Fisheries and Aquaculture Journal

Pandey et al., Fish Aqua J 2017, 8:2
DOI: 10.4172/2150-3508.1000203

Research Article Open Access

Features of Sperm Motility and Circadian Rhythm in Japanese Anchovy (*Engraulis japonicus*)

Dipak Pandey[1,2], **Yong-Woon Ryu**[2] **and Takahiro Matsubara**[2*]

[1]*The United Graduate School of Agricultural Sciences, Bioresource Production Science, Ehime University, Japan*

[2]*South Ehime Fisheries Research Center, Ehime University, Japan*

[*]**Corresponding author:** Dr. Takahiro Matsubara, South Ehime Fisheries Research Center, Faculty of Agriculture, Ehime University, 25-1 Uchidomari, Minamiuwa-gun, Ainan, Ehime 7984206, Japan, Tel: (+81) 895-737112; Fax: (+81) 895-737113; E-mail: matsu@agr.ehime-u.ac.jp, b741012u@mails.cc.ehime-u.ac.jp

Received date: May 03, 2017; **Accepted date:** May 24, 2017; **Published date:** May 28, 2017

Abstract

Japanese anchovy (*Engraulis japonicus*) is a commercially and ecologically important fish that exhibits group synchronous and multiple spawning. However, the reproductive characteristics of the male in this species, especially sperm features and activation, are still largely unknown. In this study, we confirmed that features of the sperm and characteristics of the activations, regarding sperm motility and moving velocity. The average size of the sperm was 51 ± 1.3 μm in total length and possessed a normal structure with clockwise, anticlockwise, and linear motion. The initial motility at one minute after activation in seawater was 75 ± 12% during spawning time in this species (21:00–22:00), and the initial moving velocity (196 ± 26 μm/sec) remained constant for fifteen minutes post activation. While, comparatively low motility (30 ± 10%) was found until 17:00, and the sperm was almost immotile in the morning (08:00–09:00). Swimming ability was also confirmed with sperm that swam for more than one hour in seawater without an exogenous energy supply derived from the ovary in females, suggesting the trigger for sperm activation in multiple spawning fish is possibly species dependent. This report is the first to demonstrate time specific activation, that is, circadian rhythm, in teleost males.

Keywords: *Engraulis japonicus*; Sperm motility; Synchronous spawning; Circadian rhythm

Introduction

Japanese anchovy is a small pelagic fish belonging to the order Clupeiformes and is widely distributed around Japan. This species is commercially important to fisheries in Japan [1], China [2], Korea [3], and Taiwan [4], and furthermore, it plays an important role as a key member of marine ecosystems [5]. Japanese anchovy is known as a multiple spawning fish having a long spawning season [6,7] and spawn oval-shaped pelagic eggs. Females spawn periodically at intervals of two or more days during the spawning period [8], and the spawning rhythm is regulated by ambient water temperature [9]. However, the reproductive characteristics of the male of this species, especially sperm activity, have yet to be clarified.

To date, research on sperm in fish has centered on initiation mechanisms of motility [10] and sperm quality for artificial fertilization [11]. However, the acquisition mechanisms of sperm motility have not yet been demonstrated. In Japanese eel, it has been shown that spermatogenesis completes in an *in vitro* culture with 17α, 20β-dihydroxy-4-pregnen-3-one (DHP), and the sperm is morphologically the same as functional sperm naturally matured from males, but the sperm does not move in seawater (SW) [12]. In addition, *in vitro* incubation of sperm, which was from artificially matured males of Japanese eel with bicarbonate medium, showed increased sperm motility [13]. These results indicate that spermatogenesis and acquisition of sperm activation mechanism are controlled by different mechanisms.

In oviparous teleosts, spermatogenesis is generally known to take a long time (several weeks to months), in which spermatozoa are released at the same time and place with female ovulation [14]. Therefore, the intrinsic quality and quantity of both gametes affect the success of fertilization. Motility is one important function of the male gametes (sperm), which allows them to actively reach and penetrate the female gametes (eggs). A teleost egg is covered with a thick envelope called a chorion, which has a narrow pore designated as a micropyle that helps to avoid polyspermy [15]. Sperm can only enter and reach the ooplasmic membrane through the micropyle [16,17]. Thus, sperm motility might directly influence fertilization.

In most external fertilization types of teleost, sperm remains quiescent in the seminal plasma and becomes transiently motile when released into hypotonic fresh water or hypertonic seawater depending on the spawning environment [18]. In most freshwater species, sperm usually moves for less than 2 min and high activation is observed for less than 30 s [19]. Meanwhile, longer durations of sperm movement have been reported in various marine fish, including *Anarhichas minor* [20] and *Abramis brama orientalis* [21], and the time might be related to their reproductive strategies. It is known that several factors affect sperm motility, such as pH, temperature, ions, osmolality, and ovarian fluid (OF) [22]. Ovarian fluid is slightly viscous fluid found in the gonad cavity of oviparous fishes after ovulation. Ovarian fluid has been studied for its role in fish reproduction, its chemical composition, novel proteins and utility in testing for the presence of fish diseases [23]. There are also some reports on sperm being activated by ovarian fluid, which is extruded with ovulated eggs in Rainbow trout, *Oncorhynchus mykiss* [24] and Arctic charr, *Salvelinus alpinus* (L.) [25,26]. However, such information about the sperm activation of male Japanese anchovy is not well known.

Although understanding of sperm activation in teleost species is important, especially for commercial seed production, it is still a

largely unknown area due to the technical difficulties of analyzing sperm motility. A microscope connected to a computer analyzing system (CASA: - is commonly used to evaluate sperm motility in fish, such as common carp [27], African catfish [28], and zebrafish [29]. The aim of the present study is to clarify the features of anchovy sperm and time course changes in sperm motility using such a CASA system, which should provide basic knowledge about male reproduction of a multiple spawning teleost. Moreover, the effect of OF on motility was also examined to verify the activation system of the sperm.

Materials and Methods

Experimental animals

Juvenile Japanese anchovy captured by a commercial fishing boat were transferred to a sea surface aquaculture cage (5 × 5 × 5 m) located in Mishou Bay in Southern Ehime in December 2014. Captive reared anchovy were transferred to a 30-ton aquarium at the South Ehime Fisheries Research Center, Ehime University, and reared under natural photoperiod and water temperature conditions (19.5–20.9°C). About two hundred adults (body weight range: approximately 10–12 g) were placed into two one-ton tanks. Fish were fed 40 g of commercial feed (Otohime S2; Nissin-Marubeni, Japan) per day, which corresponded to about 2% of body weight, under a controlled photoperiod of 14L:10D and reared from May to June 2015.

Sampling

Sampling was carried out at four different time points (Table 1). All males from each time sampling point were examined. Gonads were excised and weighed to calculate the gonadosomatic index (GSI; [gonad weight/body weight] × 100). Intra-testicular semen was collected by syringe after making an incision in the posterior part of the excised testes and stored in a closed test tube (1.5 mL) on ice until use (up to 1 h). Sperm features were individually assessed from all sampled males. Stripped semen was diluted with Hank's solution, which is an inhibiting solution of sperm motility (Nacalai Tesque, Kyoto, Japan) containing calcium and magnesium, at a ratio of 1:9 (semen:solution) and used within 1 h. Ovarian fluid was collected from 5 ovulated females sampled at 21:00, the spawning time in this species. After expelling the eggs onto a plastic plate by gentle abdominal pressure, transfer in a sieve (mesh size 1 mm^2) and the OF was poured off, collected and store at 4°C for analysis or -30°C for lateral use. The obtained OF was then diluted with Hank's solution at a ratio of 1:1 before use.

	Sampling Point			
	08:00 - 09:00	**12:00 - 13:00**	**16:00 - 17:00**	**20:00 - 21:00**
Total sample fish	19	15	20	21
Total male	7	6	12	9
Physiological status of female (Pandey et al., In press)		Final oocyte maturation		Ovulation and spawning
All experimental fishes were reared under 14L-10D photoperiod (light, from 05:00 to 20:00) with water natural temperature (19.5°C ~ 20.9°C).				

Table 1: Sampling information of Japanese anchovy in this study.

Sperm motility analysis

Each 5 µL of semen diluent was added to 500 µL of filtered SW and mixed gently. Then, 6 µL of the sperm suspension was pipetted into a Standard Count 2 Chamber slide (Leja products B.V., GN Nieuw Vennep, Netherlands) and observed under a microscope (VANOX-T; Olympus, Japan) connected to a digital high speed camera (HAS-L1 Ver. 2.14; Detect Inc., Japan). Video tracking was carried out at 1, 5, 10, 15, 45, and 60 min post activation. Each video was recorded at 100 frame per second (FPS) at a resolution of 640 × 680 CFG. Sperm motility was analyzed using capture video under sperm tracking software DIPP-Motion Pro (Ditect Inc., Japan).

Statistical analysis

All data are presented as mean ± standard error (SEM) and were subjected to one-way ANOVA followed by the Tukey and Kramer HSD test. Statistical significance was set at $P \leq 0.05$. All statistical analyses were performed using SAS 10.0 packed in Jump (SAS; Cary, NC, USA).

Results

Morphology of Japanese anchovy sperm

The sperm of the Japanese anchovy was quite small and moved in a linear, semi linear, and circular direction. The total length of the sperm was approximately 51 ± 1.3 µm including the oval head (3.0 ± 0.2 µm), mid piece (1.7 ± 0.1 µm), and tail (48.3 ± 1.7 µm) (Figure 1). There were no changes in morphological features at the different sampling times (data not shown).

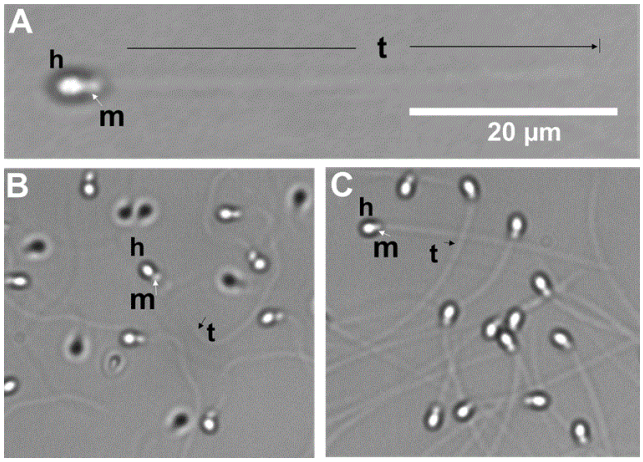

Figure 1: Japanese anchovy sperm under a light microscope. A; single sperm including head, mid piece and tail, B; motile sperm at 1 min post activation, C; Motionless sperm after 60 min post activation. h: head, m: mid piece, t: tail.

Gonadosomatic index

Our previous study showed that the GSI in female Japanese anchovy is approximately 6 to 8 when they are undergoing final maturation (FOM), and reaches 20 at the spawning time around 21:00 under natural environmental conditions [30]. In this experiment, there was no significant ($p>0.05$) difference in the GSI of the males between the sampling times (Figure 2). However, the values were slightly higher and reached 11 ± 2 at 17:00 and 21:00 rather than 09:00 and 13:00, indicating the GSI was higher before spawning.

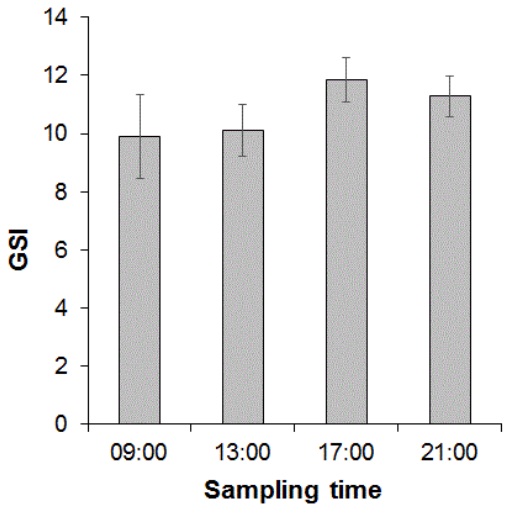

Figure 2: Changes in male gonadosomatic index (GSI) at different sampling times.

Sperm motility and moving velocity

The percentage of motile sperm significantly differed among the four groups: highest motility was confirmed at 21:00 ($P<0.0001$, Figure 3). Initial motility, at 1 min after SW dilution, at the sampling times of 9:00, 13:00, 17:00, and 21:00 were 4 ± 5%, 12 ± 9%, 28 ± 7%, and 75 ± 12%, respectively. Motility decreased in accordance with time (minutes post activation) and became almost inactive after 60 min post activation. On the other hand, the moving velocity of the motile sperm was not significantly different between the time sampling points, even though the values were slightly higher close to the spawning time (Figure 4). The average moving velocity at 1 min post activation was 123 ± 17, 135 ± 17, 127 ± 25, and 196 ± 26 µm/s, respectively. The moving velocity remained constant for 15 min post activation, and reached zero after 60 min post activation.

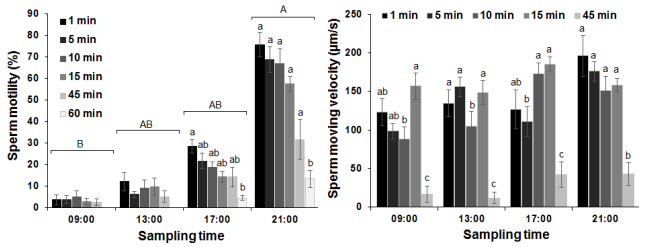

Figure 3: Variations in sperm motility and moving velocity under seawater activation by sampling time and duration. Data are expressed as mean ± standard error. Means sharing a letter superscript are not significantly different ($P<0.05$).

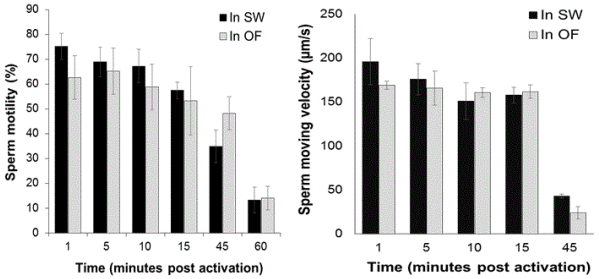

Figure 4: Effect of ovarian fluid on motility and moving velocity in different tracking point post activation. Data are expressed as mean ± standard error.

Effect of ovarian fluid on sperm motility and moving velocity

Comparisons of two activation mediums, SW and SW + OF, on sperm motility and moving velocity showed no significant differences (Figure 4). Initial motility at 1 min post activation for SW and SW + OF was 75 ± 12% and 62 ± 20%, respectively. The levels continuously decreased in accordance with time and reached 13 ± 12% and 14 ± 11%, respectively, at 60 min post activation. On the other hand, the initial moving velocity was 196 ± 26 µm/s in SW and 169 ± 10 µm/s in SW + OF, remained constant until 15 min post activation, and then decreased.

Discussion

Sperm motility in teleosts can be evaluated using a number of methods as follows: ratio of motile sperm, moving speed, and motile duration. In this study, we accurately analyzed the ratio of moving spermatozoa, moving speed, moving style whether straight or circular or both, and moving duration using a CASA system to clarify the features and characteristics of activation in sperm of Japanese anchovy.

Japanese anchovy is known as a multiple spawning fish [6] and the females spawn at intervals of two days or more [8]. Thus, spawning occurs in group stocking tanks every day under appropriate rearing conditions. In our previous report about final oocyte maturation in Japanese anchovy, spawning occurred from 21:00 to 22:00 every day under captive conditions as follows: 14L:10D photoperiod in water of 19.5 to 20.9°C. Moreover, final oocyte maturation started from 13:00 to 15:00 and progressed to reach ovulation by around 21:00 [30]. Group synchronized spawning of Japanese anchovy is also reported under wild [6] and captive conditions [30]. It is noteworthy that circadian rhythm in sperm motility was also observed in each sampled male under the same captive condition in this study, revealing that male anchovy has a daily rhythm in acquisition of sperm motility. Moreover, this finding suggests that all mature males can synchronously participate in fertilization at the same time of spawning. As shown in Table 2, time duration of sperm movements in Japanese anchovy is quite longer, at approximately 60 min, compared with other fish species. The characteristics of the spermatozoa may ensure successful fertilization in independently spawned unfertilized eggs without pairing behavior. In addition, this characteristic may enable a high genetic variability because a clutch of eggs from one female can be fertilized by spermatozoa, suspended in the surrounding seawater, from many males.

	Duration of motility (s or min)	**Initial velocity (μm/s)**	**References**
Fresh-water fish species			
Esox lucius	60-80 s	160-170	[37]
Oreochromis mossambicus	>30 min	70-80	[38]
Anguilla anguilla	<30 min	120-160	[39]
Carassius auratus	3 min at 18-21°C	ND	[40]
Cyprinus carpio	200 s	140	[41]
Salmo irideus	60-105 s	ND	[42]
Sea-water fish species			
Thunnus thynnus	140 s	215-230	[43]
Merluccius merluccius	400-500 s	65-130	[43]
Gadus morhua	700-800 s	130	[43, 44]
Dicentrarchus labrax	50-60 s	120	[45]
Acipenser persicus	1.5-5 min at 15-20°C	ND	[46]
Anarhichas minor	>2 days	40-50	[20]
Scophthalmus maximus	600 s	220	[47]
Takifugu niphobles	50 s	160	[48]
Oncorhynchus mykiss	30 s	220	[49]
Abramis brama orientalis	20 min	ND	[21]
Engraulis Japonicus	**60 ± 11 min at RT**	**196 ± 26**	**Present result**

* Duration of motility refers to the period of time from activation to complete inactivation where no single sperm is seen to be active. Temperature of experimental test is indicated in °C; RT refers to 'room temperature'.

**Initial velocity refers to the earliest value of sperm moving velocity at activation. ND: not defined.

Table 2: Experimental values for duration of motility and initial velocity for spermatozoa of various fish species.

In Pacific herring, which belongs to the same order (Clupeiformes) as Japanese anchovy, intensive studies related to the initiation of sperm motility have been conducted [10,31]. Although the Pacific herring sperm did not move in seawater when released for spawning, they started moving near unfertilized eggs through the action of herring sperm activating protein (HSAP) contained in OF [32], and entered an egg micropyle through the action of sperm motility initiating factor (SMIF), which is present near the micropyle [31,33]. Also, the functional relationship of these two physiological activation factors in fertilization is discussed in Pacific herring [10]. Such activation is also observed in the nest-building marine sculpin *Hemilepidotus gilberti* in the presence of OF, resulting in an increased period of sperm motility of up to 90 min, six times longer than in SW alone [34]. Sperm of freshwater species, such as bullhead *Cottus gobio* L. [35] and the wolf fish *Anarhichas minor* Olafsen [20], also showed an extended period of sperm movement in the presence of OF, with motility lasting for 2 h in the bullhead and 48 h in the wolf fish. In contrast, ovarian fluid did not influence sperm motility in fifteen-spined stickleback *Spinachia spinachia* [36]. In this study, the moving ability of the sperm also not change in the presence of OF, indicating that OF may not influence activation of sperm in Japanese anchovy. This finding may be related to species-specific egg characteristics, for example, the eggs of Japanese anchovy are pelagic and non-adhesive, whereas herring eggs are demersal and adhesive. However, the mechanism is not clear, and thus should be verified with further research.

In this study, we have clarified the process in which spermatozoa acquire motility during a time period of approximately half a day. It is suggested that the process occurs concomitant with female final oocyte maturation. Synchronous functionalization of both female and male gametes is suggested to ensure efficient fertilization in Japanese anchovy. Moreover, long duration sperm moving seems likely to guarantee a group synchronous non-pairing spawning style. To show the rhythm is possible among teleost species having multiple spawning characteristics, further research is necessary to determine such a rhythm of sperm motility in other species. Finally, this is the first report showing the time course of acquisition of sperm motility in a multiple spawning teleost.

Acknowledgement

We would like to thank the entire team at SEFRC for their kind co-operation and help during this research work and we are grateful to Dr.

Rie Goto (Ehime University), Dr. Haruhisa Fukada (Kochi University), Dr. Kazuhiro Mochida (National Research Institute of Fisheries and Environment of Inland Sea, Hiroshima), and Dr. Sayumi Sawaguchi (Japan Fisheries Research and Education Agency, Yokohama) for their kind suggestions. We also thank Dennis Murphy (The United Graduate School of Agriculture Sciences, Ehime University) for his critical reading of this manuscript. This work is included in the doctoral dissertation for The United Graduate School of Agricultural Sciences, Ehime University.

References

1. Aoki I, Miyashita K (2000) Dispersal of larvae and juvenile of Japanese anchovy Engraulis japonicus in the Kuroshio Extension and Kuroshio-Oyashio transition regions, western North Pacific Ocean. Fish Res 49: 155-164.
2. Chiu TS, Young SS, Chen CS (1997) Monthly variation of larval anchovy fishery in I-lan Bay NE Taiwan with an evaluation for optimal fishing season. J Fish Soc Taiwan 24: 273-282.
3. Kim JY, Kang YS, Oh HJ, Suh YS, Hwang JD (2005) Spatial distribution of early life stages of anchovy (Engraulis Japonicus) and hairtail (Trichiurus lepturus) and their relationship with oceanographic features of the East China Sea during the 1997–1998 El Nino event. Estuar Coast Shelf Sci 63: 13-21.
4. Zhao X, Wang Y, Dai F (2008) Depth-dependent target strength of anchovy (Engraulis japonicus) measured in situ. ICES J Mar Sci 65: 882-888.
5. Takasuka A, Oozeki Y, Kimura R, Kubota H, Aoki I (2004) Growth-selective predation hypothesis revisited for larval anchovy in offshore waters: cannibalism by juveniles versus predation by skipjack tunas. Mar Ecol Prog Ser 278: 297-302.
6. Funamoto T (2001) Maturation and spawning of Japanese anchovy. Nippon Suisan Gakkaishi 67: 1129-1130.
7. Tsuruta Y (1993) Geographical variation in the reproductive traits of Japanese anchovy as related to offshore distribution. Bull Jpn Soc Fish Oceanogr 57: 368.
8. Yoneda M, Kitano H, Selvaraj S, Matsuyama M, Shimazu A (2013) Dynamics of gonadosomatic index of fish with indeterminate fecundity between subsequent egg batches: application to Japanese anchovy Engraulis japonicus under captive conditions. Mar Biol 160: 2733-2741.
9. Yoneda M, Kitano H, Tanaka H, Kawamura K, Selvaraj S, et al. (2014) Temperature- and income resource availability-mediated variation in reproductive investment in a multiple-batch-spawning Japanese anchovy. Mar Ecol Prog Ser 516: 251-262.
10. Cherr GN, Morisawa M, Vines CA, Yoshida K, Smith EH, et al. (2008) Two egg-derived molecules in sperm motility initiation and fertilization in the Pacific herring (Clupea pallasi). Int J Dev Biol 52: 743-752.
11. Ochokwu IJ, Apollos TG, Oshoke JO (2015) Effect of egg and sperm quality in successful fish breeding. IOSR-JAVS 8: 48-57.
12. Miura T, Ando N, Miura C, Yamauchi K (2002) Comparative studies between in vivo and in vitro spermatogenesis of Japanese eel (Anguilla japonica). Zool Sci 19: 321-329.
13. Ohta H, Ikeda K, Kagawa H, Tanaka H, Unuma T (1999) Acquisition and loss of potential for motility of spermatozoa of the Japanese eel Anguilla japonica. UJNR Technical Report 28: 77-81.
14. Babin PJ, Cerda J, Lubzens E (2007) The fish oocyte: from studies to biotechnological applications. Springer.
15. Murata K (2003) Blocks to polyspermy in fish: a brief review. Aquaculture and pathobiology of crustacean and other species. Proceedings of the Thirty-second US Japan Symposium on Aquaculture Panel Symposium, Davis and Santa Barbara, California USA.
16. Yanagimachi R (1957) Some properties of the sperm-activating factor in the micropyle area of the herring egg. Annot Zool Japon 30: 114-119.
17. Yanagimachi R, Cherr G, Matsubara T, Andoh T, Harumi T, et al. (2013) Sperm attractant in the micropylar region of fish and insect eggs. Biol Reprod 88: 1-11.
18. Morisawa M (1994) Cell signaling mechanism for sperm motility. Zool Sci 11: 647-662.
19. Kime DE, Van Look KJW, McAllister BG, Huyakens G, Rurangwa E, et al. (2001) Computer assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comp Biochem Physiol 130: 425-433.
20. Kime DE, Tveiten H (2002) Unusual motility characteristics of sperm of the spotted wolf fish. J Fish Biol 61: 1549-1559.
21. Gosteeva MN (1957) Ecological and morphological characteristics of development of Aral Bream, Abramis brama orientalis (Berg). Trudy Institute Morfologia Zhivotnykh AN USSR 20: 121-147.
22. Alavi SMH, Cosson J (2006) Sperm motility in fishes, (II) Effects of ions and osmolality: a review. Cell Biol Int 30: 1-14.
23. Sawyer ES, Sawyer PJ, Janmey PA (2003) Method of using fish ovarian fluid for culture and preservation of mammalian cells. United States Patent: US006562621 B1 (Invention).
24. Dietrich GJ, Wojtczak M, Slowinska M, Dobosz S, Kuzminski H, et al. (2008) Effects of ovarian fluid on motility characteristics of rainbow trout (Oncorhynchus mykiss Walbaum) spermatozoa. J Applied Ichthyology 24: 503-507.
25. Turner E, Montgomerie R (2002) Ovarian fluid enhances sperm movement in Arctic charr. J Fish Biol 60: 1570-1579.
26. Urbach D, Folstad I, Rudolfsen G (2005) Effect of ovarian fluid on sperm velocity in Artic charr (Sylvanius alpinus). Behavior Ecology and Sociobiology 57: 438-444.
27. Christ SA, Toth GP, McCarthy HW, Torsella JA, Smith MK (1996) Monthly variation in sperm motility in common carp assessed using computer-assisted sperm analysis (CASA). J Fish Biol 48: 1210-1222.
28. Rurangwa EF, Volckaert AM, Huyskens G, Kime DE, Ollevier F (2001) Quality control of refrigerated and cryopreserved semen using computer-assisted sperm analysis (CASA), viable staining and standardized fertilization in African catfish (Clarias gariepinus). Theriogenology 55: 751-769.
29. Wilson-Leedy JG, Ingermann RL (2007) Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology 67: 661-672.
30. Pandey D, Ryu YW, Goto R, Matsubara T (2017) Morphological and biochemical changes of oocytes during final oocyte maturation in Japanese anchovy (Engraulis japonicus). Aquacult Sci 65: 29-40.
31. Cherr G, Vines CA, Smith HE, Pillai M, Griffin F, et al. (2015) Sperm motility initiation in pacific herring. Flagellar Mechanics and Sperm Guidance 4: 208-224.
32. Morisawa M, Tanimoto S, Ohtake H (1992) Characterization and partial purification of sperm- activating substance from eggs of the herring, Clupea palasii. J Exp Zool 264: 225-230.
33. Griffin FJ, Vines CA, Pillai MC, Yanagimachi R, Cherr GN (1996) Sperm motility initiation factor is a minor component of the pacific herring egg chorion. Dev Growth Diff 38: 193-202.
34. Hayakawa Y, Munehara H (1998) Fertilization environment of the non-copulating marine sculpin, Hemilepidotus gilberti. Env Biol of Fishes 52: 181-186.
35. Lahnsteiner F, Berger B, Weismann T, Patzner RA (1997) Sperm structure and motility of the freshwater teleost Cottus gobio. J Fish Biol 50: 564-574.
36. Elofsson H, Look KV, Borg B, Mayer I (2002) Influence of salinity and ovarian fluid on sperm motility in the fifteen-spined stickleback. J Fish Biol 63: 1429-1438.
37. Hulak M, Rodina M, Alavi SMH, Linhart O (2008) Evaluation of semen and urine of pike (Esox lucius L.): ionic composition and osmolality of the seminal plasma and sperm volume, density and motility. Cybium 32: 189-190.

38. Morita M, Takemura A, Okuno M (2004) Requirement of Ca2+ on activation of sperm motility in euryhaline tilapia (Oreochromis mossambicus). J Exp Biol 207: 337-345.
39. Woolley DM (1998) Studies on the eel sperm flagellum. The kinematic of normal motility. Cell Motility and the Cytoskeleton 39: 233-245.
40. Suzuki R (1959) Sperm activation and aggregation during fertilization in some fishes: III Non species specificity of stimulating factor. Annotation Zoology Japan 32: 105-111.
41. Musselius VA (1951) How to store carp milt and to determine its quality. Rybnoe Khozyaistov 27: 51-53.
42. Dorier A (1951) Conservation of the vitality and fertile power of the spermatozoids of rainbow trout. Trav Labor Hydrob Piscic Grenoble Annees 75-85.
43. Cosson J (2008a) Methods to analyze the movements of fish spermatozoa and their flagella in fish spermatology. Alavi SMH, Cosson JJ, Coward K and Rafiee G (eds.) Oxford: Alpha Science 63-101.
44. Cosson J (2008b) The motility apparatus of fish spermatozoa. In Fish Spermatology. Alavi SMH, Cosson JJ, Coward K and Rafiee G (eds.) Oxford: Alpha Science 281-316.
45. Abascal FJ, Cosson J, Fauvel C (2007) Characterization of sperm motility in European sea bass. The effect of heavy metals and physicochemical variables on sperm motility. J Fish Biol 70: 509-522.
46. Alavi SMH, Cosson J, Karami M, Abdolhay H, Amiri BM (2004) Chemical composition and osmolality of seminal fluid of Acipenser persicus; their physiological relationship with sperm motility. Reproduction 35: 1238-1243.
47. Dreanno C, Seguin F, Cosson J, Suquet M, Billard R (1999) Metabolism of turbot (Scophthalmus maximus) spermatozoa: relationship between motility, intracellular nucleotide content and mitochondrial respiration. Mol Rep and Dev 53: 230-243.
48. Oda A, Morisawa M (1993) Rises of intracellular Ca2+ and pH mediate the initiation of sperm motility by hyper osmolality in marine teleosts. Cell Motility and the Cytoskeleton 25: 171-178.
49. Christen R, Gatti JL, Billard R (1987) Trout sperm motility: the transient movement of trout sperm is related to changes in the concentration of ATP following the activation of the flagellar movement. European Journal of Biochemistry 166: 667-671.