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Survival of ovulated oocytes of the European catfish (*Silurus glanis*) after *in vivo* and *in vitro* storage or exposure to saline solutions and urine

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Abstract

Survival of *Silurus glanis* ovulated oocytes (ova) measured by the capacity of normal development *i.e.* percentage of normal and abnormal hatched larvae after *in vivo* and *in vitro* storage and exposure to sperm activating or immobilizing solutions and urine was studied. In the case of ovulated oocytes left in the ovaries, total hatching and abnormal hatched larvae (in % of hatching) were respectively 74 and 8.2% immediately after ovulation, 77 and 8.6% after 2 h, 54 and 18% after 4 h, and 38 and 50% after 6 h of storage. Four and 6 h stay of ovulated oocytes in ovaries resulted in a significant increase of abnormal hatched larvae ($p<0.01$). For the ova stored *in vitro*, the capacity to undertake a full embryonic development after fertilization was not significantly changed either after 8.5 h at 19°C or 3.5 h at 25°C; there was no ova survival at all after 3.5 h storage at 8°C, 12 h at 19°C and 8.5 h at 25°C. There was a significant increase of abnormal larvae after 3.5 h at 25°C (74%, $p<0.001$) and after 8.5 h at 19°C (37%, $p<0.05$). Exposure of non inseminated ova to water buffered to pH 7.0 (5 mM Tris-HCl) to sperm activating solution (17 or 41 mM NaCl, 5 mM Tris-HCl, pH 8.0) or to immobilizing solution (200 mM NaCl, 30 mM Tris-HCl, pH 7.0) resulted in a regular and rapid decrease of their capacity of development; this was 10% of normal hatched larvae within 4 min in water, and within 6-8 min in the NaCl solutions. A similar situation was observed after exposure to urine with a loss of embryonic development of 30% within 3 min. These results indicate that all steps of the whole procedure of gamete collection and artificial insemination should be carried out rapidly as soon as possible after ovulation.

Keywords: *Silurus glanis*, ovulated oocyte, ova, hatching, activating solution, immobilizing solution, urine.

Survie d'ovules chez le poisson-chat européen Silurus glanis *conservés* in vivo *et* in vitro *ou dilués dans différentes solutions salines et dans de l'urine.*

Résumé

La survie des ovules de *Silurus glanis* conservés *in vivo* (dans l'ovaire), *in vitro* ou dilués dans des solutions d'immobilisation ou d'activation du sperme et dans l'urine est évaluée par le pourcentage d'éclosion de larves normales et anormales. Dans le cas d'ovules laissés dans les ovaires, les pourcentages totaux d'éclosion et ceux de larves anormales sont respectivement de 74 et 8,2 % immédiatement après l'ovulation, 77 et 8,6 % après 2 h de séjour, 54 et 18 % après 4 h et 38 et 50 % après 6 h. Après 4 et 6 h de séjour des ovules le pourcentage total d'éclosion diminue de façon significative et celui de larves anormales augmente. Dans le cas d'ovules conservés *in vitro* et non dilués, la fertilité initiale n'est pas modifiée après 8,5 h à 19°C ou 3,5 h à 25°C mais aucune survie n'est observée après 3,5 h à 8°C, 12 h à 19°C et 8,5 h à 25°C. Des ovules non fécondés, placés dans les solutions d'activation (17 ou 41 mM NaCl, 5 mM Tris-HCl, pH 8,0) ou d'immobilisation (200 mM NaCl, 30 mM Tris-HCl, pH 7,0) du sperme subissent une rapide diminution de leur capacité à donner lieu à un développement embryonnaire. Le taux d'éclosion est de 10 % de larves normales, après 4 min dans de l'eau douce tamponnée (5 mM Tris-HCl,

pH 7,0), après 6-8 min dans les solutions de NaCl. Le pourcentage d'éclosion est réduit de 30 % lorsque les ovules sont placés dans l'urine pendant 3 min. Les étapes de l'ensemble de la procédure de récolte des gamètes et d'insémination artificielle doivent être effectuées le plus rapidement possible après l'ovulation.

Mots-clés : *Silurus glanis*, ovule, éclosion, urine.

INTRODUCTION

The survival time of ovulated oocytes (or ova) is usually short after ovulation in many fish species. In the common carp (*Cyprinus carpio*) results of Saad and Billard (1987) indicated that the ova survival decreased regularly after ovulation and that no fertilization was acheived after 6 h of *in vitro* storage. In the European catfish (*Silurus glanis*) Linhart *et al.* (1991) showed that non-fertilized eggs stored under aerobic conditions at 17-18°C were still fertile 3 h after stripping with percentage of fertilization similar to eggs fertilized immediately after ovulation. In *Clarias gariepinus* the latency time, between hormone injection and egg collection and duration of *in vivo* viability of ova was dependent on water temperature (Hogendoorn and Vismans, 1980). The highest percentage of hatching was observed when ova stripping took place 21, 11 or 7 h after hypophysation at 20, 25 or 30°C, respectively. A sharp decrease in hatching percentage was observed when stripping occurred 30 h after injection at 20°C or 8-9 h at 30°C, indicating that significant ageing of ova occurred about 8-9 h after ovulation at 20°C and less than 2-3 h at 30°C. After injection with DOCA (0.05 $mg.kg^{-1}$), the optimal latency time for ova stripping was 14 h at 25°C (Richter and Van Den Hurk, 1982). When stripping was performed 16.5 h after injection both a decrease in hatching percentages and a rise in the proportion of deformed larvae were observed. After induced ovulation with HCG (1 500 $IU.kg^{-1}$), the optimal latency time was 12 h at 29°C in *Heterobranchus longifilis* (Legendre, 1992) and ageing of ova occurred rapidly after ovulation: the proportion of deformed larvae significantly increased 2 h after ovulation and after 4 h the hatching percentage dropped from 92 to 36%. Mollah and Tan (1983) reported a good ova survival during 10 h between ovulation and egg-stripping in *Clarias macrocephalus* treated with HCG (2 $IU.kg^{-1}$) at 26-31°C. Egg viability decreased significantly at 12 h post-ovulation, with little or no hatching at 20 h.

The present paper reports studies on European catfish ovulated oocytes survival stored *in vivo* inovarian cavity and *in vitro* in aerobic condition and in various solutions to which the ova are exposed during the process of artificial insemination (sperm activating or immobilizing solutions and urine).

MATERIAL AND METHODS

The experiments were carried out at the Prerov Fish Farm, Hodonin Czech Republic in 1991, at the Research Institute of Fish Culture, Vodnany Czech Republic in 1992, 1994 and at the Anjou Fish Culture, Morannes France in 1993. Fish were reared in finishing ponds. During the pre-spawning period the 4-8 years old broodfish were captured and kept separate in small ponds (0.1-0.2 ha) supplied with fodder fish (common carp) and transferred at spawning time in the hatchery in 1 000 l tanks divided into several compartments with a water flow rate of 0.2 $l.s^{-1}$ at 22-24°C. The spermiation and ovulation were stimulated by carp pituitary homogenates injected intramuscularly at doses 5 $mg.kg^{-1}$ of body weight (Linhart and Billard, 1995). Females were checked for ovulation every 2 h starting 18 h (410 degree-hours) post stimulation.

The sperm was collected 24/48 h after hypophysation into a 20 ml Lure syringe containing 10 ml of the immobilizing medium for European catfish spermatozoa (NaCl: 200 mM, Tris: 30 mM, pH 7.0; Linhart *et al.*, 1987; Saad and Billard, 1995). Sperm pooled from individual males was stored at most 2 h in refrigerator at 4-6°C prior to fertilization. Before injection and gamete collection, the males and females were anesthetized in a solution of 2-phenoxyethanol (1:1 000).

Survival of ovulated oocytes after short-term storage *in vivo* in ovarian cavity

Ovulated oocytes left after ovulation in the ovarian cavity were stripped-out in batches of about 100 g each, every 2 h, over a period of 6 h. Sampling time expressed in degree hours (°h) after the hormone injection were 490, 540, 590 and 648 (*table* 1). In practical hatchery conditions 490°h is a standard time between injection and stripping at 22-24°C.

Tests were carried out with three different females and incubation of eggs were made in triplicate. Sperm from 3 males was collected in the immobilizing solution and mixed, and 200 μl (5.10^7 spermatozoa) were dropped on 20 g of ova (about 3 200); 15.600 spermatozoa per ovum) and immediately covered and mixed with 20 ml of the activating solution. Five minutes later, 3 batches of about 300 ova from each female were placed into three (15 cm diameter) plastic incubators in a tank at 23-25°C until hatching. The ova viability was estimated by the success of fertilization followed by embryonic development i.e. by the percentage of hatching from

the total number of eggs and the percentage of abnormal hatched larvae expressed in percentage of all hatched larvae.

Short-term storage of ova (*in vitro*)

In the first experiment (*table* 2) 10 g ova (about 1 600) from 2 females were placed undiluted in 8-10 cm in diameter glass Petri dishes and stored in aerobic conditions at 8, 19 and 25°C and for each temperature during 0 (control), 3.5, 8.5 and 12 hours.

In the second experiment (*fig.* 1) batches of about 60 ova from 7 females were individually placed, undiluted, in Petri dishes and stored in aerobic conditions at 19°C. Ova survival tests were carried out in triplicate for each females and measured, as above, 2 h after stripping and 1-2 times over a period of at most 14 h (*fig.* 1). A pool of sperm from 3 males was used to fertilize the ova. The sperm diluted >0.9/l in the immobilizing solution (100 μl; 3 to 1.10^7 spermatozoa per egg) was poured on the eggs and 15 ml of the activating solution were immediately added and mixed. Five minutes later, the diluent was discarded, and replaced by fresh water. The eggs in Petri dishes were transferred in an incubation tank at 21-23°C until hatching.

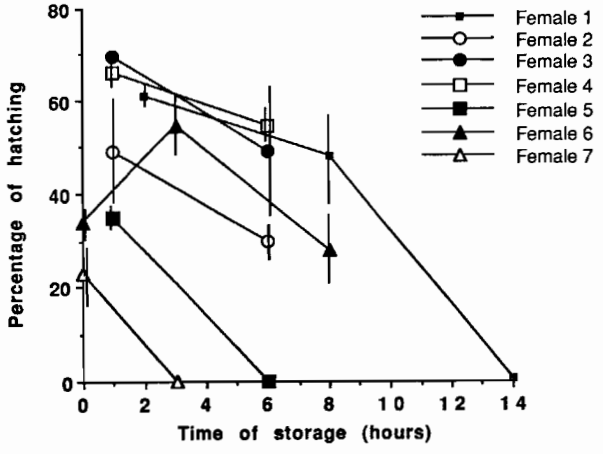

Figure 1. – Percentage of hatching with SD after short-term *in vitro* storage of European catfish ova from 7 females at 19°C. The first sample was tested within a delay of 2 h after stripping.

Changes of hatching after ova exposure to sperm activating or immobilizing solutions and urine

Activating solution

Triplicate batches of 60-100 ova placed at 20°C in Petri dishes of 10 cm diameter were diluted with 10 ml of activation solutions made of various NaCl concentrations, 0, 17 and 41 mM with 5 mM Tris-HCl, pH 8.0. At increasing time, over a period of 8 min after ova dilution, sperm was added: $2.8.10^7$ spermatozoa for experiment 1 (*fig.* 2) and the 1.10^7 spermatozoa for experiment 2 (*fig.* 3).

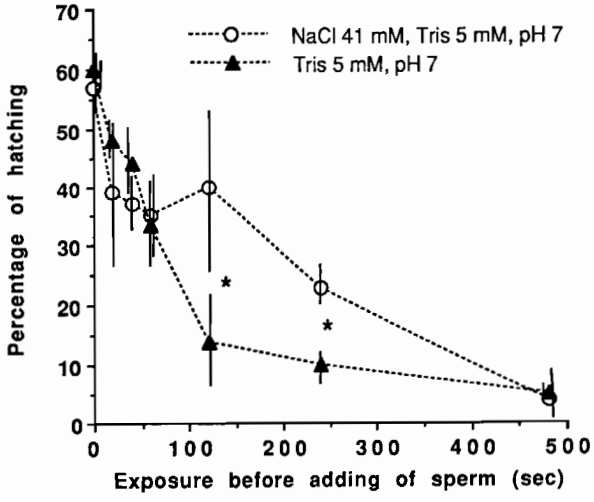

Figure 2. – Changes in the fertility of European catfish ova diluted in buffered fresh water and 41 mM NaCl solution (* $p<0.05$) for increasing time (0-8 min) before fertilization.

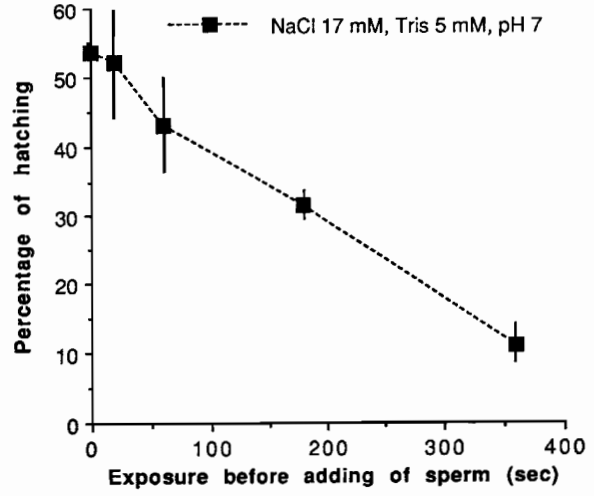

Figure 3. – Changes in the fertility of European catfish ova after dilution in 17 mM NaCl 5 mM Tris-HCl, pH 8.0 solution during several min before fertilization.

Immobilizing solution

Ten grams of ova were placed in triplicate in large Petri dishes and mixed with 10 ml of immobilizing solution (200 mM NaCl, 30 mM Tris, pH 7.0) at 19°C. After 0, 20 seconds, 1, 3 and 9 min exposure, 60-100 ova were removed and fertilized by mixing with $2.8.10^7$ spermatozoa and 10 ml freshwater in 10 cm Petri dishes.

Urine

A pool of urine (pH 5.8, 54 mOsmol.kg^{-1}) was collected from males just before stripping females and stored for few minutes at 18-19°C. Ten grams of ova (about 1 600) were mixed with 10 ml urine in Petri dishes in triplicate. After 0, 20 seconds, 1, 3 and 9 min exposure 60-100 ova were removed from urine and fertilized by mixing with $2.8.10^7$ spermatozoa and 10 ml freshwater.

Statistical significance was assessed using comparison of variances, Student's *t*-test or one-way analysis of variance, followed by Tukey's multiple comparison test. Values are given ± SD.

RESULTS

Survival of ovulated oocytes after short-term storage *in vivo* in ovarian cavity

The survival of ovulated oocytes in ovaries after ovulation was highly variable between females. The average ova survival measured as percentage of total hatched larvae was 74% after the first stripping and respectively 77%, 54% and 38%, 2-4-6 hours later. There was a significant decrease ($p<0.01$) of hatching percentage after 4 and 6 h (*table* 1). The percentages of hatched abnormal larvae (in % of total hatching) were 8.2 soon after ovulation, 8.6 after 2 h, 18 after 4 h and 50 after 6 h of storage ($p<0.01$).

Table 1. – Changes with time of the viability of European catfish oocytes left in the ovarian cavity (*in vivo* storage) after ovulation. Intra group means with a common superscript (*a*) are not significantly different. Means with a common superscript (*b*) are statistically different ($p<0.01$) of controls (time 0).

Storage time (h)	Degree hours post injection	Female	Hatching (%)	Abnormal larvae in % of hatched larvae
		A	79.4 ± 5.7	7.2 ± 3.9
0	490	B	71.2 ± 5.1	6.3 ± 2.6
		C	72.3 ± 9.0	11.3 ± 1.8
		mean	74.5 ± 7.0^a	8.2 ± 3.4^a
		A	83.3 ± 4.4	6.7 ± 3.7
2	540	B	70.7 ± 17.3	14.3 ± 2.6
		C	78.8 ± 6.1	4.9 ± 1.3
		mean	77.5 ± 10.9^a	8.6 ± 4.9^a
		A	61.3 ± 7.6	20.1 ± 4.9
4	590	B	38.9 ± 3.3	20.1 ± 2.9
		C	62.4 ± 5.2	13.6 ± 4.5
		mean	^{b}54.2 ± 12.5^a	^{b}17.9 ± 4.9^a
		A	16.7 ± 1.8	74.9 ± 8.4
6	648	B	36.5 ± 3.7	50.1 ± 4.3
		C	60.6 ± 7.4	25.1 ± 4.0
		mean	^{b}37.9 ± 19.5	^{b}50.0 ± 22.2^a

Short-term storage of ova (*in vitro*)

Data of experiment 1 shows that the ova survival at 19°C measured as percentage of hatching was highly variable between females and decreased regularly during storage (*fig.* 1). Only female 6 showed a higher ova survival after 3 h storage compared to control. In experiment 2 (*table* 2), after 3.5 hours storage at 19°C, undiluted ova showed a higher survival (54%) than the control, fertilized immediately after stripping (35%, $p<0.01$). It should be underlined that only 2 females were used in this experiment and that the ova were of poor quality as shown by the low hatching rate. There was no survival at all after 3.5 h and beyond at 8°C, 12 h at 19°C and 8.5 h at 25°C. There was a significant increase in the percentage of abnormal larvae after the ova had been stored 3.5 h at 25°C (74%, $p<0.001$) and after 8.5 h at 19°C (37%, $p<0.01$).

Table 2. – Changes with time and temperature in the survival of European catfish ovulated oocytes stored undiluted for 0 (control), 3.5, 8.5 and 12 h in Petri dishes at 8, 19 and 25°C, before fertilization. Means with a common superscript (*b*) are statistically different ($p<0.01$) of control. Means with a common superscript (*c*) are statistically different ($p<0.01$) of 3.5 h storage at 19°C. Eggs of two females were incubated in triplicate samples.

Temperature (°C)	Time of storage (h)	Hatching (%)	Abnormal larvae in % of hatched larvae
Control	0	35.2 ± 2.6	9.9 ± 1.7
	3.5	0	
8	8.5	0	
	12.0	0	
	3.5	^{b}53.7 ± 6.2	6.9 ± 2.1
19	8.5	27.3 ± 6.8	^{bc}37.0 ± 7.7
	12.0	0	
	3.5	41.5 ± 4.9	^{b}73.7 ± 8.9
25	8.5	0	
	12.0	0	

Effects of ova exposure to the sperm activating or immobilizing solutions and urine prior to fertilization

The capacity of development of ova was entirely loss in less than 10 min after immersion of ova into buffered fresh water (5 mM Tris-HCl, pH 7.0) and NaCl solution (41 mM NaCl, 5 mM Tris-HCl, pH 7.0) (*fig.* 2). After 120 and 240 seconds exposure the hatching percentage was found significantly higher in NaCl solution compared to buffered fresh water. An additional experiment was carried out with an activating solution of intermediate osmotic pressure (17 mM NaCl, 5 mM Tris-HCl, pH 7.0) and showed

(*fig.* 3) a slightly better ova survival during the first 40 seconds compared to the 41 mM NaCl solution.

The ova exposed to immobilizing solution (200 mM NaCl, 30 mM Tris-HCl, pH 8.0) showed a rapid loss of subsequent hatching within the first minute and was 50 and 10% at respectively 3 and 9 min (*fig.* 4). The ova exposed to urine showed significantly regular decrease of hatching which reached nearly 15% after 3 min and 0% after 9 min.

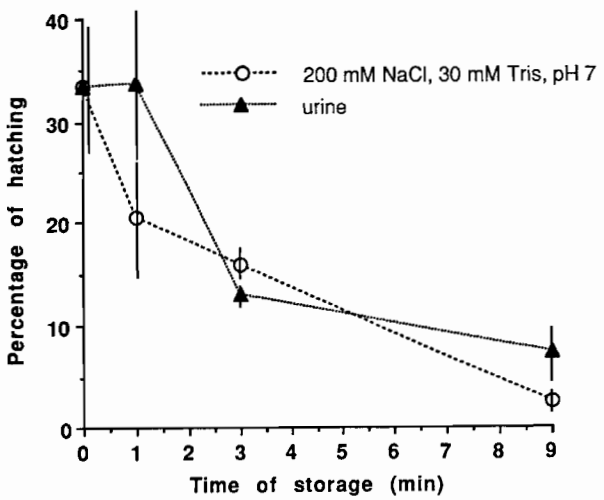

Figure 4. – Changes in the fertility of European catfish ova after incubation in the sperm immobilizing solution (1:1; 200 mM NaCl, 30 mM Tris, pH 7.0) and urine (1:1) at 18-19°C.

DISCUSSION

These results show that *Silurus glanis* ovulated oocytes lose progressively their capacity to undergo fertilization/embryonic development once ovulated; this occurs when the oocytes are left in ovarian cavity or stripped and stored undiluted *in vitro* as reported in other fish species, for instance the common carp (Linhart *et al.*, 1995). The increase of the percentage of abnormal larvae with time and temperature of ova storage can be related to phenomenon of ageing as often reported (Bromage, 1995). The increase of hatching percentage after 3.5 h of ova storage in the case of few females having poor quality eggs may also be due to ageing phenomenon leading for instance to some delay in the reorganization of chromosomes after sperm chromatine decondensation in egg cytoplasm and in the process of synchronization of the 2nd meiotic division of the egg.

This increase of abnormal larvae was not as dramatic as in *Clarias garipienus* (60% abnormal larvae after 6 h; Richter and Van Den Hurk, 1982) and *Heterobranchus longifilis* (20% of abnormal larvae after 2 h; Legendre, 1992). The ova were very sensitive to fresh water, various saline solutions and urine. The survival of ova was very short, 40 sec to 2 min in fresh water and 2-4 min in saline solutions. This was also reported for some other warm water species. The ova of common carp fertility was lost 3 minutes following immersion in fresh water, probably due to the closing of micropyle, but was retained for 8 min when kept in the sperm activating solution. In silver carp, ova loss their capacity for fertilization and development after 30-40 seconds in water and after more than 50-70 seconds in saline solution (Mikodina and Makeyeva, 1980). In the sperm immobilizing solution and in urine (54 $mOsmol.kg^{-1}$) the loss of ova fertility was very rapid, possibly as a result of osmotic shock. Other factors may also be involved in the loss of ova fertilizability and development. In goldfish Hsu and Goetz (1993) preserved ova fertilization for 30 min in Ringer solution with Tris pH 7.3 and 0.5 $mg.ml^{-1}$ soybean trypsin inhibitor compared with control Ringer-Tris pH 7.3 or freshwater. The soybean trypsin inhibitors probably block chorion expansion and preserve the fertility of eggs. The loss of fertility was also related to chorion expansion in carp (Renard *et al.*, 1990).

Practically, in hatchery conditions mixing ova with urine must be avoided. In that case good quality ova can survive at 19°C *in vitro* or *in vivo* in ovarian cavity only for a few hours prior to fertilization and cannot be exposed even for short periods of time to water or saline solutions. In the procedure of artificial insemination the spermatozoa brought to eggs with the immobilizing solution must be activated rapidly after the eggs are diluted.

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