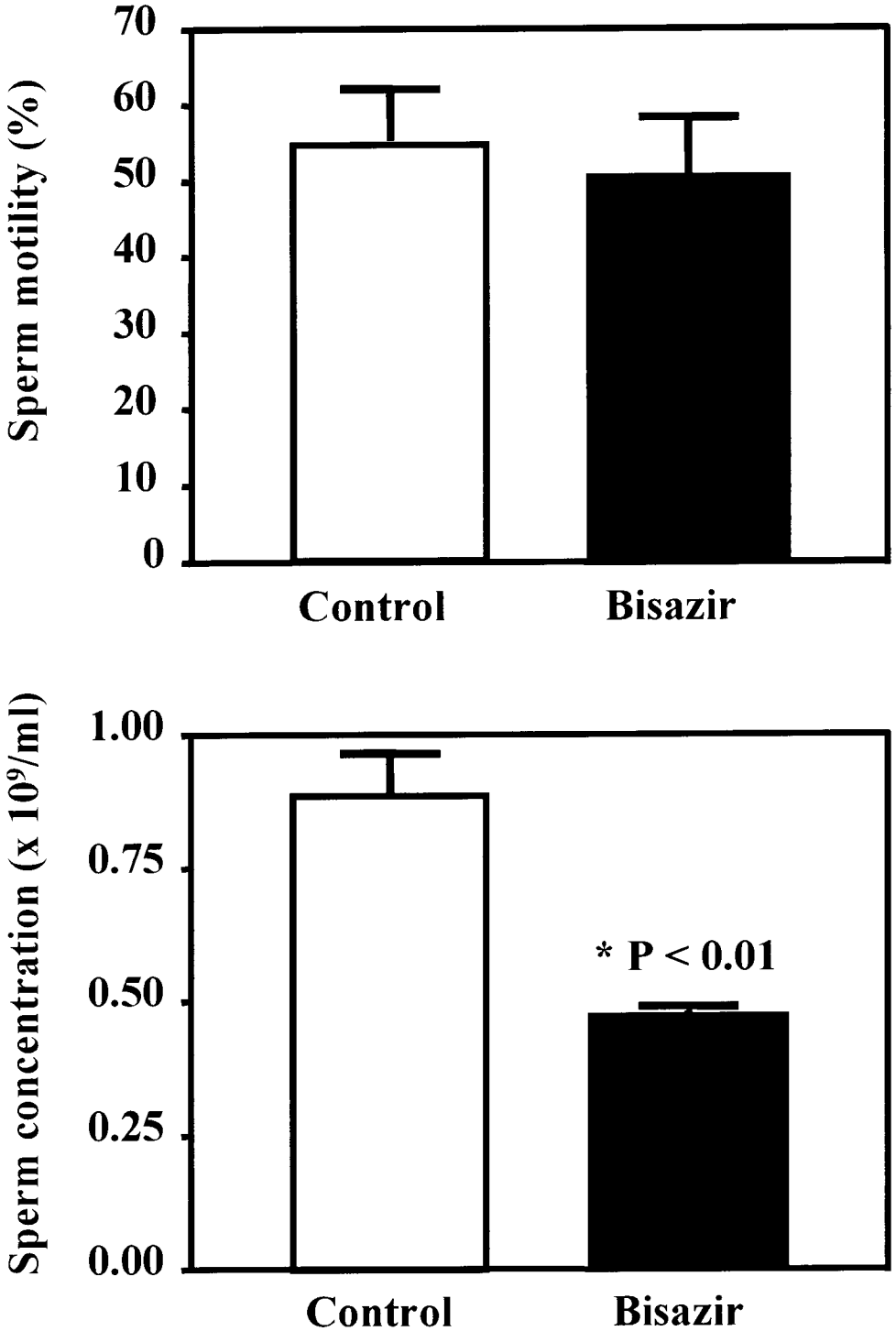

Figure 2: Sperm motility (A) and concentration (B) of control (n=13) and bisazir-treated (n=11) males.

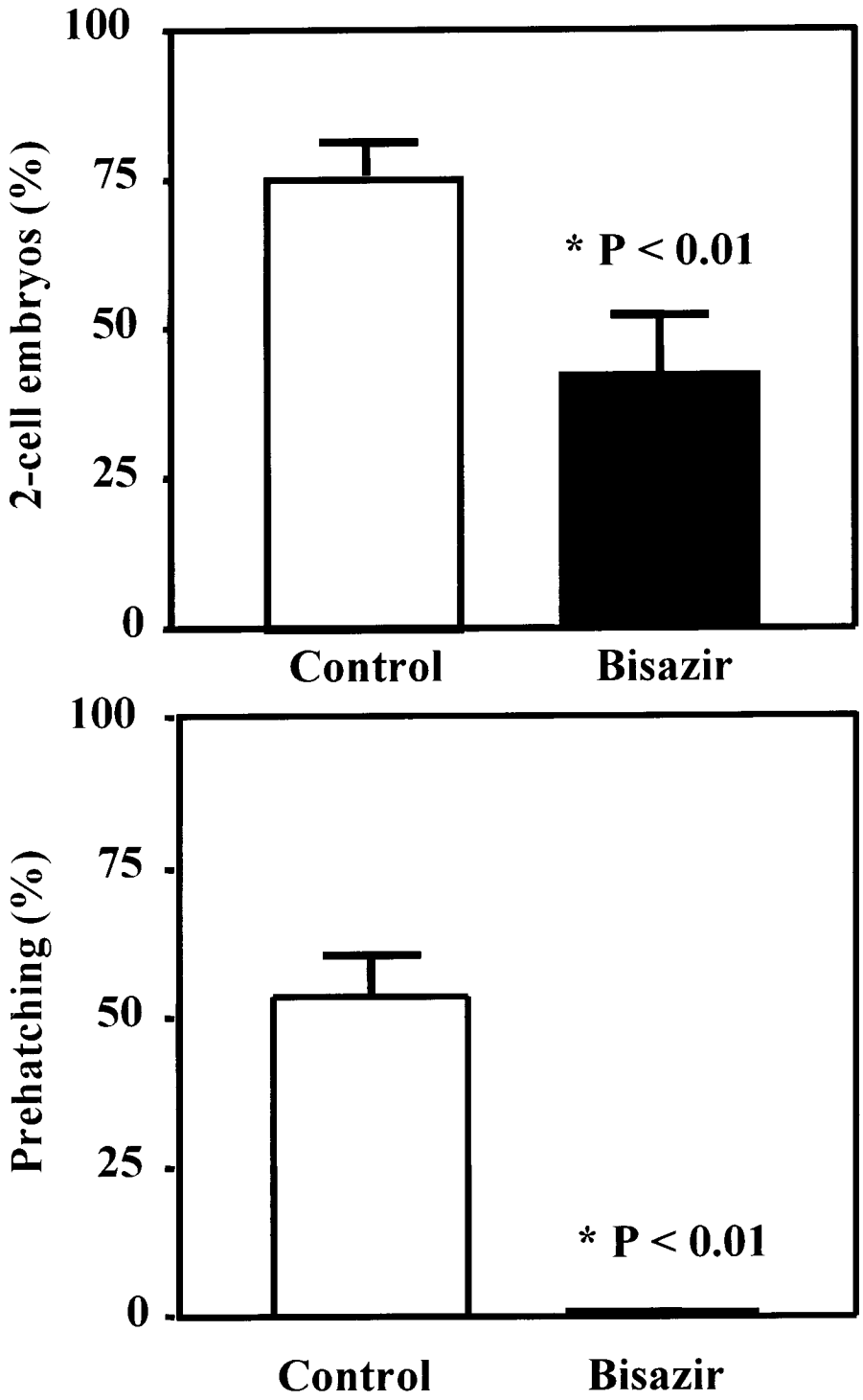

Figure 3: Fertilization rates measured at 2-cell embrys (A) and at pre-hatching (B) of control and bisazir-treated sea lamprey.