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The sperm motility in marine teleosts as a tool to evaluate the toxic effects of xenobiotics

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The possibility of using the sperm of teleosts as a model system for ecotoxicological assessments has been explored by evaluating sperm motility parameters: (1) time to reach the maximum motility value (activation time), (2) maximum motility value, (3) duration of maximum motility value, and (4) total time of motility (until class 0). Sperm of *Dicentrarchus labrax*, *Sparus aurata*, *Diplodus puntazzo* and *Pagellus erythrinus* were analysed and compared. The effects of dimethylsulfoxide, ethylene glycol, propylene glycol, glycerol and methanol on sperm motility in these marine species were investigated. Among the systems tested, sperms of *S. aurata* and *D. labrax* were the most sensitive to the tested xenobiotics and *S. aurata* spermatozoa were shown to be easier to manage for ecotoxicological assays.

Keywords: marine teleosts; sperm motility; xenobiotics; ecotoxicological assays

1. Introduction

Toxicity tests to assess ecosystem contamination are now used alongside, if not to replace, analytical methods that directly search for pollutants, because toxicity tests provide more accurate information about the actual effect of the toxic on natural ecosystems, even at low concentrations. Factors that must be taken into account when determining the value of a biological system as an indicator in toxicity tests are the sensitivity of the system to different pollutants, and the year-round availability of the organism and its gametes and embryos.

Because of their high sensitivity, the gametes and embryos of aquatic organisms are commonly used in ecotoxicological tests to assess the quality of waters and sediments in areas subject to anthropogenic effects [1–7].

Reproductive capacity is, in fact, a key factor in the survival of a species, therefore, these biological systems can serve as valuable tools in assessing the environmental risk posed by chemical contamination.

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Spermiotoxicity studies have been widely used in many biological systems, confirming the high sensitivity of the sperm of different aquatic species to tested contaminants [8–13].

Among the factors that promote the use of sperm in ecotoxicological studies is the rapidity of exposure and evaluation: standardised protocols involve a short (minutes) exposure to the toxic, followed by assessment of the fertilisation ability of the spermatozoa. Recent studies have shown a good correlation between sperm motility and fertilisation ability in many aquatic organisms [14–18], consequently, analysis of the parameters characterising the sperm motility may be an efficient method for the early identification of potentially damaging events in aquatic ecosystems.

The use of fish spermatozoa has number of advantages: (1) brood fish can be easily made available all year long for some commercially farmed species; (2) fish sperm is easy to collect and can be safely stored for a short time until investigation; (3) because fish sperm featuring external fertilisation are usually immotile in the seminal fluid, it is easy to trigger motility by controlled transfer to a competent swimming medium; and (4) fish sperm cells show largely homogenous behaviour, all spermatozoa can be activated at the same time while swimming with very similar characteristics at a certain point post activation [18].

Moreover, fish sperm motility has been reported to be clearly influenced by various xenobiotic substances [19,20].

Dicentrarchus labrax and *Sparus aurata* are widespread fish species with great economic–commercial value in the fishing industry and in aquaculture, and they are the species more commonly bred in Italian marine fish farms [9,21]. *Diplodus puntazzo* is the third most commonly farmed marine species in Italian aquaculture [22], while *Pagellus erythrinus* is an important species for fisheries and is one of the most promising species for the diversification of marine aquaculture in the Mediterranean Sea [23].

In addition, the euryhaline coastal species *D. labrax* and *S. aurata* are particularly affected by anthropic pollution, and are therefore representative species of the coastal marine environment and promising candidates for ecotoxicological assessment.

Dimethylsulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), glycerol (GlOH) and methanol (MetOH) are the most common agents used during cryopreservation procedures to protect cells from damage induced by exposure to low temperatures; moreover, DSMO, EG, PG and MetOH are also frequently used as solvents for relatively hydrophobic substances.

The Organization for Economic Cooperation and Development (OECD) [24] currently recommends the use of DSMO, EG, PG and MetOH in aquatic toxicity testing to help achieve a more effective dispersion of some toxicants. Besides, recent observations have shown that some solvents may affect the reproduction of certain fish species [25].

The objective of this study was to evaluate the toxic effects of DMSO, EG, PG, GlOH and MetOH on the spermatozoa of the marine teleosts *D. labrax*, *S. aurata*, *D. puntazzo* and *P. erythrinus*, as a preliminary step in the development of an ecotoxicological assay for monitoring aquatic environments.

This evaluation is essential to calibrate their use as both cryoprotectants and solvents, first, to avoid their intrinsic toxicity compromising cryopreservation procedures, and second, for correct determination of the toxicity of chemical substances in ecotoxicological assays.

2. Materials and methods

2.1. *Animals*

Four species of marine teleosts were used: European seabass (*D. labrax*), gilthead seabream (*S. aurata*), sharpsnout seabream (*D. puntazzo*) and common pandora (*P. erythrinus*).

2.2. *Collection, transport and motility evaluation*

For all species considered, seminal fluid was obtained by abdominal stripping of at least 10 adult mature males bred in Mediterranean fish farms and previously anesthetised with 200 ppm phenoxyethanol. Semen was collected individually and samples contaminated with faeces or urine were discarded.

Samples maintained at a temperature of 3 ± 1 °C during transport to the laboratory.

Aliquots of each sample were activated by 1:100 dilution with filtered and autoclaved artificial seawater (FSW), prepared according to ASTM 2004 [26], activated sperm was then kept at room temperature (22 ± 2 °C).

According to the method of Fabbrocini et al. [14], sperm was evaluated by taking into account the percentage of sperms with rapid, vigorous and linear (RVL) motility and the results were expressed in terms of motility classes.

Semen that showed better motility was mixed in homogeneous pools and used in subsequent experimental phases, whereas semen samples showing low motility classes were discarded.

After formation of the homgenous pools, a semen aliquot for each species was diluted and maintained at 22 ± 2 °C to evaluate the trend in motility, by recording the percentage of RVL sperms from 10 s to 70 min after activation. This analysis allowed us to evaluate the following sperm motility parameters: (1) time to reach the maximum motility value (activation time), (2) maximum motility value, (3) duration of maximum motility value and (4) total time of motility (until class 0).

2.3. *Short-term storage*

Undiluted semen aliquots, stored at 3 ± 1 °C in the dark, were activated 6, 24, 48 and 72 h after sampling to evaluate the effect of short-term storage. The maximum motility value and the total duration of motility were recorded for each activation.

2.4. *Toxicity test*

The following xenobiotics (final concentrations in % v/v) were tested: DMSO (5, 7, 10%), EG (5, 7, 10%), PG (5, 7, 10%), GlOH (5, 7, 10%) and MetOH (2, 4, 6%).

The semen, diluted 1:6 with a solution of 1% NaCl (v/v) containing the xenobiotics, was incubated for 30 min at a temperature of 22 ± 2 °C. After exposure to the xenobiotics, aliquots of sperm were activated in FSW (1:100 final dilution) and the maximum motility values were recorded. Two controls were used, undiluted semen (control) and semen diluted in 1% NaCl without cryoprotectants (control 1% NaCl).

2.5. *Statistical analysis*

The data are expressed as the mean of at least five replicates of each experiment performed in triplicate ($n \geq 15$).

Analysis of variance (ANOVA) was applied to the data to determine significant differences (the significance level was set at $p < 0.05$).

For each species, the responses of the sperm motility parameters to the tested xenobiotics were corrected for effects in controls by applying Abbott's formula [26], thus obtaining the effect percentage.

3. Results

Figure 1 shows, for each species, the motility trend of sperms activated immediately after pool formation and maintained at 22 ± 2 °C. The sperm of four species showed differences in all motility parameters evaluated.

Maximum sperm motility values were reached within a few seconds for all species analysed. *D. labrax* showed the shortest activation time, reaching the maximum motility value 10 s after activation; the longest activation time was recorded with *P. erythrinus* sperm, which reached the maximum motility value 1 min after the activation; *S. aurata* and *D. puntazzo* showed an intermediate time of 30 s.

The maximum motility value for all species was higher than class 4 (RVL sperms 80%); *D. labrax* and *S. aurata* showed values close to class 5(4.96 ± 0.14 and 4.92 ± 0.19, respectively); the maximum sperm motility value was lower in *D. puntazzo* (4.63 ± 0.31) and *P. erythrinus* (4.28 ± 0.26).

The duration of the maximum motility value was: 9 min in *S. aurata*, 4 min in *P. erythrinus,* 50 s in *D. labrax* and 30 s in *D. puntazzo*.

The total duration of sperm motility was higher for *P. erythrinus* (30 min) and *S. aurata* (25 min), medium in *D. puntazzo* (10 min) and very short in *D. labrax* (< 3 min).

Table 1 shows the maximum motility values and total motility duration for sperm stored at 3 ± 1 °C in the dark and activated 6, 24, 48 and 72 h after sampling.

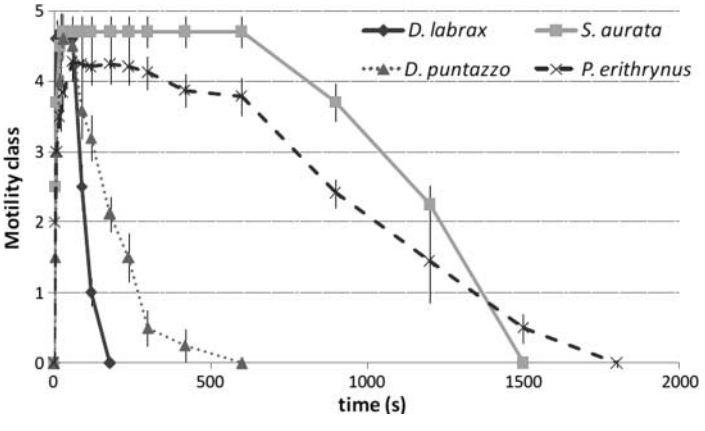

Figure 1. Sperm motility trend of *Dicentrarchus labrax*, *Sparus aurata*, *Diplodus puntazzo* and *Pagellus erythrinus*, activated immediately after the pool formation and kept at 22 ± 2 °C.

Table 1. Effects of semen storage at 3 ± 1 °C in the dark on maximum motility (class) and total motility duration (min).

		Storage time (h)				
Species	Parameter	0	6	24	48	72
Dicentrarchus labrax	Maximum motility value	5	4.5	1.5	1	0.5
	Total motility duration	3	3	3	2	1
Sparus aurata	Maximum motility value	5	5	5	5	4
	Total motility duration	25	25	25	25	20
Diplodus puntazzo	Maximum motility value	4.5	4	3.5	3	2.5
	Total motility duration	10	7	7	7	5
Pagellus erythrinus	Maximum motility value	4.5	4	3.5	3.5	3.5
	Total motility duration	30	30	25	25	20

Table 2. Effects of inhibitor medium on maximum motility (class).

Species	Control 0 h	Control 30 min	Control 1% NaCl 30 min
Dicentrarchus labrax	5	2.5	5
Sparus aurata	5	5	3.5
Diplodus puntazzo	4.5	4.5	4
Pagellus erythrinus	4.5	3	3.5

The spermatozoa of *S. aurata* and *P. erythrinus* showed good resistance to cold storage, with a maximum motility value greater than class 3. Total durations of motility were quite similar to those of semen activated immediately after pool formation.

D. puntazzo sperm showed lower tolerance to storage at $3 \pm 1\,^{\circ}C$ (up to 48 h) compared with sperms of *S. aurata* and *P. erythrinus*: longer storage times, in fact, led to substantial reductions of the maximum sperm motility value (less than class 3) and total duration of motility (5 min at 72 h of storage).

Finally, sperm of *D. labrax* showed high sensitivity to cold storage, maintaining good motility characteristics for just 6 h. Storage times longer than 6 h induced large losses in terms of maximum motility value and total motility duration.

Table 2 shows maximum sperm motility values recorded for all analysed species in control toxicity tests: (1) Control 30 min in 1% NaCl, (2) Control 30 min without dilution and (3) Control 0 h, i.e. semen activated immediately after collection.

Incubation of the semen without dilution for 30 min at room temperature caused a drastic reduction in sperm motility in *D. labrax* (class 2.5) and less reduction in *P. erythrinus* (class 3); while no significant effect was recorded for the sperm of *S. aurata* and *D. puntazzo* (classes 5 and 4.5, respectively).

Sperm diluted 1:6 in 1% NaCl and incubated under the same experimental conditions (Control NaCl 1% 30 min), showed that were of good quality in terms of maximum motility value (minimum 3.5 class for all species analysed); in fact, with *D. labrax* and *P. erythrinus*, motility losses were lower (class 5 and 3.5, respectively) than with Control 30 min; whereas in *S. aurata* and *D. puntazzo* there was a reduction in the maximum motility value (classes 3.5 and 4, respectively).

Figure 2 shows the effects of xenobiotics on the maximum motility values recorded for each species after incubation for 30 min and expressed as effect percentages.

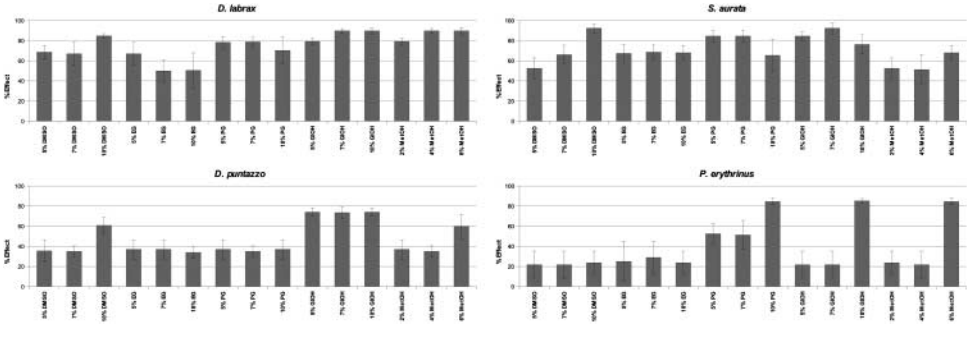

Figure 2. Effects of 30 min incubation at $22 \pm 2\,^{\circ}C$ in dimethylsulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), glycerol (GlOH) and methanol (MetOH) on maximum sperm motility values of *Dicentrarchus labrax*, *Sparus aurata*, *Diplodus puntazzo* and *Pagellus erythrinus*, expressed as effect percentages.

In *D. labrax*, all tested xenobiotics induced reductions in motility, with effect percentages > 50%. The increase in xenobiotic concentration induced significant increases in the effect percentage with DMSO, GlOH and MetOH, whereas it had no effects with EG and PG.

For *S. aurata* sperm, all xenobiotics, even at the lowest concentrations tested, induced effect percentages >50%; significant increases in effect percentages were recorded only for DMSO and MetOH when the concentration increased.

The sperm of *D. puntazzo* showed lower sensitivity than *D. labrax* and *S. aurata*. EG and PG were less toxic, giving effect percentages < 40% even at the highest concentrations tested.

With DMSO and MetOH, an increase in the test concentration (10% and 6%, respectively) induced a significant increase in the effect percentage (60%).

GlOH was the only xenobiotic to record an effect percentage of 74% at the lowest concentration tested (5%), but an increase in the concentration of GlOH did not induce a significant increase in the effect percentage.

P. erythrinus sperm was the least sensitive to the tested xenobiotics, except for GlOH. Exposure to DMSO and EG, even at the highest concentrations tested (10%), led to effect percentages of < 30%; with GlOH and MetOH, the highest effect percentages (> 80%) were recorded only at the maximum tested concentrations (10% and 6%, respectively). PG was the only xenobiotic to induce an effect percentage > 50% at the lowest tested concentration (5%); effect percentage values were > 80% at 10% PG.

4. Discussion

The sperms of marine teleosts selected and analysed here showed different features, especially regarding duration of the maximum motility value and the total duration of motility, these parameters were high for *S. aurata* and *P. erythrinus*, intermediate for *D. puntazzo* and very short for *D. labrax*.

The results obtained for *D. labrax* and *S. aurata* confirm previous studies with these species [14,27,28], although for *D. labrax* there are some differences from the results reported by Fauvel et al. [29].

The total duration of motility for *D. puntazzo* was higher that reported by Papadaki et al. [30].

For *P. erythrinus*, differences in total time of sperm motility were recorded with respect to the data of Lechekhab [31], with a total motility duration ranging from 35 min (in August) to 60 min (in July) reported for adults males bred in Algeria.

Differences among studies are probably related to small differences in the activation media, especially osmolarity.

Of the parameters analysed, the use of sperm motility as a biological indicator is limited by its short duration which leads to an unsatisfactory incubation time for the sample under test.

Regarding the cold storage of sperm, a decreasing scale of tolerance was found, as follows: *S. aurata* > *P. erythrinus* > *D. puntazzo* > *D. labrax*.

Analysis of semen motility after cold storage is essential to assess the possible medium- to long-term conservation of sperm, without altering its physiological function, and to increase its period of use.

Incubation of sperm in 1% NaCl inhibitor does not significantly alter sperm quality in terms of the maximum motility value.

D. labrax sperm has been the subject of numerous studies aimed at identifying a diluent inhibitor able to preserve its short motility. Several authors have developed diluents of similar ionic composition and osmolality as the seminal plasma [30,32–34], in which spermatozoa are immotile, whereas others have provided a medium that inactives the spermatozoa to maintain potential

motility [27]. The results obtained in this study confirm the ability of 1% NaCl solution to preserve the quality of *D. labrax* semen for 30 min at room temperature.

For the other species analysed here, few studies have been conducted on sperm motility and the possibility of sperm storage in media able to preserve movement quality.

The 1% NaCl solution was tested to assess its possible use in the toxicity test. Incubation of sperm (30 min at room temperature) in this inhibition medium reduced losses in terms of maximum sperm motility value recorded for *D. labrax* and *P. erythrinus*, and induced a low reduction in maximum sperm motility value for *S. aurata* and *D. puntazzo* (three classes of motility in *S. aurata*, from 100 to 65% of RVL spz; one class of motility for *D. puntazzo*, from 90 to 80% RVL spz). However, the losses for these two species have been reduced.

Based on these results, the toxicity test was conducted for all four species by using the inhibitor diluent, which allows us to overcome the short duration of sperm motility.

The lowest concentration of DMSO tested (5%, corresponding to 55 $g \cdot L^{-1}$) induced effect percentages of $> 50\%$ in *D. labrax* ($69 \pm 7\%$) and *S. aurata* ($53 \pm 10\%$), although it induced a lower toxic effect in *D. puntazzo* ($35 \pm 11\%$) and *P. erythrinus* ($22 \pm 20\%$). A similar result was also recorded with 5% EG (corresponding to 55.5 $g \cdot L^{-1}$) and 2% MetOH (corresponding to 15.8 $g \cdot L^{-1}$), with effect percentages $> 50\%$ in *D. labrax* and *S. aurata* ($67 \pm 12\%$ and $67 \pm 9\%$ for EG and $79 \pm 10\%$ and $53 \pm 3\%$ for MetOH), and lower in *D. puntazzo* and *P. erythrinus*.

With the other xenobiotics, *D. labrax* and *S. aurata* were confirmed as the most sensitive systems, with effect percentages of $78 \pm 6\%$ and $85 \pm 6\%$, respectively, for 5% PG (51.8 $g \cdot L^{-1}$), and $79 \pm 3\%$ and $85 \pm 5\%$ for 5% GlOH (63 $g \cdot L^{-1}$).

The spermatozoa of *D. puntazzo* showed high sensitivity to GlOH, with an effect percentage of $74 \pm 4\%$ at 5% GlOH. By contrast, *P. erythrinus* sperm were more sensitive to PG with an effect percentage of $53 \pm 10\%$ at 5% PG.

Recorded sensitivity levels for these four species are broadly comparable with LC_{50} values found in the literature for two biological systems commonly used in ecotoxicology: *Oncorhynchus mykiss* juveniles and *Daphnia magna* neonates [35,36] (Table 3).

In this study, the preliminary toxicity tests with cryoprotectant xenobiotics that usually show very low toxicity, gave positive indications for the use of sperm motility to evaluate polluted samples.

The incubation time of inactive sperm in the xenobiotic is a key factor for the assessment and improvement of test sensitivity, and different authors have developed methodologies that differ for this parameter [9,11,19,20,52].

For the sperm motility endpoint, development of appropriate incubation protocols will enable greater sensitivities [19].

In relation to the physiological characteristics and the motility parameters, the investigated systems seem to be suitable for ecotoxicological applications.

D. labrax and *S. aurata* sperm were particularly sensitive to the tested toxic substances and might therefore be good candidates for ecotoxicological assessments.

Table 3. Values of 50% lethal concentration (LC_{50}) recorded in the literature for juveniles of *Oncorhynchus mykiss* and neonates of *Daphnia magna* exposed to dimethylsulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), glycerol (GlOH) and methanol (MetOH).

	Xenobiotics [Ref]				
Species	DMSO	EG	PG	GlOH	MetOH
O. mykiss	32.3	22.8–50	51.6	51–75 (LC_{100})	19
(96-h LC_{50}, $g \cdot L^{-1}$)	[37]	[38–41]	[42]	[43]	[39]
D. magna	24.6 ± 19.1	46.3–54.7	43.5	> 10	13.24
(48-h LC_{50}, $g \cdot L^{-1}$)	[36]	[39,40,44–47]	[48]	[49,50]	[51]

The sperm of gilthead seabream is probably more suitable for ecotoxicological test because of its specific characteristics, availability and easy management. Its use might also be coupled to the use of gilthead seabream embryonic stages, already proposed as a new biological system for environmental investigations [53].

The use of computer-assisted sperm analysis (CASA) [8] will help in the validation of these indicators for their use in toxicity tests for natural ecosystems monitoring.

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