GREAT LAKES FISHERY COMMISSION

Project Completion Report[1]

Regulation of function in spermatozoa of the sea lamprey (*Petromyzon marinus*). The first step to contraception.

by:

Konrad Dabrowski[a], Jan Glogowski[c], Andrzej Ciereszko[c], Lin Feng[a], Jacques Rinchard[a], David Stetson[b], Susan Christ[d], and Gregory Toth[d]

[a]School of Natural Resources
[b]Department of Zoology
The Ohio State University
Columbus, OH 43210-1085

[c]Insitute of Animal Reproduction and Food Research
Polish Academy of Sciences
Olsztyn, Poland

[d]Environmental Protection Agency
Cincinnati, OH 45268

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Final Report to the
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Project title: Regulation of Function in Spermatozoa of the Sea Lamprey (*Petromyzon marinus*). The First Step to Contraception.

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

Co-Investigators: Jan Glogowski, Andrzej Ciereszko
Institute of Animal Reproduction and Food Research
Polish Academy of Sciences

Lin Feng, Jacques Rinchard
School of Natural Resources
The Ohio State University

David Stetson,
Department of Zoology
The Ohio State University

Susan Christ, Gregory Toth
U. S. Environmental Protection Agency

Introduction

The invasion of the sea lamprey (*Petromyzon marinus*) in the Great Lakes had a devastating impact 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 all the Great Lakes might be impared by lamprey attack, if lake whitefish are attractive as hosts. 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 lamprey are mainly treatment of streams with lampricides to kill larvae and 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 ongoing in order to create an integrated control program.

Quality and quantity of sperm are important components of reproductive success in this species and may be a potential target for control of its reproduction (Kobayashi and Yamamoto 1994).

Lamprey gametes are difficult to handle and basic information on this issue is lacking. Better understanding of this species' gamete biology is important both for development of laboratory techniques for studies of reproduction and for future control of sea lamprey reproduction. 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 a lack of knowledge on the biology of fertlization in the sea lamprey, we do not have good understanding of the gamete interaction 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.

Objectives

General To understand male sea lamprey reproductive physiology and identify potential targets for control of reproduction.

Specific

1. To describe the ultrastructure of spermatozoa and characterize sperm motility;
2. To assay proteolytic enzyme activity in sperm acrosomes;
3. To evaluate cryopreservation techniques on the viability of spermatozoa;
4. To determine the effects of storage time of lamprey eggs at optimized temperature on their fertilizing ability;
5. To determine optimal sperm/egg ratio for fertilization of sea lamprey;
6. To determine the effect of time after sperm release to water on fertilization of sea lamprey eggs;
7. To correlate fertilization successes estimated at 3 min (cytoplasmic bleb test) and 5 hours (2-cell embryos), and hatching rate;
8. To evaluate fertilization rate of spermatozoa treated with gossypol;
9. To evaluate the quality of semen and levels of plasma sex steroids of sea lampreys injected with different doses of gossypol; and
10. To evaluate fertilization rate of spermatozoa of lampreys sterilized with bisazir.

1997

Induced spawning results

Lampreys were obtained from the Cheboygan River, Michigan (Mrs. Kim Fredricks), the St. Marys River, Michigan (Mr. Mike Twohey, Marquette Biological Station), and Cattaraugus Creek, Springville, New York (Lake Erie population). Animals were cooled with ice during transport (10-24 h) and then gradually acclimated to 14°C on arrival. In the first phase of the

study (funded by LEPF[1]), animals were held for 5 days, then weighed, and individually tagged with plastic tags attached behind the dorsal fin. Males and females were divided to two sub-groups, 8 animals of each sex were injected with 100 µg/kg of luteinizing hormone releasing hormone (LHRH) and 10 mg/kg of pimozide (Sigma Chem., St Louis, MO) dissolved in 0.7% sodium chloride. Four animals were injected with a vehicle and four were not injected. The second injection of only 200 µg LHRH/kg was applied 8 days later. The first males spermiated 16 days after first injection and all of them at 22 days when water temperature had already reached 19°C. The latter coincided with the first 25% of spermiating males in control groups (Fig. 1).

Sperm biochemical characteristics

Sperm volume, concentration, motility and acrosomal amidase activity against trypsin- and chymotrypsin-like synthetic substrates were determined (Ciereszko and Dabrowski 1993; Ciereszko et al. 1994, 1996a,b). Milt volume in males **(n = 15) with a mean weight** of 264 g (**weight range**, 204-386 g) was 3.3 ± 1.4 ml (mean ± S.D.), sperm concentration was 1.03 ± 0.4 x 10^9 cm^{-3}, and motility 52 ± 25% (n=15). No trypsin-like amidase activity was found, whereas chymotrypsin-like activity amounted to 1.12 ± 0.74 µU/10^6 spermatozoa. The preliminary characteristics of this activity have been established (Fig. 2). No hyaluronidase activity was found in lamprey sperm, in contrast to boar sperm used as a control (Harrison 1988). Potassium (10.5 ±. 0.6 mM) and sodium (106.3 ± 12.8 mM) were two major ions in the seminal plasma (Table 1), whereas protein concentration was 0.43 ± 0.18 g/L.

Preliminary data was collected displaying the effect of sperm concentration and time after sperm activation on fertilization rate (Table 2).

Sperm ultrastructure

Data collected in 1996 is the first information on the basic characteristics of sea lamprey sperm (Fig. 3). In our ultrastructural studies we utilized experience gained in similar studies on the acrosomal sperm of the lake sturgeon (*Acipenser fulvescens*) (Ciereszko et al. 1994) and the anacrosomal sperm of muskellunge (*Esox masquinongy*) (Lin et al. 1996). In accordance with earlier findings of Jaana and Yamamoto (1981) and Kobayashi and Yamamoto (1994) for spermatozoa of *Lampetra japonica*, we described changes in the sperm head. We identified the dynamic changes in the acrosomal vesicle of activated spermatozoa, but were unable to observe the discharge of the acrosomal filament (AFD) (Fig. 3a,b). Kobayashi (personal communication, 1997) was also unable to control the AFD process and associated it with the maturity of individual males. Treatment with "egg water" (substances washed out from ovulated eggs), used to activate spermatozoa, resulted in significant changes in the duration of sperm motility and

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[1] Part of the research presented here was completed with the funding provided by The Lake Erie Protection Fund, project "Induced spermiation and characterization of spermatozoa of the sea lamprey."

swimming characteristics (Fig. 4), but did not result in AFD in our preliminary studies with sea lamprey.

Sperm characteristics from other lamprey species have been studied in non-parasitic *Lampetra planeri* and *Mordacia mordaxas* as well as in parasitic, *L. fluviatilis*, *L. japonica*, and *M. praecox* (Jamieson 1991). Longitudinal sections of the sea lamprey sperm head revealed the content of the acrosomal vesicle attached to the inner surface of the vesicle membrane, however, it was distinct from the subacrosomal ring (Fig. 3c,d). The acrosomal vesicle of the sea lamprey sperm seemed to differ from the subovoidal shape of *L. planeri* (Stanley 1967) and the flattened shape of *L. japonica* (Jaana and Yamamoto 1981). The latter authors, who described testicular sperm discharging the acrosomal filament (central fiber), suggested that this may occur at the time of fixation. Kille [1960; including observations by Ballowitz (1905)] concluded that most spermatozoa extrude the filament when they come into contact with the surface of egg chorion or simply glass.

Sperm motility

Prolonged sperm motility may be important in determining the reproductive advantage of lamprey over other aquatic animals. Spermatozoa of freshwater fish have a very short duration of motility as compared to marine organisms. For example, rainbow trout (*Oncorhynchus mykiss*) spermatozoa are motile for only about 30 s (Billard 1992). Kobayashi (1993) reported that about 40% of spermatozoa of *L. japonica* were motile for 5 minutes after activation, which is almost as long as in the lake sturgeon (Ciereszko et al. 1996a). The prolonged motility may contribute to an enormous fertlization success in lampreys.

We have observed that indeed the spermatozoa of the sea lamprey remained motile for up to 7 min after activation (unpublished). Furthermore, with the help of the computer-assisted sperm motion analysis (CASA), we demonstrated that the motility is significantly extended and the swimming velocity increased when the sperm-activating protein (or peptide) (SAP-1) was present (Fig. 4).

Current understanding of the fertilization process

The acrosome is a structure that serves a role in the spermatozoan's (1) species specific recognition of egg, (2) its further penetration through the egg envelopes, and (3) its fusion with the egg nucleus. We observed a gradual expansion of the acrosomal vesicle in lamprey spermatozoa. However, the process was not related to the presence of "egg water" (Fig. 3). The acrosome is characterized by heterogenous contents of the acrosomal vesicle and in some species of fish (sturgeon, Ciereszko et al. 1994; lamprey, Jaana and Yamamoto 1981), includes a penetrating perforatorium, i.e. acrosmal filament or central fiber. (Jamieson 1991). The disruption of the apical plasma membrane and the membrane of the acrosomal vesicles is called the "acrosomal reaction" (AR). These processes are associated with an influx of Ca^{2+} and Na^{2+} and cause a membrane depolarization and intracellular alkalinization. The AR, usually triggered at the egg surface, leads to the release or exposure of a number of hydrolytic enzymes associated with the acrosome. The spermatozoan's penetration of egg vitelline envelopes without a distinguished

micropyle, when flagellum activity ceased (Kobayashi and Yamamoto 1994) in lamprey, must be facilitated by the acrosomal hydrolyzing enzymes (Fig. 5).

Sperm cryopreservation

In the present experiment we utilized several cryoprotectants and procedures based on their success with sturgeon sperm (excellent motility, Ciereszko et al. 1996a) or mammalian sperm (Sankai et al. 1994). Milt from four males (density 0.91 x 10^9/ml; motility ± 60%) was diluted 1:1 with extender plus cryoprotectant and immidiately frozen (variant I), or extender was added first followed with a 60 - 90 min equilibration and then cryoprotectant added in small portions (variant II). Dimethylsulfoxide (DMSO), glycerol and dimethylacetamide (DMA) were used as cryoprotectants. No motility or egg fertilization was observed after thawing, indicating that neither of the variants resulted in viable spermatozoa. Microscopic examination of cryopreserved sperm (Fig. 6) revealed that the central canal of the sperm head was dilated (black thin arrow) or the central fiber was missing or fragmented. Longitudinal sections through the posterior region of sperm showed no mitochondria and missing or broken outer plasma membranes (white arrow heads). This suggests complete disintegration of the motility apparatus of flagellum. The acrosomal region showed dispersion of the floculent material of the vesicle and explosion of the anterior membrane of the vesicle (black arrow heads).

1998

Animal handling and fertilization

Sea lampreys were obtained from Cheboygan River, Michigan (Lake Huron Biological Station) and air-shipped to Columbus, Ohio. They were kept in the aquaculture facility of the School of Natural Resources, The Ohio State University. Fish were kept in the dark 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 storage at 15°C for eggs and on ice for milt. 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 fertilization rate (2-cell embryos) (Fig. 7), eggs were transferred to California-type hatching trays.

Fertilization rate in most experiments was estimated by calculation of percentage of fertilization at the 2-cell embryos stage (5 hours after fertilization). Additionally, fertilization rate was assessed 3 minutes after fertilization (Fig. 8) (perivitelline space or cytoplasmic bleb test, CBT) or at hatching.

Sperm concentration was estimated microscopically using a Double Neubauer Counting Chamber (Ciereszko and Dabrowski 1993).

Effects of storage time of lamprey eggs at optimized temperature on their fertilizing ability.

We found that when eggs were stored at 15°C, fertilizing ability amounted to 95.8 ± 14.0% (mean ± S.D.), 70.8 ± 6.4% and 19.8 ± 7.3% after one, two or three days of storage, respectively (Fig. 9). Storage of eggs at 15°C was superior to storage on ice where only 52.7 ± 12.1%, 18.4 ± 17.6% and 7.3 ± 7.3% of fertilization rates were recorded, after after one, two or three days of storage, respectively. Consequently, in other experiments we used eggs obtained either on the day of collection or after one-day storage at 15°C. Eggs of lower quality (without stickiness, presumably overripe) were not suited for storage at 15°C. Although fertilization rate of these eggs was very good on the day of collection (91.4 ± 4.3%) it declined dramatically after storage, to 50.6 ± 7.3% and 0.5 ± 0.5% after one and two days of storage, respectively (Fig. 9).

Determination of optimal sperm/egg ratio for fertilization of sea lamprey.

Establishment of optimal sperm/egg ratio is important in order to use known and optimal number of spermatozoa for fertilization experiments rather than use an excess of sperm. In this experiment, we tested effects of sperm number on fertilization success. An increase of fertilization rate was observed up to 50,000 spermatozoa per egg (Fig. 10). For this reason, a constant sperm/egg ratio (1:50,000) was used in our studies. This allowed us to perform fertilization trials at a controlled condition in regard to the gamete ratio.

Determination of the effect of time after sperm release to water on fertilization of sea lamprey eggs.

Unlike in teleost fish where eggs usually have to be fertilized within a few minutes (or less) after release to water, fertilizing ability of sea lamprey eggs is markedly prolonged. However, detailed data on this issue are lacking. We found that after one, two or three hours of egg exposure to water at 22°C, the fertilization rate was 85.2 ± 11.2%, 60.3 ± 15.5% and 31.5 ± 10.7%, respectively (Fig. 11). In some cases fertilization rate after one hour of water exposure was as high as 94.8% - 96.4%. This may better reflect initial quality of eggs obtained under laboratory conditions. We speculate that at water temperatures characteristic for natural spawning (11 - 15°C), survival of eggs may be significantly higher after an extended period following the eggs release to water. Therefore, released lamprey eggs will be capable of fertilization during several hours. Long viability of sea lamprey gametes in freshwater (much longer than in eggs of most fishes) may contribute to the enormous reproductive success of this species.

Correlations among fertilization successes estimated at 3 min (cytoplasmic bleb test) and 5 hours (2-cell embryos) and hatching rate.

A reliable and quick estimation of fertilizing success is a prerequisite for fertilization experiments. We estimated fertilization rate 2-3 minutes after fertilization (perivitelline space

and cytoplasmic bleb test), five hours after fertilization (2-cells stage) and at hatching in order to find the earliest estimation of fertilization success. Both early assessments of fertilization correlated significantly (r = 0.92 and 0.93, P < 0.0001) with the percentage of live embryos at hatching (Figs. 12, 13). For this reason, both tests can be employed for the quick estimation of fertilization success. Both are cost effective and less time-consuming than estimation of the percentage of live embryos at hatching.

Evaluation of fertilization rate of spermatozoa treated with gossypol

Effect of 30 minutes preincubation of lamprey semen with 100μM gossypol acetate on sperm fertilizing ability (mean ± SEM) was examined. Control sperm suspension contained: 1% ethanol, or gossypol with the same final concentrations in fertilization medium as in experimental variants (n = 4). We observed 56.5 ± 21.2% and 57.6 ± 22.0% fertilization rate in control samples without gossypol and with gossypol introduced *in vitro* with sperm, respectively. This indicates that low concentrations of gossypol in fertilization medium did not affect sperm or egg fertility. However, preincubation of sperm with 100 μM gossypol for 30 minutes produced a significant decrease of fertilization rate to 28.4 ± 10.7% (Fig. 14). These results indicate that gossypol can reduce *in vitro* fertilizing ability of sea lamprey spermatozoa.

Evaluation of the quality of semen and levels of plasma sex steroids of sea lampreys injected with different doses of gossypol.

Effects of injecting lampreys with different concentrations of gossypol (25, 50, 100 and 200 mg/kg of body weight) on semen quality and levels of plasma sex steroids (testosterone, progesterone, estradiol-17β and 17,20β-dihydroxy-4-pregnen-3-one) were investigated. Two lots of lamprey (average weight : 227.8 ± 21.3 g) from the Lake Huron Biological Station were used in this experiment. The first lot was divided in three groups and fish were injected with 0.2 ml/kg 50% ethanol as control group 1 (n = 12) or with gossypol diluted in ethanol in doses of 100 (n = 12) and 200 mg/kg (n = 13). The second lot also was divided into three groups and fish were injected with 0.2 ml/kg 50% ethanol as control group 2 (n = 12) or gossypol in doses of 25 (n = 12) and 50 (n = 12) mg/kg. Sperm motility, concentration and volume were recorded after 31, 36 and 40 days and 24, 28 and 33 days in lots 1 and 2, respectively. Fertilization rates were measured after 36 and 40 days and 28 and 33 days in lots 1 and 2, respectively. Blood was collected from the caudal vessel into a heparinized syringe before treatment and after 40 and 33 days in lots 1 and 2, respectively. Plasma levels of estradiol-17β (E2), testosterone (T), progesterone (P) and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) were measured by radioimmunoassay in plasma extracts (method similar to Ottobre et al. 1989). All plasma sex steroids were measured by radioimmunoassay in plasma extracts (method similar to Ottobre et al., 1989).

At the end of the experiment, the testes were removed, fixed in Bouin's solution, embedded in paraffin, and stained with Mayer's hamatoxylin and eosin for histological examination.

High mortality was observed at the day of intraperitoneal injection in the groups of 200 mg/kg (84.6%), 100 mg/kg (41.7%) and 50 mg/kg (25%). These results indicate that a single injection of a high dose of gossypol is toxic for lampreys. Semen from gossypol-treated males was yellow and contained yellow sediment. Most likely it was a gossypol acetate. At each sampling date, sperm motility was higher in control groups than in treated groups but no significant differences were observed between the groups (Fig. 15). No significant differences in sperm concentration and volume were found between gossypol-treated and control lampreys (Fig. 15). Sperm from gossypol-treated males tended to have lower fertilizing abilities, but these differences were not significant (Fig. 16).

Although there was some variation among groups in the plasma levels of the four sex steroids examined, no significant differences were observed (Fig. 17). However, there was a slight decrease in plasma E2 concentrations associated with the increase of the concentration of gossypol (Fig. 17). The low concentrations of gossypol (25 and 50 mg/kg) induced an increase of the P levels whereas the high concentrations (100 and 200 mg/kg) decreased those levels (Fig. 17). Finally, as revealed at the light microscopic level, the structure of the testes in gossypol-treated lampreys at the end of the treatment appeared to be unchanged from those of control lampreys.

Our results indicate, that a single injection of gossypol at different doses did not significantly affect sperm characteristics and the levels of sex steroids. However, the slight decrease of E2 levels, a major steroid associated with the spermiation in sea lamprey spermatogenesis (Sower 1990), could be due to an action of gossypol on the hypothalamo-hypophysial axis, on Leydig cells or on interrenal cells. Gossypol at high doses is toxic for lampreys. Prolonged treatments and further *in vitro* studies are required to confirm the effects of gossypol in male sea lamprey spermatogenesis.

Evaluation of fertilization rate of lamprey spermatozoa sterilized with bisazir.

Bisazir is currently used in sterilization programs of male sea lamprey. Our results indicate that semen characteristics of lampreys injected with bisazir did not differ from controls. Sperm concentrations were 0.545 ± 0.279 and 0.557 ± 0.255 x 10^9 spermatozoa and sperm motilties were 55.0 ± 13.3% and 52.5 ± 15.5% for control and bisazir-injected lampreys respectively (Fig. 18). Spermatozoa from bisazir-injected lampreys were able to fertilize eggs and to induce first divisions of the zygote. Fertilization rate at 2-cells stage of these spermatozoa were not different from control sperm. However, sperm of bisazir-treated lampreys produced no live embryos at hatching. At the same time, 62.5 ± 14.9% of live embryos at hatching were observed in the control group. We found a significant correlation between fertilization rate estimated at 2-cells stage and at hatching for control sperm (Fig. 19). This confirmed our earlier findings. Our results also confirm a usefulness of bisazir for sterilization of male lampreys. Sterilized lampreys produce sperm with characteristics (sperm motility and concentration) similar to control lamprey. This sperm can fertilize eggs and induce first cell divisions of the zygote, but later stages of embryonic development are impaired, likely due to damage to the sperm genome.

Significance of results to the Commission's Sea Lamprey Program

This is the first report on physiological parameters related to sea lamprey sperm. Sperm characteristics (volume, density, motility, seminal plasma osmolality, ionic concentration) were described in males induced to spermiate with gonadotropin releasing hormone injections. Sperm volumes for individual males, morphological and motion parameters (computer assisted sperm motion analysis; CASA) such as velocity and linearity, were established for spermatozoa activated in water or with "gynogamone", egg-associated chemotactic substance(s). A preliminary characteristic of acrosomal enzymes of lamprey sperm was provided. These data are essential to verify the contraceptive effect of bisazir, which is already in use, as well as to establish conditions for tests with new sterillants.

Data collected in 1997-1998 provide the first information on the basic characteristic of sea lamprey sperm. We confirmed that, as in other lamprey and ancient fishes, dynamic changes take place in the acrosomal vesicle of activated spermatozoa (in hypotonic solution). Specifically, we observed during activation with "egg water" (substances washed out from ovulated eggs) numerous occurances of exocytosis in acrosomes. However, we were unable to induce the discharge of the acrosomal filament.

Current understanding of the fertilization process includes specific roles of the acrosome in (1) recognition of species specific egg, (2) its further penetration through the egg envelopes, and (3) its fusion with the egg nucleus. In sturgeon (Ciereszko et al., 1996) and some lamprey species, a penetrating perforatorium, i.e. acrosomal filament, extends backwards from the acrosome through the nucleus. The disruption of the apical plasma membrane and the membrane of the acrosomal vesicle is called the "acrosomal reaction" (AR).

Gamete collection by stripping followed by artificial fertilization have been only fragmentarily studied in sea lamprey. Good quality oocytes (with stickiness at fertilization) need to be stored in optimized conditions to retain their maximal fertilizing ability. Several end points for fertilizing ability need to be recognized in order to determine (1) sperm penetrating ability through the jelly coat and vitelline envelopes, (2) multi cell embryo formation and (3) hatching of normal larvae. These criteria are essential for determining genetic sterility of treated male lampreys with maintained physiological capacity (motility and AR) of their spermatozoa.

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Table 1 Osmolality (miliosmomole/kg) and ionic concentrations (mM) in seminal plasma of two teleost fish, lake sturgeon (*Chondrostei*) and sea lamprey (1996). Data are presented as a mean ± SD.

	Species			
	Oncorhynchus mykiss[1]	*Esox masquinongy*[2]	*Acipenser fulvescens*[3]	*Petromyzon marinus*
Osmolality	220.8±69.7	284.3±10.0	---	249.0±20.0
P	2.66±2.92	5.57±5.57	3.90±0.38	0.66±0.28
K	20.01±4.54	23.69±1.69	5.82±0.49	10.48±2.58
Na	67.61±13.64	131.96±2.96	25.65±2.79	106.3±12.8
Ca	1.39±0.47	2.11±0.19	0.16±0.05	0.57±0.08
Mg	1.01±0.27	1.45±0.61	0.25±0.02	1.24±0.28
Cl	43.07±11.62	132.32±7.02	5.41±2.79	---

[1]Ciereszko and Dabrowski, unpublished data for 2 year old males of Mount Shasta Strain.
[2]Lin et al. (1996); saline injected group.
[3]Toth et al. (1997).

Table 2 Sperm and egg viability tests.

A. Test for egg quality in lamprey (June 29, 1996).

No.	Female wt. (g)	Eggs (%)	Fertilization (%) (duplicate)
1			16.4
2	207	20.7	33.7
3	330	29.3	0
4	318	24.2	44.1
5			14.2

*Combined sperm from 3 males; motility 60 $\pm$ 10%; sperm density 2.10^6/egg.

B. Test for "delayed" fertilization of eggs released to fresh-water (n=3)

Time (min)	0	10'	60'
Fertilization (%)	17.2 $\pm$ 8.7	0.92 $\pm$ 0.27	0

*Test on July 15, 1996.

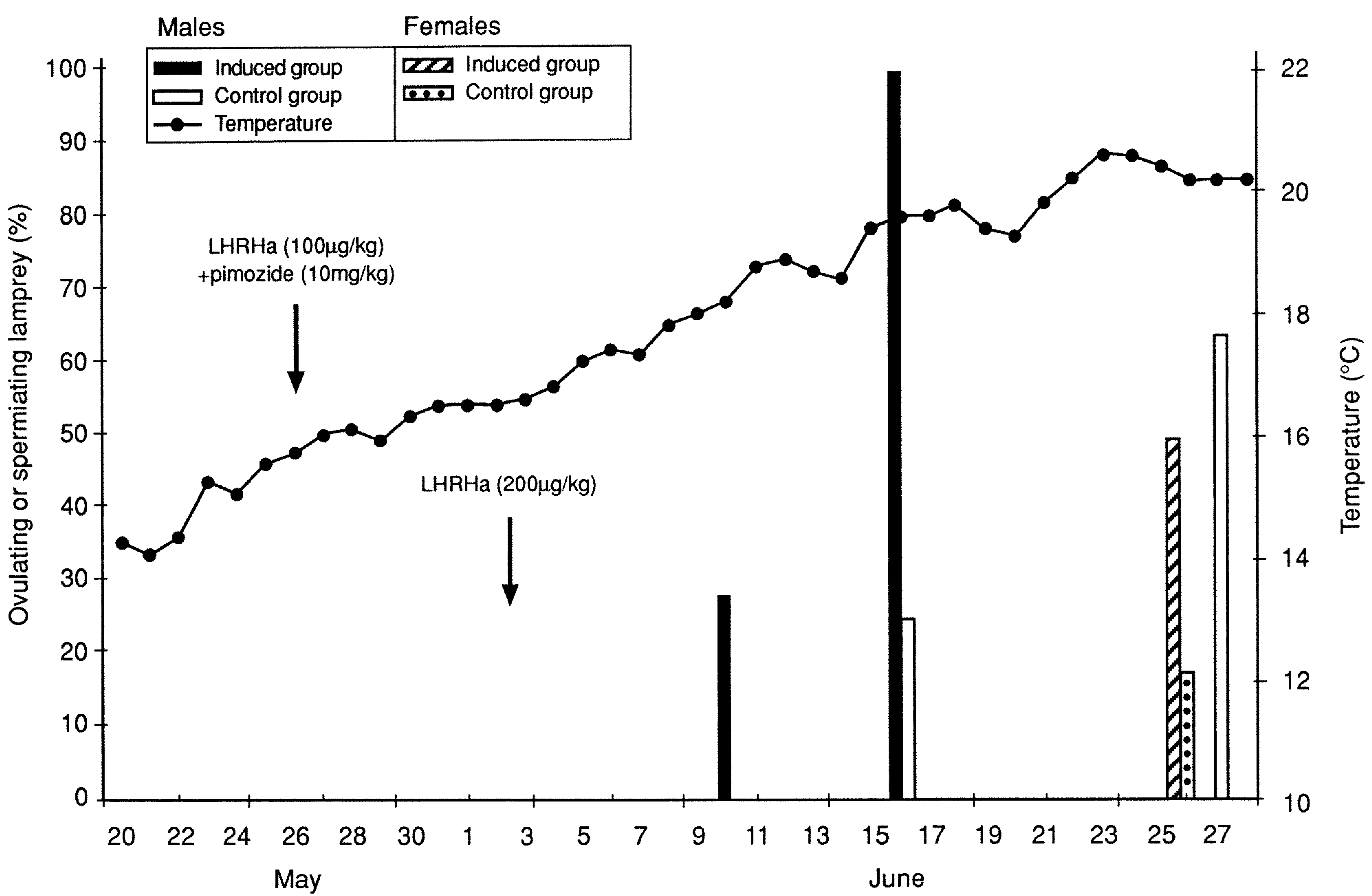
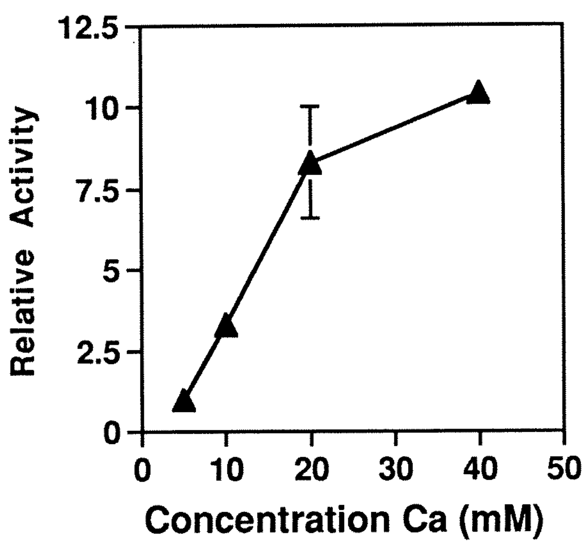
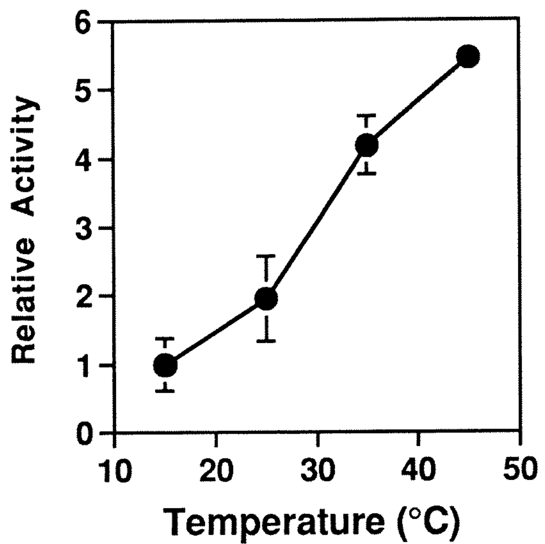


Fig. 1 Stimulation of spermiation or ovulation in sea lamprey by injection of LHRHa and pimozide.





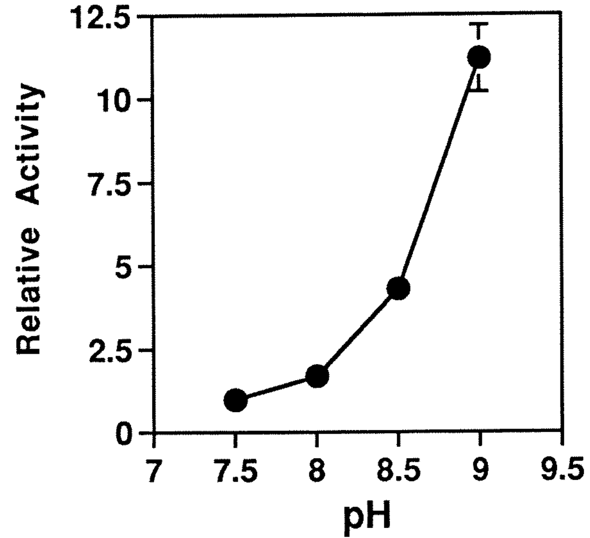
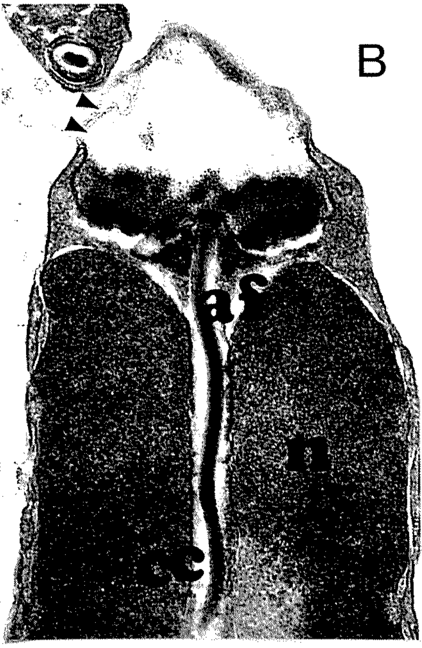


Fig. 2 Chymotrypsin–like activity of sea lamprey spermatozoa — optimization study.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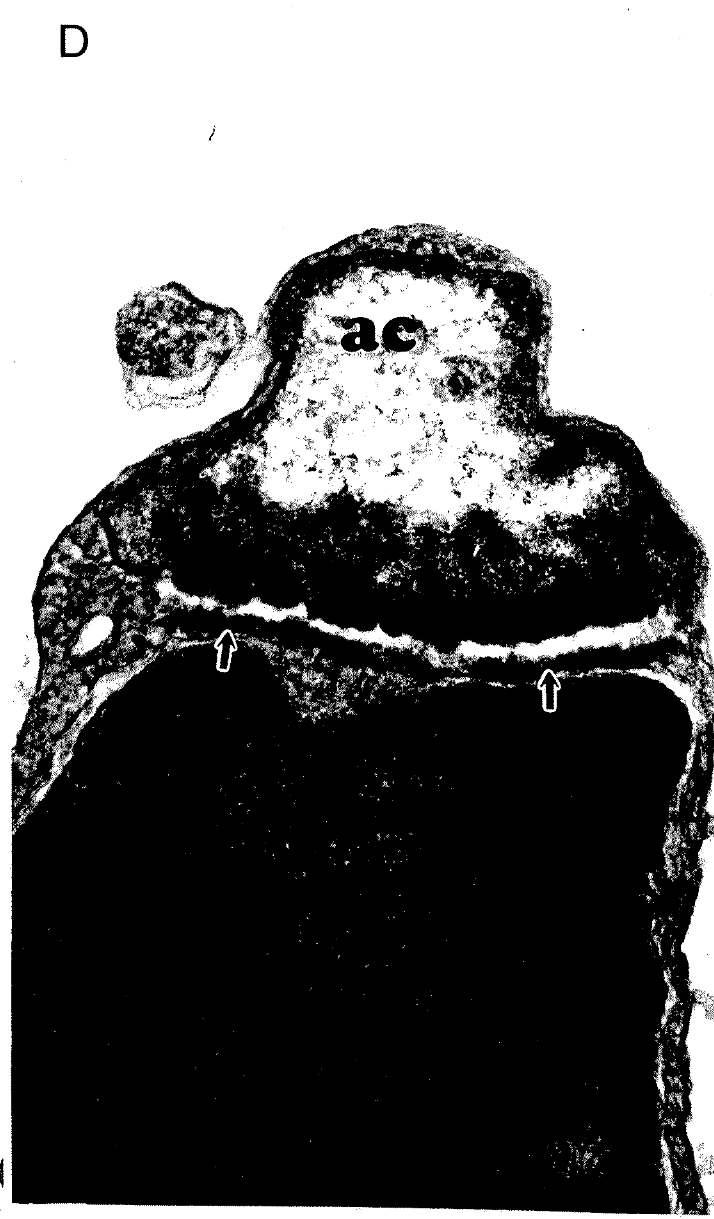
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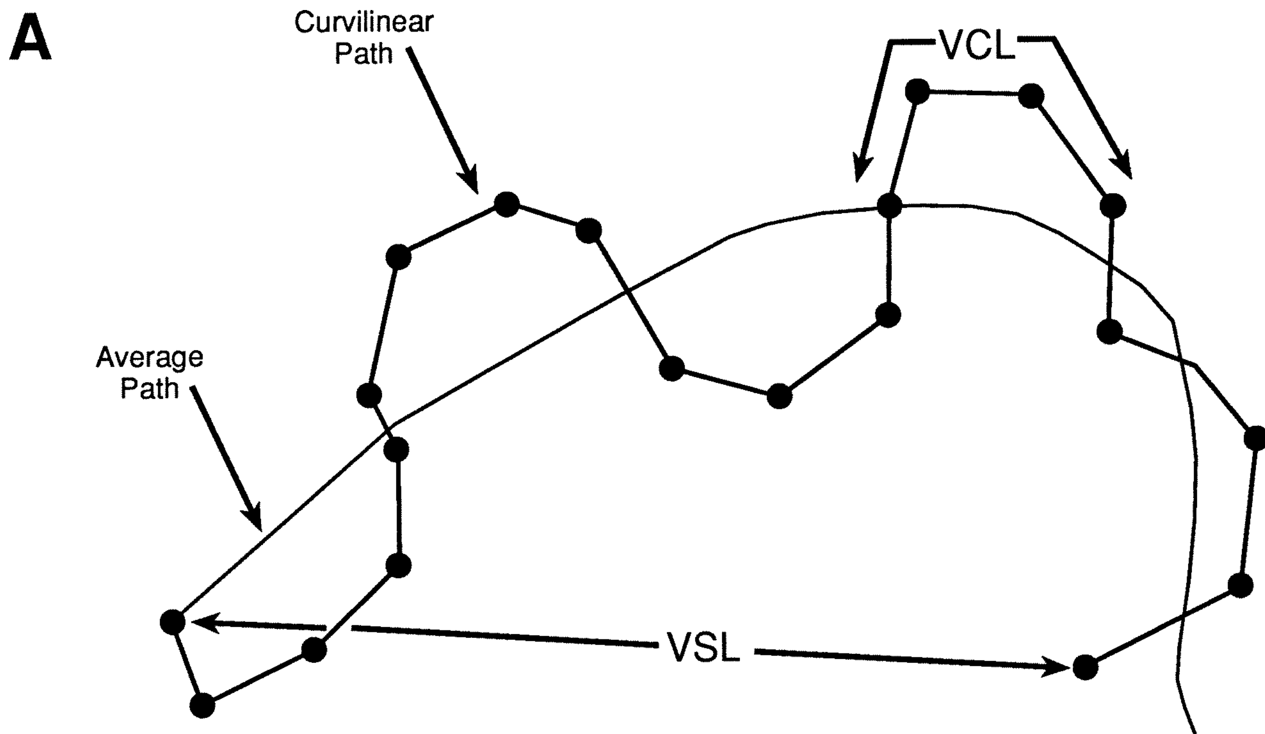


B Sea lamprey sperm motility filmed at 200 Hz and assessed on a miminum 100 cells (n = 3-4)

Activation medium	Diluted Ringer (LR)	"Egg water"
Motile cells (%)		
0 min	35.0 ± 20.9	28.6 ± 15.4
3 min	7.8 ± 3.1	44.2 ± 13.9
Curvilinear velocity (μm/s)		
0 min	276 ± 41	321 ± 34
3 min	308 ± 34	306 ± 51
Straight line velocity (μm/s)		
0 min	40.9 ± 7.5	39.3 ± 2.4
3 min	35.4 ± 1.6	39.5 ± 5.0

Fig. 4 Using CASA for sperm motion studies. (A) An example of path of the spermatozoan; VSL-straight line velocity; VCL-curvilinear velocity. (B) Observation of VCL and VSL of the sea lamprey spermatozoa activated in the diluted Ringer solution (Kobayashi 1993) and "egg water" containing the sperm activating factor or the "gynoamone."

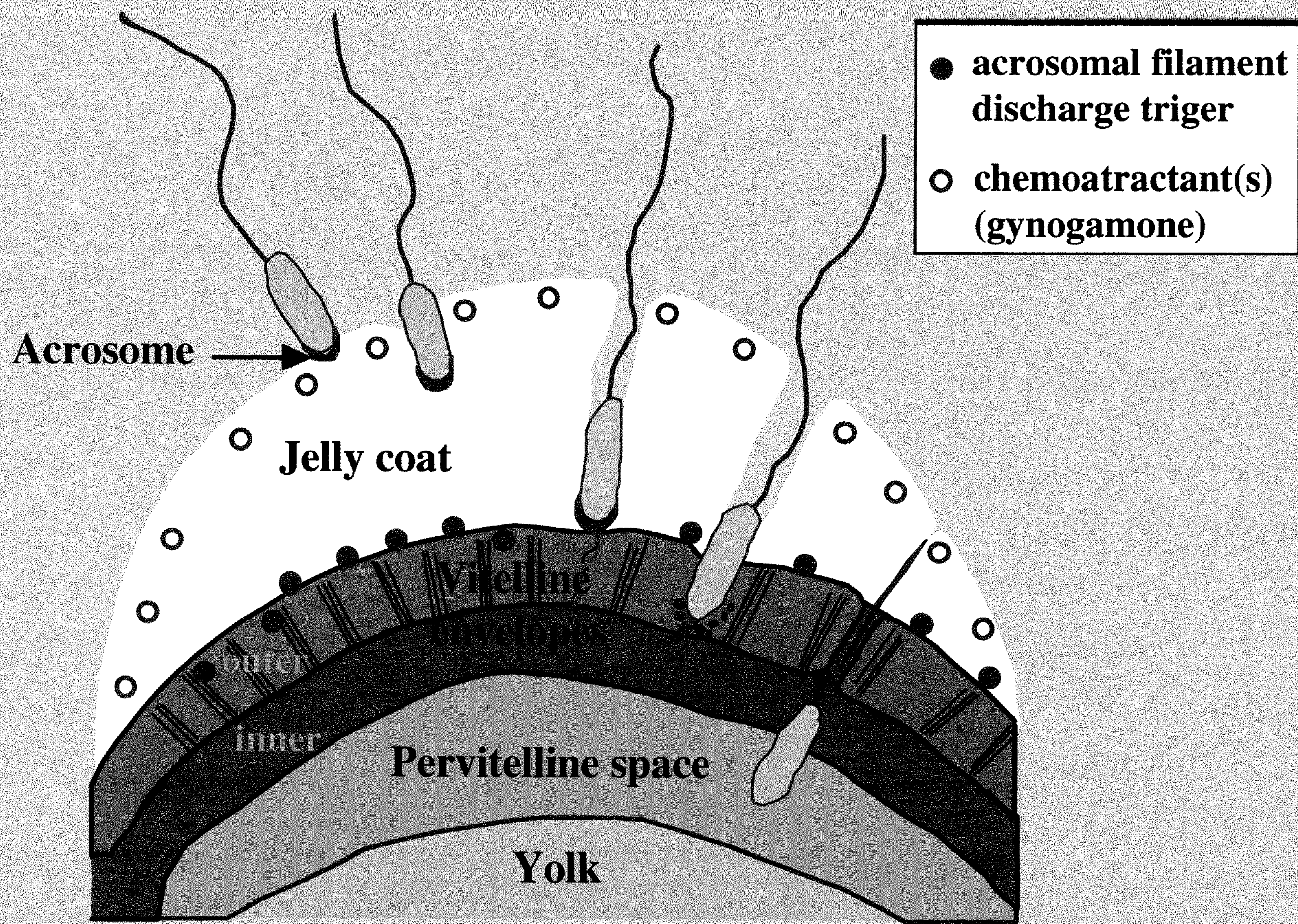


Fig. 5. Schematic representation of a spermatozoa penetrating oocyte membranes in lamprey.

Fig. 6 Electron micrographs of cryopreserved spermatozoa of sea lamprey (see text for description).

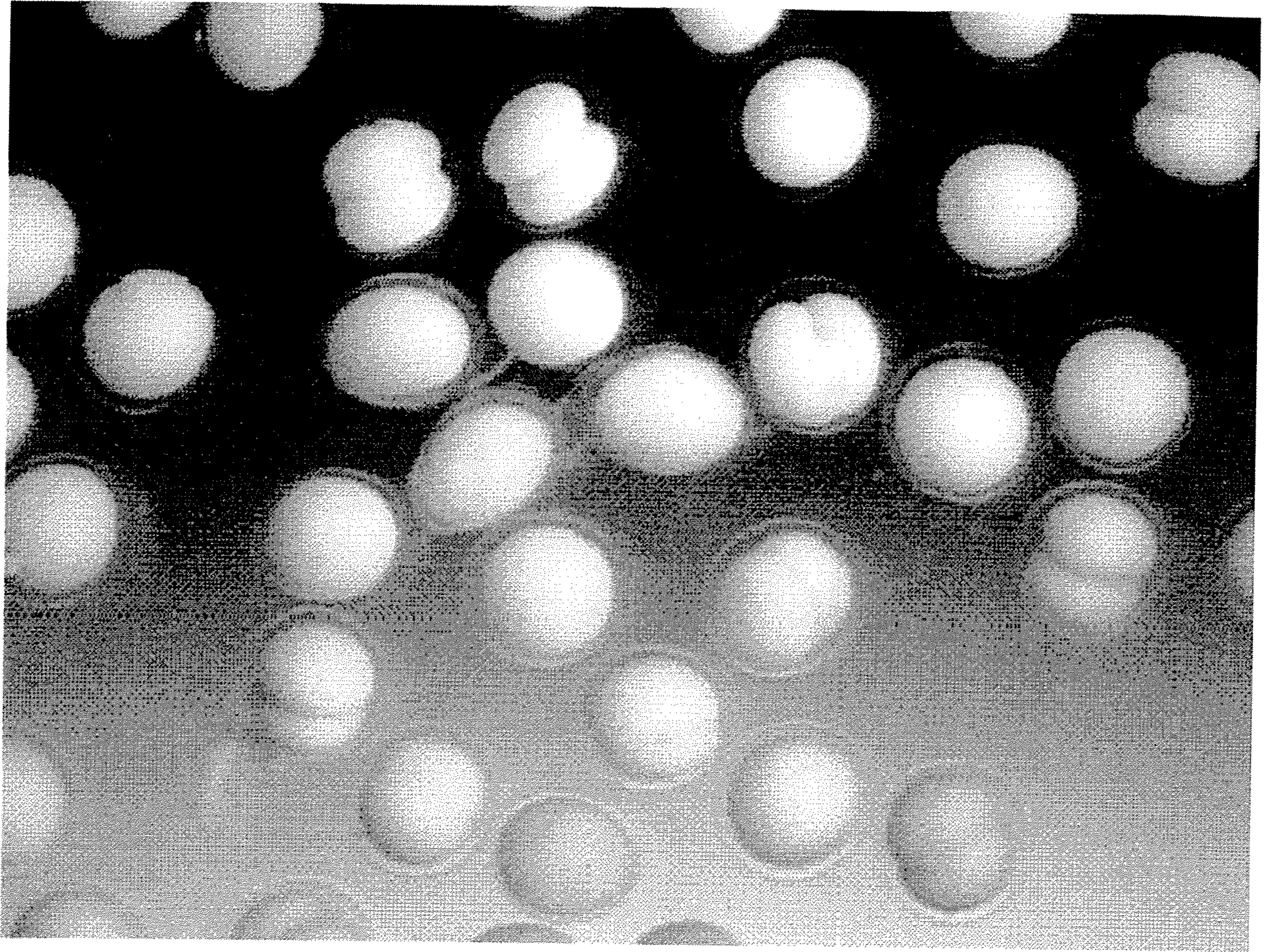


Fig. 7. Unfertilized eggs and 2-celled embryos of sea lamprey five hours after fertilization.

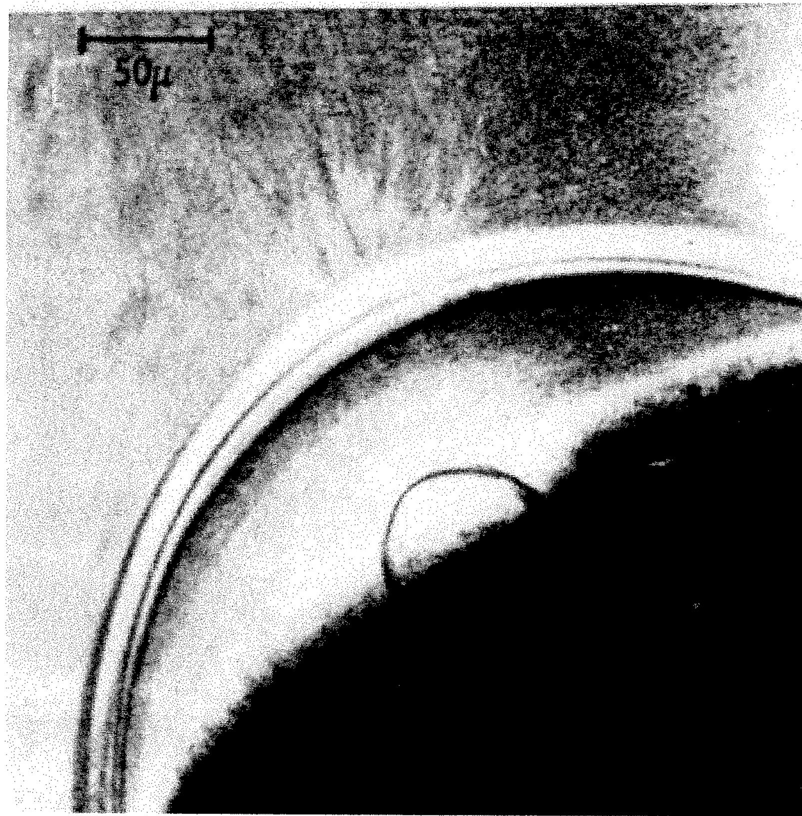


Fig. 8. **Animal pole of the lamprey egg showing cytoplasmic bleb 3 min after fertilization at 20°C (Kille, 1960).**

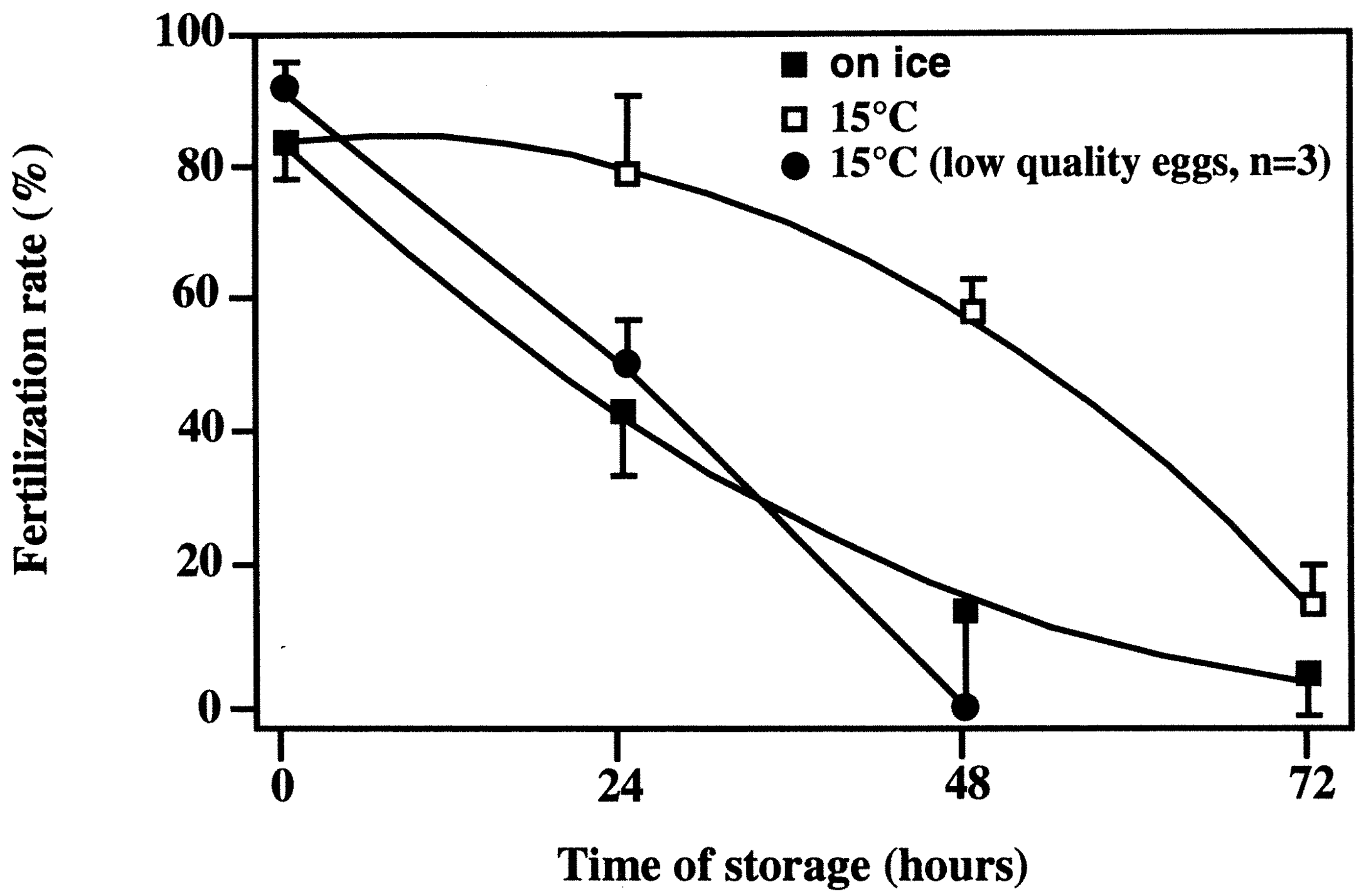


Fig. 9. Ability to fertilize (mean ± SEM) of sea lamprey eggs in relation to time of storage on ice or at 15°C (n=4).

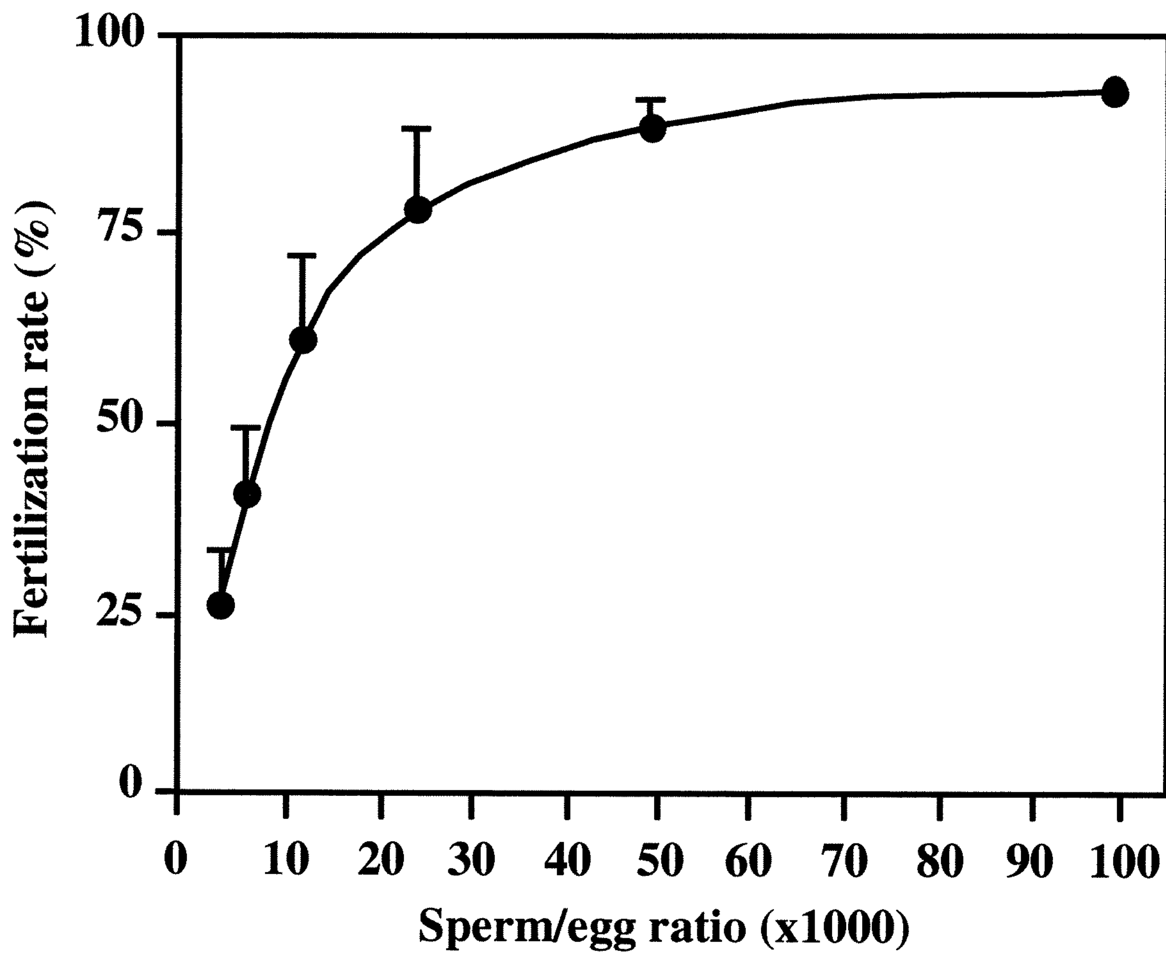


Fig. 10. Fertilizing rate (mean + SEM) of sea lamprey eggs in relation to number of spermatozoa per egg (n=4).

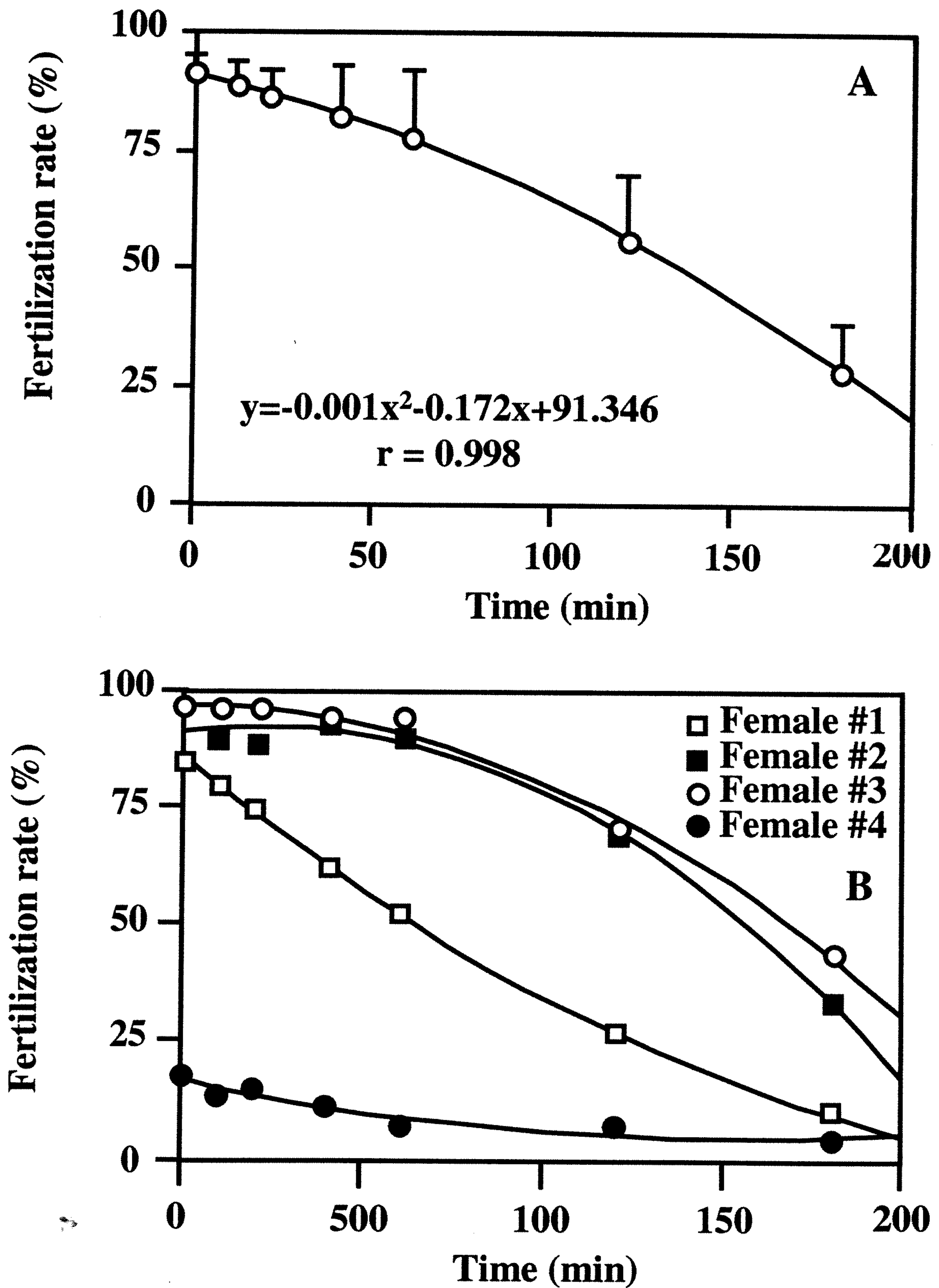


Fig. 11. Fertilizing ability of sea lamprey eggs in relation to time of exposure to water. A-Mean values of 3 females with superior egg quality, B-Individual females.

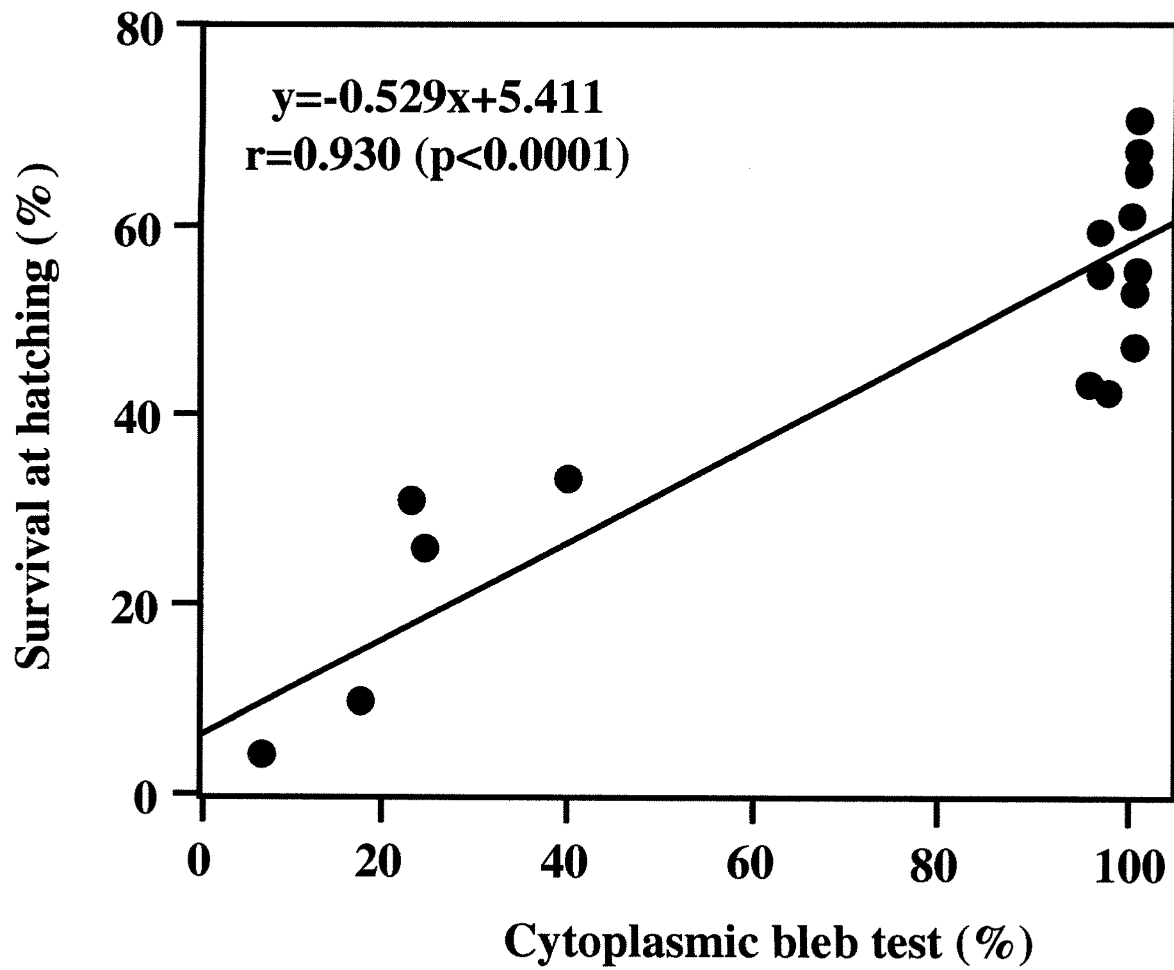


Fig. 12. Regression between cytoplasmic bleb test (CBT) and embryo survival at hatching.

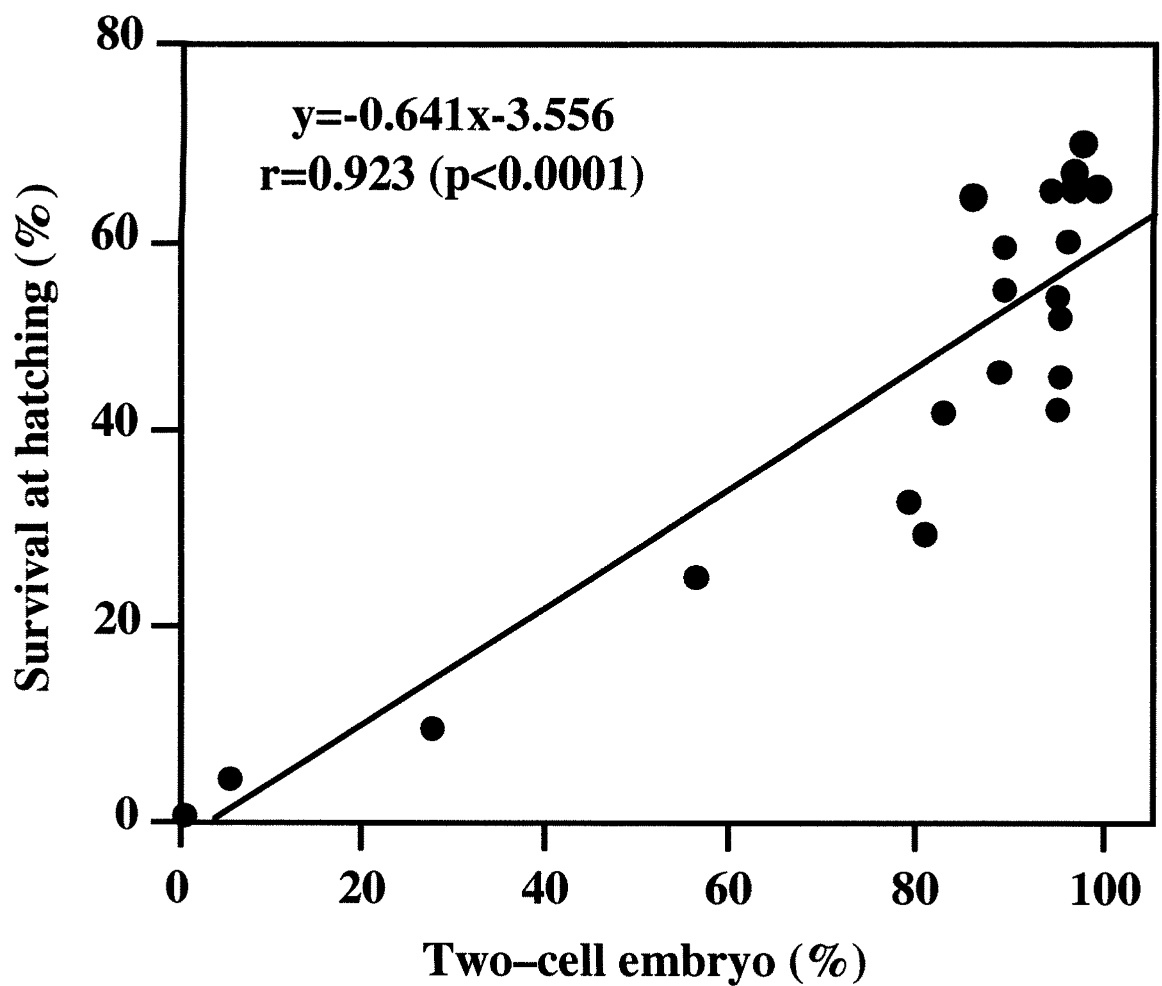


Fig. 13. Regression between percentage of embryos at 2-cell stage and embryo survival at hatching.

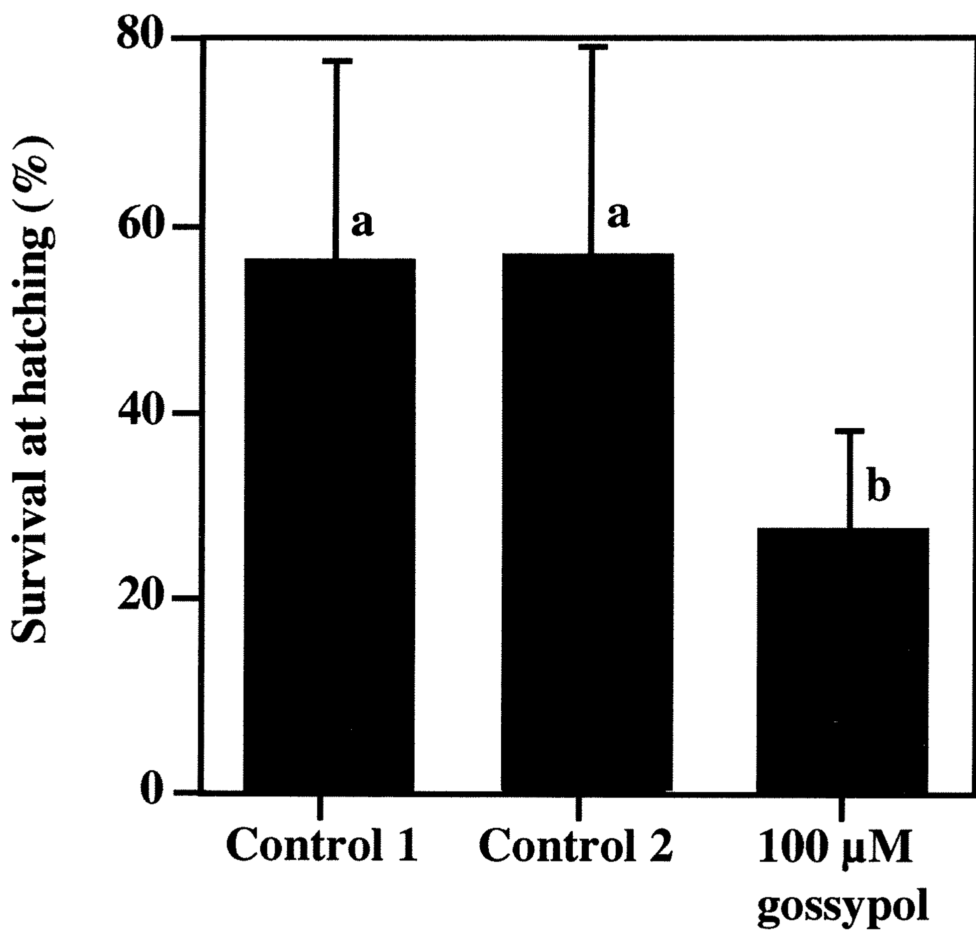


Fig. 14. ***In vitro*** **effect of gossypol exposure on sea lamprey hatching rate.**

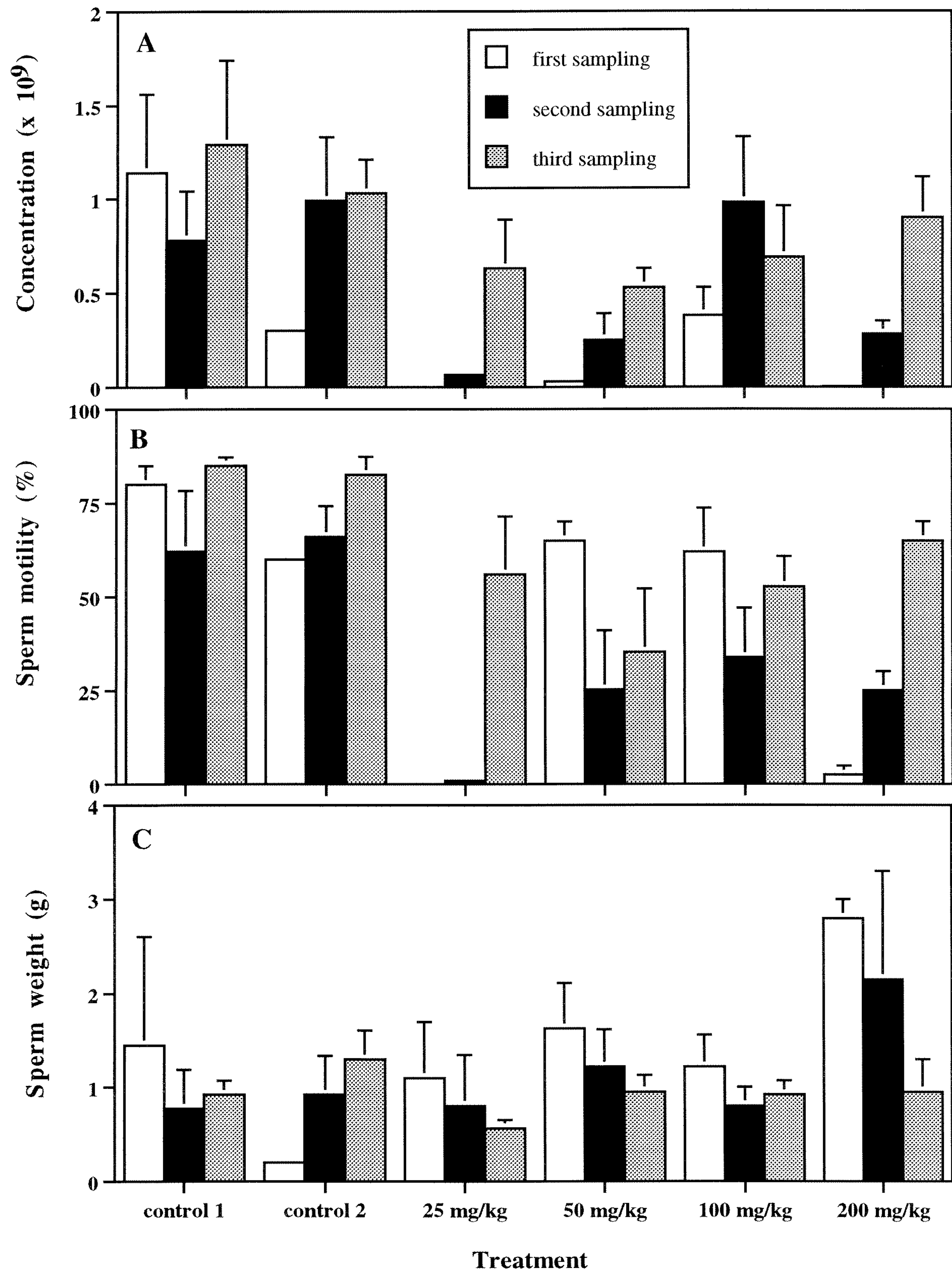


Fig. 15. Effect of gossypol on semen characteristics of male sea lamprey. A-Sperm concentration, B-Sperm motility and C-Milt weight.

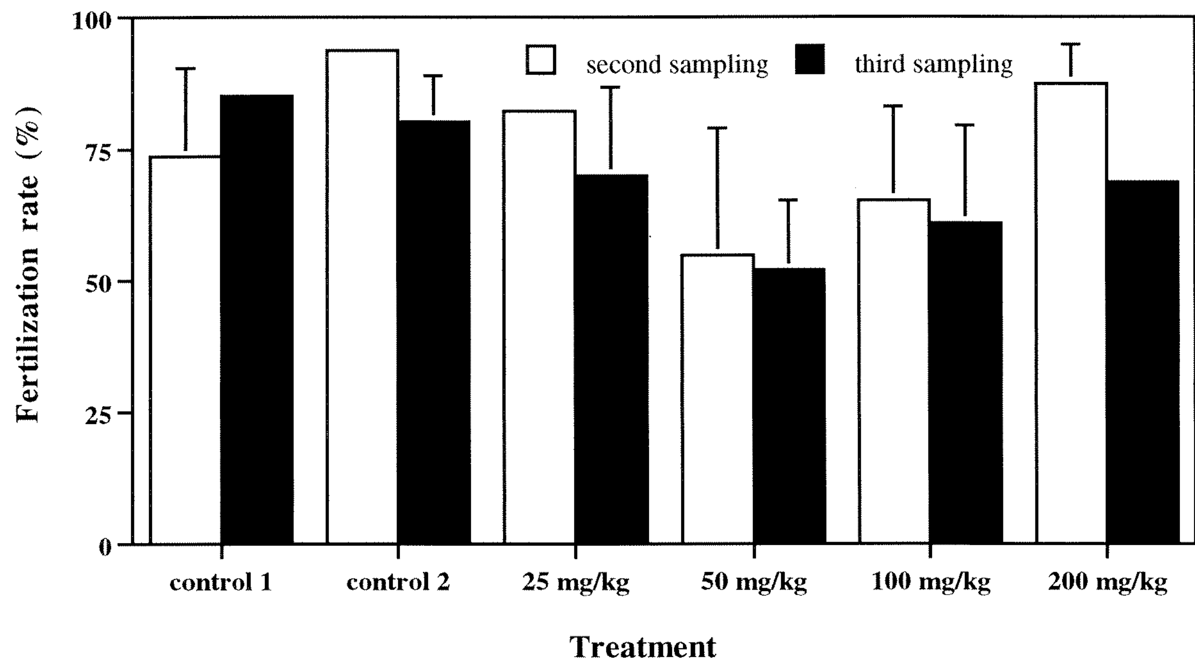


Fig. 16. Effect of gossypol on fertilization rate of sea lamprey spermatozoa

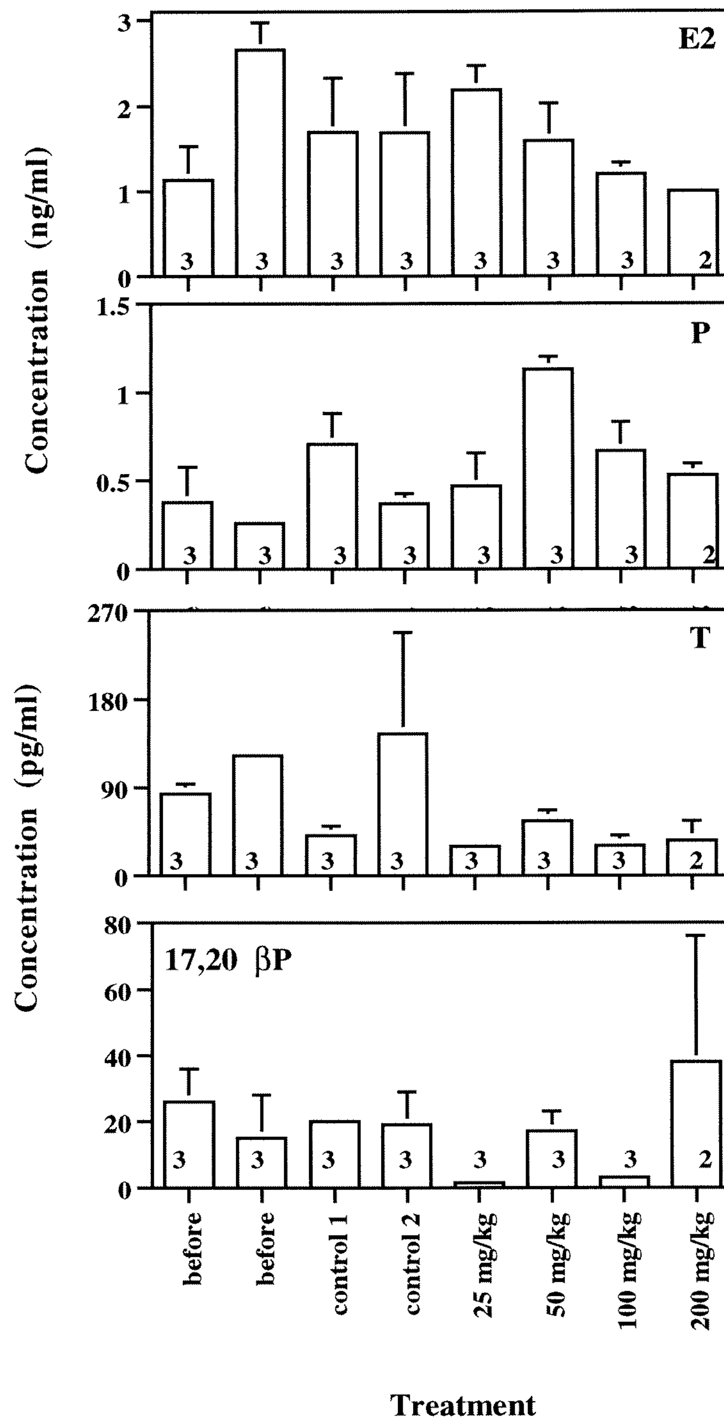
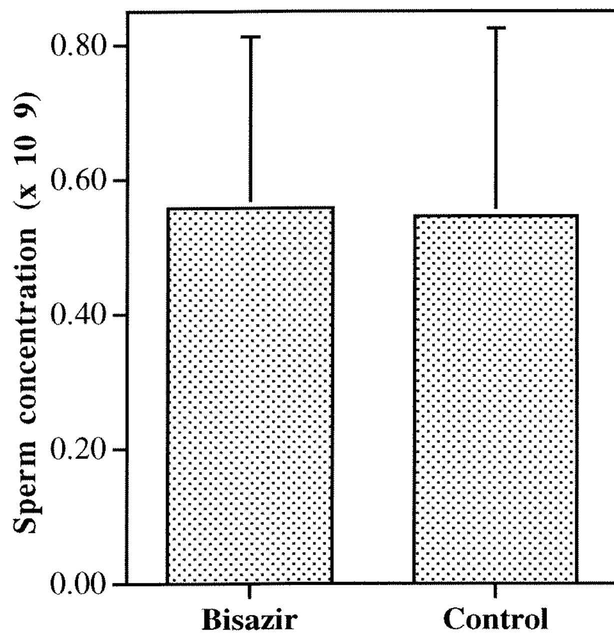
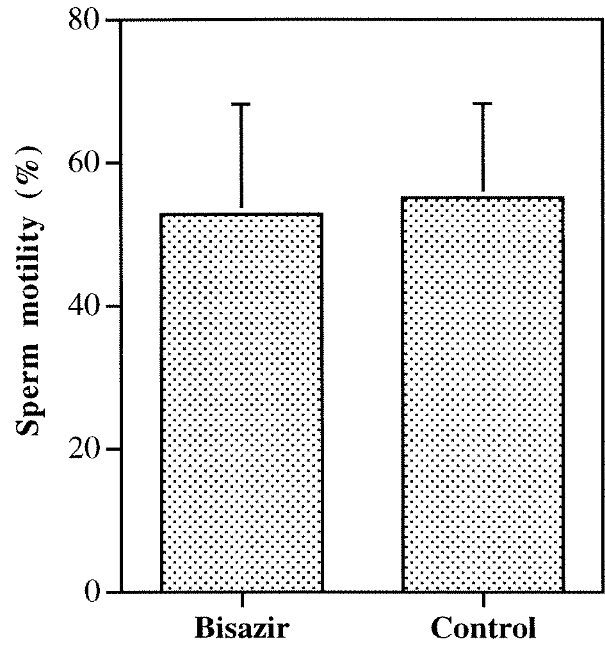
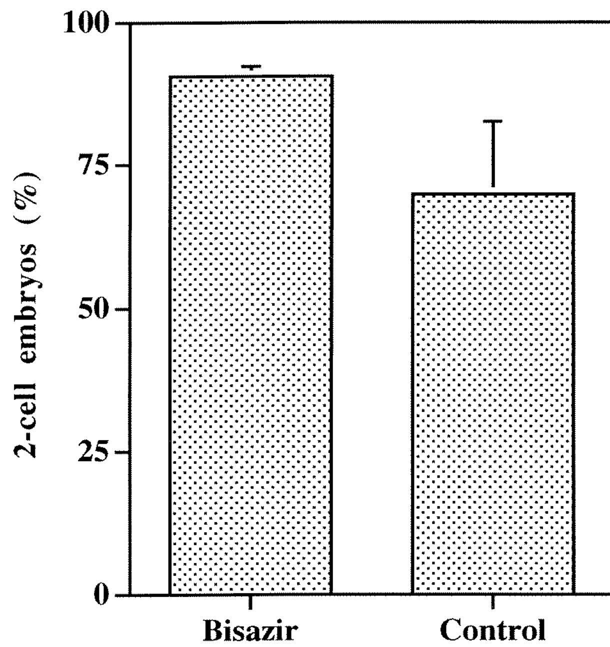


Fig. 17. Effect of gossypol on plasma sex steroids in male sea lamprey. Numbers of fish in each group are depicted at the base of each bar. Error bars are not indicated when too small







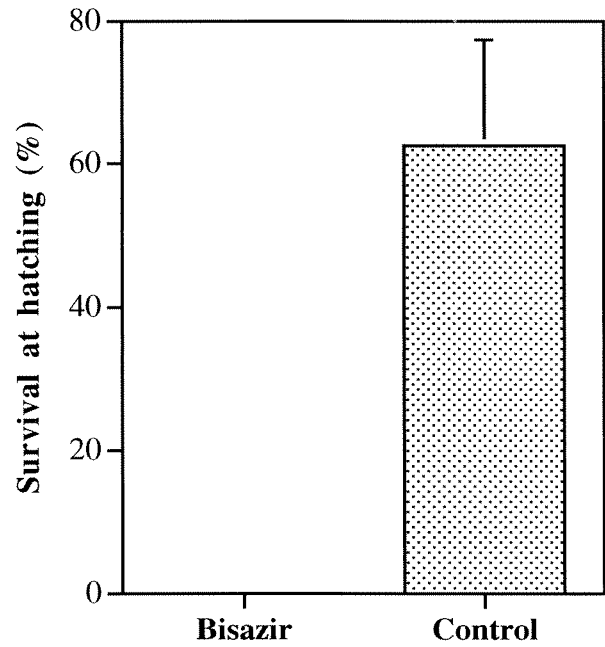


Fig. 18. **Sperm characteristics of milt from control and bisazir-treated sea lampreys.**

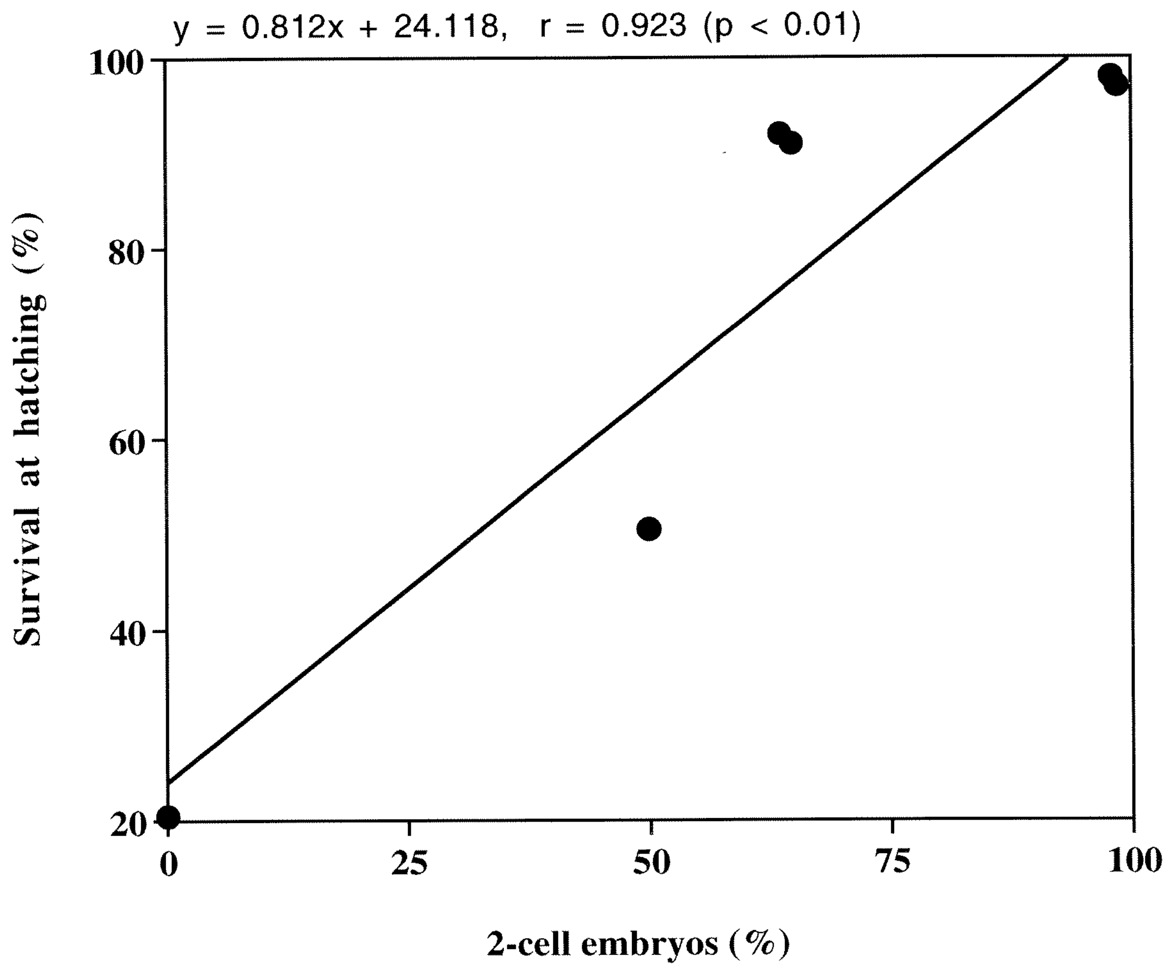


Fig. 19. Regression between fertilization rates estimated at 2-cell stage and at hatching (data for 6 fish of control from bisazir experiment)