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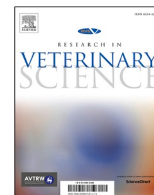

Comparative evaluation of the effects of different activating media and temperatures on European eel (*Anguilla anguilla*) sperm motility assessed by computer assisted sperm analysis

Alberto Elmi, Antonio Casalini, Martina Bertocchi, Pietro Emmanuele, Camilla Aniballi, Albamaria Parmeggiani, Nadia Govoni, Domenico Ventrella *, Oliviero Mordenti, Maria Laura Bacci

Department of Veterinary Medical Sciences, Alma Mater Studiorum Università di Bologna, Ozzano dell'Emilia, 40064, BO, Italy

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A B S T R A C T

The European eel is a critically endangered teleost fish with very poor success rate for captive breeding and artificial reproduction. Therefore, to support its conservation, new strategies are needed to ensure fertilization. Objective analysis of sperm motility may be critical as it potentially represents one of the most important reproductive quality parameters. Spermatozoa acquire motility once in contact with hyperosmotic solutions as saltwater, yet the exact mechanisms and the role of temperature are still to be clarified. The main aim of the study was to assess the effects of 3 activating media (artificial sea water, tank water and commercial Actifish®) at 4 and 20 °C on sperm motility, by means of computer assisted sperm analysis. Secondary aim was to test 2 different concentrations of Actifish® mimicking sea water pH/osmolality, at 4 °C. The results suggested how both temperature and activating media have effects on spermatozoa motility and kinematics, with temperature mainly acting upon interaction with the media type. The samples activated with tank water at 20 °C showed the poorest motility outcomes (mean 38.1%), while the ones activated with Actifish® diluted 1:4 and artificial sea water, at 4 °C, the highest (means 51.8 and 51.5% respectively). Additionally, diluting Actifish® to reach same pH and osmolality of seawater led to worse motility outcomes, suggesting that composition may be the critical factor for activation rather than osmolality itself.

1. Introduction

The European eel (*Anguilla anguilla*) is a teleost fish with a complex catadromous life cycle, spending most of their lives in fresh water and migrating to the sea to breed, that also includes metamorphosis from the leptocephalus stage to silvering eels. Due to the long life cycle, overfishing, habitat degradation and climate changes, this species is included in the International Union for Conservation of Nature (IUCN) red list as "critically endangered" (Pike et al., 2020). In order to support the conservation of this species and to meet the ever-increasing food market demand, artificial reproductive technologies are needed to support fertilization (Emmanuele et al., 2020). When compared to the Japanese eel species (*Anguilla japonica*), for which breeding facilities successfully and relatively easily produce offspring (Kagawa et al., 2005), breeding protocols for the European eel only started being developed in the last decades (Palstra et al., 2005; Asturiano et al., 2007; Di Biase et al., 2016; Asturiano et al., 2016; Politis et al., 2018; Herranz-Jusdado et al., 2019). Fertilization in eel, as reported for the majority of other fishes, is external: once issued, sperm must be activated by specific environment clues such as osmolality, pH, ionic/gaseous composition and temperature (Ohta et al., 2001). As reported in mammals (Elmi et al., 2019), sperm motility is one of the key parameters to evaluate gamete quality in teleost fishes (van der Horst, 2021), and several factors, both individual-related (age, hormonal synchronization treatment, type of feed) and environmental-related, can influence it (Morisawa, 2008; Mordenti et al., 2016; Kowalski and Cejko, 2019). In captive male eels, weekly injections of human chorionic gonadotropin (hCG), or similar gonadotropins (Peñaranda et al., 2018), are necessary in order to induce spermiation, with a reported gradual increase in sperm quality starting from the onset of spermiation (week 5) up to weeks 8/11 of hCG treatment

* Corresponding author.
E-mail address: domenico.ventrella2@unibo.it (D. Ventrella).

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(Khan et al., 1987; Asturiano et al., 2005, 2006). When looking at environmental influences, both diet and husbandry conditions are capable of modifying seminal plasma composition and sperm content, thus chancing the overall quality of eel sperm material (Baeza et al., 2015; Vílchez et al., 2016b; Locatello et al., 2018). As previously stated, eel spermatozoa are quiescent within the spermiduct and only became motile once in contact with an hyperosmotic solution, going from an osmolality of 300/350 mOsm/Kg (seminal plasma) up to 600–1800 mOsm/L (seawater) (Asturiano et al., 2004; Morisawa, 2008; Dzyuba and Cosson, 2014; Gallego and Asturiano, 2019). Upon activation, they remain motile for a maximum of 15 min, with the best motility characteristics being exhibited only during the first one (Gallego et al., 2014). It has been reported how, in the European eel sperm, the extracellular pH influences motility, as in other marine species, with the optimum activity at a pH of 7.8–8.2; on the other hand low seawater pH (acidification) determines a strong decrease in sperm motility (Garzón et al., 2008; Pérez et al., 2020). Ionic seminal plasma composition (Asturiano et al., 2004), and the variation of intracellular concentration of some ions, seem to be involved in activation of eel sperm (Huang et al., 2011). In particular, the percentage of motile spermatozoa (total motility) is influenced by seminal plasma potassium concentrations (Ohta et al., 2001; Vílchez et al., 2017) but not by calcium (Pérez et al., 2016), with the latter acting as modulator of velocities and beat frequency. Sodium is also involved in eel sperm motility: removal from the seminal plasma induces reduction in motility and also sperm head area (Vílchez et al., 2016a). Regarding temperature, 18–24 °C is the optimal range reported for the *Anguilla japonica*, with a decrease in motility when spermatozoa are exposed to higher temperatures (Huang et al., 2011). As for the European eel, the optimum temperature for spermiation and spawning is 20 °C (Gallego et al., 2015; Peñaranda et al., 2016), yet short term storage temperature from 4 °C to 20 °C do not seem to affect spermatozoa (Asturiano et al., 2016; Beirão et al., 2019). Literature regarding the analysis of morph-functional characteristics of European eel milts reports a laboratory working temperature of 4 °C, probably related to an easier standardization of refrigerated procedures (Gallego et al., 2013; Merino et al., 2023). To preserve and store fish semen, several studies were conducted aimed at developing refined cryopreservation protocols capable of maintaining semen quality (Magnotti et al., 2018; Figueroa et al., 2020; Magnotti et al., 2022). Taking into account the physico-chemical composition of the eel seminal plasma, an isosmotic extender medium named P1 was previously designed and tested, with the same pH of seminal plasma (8.5) in order to prevent spermatozoa activation (Asturiano et al., 2004; Peñaranda et al., 2010). Other formulations of extenders have also been proposed with the final goal of cryopreserving spermatozoa for a long term (Herranz-Jusdado et al., 2019). Generally speaking, sperm motility and its kinematics represent an essential tool to assess sperm quality in several fish species including eel. Nonetheless, on the bases of the aforementioned factors, it is fundamental to standardize the protocol for motility evaluation in order to obtain reliable data (Gallego et al., 2013; Dzyuba and Cosson, 2014; Gallego and Asturiano, 2019). For this reason, the use of computer assisted sperm analysis (CASA), capable of providing an objective assessment, is pivotal and has already been set up for this species (Gallego et al., 2013, 2014, 2015). What is partially missing, on the basis of the available literature, is a well characterized activation protocol. Indeed, the only published work, regarding sperm motility and European eel, use artificial seawater, as for other marine species, sometimes adding 2% bovine serum albumin (*w/v*) (Herranz-Jusdado et al., 2019).

The present study therefore aimed to investigate the effects of a commercial fish extender, also used as an activator, called Actifish® (IMV-Technologies, L'Aigle, France) and the reproduction tank water in comparison to artificial sea water on European eel sperm motility, assessed by CASA, at two different temperatures (4 °C and 20 °C). The second experiment was to test two different concentrations, with different osmolalities, of Actifish® at 4 °C on eel spermatozoa motility, in comparison with standard artificial sea water.

2. Materials and methods

2.1. Animals

Twenty-four male adult silver eels ($n = 24$; 138.4 ± 10.3 g body weight; 44.9 ± 1.1 cm body length) were included in the study. Sixteen animals were used for experimental protocol 1, the remaining 8 for experimental protocol 2. For the experimental purposes, farmed eels were transferred to the dedicated facility of the Department of Veterinary Medical Sciences (Cesenatico, Italy). All animals were weighed, individually marked by fish-tags (FLOY TAG Mod Floy T-Bar Anchor) and kept in a recirculating system with natural sea water, consisting of one fish-rearing tank (700 L) equipped with a foam separation tank (protein skimmer) and a biological filter containing plastic porous balls. A thermal regulation system, a UV-sterilizer lamp, an ozonizer and an aerator (electromagnetic air compressor) to adjust the rearing water conditions were also included in the system.

All eels were gradually acclimated, during the first 7 days, in order to reach the standard experimental conditions used in the experimental facility: temperature of 15 ± 0.5 °C, salinity of 32–35 ‰, pH 7.8–8.2, and dissolved oxygen 9 ppm (Di Biase et al., 2016). During the experimental period, eels were maintained in darkness and without feeding according to previously published protocols (Dollerup and Graver, 1985). All sampling and manipulation procedures were approved by the Ethical Committee of the University of Bologna (ID 575/2016).

2.2. Hormonal synchronization

In order to promote gametogenesis, all males were hormonally treated to induce maturation and spermiation upon weekly intramuscular injection of 1.0 IU/g hCG (Corulon, 5000 UI, Intervet, Segrate, Milan, Italy) as previously described (Asturiano et al., 2006; Mordenti et al., 2014). Prior to injection, animals were anaesthetized by immersion in water added with phenoxyethanol (400 ppm). Males were injected every week up to spermiation and checked by hand stripping. During the spermiation period, starting from week 10–17 of hCG treatment, fishes were sampled 24 h after hormone administration.

2.3. Milts collection and evaluation (concentration and viability)

Anaesthesia was induced as described in the section above. Once anaesthetized, eels were placed in a dry cloth and milt collected by applying delicate pressure on the abdomen after accurate cleaning of the urogenital area to avoid any contamination by urine or faeces (Locatello et al., 2018). Ejaculates were immediately diluted 1:10 with P1 medium (NaCl 125 mM, $NaHCO_3$ 20 mM, KCl 30 mM, $MgCl_2$ 2.5 mM, $CaCl_2$ 1 mM; Asturiano et al., 2005), added with 2% BSA and final pH of 8.5 (Peñaranda et al., 2010). Samples, maintained at 4 °C, were transferred within 1 h from collection site to the physiology laboratories of the Department of Veterinary Medical Sciences of the University of Bologna (Ozzano dell'Emilia, Italy).

Upon arrival, one aliquot of each sample was further diluted 1:100 or 1:1000 in P1 medium to measure sperm concentration using a Thoma haemocytometer with 400× magnification and contrast phase microscope (Nikon Eclipse E600; Nikon Corporation, Tokyo, Japan). The number of spermatozoa lying in 5 bigger squares (0.2 × 0.2 × 0.1 mm each) of the haemocytometer was averaged and used for concentration calculation. Results were reported as spermatozoa $\times 10^9$/mL.

Viability was assessed using the Eosin-Nigrosin staining method (Elmi et al., 2017): 10 μL of staining solution were added to 10 μL of each sample (diluted as for the assessment of sperm concentration), and 5 μL were immediately smeared on a glass microscope slide for analysis. The percentage of live cells (undyed spermatozoa/all spermatozoa) was evaluated on a minimum of 400 cells.

Sperm concentration and viability were assessed to have an overall idea of the quality of the collected milts prior inclusion in the experimental protocols.

2.4. Experimental protocol 1

Sixteen ejaculates ($n = 16$) collected from sixteen male eels were used to test three different activating media at two temperatures (4 °C and 20 °C):

- ASW: artificial seawater (sea salts 3.7%, Sigma-Aldrich, Saint Louis, MO, USA), added with 2% BSA, pH corrected to 8.2;
- TW: tank water, taken directly from the breeding tanks;
- Actifish®: a commercially available activating medium (IMV Technologies, L'Aigle, France) diluted 1:4 with bi-distilled water.

Both pH (SevenExcellence pH-meter S400, pH electrode InLab Expert Pro-ISM; Mettler Toledo, Columbus, Ohio, US) and Osmolality (The Advanced® Micro-Osmometer Model 3300, A. De Mori, Milan, Italy) were tested for each activating medium, freshly made on the day of each testing (Table 1).

From each of the 16 ejaculates, 6 experimental samples were prepared (96 samples in total) by suspending a fixed number of spermatozoa in 200 μL of P1 extender (final concentration 2×10^7 spz/mL), then kept for 1 h at either 4 or 20 °C. Each sample was then activated adding 800 μL (Pérez et al., 2016) of the tested medium, also kept either at 4 or 20 °C (± 0.3 °C) in refrigerated incubators (RI-150EU; Thermo scientific, Waltham, Massachusetts, US), and motility was objectively assessed within 10 s. All the material used for the experiment, including the detachable microscope stage and pipette tips, were kept at the correct temperature (4 or 20 °C) using the previously described refrigerated incubator.

2.5. Experimental protocol 2

Eight ejaculates ($n = 8$) collected from 8 male eels were used for the second experiment, with three experimental samples per ejaculate ($N = 24$) prepared as previously reported.

In order to find the Actifish® dilution showing similar osmolality to the one recorded for ASW, serial dilutions were preliminary performed and checked for pH and osmolality as described above (Table 2).

The chosen dilution of Actifish® to be tested and compared to ASW and Actifish® 1:4, only at 4 °C, was 1:2. Activation was performed as for experiment 1.

2.6. Evaluation of sperm motility

Spermatic objective motility, both total and progressive, and its kinematics were assessed using a Computer Assisted Sperm Analysis (CASA; Hamilton Thorne CEROS II; Animal Motility II, Software Version 1.9, Beverly, MA, USA) with dedicated fish software. Analyses were performed as previously reported (Gallego et al., 2013). Frame capture speed was set at 60 Hz, with a frame count of 45. The system is equipped with a 10× Zeiss objective, with objective magnification X of 1.2 and Y of 1.21, and a high-definition digital camera used at 300 gain and 8 ms exposure. To evaluate the effects of temperature on eel sperm motility, activating media, plastic labware, Leja 4 chambers slides (IMV Technologies, L'Aigle France) and the removable CASA stage were kept at either 4 °C or 20 °C for 1 h prior to analyses, just like the experimental samples. Motility was always acquired within 10 s from activation. A total of at least 500 spermatozoa were captured for each sample, with analyzed parameters being: total motility (tMot, %); progressive motility (pMot, %), slow motility (Slow, %), static spermatozoa (Static, %), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s) average path velocity (VAP, μm/s), distance average path (DAP, μm), distance curved line (DCL, μm), distance straight line (DSL, μm), percentage of linearity (LIN, %), percentage of straightness (STR, %) Wobble coefficient (WOB, %), mean amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz).

Table 1
pH and osmolality of the different activating media used in experiment 1. Data are reported as Means ± SD.

Activating medium	pH	Osmolality (mOsm)
ASW	8.25 ± 0.10	999.67 ± 12.50
TW	8.14 ± 0.02	957.50 ± 3.54
Actifish® (1:4)	8.53 ± 0.20	592.75 ± 16.74

ASW: artificial sea water, TW: tank water.

Table 2
pH and osmolality of the different Actifish® dilutions tested prior to experiment 2.

Actifish® dilutions	pH	Osmolality (mOsm)
undiluted	8.29	2399
1:2	8.30	1174
1:3	8.32	798
1:4	8.33	613

2.7. Statistical evaluations

Statistical evaluations and graphical representations were performed using R 3.0.3 (the r Foundation for Statistical Computing) and GraphPad Prism v.8 (GraphPad Software Inc., San Diego, CA, USA). Data were reported as mean and standard deviations (SD). Normal distributions were assessed by means of Shapiro–Wilk tests, while the equality of variances in the two groups by means of Levene's test. For experiment 1, two-way ANOVAs were performed, with activating media, temperature and their interaction set as factors, followed by post-hoc Tukey's multiple comparison test. For experiment 2, either parametric (one-way ANOVA with Geisser-Greenhouse correction) or non-parametric (Friedman test) tests were performed, again followed by post-hoc Tukey's multiple comparison test. The significance level for all statistical analyses was set at $p < 0.05$.

3. Results

A total of twenty-four milts were included in the experiments, 16 for experiment 1 and 8 for experiment 2. The ejaculates used in the present study showed very good sperm viability (94.2 ± 3.4%) and high variability in terms of concentrations, ranging from 2.11×10^8 up to 4.83×10^9 spermatozoa/mL.

3.1. Experiment 1

Data regarding percentages of total motility (tMot), progressive motility (pMot), slow spermatozoa (Slow) and static sperm (Static) are reported in Fig. 1, alongside with the results of the post-hoc tests. The two-way ANOVA analysis highlighted how tMot was influenced by both temperature ($p = 0.016$) and the activating medium ($p = 0.0012$), also confirmed by the % of static spermatozoa with a p = 0.016 for temperature and p = 0.0012 for activating media. The effects of temperature, activating media and their interaction were also statistically significant when looking at pMot ($p = 0.0095$, $p < 0.0001$ and $p = 0.0349$ respectively). The samples activated with TW at 20 °C showed the lowest percentages of both total and progressive motility when compared to the others. The percentage of slow spermatozoa was influenced by temperature ($p = 0.0253$) and by its interaction with activating media ($p = 0.0108$), but not by media themselves ($p = 0.0733$). Also in this case, activation with TW at 20 °C led to the worst results.

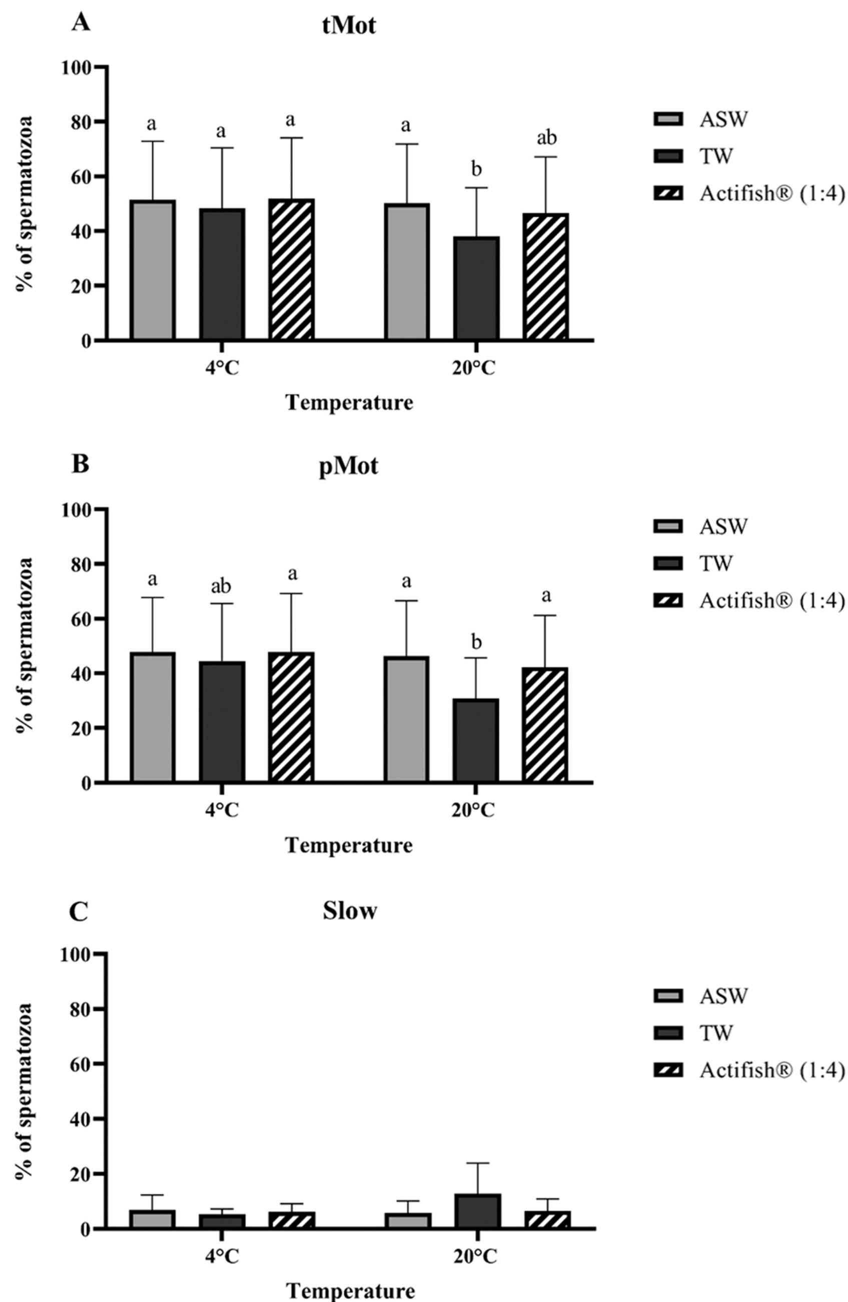

Fig. 1. Experiment 1: effects of the different activating media (ASW = Artificial sea water, TW = tank water, Actifish® 1:4) at two different temperatures (4 °C and 20 °C) on A = sperm total motility (tMot), B = progressive motility (pMot), C = slow spermatozoa (Slow). Different letters represent statistically relevant differences ($p < 0.05$) upon two-way ANOVA followed by Tukey's post-hoc multiple comparison test. Data are reported as mean ± SD, $n = 16$.

The p values of the two-way ANOVAs regarding kinematic parameters, reported in Table 3, highlighted that temperature, activating media and their interaction were statistically significant for STR, WOB and LIN. On the other hand, DAP, DCL, DSL, VAP, VSL, VCL and BCF were statistically influenced only by activating media and their interaction with temperature. Finally, ALH showed a statistically relevant difference only for the activating media.

The means ± SD of the kinematic parameters are reported in Table 4, alongside with the results of the post-hoc tests.

3.2. Experiment 2

The one way ANOVA performed on Experiment 2 data was statistically significant for tMot ($p = 0.0050$), pMot ($p = 0.0006$), Slow ($p = 0.0001$) and Static spermatozoa (p = 0.0050), as shown in Fig. 2. Activation with Actifish® diluted 1:2 led to the worst results in terms of total and progressive motility as well as the percentage of slow spermatozoa, when compared to ASW and 1:4 dilution.

Data regarding the kinematic parameters of Experiment 2 are reported, as mean ± SD, in Table 5: all parameters showed statistically significant differences upon ANOVA analysis. The outcomes of the *post hoc* analysis are also included as letters in Table 5. Actifish® 1:2 let to statistically relevant lower results with regards to velocity (VAP, VCL, VSL), distance (DAP, DCL, DSL) and WOB. Both tested concentrations of Actifish® (1:2 and 1:4) showed statistical differences when compared to ASW with regards to LIN, STR and ALH. Finally, Actifish® 1:2 differed from its 1:4 dilution but not from ASW for BCF.

Table 3
P-values resulting from the two-way ANOVAs for the different kinematic parameters.

Kinematics	Temperature	Activating media	Interaction
VAP, µm/s	0.1185	0.0005*	0.0003*
VCL, µm/s	0.2584	0.0405*	0.0176*
VSL, µm/s	0.1008	0.0038*	0.0021*
DAP, µm	0.1608	0.0021*	0.0034*
DCL, µm	0.9910	<0.0001*	0.0052*
DSL, µm	0.1166	0.0014*	0.0007*
LIN, %	0.0351*	0.0004*	0.0033*
STR, %	0.0153*	0.0014*	0.0069*
WOB, %	0.0451*	<0.0001*	0.0008*
ALH, µm	0.3872	<0.0001*	0.0701
BCF, Hz	0.1176	0.0003*	0.0422*

* = $p < 0.05$.

Table 4
Descriptive statistics of the kinematic parameters (mean ± SD) and post-hoc tests results (n = 16).

Kinematics	T °C	ASW	TW	Actifish® 1:4
VAP, µm/s	4	103.0 ± 12.4^{ab}	103.6 ± 12.3^{ab}	94.9 ± 12.7^{bc}
	20	107.4 ± 11.7^{a}	85.9 ± 17.3^{c}	95.4 ± 9.8^{bc}
VCL, µm/s	4	153.5 ± 11.7^{ab}	156.8 ± 10.2^{ab}	141.7 ± 11.8^{b}
	20	157.5 ± 11.9^{a}	144.0 ± 12.1^{b}	145.7 ± 11.1^{b}
VSL, µm/s	4	92.8 ± 11.4^{ab}	93.2 ± 11.9^{abc}	85.8 ± 12.9^{c}
	20	96.8 ± 11.4^{a}	74.9 ± 18.4^{bc}	85.9 ± 9.9b^{c}
DAP, µm	4	66.1 ± 7.5^{ab}	66.6 ± 7.8^{abc}	61.7 ± 7.8^{c}
	20	69.1 ± 6.3^{a}	56.51 ± 0.1^{bc}	62.0 ± 6.0^{bc}
DCL, µm	4	98.8 ± 6.6^{ab}	101.2 ± 6.7^{a}	92.3 ± 6.8^{c}
	20	101.9 ± 5.9^{a}	95.5 ± 6.0^{ac}	94.9 ± 6.2^{bc}
DSL, µm	4	59.5 ± 6.9^{a}	59.7 ± 7.5^{a}	55.8 ± 7.9^{ab}
	20	62.0 ± 6.4^{a}	49.0 ± 11.1^{b}	55.8 ± 6.2^{ab}
LIN, %	4	59.4 ± 3.8^{a}	57.9 ± 4.7^{ab}	59.5 ± 6.0^{a}
	20	59.8 ± 4.3^{a}	49.6 ± 9.0^{b}	58.1 ± 5.2^{a}
STR, %	4	88.7 ± 1.7^{a}	87.6 ± 2.8^{a}	88.6 ± 4.2^{a}
	20	87.9 ± 2.8^{a}	82.3 ± 5.8^{b}	88.3 ± 4.0^{a}
WOB, %	4	66.5 ± 3.9^{a}	65.1 ± 4.3^{a}	66.3 ± 4.7^{a}
	20	67.3 ± 3.4^{a}	58.2 ± 7.5^{b}	64.9 ± 3.9^{a}
ALH, µm	4	5.6 ± 0.3^{a}	5.6 ± 0.2^{a}	5.1 ± 0.3^{c}
	20	5.5 ± 0.3^{ab}	5.7 ± 0.4^{a}	5.3 ± 0.6^{bc}
BCF, Hz	4	31.2 ± 3.0^{b}	34.1 ± 2.7^{a}	33.7 ± 1.4^{a}
	20	33.1 ± 3.2^{ab}	33.4 ± 1.8^{ab}	34.7 ± 2.2^{a}

Superscript letters indicate differences, for the given parameter, between the different activating media and temperatures ($p < 0.05$).

4. Discussion

Fish are exposed to a wide range of temperatures, especially during reproduction, and this parameter has a critical role in controlling sperm physiology at many levels such as enzymatic activities, membrane permeation and energetic metabolism (Cosson, 2019). For this reason, the main aim of the present study was to test the effects of the exposure to two different temperatures during eel sperm activation and analyses: 4 °C, which is the most commonly used temperature for *in vitro* management of fish and European eel milt (Asturiano et al., 2016; Herranz-Jusdado et al., 2019), and 20 °C, that is the temperature used for the reproduction tanks in eels breeding facilities (Di Biase et al., 2017; Emmanuele et al., 2020; Mordenti et al., 2018; Peñaranda et al., 2016). Additionally, the choice of the activating media plays a pivotal role in the management of spermatozoa, especially in fish species where the milt is activated upon environmental modifications (Kowalski and Cejko, 2019; Morisawa, 2008). Therefore, since data regarding the effects of different activating media in the chosen species are lacking, we tested three different media: artificial sea water (ASW), tank water collected directly from the breeding tank (TW) and Actifish®, a commercially available activating medium diluted 1:4. ASW acted as a standardized control, as it can be freshly made on the day of each analysis without altering its composition by diluting commercially

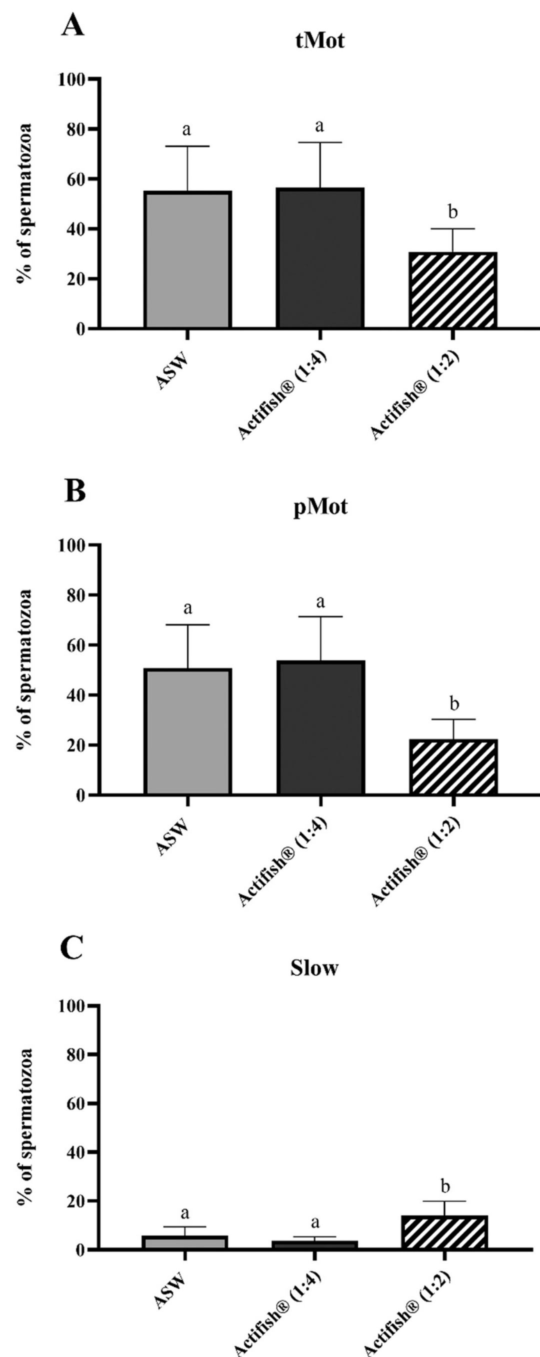

Fig. 2. Experiment 2: effects of the different activating media (ASW = Artificial sea water, Actifish® 1:4 = Actifish diluted 1:4, Actifish® 1:2 = Actifish diluted 1:2) on A = sperm total motility (tMot), B = progressive motility (pMot), C = slow spermatozoa (Slow). Different letters represent statistically relevant differences ($p < 0.05$) upon one-way ANOVA with Geisser-Greenhouse correction or Friedman test followed by Tukey's post-hoc multiple comparison test. Data are reported as mean ± SD, $n = 8$.

available salts into bi-distilled water. On the other hand, TW was included as it represented the "medium" in which fertilization normally happens in the used breeding facility, despite its composition being variable and almost impossible to standardize as directly taken from the sea. The inclusion of a commercially available extender/medium with proven benefits in other species was done to try and set up a protocol that may be not only repeatable and standardizable, but also potentially beneficial to reproductive outcomes. The data of the present study seem

Table 5
Descriptive statistics of the kinematics (mean ± SD) and results of post-hoc tests, n = 8.

Kinematics	ASW	Actifish® 1:4	Actifish® 1:2
VAP, μm/s	107.1 ± 10.6^{a}	107.6 ± 7.1^{a}	73.9 ± 9.8^{b}
VCL, μm/s	158.3 ± 12.5^{a}	154.6 ± 6.3^{a}	129.1 ± 10.9^{b}
VSL, μm/s	95.8 ± 11.0^{a}	98.7 ± 7.4^{a}	62.6 ± 11.1^{b}
DAP, μm	69.2 ± 7.0^{a}	70.7 ± 5.3^{a}	48.0 ± 6.9^{b}
DCL, μm	102.4 ± 8.3^{a}	101.7 ± 4.7^{a}	84.6 ± 8.2^{b}
DSL, μm	61.7 ± 7.4^{a}	64.6 ± 5.4^{a}	40.8 ± 7.5^{b}
LIN, %	60.0 ± 3.4^{a}	63.2 ± 3.1^{b}	46.5 ± 5.5^{c}
STR, %	88.1 ± 2.2^{a}	90.7 ± 1.7^{b}	80.9 ± 4.9^{c}
WOB, %	67.6 ± 2.9^{a}	69.3 ± 2.6^{a}	56.2 ± 3.9^{b}
ALH, μm	5.6 ± 0.3^{a}	5.1 ± 0.2^{b}	6.0 ± 0.2^{c}
BCF, Hz	31.9 ± 3.2^{ab}	34.8 ± 2.0^{a}	28.6 ± 2.0^{b}

Superscript letters indicate differences, for the given parameter, between the different activating media ($p < 0.05$).

to suggest how temperature itself affects activation and motility characteristics of spermatozoa mainly upon interaction with the type of activating medium (Tables 3 and 4): only milts activated with TW showed statistically significant differences in tMOT, STR, WOB and LIN, between 4 °C and 20 °C. Indeed, samples activated with ASW and Actifish® 1:4 did not show differences between the two tested temperatures. This is in line with what was previously reported by Herranz-Jusdado and colleagues, who tested the same two temperatures during storage of European eel milt samples without recording differences during short term (24 h) storage (Herranz-Jusdado et al., 2019). Although the temperature at which reproduction takes place is species-dependent and can influence the activation of spermatozoa (Caldeira and Soler, 2018; Dadras et al., 2017), our observations highlighted that both 4 and 20 °C are reliable for the activation of European eel sperm. Therefore, analyzing milts at 4 °C, as already described by literature, may still be useful to predict *in vivo* fertility outcomes despite natural fertilization occurring into the Sargasso sea at approximately 20 °C (Politis et al., 2017). It is important to mention that, according to the results, tank water used at 20 °C did not seem to guarantee the best functional spermatozoa outcomes. Nonetheless, TW at 20 °C represents a necessary condition when performing natural breeding in a controlled environment without compromising fertility outcomes (Di Biase et al., 2017, 2016; Mordenti et al., 2018, 2014). This finding further highlights how reproduction in the European eel is a complex process and *in vitro* findings not always necessarily reflect the natural situation. As for the choice of the best activating medium, TW at 20 °C induced a statistically relevant decrease in sperm motility and, in particular, in tMot, pMot, static spermatozoa that kinematic parameters. Considering that the pH and osmolality of TW were similar to the ones recorded for ASW, both comparable with previously reported literature (Gallego and Asturiano, 2019; Asturiano et al., 2004), it can be assumed that the difference in motility is ascribable to the ionic/salt composition of TW, obviously less standardizable. The concentrations of some extracellular ions in seminal plasma are critical in the activation of eel motility: for example, a reduction in seminal plasma extracellular potassium results in a reduction of motility both in European and Japanese eels (Ohta et al., 2001; Vílchez et al., 2017). Also seminal plasma sodium and calcium concentrations in media are involved in spermatozoa activation (Pérez et al., 2016; Vílchez et al., 2016a), further strengthening the importance of ions and salt composition. The present study, to the best of the authors' knowledge, represents the first report on the use of the commercial medium Actifish® (IMV-Technologies) in European eel. Actifish® has already been tested in other species, such as rainbow trout (Haffray et al., 2008), and is commonly used according to the manufacturer's instructions in numerous other species to improve fertilization rates. Despite some fluctuations, all the tested activating media (ASW, TW, Actifish® 1:4 and Actifish® 1:2) showed pH and osmolality compatible with what previously reported by literature for this species (Pérez et al., 2020). The dilution used for experiment 1 (1:4), was chosen based on what recommended for other teleost, despite osmolality being considerably lower than the ones recorded for both the ASW and TW. Actifish® 1:4, showed very similar results when compared to ASW (used as a standardized control for this experiment) with regards to total and progressive motility, but some kinematics parameters, and in particular distances and ALH, differed. These alterations in the kinematics arrangement can be imputable to the lower osmolality, as already reported for other fish species (Alavi et al., 2021, 2011, 2009), or once again to potential differences in its composition that, for commercial purposes, is not disclosed. In order to try and understand if the differences recorded in experiment 1 between ASW and Actifish® 1:4 could be related to physical characteristics, experiment 2 was set up. In this case, Actifish® was also diluted until reaching similar pH and osmolality to ASW (pH 8.30 *vs* 8.25; osmolality 1174 *vs* 999.67). Activation and analyses were only carried out at 4 °C since experiment 1 did not suggest strong temperature influences and more literature data are available at this temperature. Looking at the results of the CASA analysis, it is clear how the 1:2 dilution of this commercially available medium induced a consistent decrease both in spermatozoa motility and kinematics parameters. Therefore, based on such results, it looks like activating media with similar osmolality and pH are still capable of influencing the motility features of milts. Overall, looking at the results of both experiments, the alterations in the activation of European eel milt seem to be linked to the composition of the activating medium. The negative results recorded in samples activated with Actifish® 1:2 (experiment 2), maybe be due to some specific ions or molecules that may become toxic or even spermicidal when more concentrated. Actifish®, for example, contains an antibiotic (gentamicin, 0.05 g/l), commonly added to several media used for ejaculates and milt manipulation to control bacterial pollution and growth. Nonetheless, the use of gentamicin in seminal doses has been demonstrated as capable of reducing sperm quality, in terms of viability and motility, both in mammals and fishes (Anel-Lopez et al., 2021; Boonthai et al., 2016). This example shows how some specific molecules may exert dose/concentration-dependent effect of fish milts, and more studies are needed to identify and clarify safety recommendations for extenders and activating media. Nonetheless, Actifish® proved to be a valid activating medium also for European eel semen, when diluted 1:4, despite minor alterations recorded in some kinematic parameters when compared to ASW. However, according to the previous literature, the key motility parameters responsible for *in vivo* fertilization rate alterations are progressive motility, VCL and VAP (Di Chiacchio et al., 2017; Gallego et al., 2017; Kanuga et al., 2012). When looking at these key parameters, Actifish® 1:4 acts similarly to ASW. Unfortunately, it is always important to remember how predicting *in vivo* reproductive outcomes only on the basis of male gametes' quality is misleading and incomplete, as it does not take into account the female contribution, that is eggs, to the process (Kommisrud et al., 2020).

5. Conclusions

Overall, the data obtained by the present study highlight how the composition of the chosen activating medium can induce variations in the most relevant qualitative and quantitative characteristics of spermatozoa motility in the European eel, and also upon interaction of the temperature. As for the latter, the commonly used temperature of 4 °C seems to be applicable to the species, with consistent results, despite 20 °C being its physiological reproduction temperature. Additionally, the data show how critical it is, for successful reproductive outcomes, to consider the quality and potentially the composition of the water used for the breeding. Trying to standardize milt handling and analyses, this study adds pivotal information to the existing knowledge regarding the reproduction of this endangered species.

Institutional review board statement

All sampling and manipulation procedures were approved by the

Ethical Committee of the University of Bologna (ID 575/2016).

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CRediT authorship contribution statement

Alberto Elmi: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft. **Antonio Casalini:** Investigation, Formal analysis, Methodology. **Martina Bertocchi:** Investigation, Methodology, Data curation. **Pietro Emmanuele:** Investigation, Methodology. **Camilla Aniballi:** Methodology, Formal analysis. **Albamaria Parmeggiani:** Supervision, Resources. **Nadia Govoni:** Supervision, Investigation. **Domenico Ventrella:** Formal analysis, Methodology, Data curation. **Oliviero Mordenti:** Supervision, Resources. **Maria Laura Bacci:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. IMV-Technologies (L'Aigle, FR) did not play any role in the design of the study or in the acquisition/interpretation of the results despite supplying the used compound.

Data availability

Data will be made available on request.

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