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Effect of temperature, volume of ova batches, and addition of a diluent, an antibiotic, oxygen and a protein inhibitor on short-term storage capacities of turbot, *Psetta maxima*, ova

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Abstract — The effect of different parameters on short-term storage capacity of turbot ova was assessed over a 45-h period after ova collection for fertilization rates and over a 9-h period after ova collection for hatching rates. Increasing the volume of ova sampling from 0.5 to 2.5 mL, as well as adding an antibiotic–antimicotic solution or oxygen did not significantly change the storage capacity of ova. Regarding the hatching rates, a higher storage ability was recorded at 8 and 13 °C, compared to 3 °C. The mean composition of the ovarian fluid was determined (n = 57 spawns). Use of a diluent mimicking the ovarian fluid significantly decreased the storage ability as assessed by the fertilization rates but did not modify the hatching rates. Diluting ova in an artificial ovarian fluid deprived of calcium significantly decreased the fertilization and hatching rates during the storage period. Furthermore, addition or not of soybean trypsin inhibitor (Sigma T 9003) to the artificial ovarian fluid deprived of calcium did not significantly change the results. Storage capacity of control batches of ova was low: at 13 °C, without any diluent and when ova were fertilized 3 h after stripping, the hatching rate was lowered to 62.4 ± 29.4 % (mean ± SD) of the initial value. © 1999 Ifremer/Cnrs/Inra/Ird/Cemagref/Éditions scientifiques et médicales Elsevier SAS

Fish / ova / short-term storage / ovarian fluid / calcium / turbot / *Psetta maxima*

Résumé — Effet de la température, du volume du prélèvement d'ovules et de l'addition d'un diluant, d'un antibiotique, d'oxygène et d'un inhibiteur de protéines sur les capacités de conservation à court terme des ovules de turbot, *Psetta maxima*. L'effet de différents paramètres sur la capacité de conservation à court terme d'ovules de turbot a été testé durant une période de 45 h après la récolte des ovules pour étudier le taux de fécondation et 9 h pour le taux d'éclosion. L'accroissement du volume du prélèvement d'ovules de 0,5 à 2,5 mL ainsi que l'ajout d'une solution d'antibiotique-antimicotique ou d'oxygène ne modifient pas significativement la capacité de conservation des ovules. Comparée à 3 °C, une capacité de conservation supérieure à des températures de 8 et 13 °C est observée sur les taux d'éclosion. La composition du liquide ovarien a été précisée (n = 57 pontes). L'ajout d'un diluant dont la composition est proche de celle du liquide ovarien a pour conséquence la diminution des taux de fécondation, mais pas celle des taux d'éclosion. De plus, la dilution dans le liquide ovarien artificiel dépourvu de calcium diminue significativement les taux de fécondation et d'éclosion. Lorsque les ovules sont placés en présence de liquide ovarien artificiel dépourvu de calcium, l'ajout d'un inhibiteur de la trypsine (Sigma T 9003) ne modifie pas les résultats observés. À 13 °C et en absence de diluant, la capacité de conservation des lots d'ovules est faible : lorsque les ovules sont fécondés 3 h après leur récolte, le taux d'éclosion n'est plus que de 62,4 ± 29,4 % (moyenne ± écart type) de la valeur initiale. © 1999 Ifremer/Cnrs/Inra/Ird/Cemagref/Éditions scientifiques et médicales Elsevier SAS

Poisson / ovules / conservation à court terme / liquide ovarien / calcium / turbot / *Psetta maxima*

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1. INTRODUCTION

The storage of fish ova after stripping can be used when gametes must be transported to synchronize gamete availability of both sexes and for gamete management including insemination operations and chromosome manipulations. Because of inadequate dehydration and toxicity of cryoprotectants, attempts to freeze fish ova have been unsuccessful up to now [31]. As a consequence, short-term storage protocols have been developed using positive temperatures (for a review, see [3] for salmonids, [24] for siluroids and [26] for carp). Most works are devoted to salmonids, showing the effect of temperature [4, 18] and composition of diluting media [2, 5] on the storage capacity of ova. The effect of both factors has also been reported in European catfish (*Silurus glanis*) [25] and in carp (*Cyprinus carpio*) [33]. Adding oxygen in containers increased the post-storage fertility of tilapia (*Sarotherodon mossambicus*) ova [14]. Spontaneous activation of goldfish (*Carassius auratus*) ova has been blocked by soybean trypsin inhibitor, providing a means to maintain ova in vitro in a viable state for a longer period [18].

In marine fish, Blaxter [7] observed that a high hatching rate of Pacific herring (*Clupea pallasii*) eggs was recorded for storing periods not exceeding 36 h. The best survival was assessed when ova of this species were stored at salinities ranging from 10 to 17 ‰. Halibut (*Hippoglossus hippoglossus*) ova stored beyond a 6-h period showed a poor development after fertilization [1].

Management methods have been established for turbot (*Psetta maxima*) sperm including short- [9] and long-term [11] storage techniques. However, turbot ova management has been restricted to the description of the effect of over-ripening on ova quality [28] and to the reduction of this process [13].

The purpose of this work was to assess the effect of parameters such as the volume of ova batches, storage temperature and addition of a diluent, an antibiotic, oxygen or a protease inhibitor on ova survival during the insemination and hatching phases.

2. MATERIALS AND METHODS

2.1. General procedure

The experiments were conducted jointly in Instituto Español de Oceanografía (Santander, Spain) and in Ifremer (Brest, France). Broodstock management, sperm and ova collection were as previously described [11]. Just after gamete collection, 0.5-mL batches of ova (approximately 500 ova) sampled from apparently good quality spawn (viability rate > 75 %; ova were considered viable when they showed a translucent aspect and a lack of perivitelline space through a dissecting microscope) were laid in 35-mL cylindrical flasks and stored at 13 °C. In order to maintain the initial quality of sperm samples for a 45-h period, sperm was previously frozen in straws [11]. Using a non-limiting ratio of 30 000 thawed spermatozoa per ovum and adding 0.25 mL seawater at 13 °C, triplicate batches of eggs were inseminated according to a standardized protocol [34], 0, 3, 9, 21, 27 and 45 h after stripping females. Fertilization rate was assessed 3 h 30 min after insemination on 200 eggs (number of four-cell stage eggs/total number of eggs). Each of the six successive experiments was repeated using four different spawns: two spawns were processed in Spain and two in France. Furthermore in Spain, duplicate batches of 0.5 mL of eggs were transferred to 1-L beakers for incubation. Fifty per cent of the seawater volume were renewed daily. In France, duplicate batches of eggs were randomly transferred into 1-L cylindroconical incubators with a 0.05-L·min^{-1} water renewal [35]. After 6 days at 13 °C, the number of larvae was exhaustively counted. Hatching rate was defined as the number of larvae divided by the number of eggs transferred to incubators.

2.2. Experimental procedure

In order to determine the effect of different parameters on short-term storage ability of turbot ova, six successive experiments were carried out.

2.2.1. Ova volume

The effect of volume was tested using 0.5- and 2.5-mL ova batches (height of the ova layer was 2 and 5 mm, respectively).

2.2.2. Temperature

To determine temperature requirements, ova samples were kept at 3 °C (on ice), 8 °C (in a cryostat) or 13 °C (bain-marie).

2.2.3. Oxygen

The survival of ova was compared under oxygen or air atmosphere. Oxygen was insufflated for 10 s through a straw inserted in the flask cap while air was expelled through a second straw.

2.2.4. Diluent

In order to determine the nature of the diluent, the composition of the ovarian fluid was studied. First, ovatocrit (cell volume × 100/total spawn volume) was determined on six spawns by measuring diameters of 30 ova (value is the mean of two diameters observed on each ovum, using a profile projector Nikon V12) for each spawn and then calculating mean ova volume, considering the cell as a sphere. Second, the composition of the ovarian fluid of several spawns (n = 57, collected from 37 females) was determined: ovarian fluids were obtained after filtration and centrifugation at 3 500 g for 5 min. The osmolality and the pH were immediately measured. The supernatant was then frozen at –28 °C until chemical analysis. Total protein, Na^+, K^+, Ca^{2+}, Mg^{2+} and glucose content were determined. All chemical analyses were performed accord-

ing to methods previously described [12]. The effect of a diluent was tested by the addition or not of 0.50 mL of an artificial ovarian fluid (NaCl 97.4 mM, KCl 7.5 mM, $CaCl_2$ 2.1 mM, $MgCl_2$ 2.6 mM, bovine serum albumin 6.5 $g \cdot L^{-1}$, $NaHCO_3$ 25 mM buffered at pH 8.0, osmotic pressure 250 $mosm \cdot L^{-1}$). In order to maintain similar volumes for insemination, 0.25 mL of seawater was added to ova batches already diluted with artificial ovarian fluid and 0.75 mL to ova batches deprived of that medium. Since the total volume was increased compared to the general procedure, 48 000 spermatozoa were added for one ovum. The fertilization capacity of control ova batches, inseminated just after ova collection, was not significantly different between the two conditions (0.75 mL of seawater or 0.25 mL of seawater + 0.50 mL of artificial ovarian fluid).

2.2.5. Antibiotic

Antibiotic–antimicotic solution (5 µL; Sigma A 5955) containing 10 000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per mL was added to half of the ova batches.

2.2.6. Calcium and soybean trypsin inhibitor

In the last experiment, the storage capacity of ova was compared between three conditions: no artificial ovarian fluid addition, 100 % artificial ovarian fluid deprived of calcium plus 2 mM ethylene glycol tetraacetic acid (EGTA) and 100 % artificial ovarian fluid deprived of calcium plus 2 mM EGTA plus 2 % soybean trypsin inhibitor (Sigma T 9003). In order to maintain similar fertilization conditions, 100 % artificial ovarian fluid deprived of calcium and 2 mM EGTA were added to control batches of ova just before fertilization. Then, 50 000 spermatozoa were added per ovum.

2.3. Statistical analysis

All data are expressed as mean ± standard deviation. After angular transformation, fertilization and hatching rates were compared using a three-way block ANOVA. When differences were significant ($\alpha = 0.05$), a Newman-Keuls a posteriori test was used for comparison. After angular transformation, correlations between the fertilization and hatching rates were tested using linear regression.

3. RESULTS

3.1. Ova volume

No significant differences were observed in fertilization or hatching rates using 0.5 or 2.5 mL ova (*figure 1*).

3.2. Temperature

The storage capacity of turbot ova was not significantly different between the three temperatures tested as revealed by the fertilization rates (*figure 2*). On the other hand, regarding the hatching rates a higher storage ability was recorded at 8 and 13 °C compared to 3 °C ($P < 0.05$).

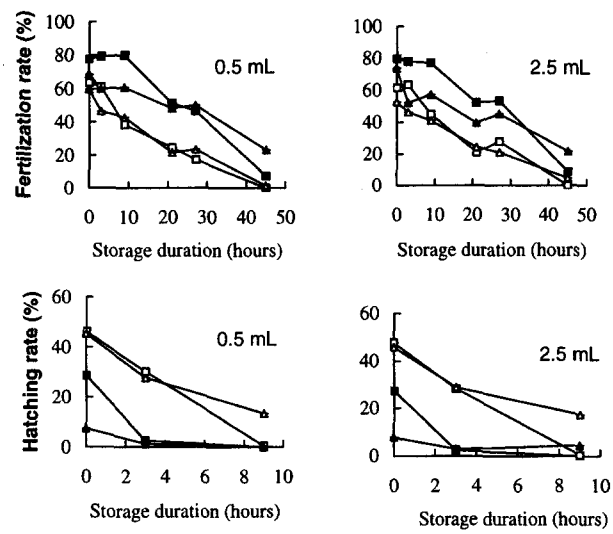

Figure 1. Effect of the volume of ova batches on the short-term storage capacity of turbot ova.

3.3. Oxygen

Adding oxygen did not increase the storage ability (*figure 3*).

3.4. Diluent

The mean ovatocrit observed on six spawns was 62.2 ± 14.1 %. The mean composition of the ovarian fluid of 57 spawns is reported in *table I*. Use of a diluent mimicking the ovarian fluid decreased the fertilization rates ($P < 0.001$) but not the hatching rates (*figure 4*).

Table I. Mean composition of the ovarian fluid of 57 spawns, collected from 37 females.

	Mean ± SD
Na^+ ($mmol \cdot L^{-1}$)	97.4 ± 22.4
K^+ ($mmol \cdot L^{-1}$)	7.5 ± 3.4
Ca^{2+} ($mmol \cdot L^{-1}$)	2.1 ± 1.4
Mg^{2+} ($mmol \cdot L^{-1}$)	2.6 ± 1.6
Cl^- ($mmol \cdot L^{-1}$)	102.9 ± 25.6
Glucose ($mmol \cdot L^{-1}$)	0.6 ± 0.3
Protein ($mg \cdot mL^{-1}$)	6.4 ± 4.2
Osmotic pressure ($mosm \cdot L^{-1}$)	234 ± 55
pH	7.60 ± 0.42

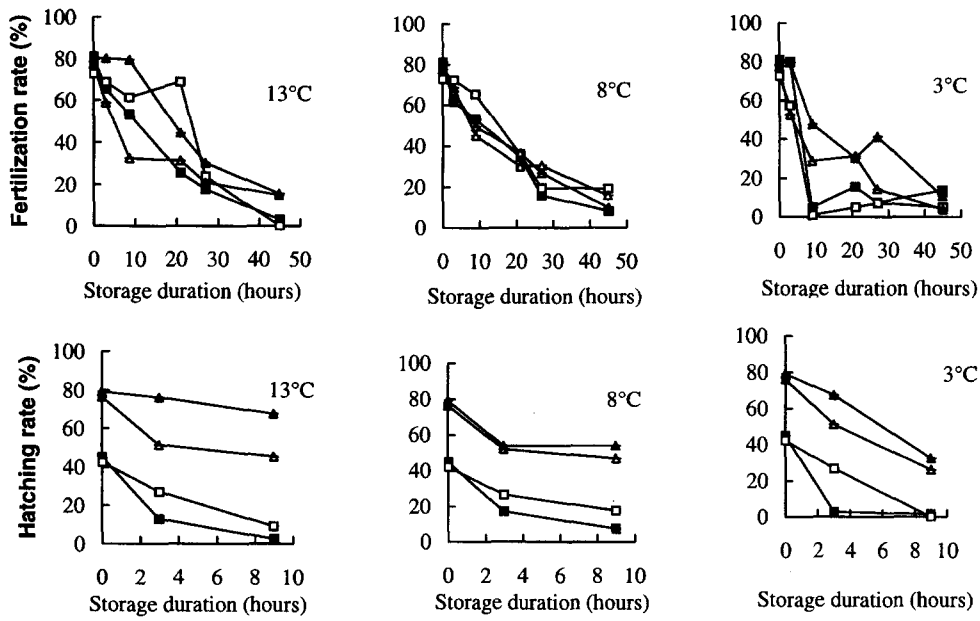

Figure 2. Effect of temperature on the short-term storage capacity of turbot ova.

3.5. Antibiotic

Adding an antibiotic did not induce any difference in storage capacity of turbot ova (*figure 5*).

3.6. Calcium and soybean trypsin inhibitor

Diluting ova in an artificial ovarian fluid deprived of calcium, significantly decreased the storage capacity (*figure 6*) as revealed by the fertilization ($P < 0.001$) and hatching rates ($P < 0.01$). A significative interaction ($P < 0.001$) was noted between blocks and media for this first parameter. Furthermore, adding an inhibitor did not change the results recorded when using an artificial ovarian fluid.

3.7. Storage capacity of control batches of ova

The mean decrease in fertilization and hatching rates of control batches of ova in each of the six

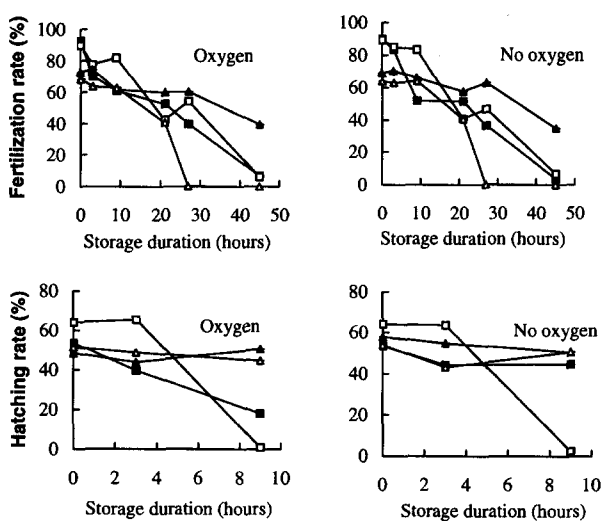

Figure 3. Effect of oxygen on the short-term storage capacity of turbot ova.

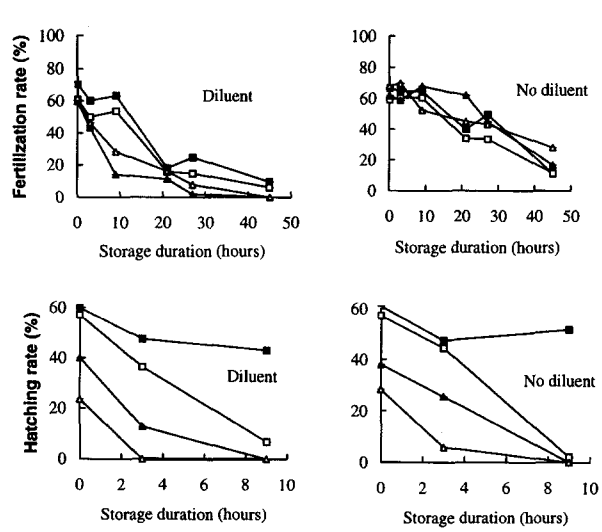

Figure 4. Effect of the presence of a diluent mimicking the ovarian fluid on the short-term storage capacity of turbot ova.

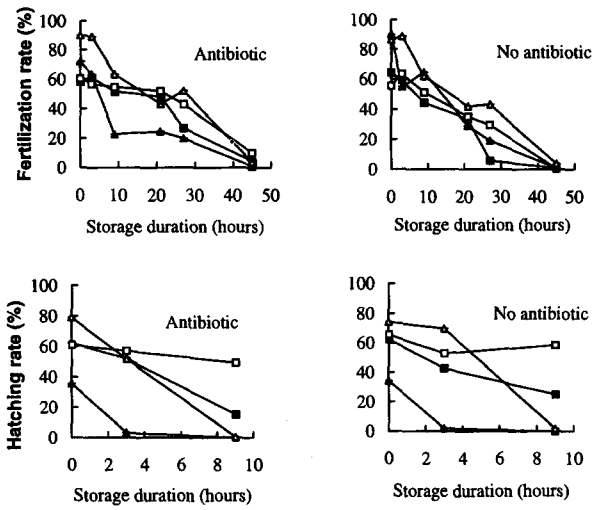

Figure 5. Effect of antibiotic on the short-term storage capacity of turbot ova.

experiments (volume: 0.5 mL; temperature: 13 °C; no diluent; $n = 24$) as a function of storage duration is reported in *figure 7*. Under these conditions, the fertilization capacity decreased significantly after a 9-h storage period ($P < 0.001$) to 80.0 ± 20.5 % of the initial value and 57.1 ± 19.3 % after 21 h. Furthermore, the hatching rate was significantly lowered to 62.4 ± 29.4 % of the initial value at 3 h and 38.1 ± 37.7 % after 9 h ($P < 0.001$). When grouping data recorded at 0, 3 and 9 h after stripping, a significant correlation was observed between fertilization rates (x) and hatching rates (y):

$y = 0.84 + 0.19x$ ($n = 72$, $P < 0.001$, R = 0.38, SE of estimate = 0.15).

4. DISCUSSION

When assessing the fertilization rate of turbot eggs, blastomers become less visible and presented more frequent morphological abnormalities as the storage period progressed, suggesting a loss of ova quality. Compared to the fertilization rate, the decrease in the hatching rate as a function of time was more precocious. This observation confirms the results reported by McEvoy [28] in the same species. The fertilization capacity of Japanese flounder (*Limanda yokohama*) ova was high for 3 days after collection, whereas the hatching rate decreased after only 24 h [16]. This delayed decrease in the fertilization rate was also reported in Pacific herring [15] and in Atlantic halibut [1]. According to observations previously published [21], this indicates that the fertilization rate of ova of some fish species is maintained for a longer period than their ability to survive the embryonic development phase. In turbot, this observation can explain the low coefficient of correlation (R = 0.38) observed between fertilization and hatching rates, when grouping data recorded at 0, 3 and 9 h after stripping.

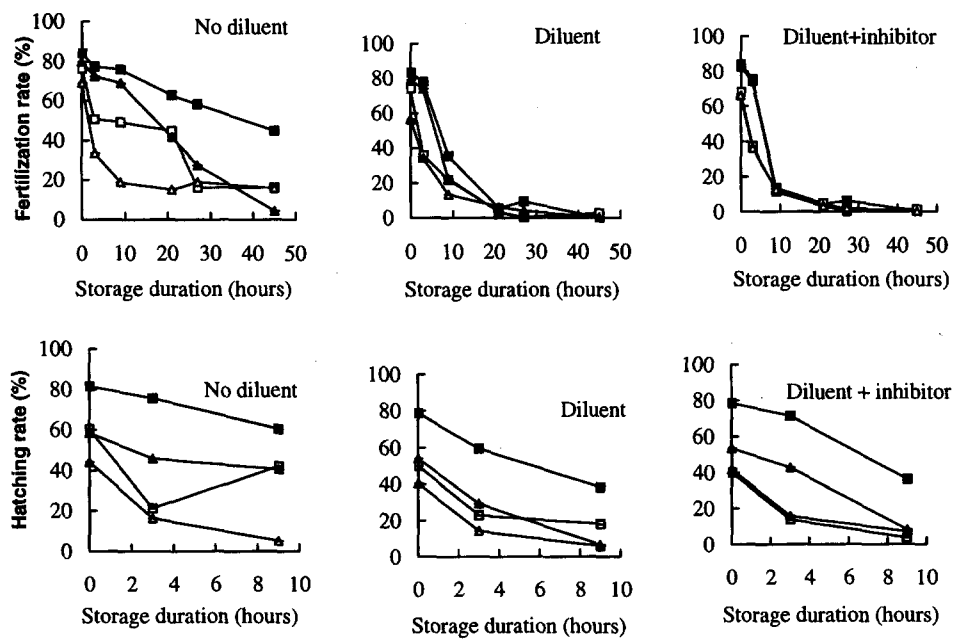

Figure 6. Effect of addition of a diluent mimicking the ovarian fluid and deprived of calcium plus 2 mM ethylene glycol tetraacetic acid (EGTA) or of a diluent mimicking the ovarian fluid and deprived of calcium plus 2 mM EGTA plus 2 % soybean trypsin inhibitor.

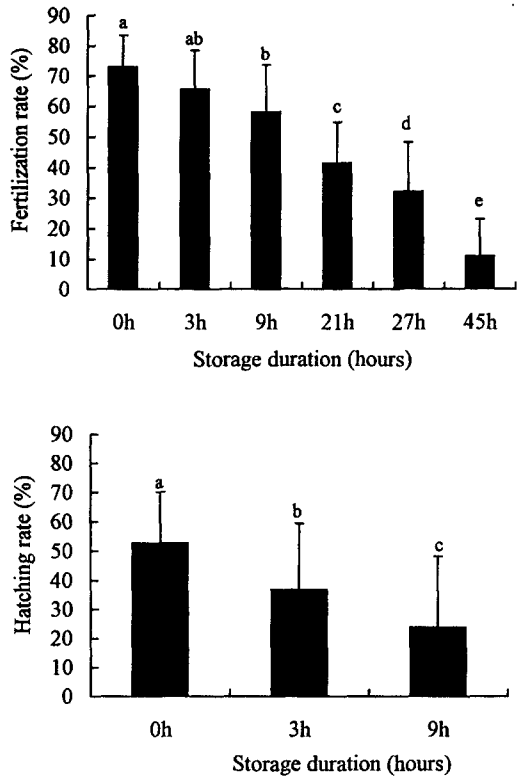

Figure 7. Changes in fertilization and hatching rates of control batches of ova (volume: 0.5 mL; temperature: 13 °C; no diluent) as a function of storage duration.

Neither increasing the volume of ova batches from 0.5 to 2.5 mL nor adding oxygen changes the storage capacity of turbot ova. This result suggests a low requirement for oxygen of turbot ova. No difference was reported in coho salmon (*Oncorhynchus kisutch*) using air or oxygen [32]. On the other hand, a better storage capacity of chum salmon ova was observed when increasing air to gamete ratio [19]. Furthermore, adding oxygen increased the survival of tilapia (*Sarotherodon mossambicus*) [14] and razorback sucker (*Xyrauchen texanus*) ova [8].

The use of an antibiotic does not change the survival of turbot ova during the storage period. Bacteria flora (from less than 10 up to 10^3 bacteria per ovum) was associated with ova of this fish species [29]. Bacteria proliferating after a 19-h storage of tilapia ova were inhibited by kanamycin sulphate [14]. However, the observed decrease in turbot ova quality during the storage period in the presence of antibiotic could rather be due to cellular lysis than to the development of bacterial flora.

Compared to 3 °C, a better survival of turbot ova was assessed at 8 and 13 °C. Decreasing storage temperature from 15 to 3 °C prolonged the survival of chum salmon ova [19]. On the other hand, a higher storage capacity of grass carp (*Ctenopharyngodon idella*) ova was reported at 22 °C than at 9 °C [36]. The optimal temperature for the storage of ova is 19 °C in European catfish (*Silurus glanis*) [25] and 20 °C in tilapia [14]. Including protecting constituents in the diluent could be necessary for the storage of turbot ova at low temperatures.

Adding an artificial ovarian fluid to turbot ova did not increase their survival. Diluting rainbow trout ova in media mimicking seminal fluid or blood plasma increased their storage capacity [5]. However, sucrose solutions in which osmotic pressure was comprised between 200 and 300 mosm·L^{-1} lowered ova survival in this species [2]. Adding a soybean trypsin inhibitor increased the storage ability of goldfish ova by blocking chorion expansion [18]. The use of this inhibitor did not increase the storage capacity of turbot ova. However, no spontaneous activation was observed during the storage period of ova of this species.

The osmotic pressure assessed in the ovarian fluid of turbot is low compared to other fish species: salmonids (from 256 to 292 mosml·L^{-1}) [22], carp (305 mosml·L^{-1}) [30] and marine lumpsucker (*Cyclopterus lumpus*, 359 mosml·L^{-1}) [10]. However, a low osmotic pressure was measured in the ovarian fluid of bleak, a cyprinidae (*Alburnus alburnus*, 237 mosml·L^{-1}) [23]. In turbot, mean pH (7.60) is also lower than values recorded in salmonids (8.4–8.8) [22] or carp (8.5) [30]. Then, high concentrations of proteins are observed in turbot compared to salmonids (from 0.95 to 2.78 mg·mL^{-1}) [22] or bleak (1.58 mg·mL^{-1}).

Compared to sperm [6, 31], in vitro survival of turbot ova is rather low: at 13 °C, without any diluent and when ova were fertilized 3 h after stripping, the hatching rate was lowered to 62.4 ± 29.4 % of the control. A low storage ability of ova was also recorded in other fish species: from 1 to 6 h in carp [26], from 2 to 12 h in Siluroids [24], from 3 to 6 h in rainbow trout (*Oncorhynchus mykiss*) and 12 h in brown trout (*Salmo trutta fario*) [27], from 1 to 9 h in cod (*Gadus morhua*) [20] and 6 h in Atlantic halibut, *Hippoglossus hippoglossus* [17]. In the ovary, McEvoy [28] showed that the storage ability of turbot ova was restricted to 10 h after ovulation at a temperature of 12–14 °C. After a 24-h storage period in the ovary, no hatching was observed by this author.

This work shows that the viability of turbot ova decreased very rapidly during in vitro storage. Except for temperature and addition of a diluent mimicking the ovarian fluid, this first set of biotests has a low effect on the storage capacity of turbot ova. Further studies are required to improve storage ability of ova of this species, regarding the effect of parameters such as the presence of cryoprotectant at a temperature near 0 °C, the composition of the diluent or the presence of light during the storage period.

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