

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

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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 $51 \pm 1.3 \mu\text{m}$ 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 $75 \pm 12\%$ during spawning time in this species (21:00–22:00), and the initial moving velocity ($196 \pm 26 \mu\text{m}/\text{sec}$) remained constant for fifteen minutes post activation. While, comparatively low motility ($30 \pm 10\%$) 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 mm²) 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 ≤ 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).

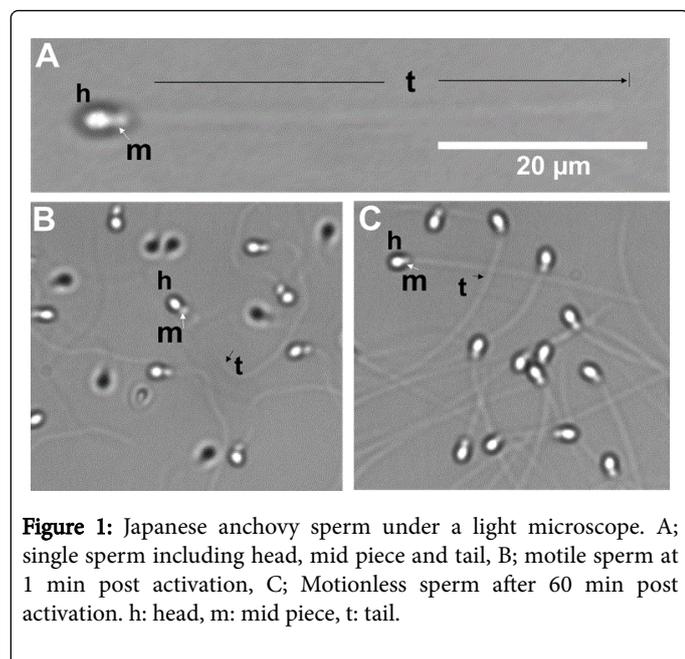


Figure 1: Japanese anchovy sperm under a light microscope. A; single sperm including head, mid piece and tail; B; motile sperm at 1 min post activation; C; Motionless sperm after 60 min post activation. h: head, m: mid piece, t: tail.

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.

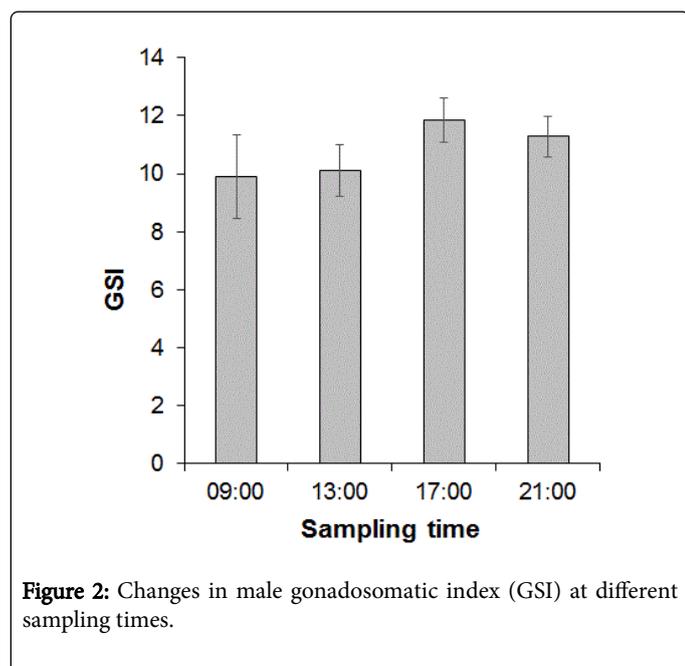


Figure 2: Changes in male gonadosomatic index (GSI) at different sampling times.

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 $4 \pm 5\%$, $12 \pm 9\%$, $28 \pm 7\%$, and $75 \pm 12\%$, 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 $\mu\text{m/s}$, respectively. The moving velocity remained constant for 15 min post activation, and reached zero after 60 min post activation.

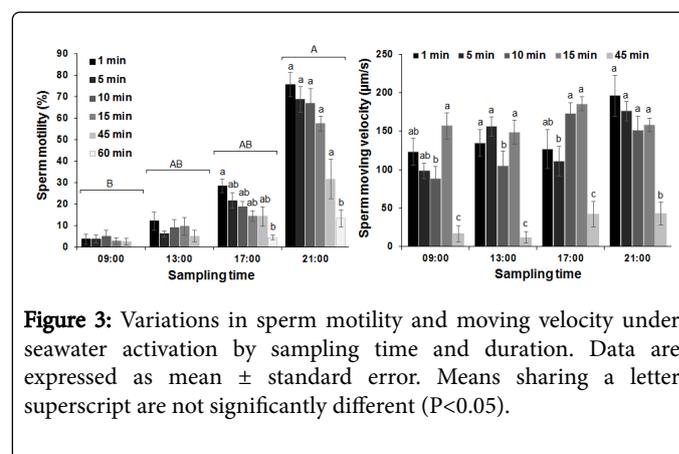


Figure 3: Variations in sperm motility and moving velocity under seawater activation by sampling time and duration. Data are expressed as mean \pm standard error. Means sharing a letter superscript are not significantly different ($P < 0.05$).

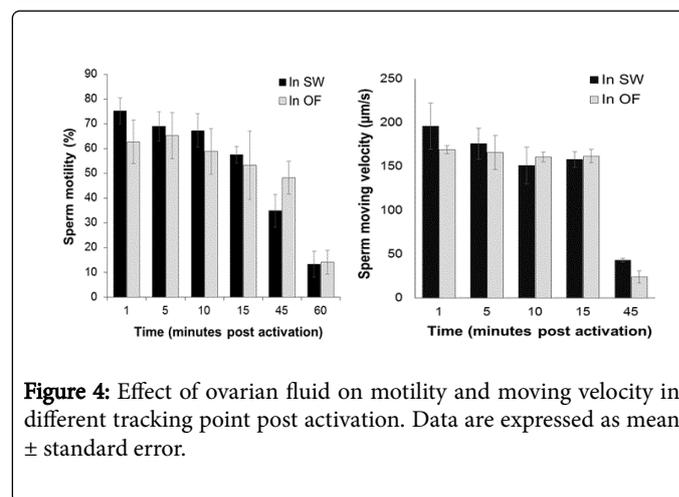


Figure 4: Effect of ovarian fluid on motility and moving velocity in different tracking point post activation. Data are expressed as mean \pm standard error.

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 $75 \pm 12\%$ and $62 \pm 20\%$, respectively. The levels continuously decreased in accordance with time and reached $13 \pm 12\%$ and $14 \pm 11\%$, respectively, at 60 min post activation. On the other hand, the initial moving velocity was 196 ± 26 $\mu\text{m/s}$ in SW and 169 ± 10 $\mu\text{m/s}$ 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 motility (s or min)	of	Initial velocity (µm/s)	References
Fresh-water fish species				
<i>Esox lucius</i>	60-80 s		160-170	[37]
<i>Oreochromis mossambicus</i>	>30 min		70-80	[38]
<i>Anguilla anguilla</i>	<30 min		120-160	[39]
<i>Carassius auratus</i>	3 min at 18-21°C		ND	[40]
<i>Cyprinus carpio</i>	200 s		140	[41]
<i>Salmo irideus</i>	60-105 s		ND	[42]
Sea-water fish species				
<i>Thunnus thynnus</i>	140 s		215-230	[43]
<i>Merluccius merluccius</i>	400-500 s		65-130	[43]
<i>Gadus morhua</i>	700-800 s		130	[43, 44]
<i>Dicentrarchus labrax</i>	50-60 s		120	[45]
<i>Acipenser persicus</i>	1.5-5 min at 15-20°C		ND	[46]
<i>Anarhichas minor</i>	>2 days		40-50	[20]

<i>Scophthalmus maximus</i>	600 s	220	[47]
<i>Takifugu niphobles</i>	50 s	160	[48]
<i>Oncorhynchus mykiss</i>	30 s	220	[49]
<i>Abramis brama orientalis</i>	20 min	ND	[21]
<i>Engraulis Japonicus</i>	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.

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