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Roles of extracellular ions and pH in 5-HT-induced sperm motility in marine bivalve

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Abstract

Factors that inhibit and stimulate the initiation of sperm motility were determined for Manila clam (*Ruditapes philippinarum*), Pacific oyster (*Crassostrea gigas*), and Japanese scallop (*Patinopecten yessoensis*). Compared with artificial seawater (ASW), serotonin (5-hydroxytryptamine creatinine sulfate, 5-HT) could fully trigger sperm motility and increase sperm velocity and motility duration. Sperm motility was decreased in ASW at pH 6.5–7.0 and suppressed at pH 4.0. In Manila clam and Pacific oyster, 5-HT could overcome the inhibitory effects of acidic pH on sperm motility. In the presence of nigericin (a K^+/H^+ exchanger), sperm motility was only triggered at pH 8.3. Testicular fluid K^+ concentrations were two- to fourfold higher than that in ASW. Sperm motility and velocity were decreased in ASW or 5-HT containing $\geq$40 mM K^+ or $\geq$2.5 mM 4-aminopyridine, suggesting K^+ efflux requirement to initiate motility. Sperm motility and velocity were reduced in ASW or 5-HT containing EGTA or W-7, suggesting that extracellular Ca^{2+} is required for Ca^{2+}/calmodulin-dependent flagellar beating. Ca^{2+} influx occurs via Ca^{2+} channels because sperm motility and velocity were decreased in both ASW and 5-HT containing T-type and L-type Ca^{2+} channel blockers. 5-HT-dependent initiation of sperm motility was associated with intracellular Ca^{2+} rise, which was comparable to that seen in ASW but was not observed in the presence of EGTA or a Ca^{2+} channel blocker. Extracellular Na^+ is also essential for sperm motility initiation via regulation of Na^+/Ca^{2+} exchange. Overall, 5-HT-dependent initiation of sperm motility in marine bivalve mollusks is an osmolality-independent mechanism and regulated by extracellular pH, K^+, Ca^{2+}, and Na^+.

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Introduction

In all animals, sperm released from the testis is in a quiescence state in the reproductive tract. Sperm acquisition of the potential for motility occurs in the sperm duct of external fertilizers (invertebrates and fish) and in the epididymis of internal fertilizers (reptiles, birds, and mammals). In external fertilizers, sperm motility is triggered after release from the genital papilla into the aquatic environment, while in internal fertilizers, sperm becomes hyperactivated when ejaculated into the female reproductive tract (Morisawa & Yoshida 2005, Yoshida *et al*. 2008, Darszon *et al*. 2011).

To understand the mechanism by which sperm motility is inhibited in the reproductive tract, it is critical to determine the biochemical constituents of the seminal fluid and compare them with those of physiological activation medium. Until now, inhibitory factors for sperm motility have been identified in mammals, fish, and invertebrates including sea urchins, ascidians, and starfish (Tosti 1994, Verma 2001). Despite the inhibitory effects of acidic pH on sperm motility in marine bivalve mollusks (Faure *et al*. 1994, Dong *et al*. 2002, Ohta *et al*. 2007, Demoy-Schneider *et al*. 2012), there is a significant lack of information to clarify the contribution of different ions. Sperm signaling in the initiation of motility mediated by pH, certain ions, or osmolality has been extensively studied in invertebrates and mammals (Darszon *et al*. 1999, 2011, Inaba 2003, Yoshida *et al*. 2008) and in fish (Alavi & Cosson 2005, 2006, Morisawa 2008). However, the signaling cascades involved in the initiation of sperm motility of mollusks are unknown.

Since the first discovery of the stimulatory effects of serotonin (5-hydroxytryptamine creatinine sulfate (5-HT) in the reproduction of marine bivalve mollusks (Matsutani & Nomura 1982), 5-HT potency to induce spawning, gamete release, and maturation of oocytes have been extensively studied in various species (Osanai 1985, Hirai *et al*. 1988, Osada *et al*. 1998, Tanabe *et al*. 2006, Yuan *et al*. 2012). 5-HT has been localized

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immunohistochemically in the gonad and its concentrations have also been determined at various stages of gonadal development (Masseau *et al*. 2002, Garnerot *et al*. 2006). Recently, Tanabe *et al*. (2010) cloned species-specific 5-HT receptors in both sperm and oocytes of Japanese scallop (*Patinopecten yessoensis* Jay), which had been detected initially by pharmacological identification (Osada *et al*. 1998).

This study investigates the potency of 5-HT to induce initiation of sperm motility in different marine bivalve mollusks: Manila clam (*Ruditapes philippinarum* Adams & Reeve), Pacific oyster (*Crassostrea gigas* Thunberg), and Japanese scallop. Potassium (K^+) was identified as a novel inhibitory factor to regulate sperm motility within the male reproductive tract. Depletion of extracellular K^+($[K^+]_e$) is shown to be associated with requirements for extracellular alkaline pH ($[pH]_e$), extracellular calcium ($[Ca^{2+}]_e$), and extracellular sodium ($[Na^+]_e$) as key determinants for the initiation of sperm motility accompanied by an increase in intracellular Ca^{2+}($[Ca^{2+}]_i$). The potential roles of Ca^{2+}, K^+, protons [H^+], and Na^+/Ca^{2+} channels were investigated using pharmacological channel blockers.

Materials and methods

Animals and sperm collection

Manila clams were purchased from Yanmar Marine Farm, Oita Prefecture, in November 2011 and June 2012. Pacific oysters were collected from Matsushima Bay, Miyagi Prefecture, in July 2012. Japanese scallops were collected from Mutsu Bay, Aomori Prefecture, in February and March 2013. All species were maintained in aquaria (90 l) at 13–15 °C (Manila clams and Pacific oysters) or 11–13 °C (Japanese scallops). They were opened by inserting a knife between the valves at the hinge arch. Care was taken to avoid injury to internal tissues. Before sperm collection, the body of each specimen was wiped dry to avoid any contamination with seawater or with mantle cavity fluid. In Japanese scallop, males were distinguished by gonad color (white testis vs orange ovary). In Manila clam and Pacific oyster, a 1 µl drop from the gonad mixed with 50 µl of artificial seawater (ASW) was observed under a microscope: in males, the sperm is very small with a distinguished head, which is partially motile in ASW. The testicular gland was opened by a small incision and released sperm were collected and kept on ice for the duration of the experiment.

Chemicals

Serotonin, 5-HT, EGTA, 4-aminopyridine (4-AP), flunarizine dihydrochloride, mibefradil dihydrochloride hydrate, Ca^{2+} ionophore A23187, Pluronic F-127, and BSA were purchased from Sigma–Aldrich. 2-(*N*-morpholino)ethanesulfonic acid monohydrate (MES) and HEPES were purchased from Dojindo Laboratories (Kumamoto, Japan). Tris (tris(hydroxymethyl)aminomethane), choline chloride, nigericin sodium salt, nifedipine, verapamil hydrochloride, W-7 hydrochloride, ammonia solution, DMSO, and K^+, Na^+, and Ca^{2+} standard solutions were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Nigericin was dissolved in ethanol. Ca^{2+} ionophore A23187, flunarizine, and nifedipine were dissolved in DMSO. Artificial sea salt was purchased from Instant Ocean Sea Salt (Blacksburg, VA, USA), comprising 468.90 mM Na^+, 10.75 mM K^+, 9.98 mM Ca^{2+}, 54.30 mM Mg^{2+}, 544.1 mM Cl^-, 27.69 mM SO_4^-, and 3.28 mM HCO_3^-, pH 8.2±0.1. All other chemicals were obtained from standard commercial sources.

Determination of ions and pH in the testicular fluid and blood plasma

Ionic constituents were analyzed in hemolymph collected from the adductor muscle and in testicular fluid using an atomic absorption spectrophotometer (A-2000 AAS Hitachi, Ltd.). Testicular fluid was collected from semen following centrifugation at 500 ***g*** for 15 min. To determine pH, 15 µl of supernatant was placed on pH-indicator strips pH 4.0–7.0 Special Indicator (Merck KGaA).

Experimental design and sperm motility assessment

Immediately after sperm collection, initiation of sperm motility was assessed in ASW. 5-HT was dissolved in distilled water at a concentration of 10^{-2} M. ASW containing 10^{-12} to 10^{-3} M 5-HT was prepared to study 5-HT stimulatory effects on the initiation of sperm motility; no buffer was used to adjust pH in these experiments. EGTA, a chelator of Ca^{2+} ions, was used to make Ca^{2+}-free ASW. NaCl and KCl were substituted by choline chloride (460 mM) and NaCl (10 mM) respectively to prepare Na^+-free and K^+-free ASW respectively. Note that '5-HT' medium in this study refers to ASW containing 10^{-5} or 10^{-3} M 5-HT.

To study the effects of pH on sperm motility in Japanese scallop, 20 mM MES (pH 4–6), 20 mM HEPES (pH 6.5–8), or 20 mM Tris (pH 8–9.5) was added to ASW to adjust pH by Docu-pH-meter (Sartorius, Goettingen, Germany). In Pacific oyster and Manila clam, ASW was buffered with 20 mM Tris. To study the effects of ions on sperm motility under testicular conditions, the pH of solutions was adjusted to 7 and 5.8 in Japanese scallop and Pacific oyster using 20 mM MES and 10 mM HEPES respectively.

Sperm motility was analyzed using a semen motility analysis system (SMAS) or by visual observation. For SMAS, sperm movements were observed using a phase-contrast microscope (BX53, Olympus) with a 20× objective and recorded with a 17.28 megapixel cooled digital color 3-CCD camera (DP73, Ditect). The recorded images were analyzed for sperm motility and velocity using an advanced software algorithm (DITECT Corporation, Tokyo, Japan). For visual observation, sperm motility was evaluated under a Nikon E600 microscope (Nikon Corporation, Tokyo, Japan) with a 20× objective to record the percentage of motility under various conditions. To evaluate sperm motility, 0.5 µl of sperm was mixed with 1 ml of a medium prepared for each treatment in a 1.5 ml eppendorf. BSA (0.1% w/v) was added to each medium to prevent sperm adhering to the glass slide; no cover slip was used. For each assessment, 25 µl of solution containing spermatozoa was placed on a glass slide and sperm motility was evaluated visually. All experiments were performed at room temperature.

Intracellular Ca^{2+} oscillations following sperm motility initiation

For Ca^{2+} imaging, 10 µl sperm of Manila clam was diluted in 20 µl ASW and suspended in a loading mixture containing 0.4% Pluronic F-127 and 40 µM Fluo-8H AM (AAT Bioquest, Sunnyvale, CA, USA) at a ratio of 1:1 (v/v). The suspension was incubated for 90 min at room temperature for dye loading and then placed on ice. Sperm suspension in ASW contained EGTA or mibefradil. Dye-loaded sperm were diluted twofold in ASW or 5-HT containing EGTA or mibefradil and loaded into the observation chamber. Ca^{2+}-imaging analysis was performed using a Olympus filter set (excitation filter BP490-500; dichromatic mirror DM505; emission filter BA510-550) and recorded on a computer connected to a digital CCD camera (ImagEM, C9100-13; Hamamatsu Photonics, Hamamatsu, Japan) at 50 frames per second using the imaging application Aquacosmos (Hamamatsu Photonics). The fluorescent signal intensity was analyzed with Bohboh software. The fluorescence intensity of transient increase (F) was normalized against the baseline fluorescence intensity (F_0) and results were shown as $(F–F_0)/F_0$.

Statistical analysis

Data were analyzed using IBM SPSS Statistics 21 and are presented as mean±S.E.M. At first, assumptions of normality and homogeneity of variance were examined using the Kolmogorov–Smirnov and the Levene's tests respectively. ANOVA was used to compare sperm motility between ASW and 5-HT at different times post activation. To determine the effects of pH and ions on sperm motility or velocity, multivariate ANOVA was used to analyze the effects of the main factors (pH/ions and ASW/5-HT) and their interactions. In all cases, significant interaction effects were observed, so the model was revised into individual ANOVA followed by Tukey's test at $P<0.05$ to investigate separately the effects of pH and various ions on sperm motility in ASW or 5-HT.

Results

Ionic composition and pH in testicular fluid and blood plasma

Ionic composition and pH were determined in testicular fluid and blood plasma of Pacific oyster and Japanese scallop (Table 1). Na^+ concentrations were lower in testicular fluid than in blood plasma but predominant compared with K^+ and Ca^{2+} ($P<0.05$). Ca^{2+} and K^+ concentrations did not differ between testicular fluid and blood plasma. There was no difference in Ca^{2+} concentrations between ASW and testicular fluid, but Na^+ concentrations were lower in testicular fluid. K^+ concentrations in testicular fluid were two- to fourfold higher than in ASW. The pH of testicular fluid was lower than that of ASW: 5.5–6.5 in Pacific oyster and 7.0 in Japanese scallop and Manila clam.

Effects of 5-HT on sperm motility and velocity

Sperm motility was measured in ASW and compared with that of ASW containing 5-HT to investigate whether 5-HT stimulates sperm motility initiation in marine bivalve mollusks. In all species, testicular sperm were in the quiescent state before dilution in ASW. Following dilution of sperm in ASW, sperm motility was triggered: 42.9±7.4% in Manila clam, 56.8±5.7% in Pacific oyster, and 78.3±2.4% in Japanese scallop evaluated at 1 min post activation (Fig. 1).

In Manila clam, sperm motility was triggered by addition of $\geq 10^{-6}$ M 5-HT ($P<0.001$, Fig. 1A). Sperm motility in ASW was increased to >60% at 15 min and decreased to <20% at 5 days post activation ($P<0.001$, Fig. 1B). In Pacific oyster, sperm motility was induced by 5-HT at $\geq 10^{-7}$ M ($P<0.001$, Fig. 1C). Sperm motility in ASW decreased to <10% at 30 min post activation ($P<0.05$, Fig. 1C). In Japanese scallop, sperm motility was triggered at $\geq 10^{-3}$ M 5-HT ($P<0.001$, Fig. 1D). In ASW, sperm motility was decreased at 15 min post activation ($P<0.05$, Fig. 1D).

In Manila clam, sperm velocity was higher in 5-HT than in ASW at 3–5 min post activation ($P<0.05$, Fig. 1E). In Japanese scallop, sperm velocity in 5-HT was higher than in ASW at 1 min to 2 h and at 1 and 2 days post activation ($P<0.05$, Fig. 1F).

These data show that 5-HT could fully trigger initiation of sperm motility in marine bivalve mollusks. In further experiments, sperm motility was induced in 10^{-5} M 5-HT for Manila clam and Pacific oyster and in 10^{-3} M 5-HT for Japanese scallop.

Table 1 pH and ionic constituents in testicular fluid (TF) and blood plasma (BP) of Pacific oyster (*Crassostrea gigas*) and Japanese scallop (*Patinopecten yessoensis*) compared with artificial seawater (ASW).

	Pacific oyster		Japanese scallop		
	TF ($n=6$)	BP ($n=5$)	TF ($n=6$)	BP ($n=4$)	**ASW**
Sodium (mM)	359.9±13.8†	417.8±21.1*	350.7±83.2†	522.6±126.4*	468.90
Potassium (mM)	17.5±1.4*	19.5±0.9*	40.8±1.3*	11.4±1.5†	10.75
Calcium (mM)	11.1±0.6*	14.3±1.6*	7.4±1.3*	8.8±0.5*	9.98
pH	5.5–6.5	ND	7.0±0.2	ND	8.1±0.2

ND, not determined. Data are mean±S.E.M. For each ion, values with different superscripts (* and †) are different between TF and BP in each species ($P<0.05$).

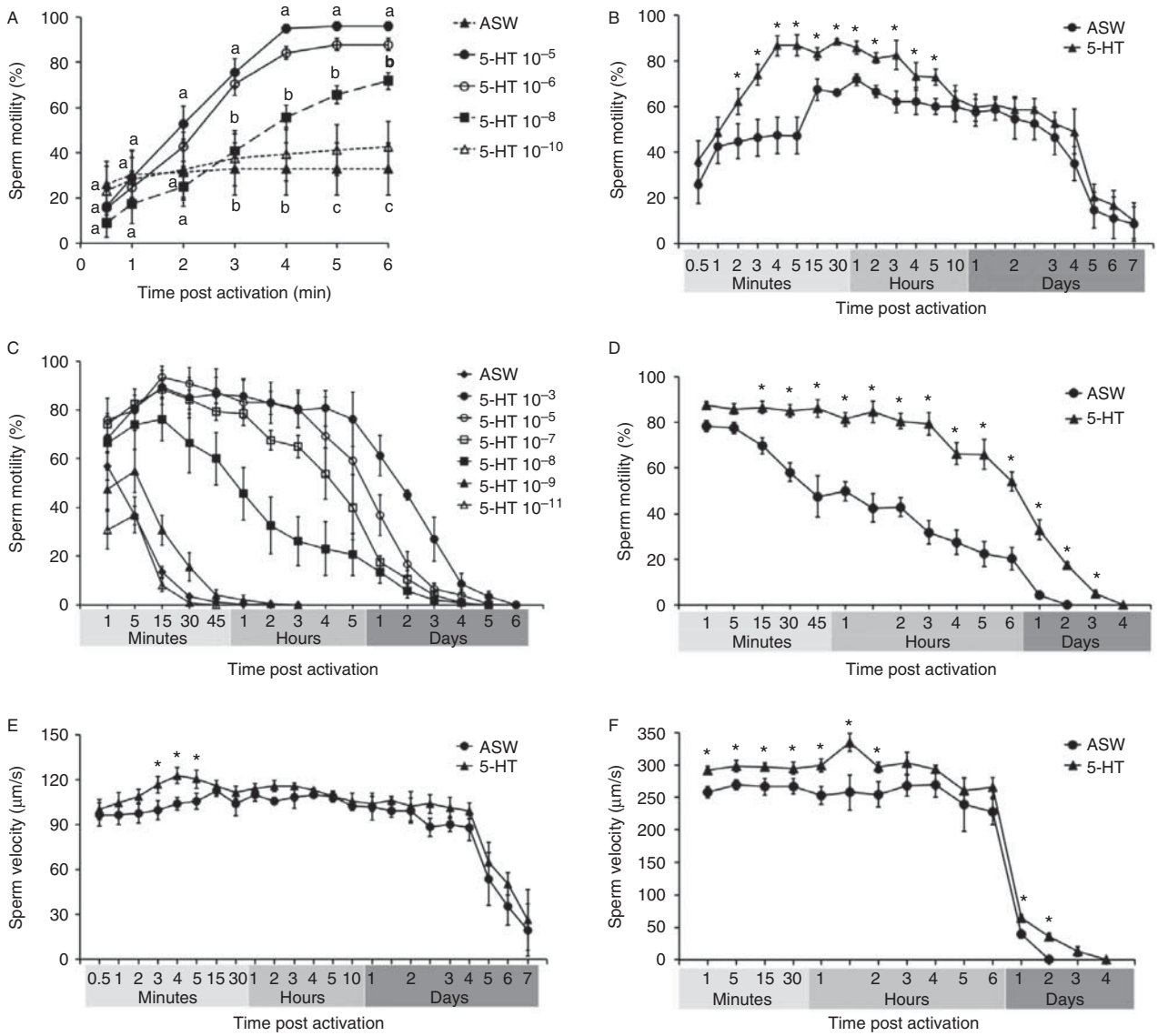

Figure 1 5-Hydroxytryptamine creatinine sulfate (5-HT) induces initiation of sperm motility and increases sperm velocity in marine bivalve mollusks. Sperm motility was activated in artificial seawater (ASW) and in ASW containing various concentrations of 5-HT. In Manila clam (*Ruditapes philippinarum*), sperm motility was triggered in $\geq 10^{-6}$ M 5-HT ($n=10$, A); duration of sperm motility in ASW was similar to that in 10^{-5} M 5-HT and percentage motility was lower ($n=7$, B). In Pacific oyster (*Crassostrea gigas*), sperm motility was triggered in $\geq 10^{-7}$ M 5-HT ($n=7$, C). In Japanese scallop (*Patinopecten yessoensis*), sperm motility decreased in ASW at 15 min and in 10^{-3} M 5-HT at 4 h post activation ($n=20$, D). Sperm velocity increased following activation of Manila clam sperm ($n=7$, E) in 10^{-5} M 5-HT and for Japanese scallop ($n=11$, F) in 10^{-3} M 5-HT. At the same time post activation, values with asterisks or with different letters are significantly different ($P<0.05$).

Effects of $[pH]_e$ on sperm motility and velocity

As testicular fluid pH was lower than that of ASW, the effects of $[pH]_e$ were studied on sperm motility initiation following activation in ASW or ASW containing 5-HT (Fig. 2). In ASW, sperm motility in Manila clam was decreased at pH 8 at 3 min and at pH 7 at $\geq$15 min post activation ($P<0.05$). In Pacific oyster, sperm motility was decreased at pH 7 at 1 min and at pH 8 at $\geq$15 min post activation ($P<0.05$). In Japanese scallop, sperm motility was decreased at pH 6 at 1 min and at pH 5 at $\geq$15 min post activation ($P<0.05$). Japanese scallop sperm motility was suppressed at pH $\leq$4.

In 5-HT, Manila clam sperm motility was decreased at pH 7 ($P<0.05$, Fig. 2). In Pacific oyster, sperm motility was decreased at pH 7 at 1 min but showed no differences among various pHs at 15–120 min post activation. In Japanese scallop, sperm motility was higher at pH 7 than pH 8 ($P<0.05$, Fig. 2).

The effects of $[pH]_e$ on sperm velocity were studied in Japanese scallop (Supplementary Figure 1, see section on supplementary data given at the end of this article).

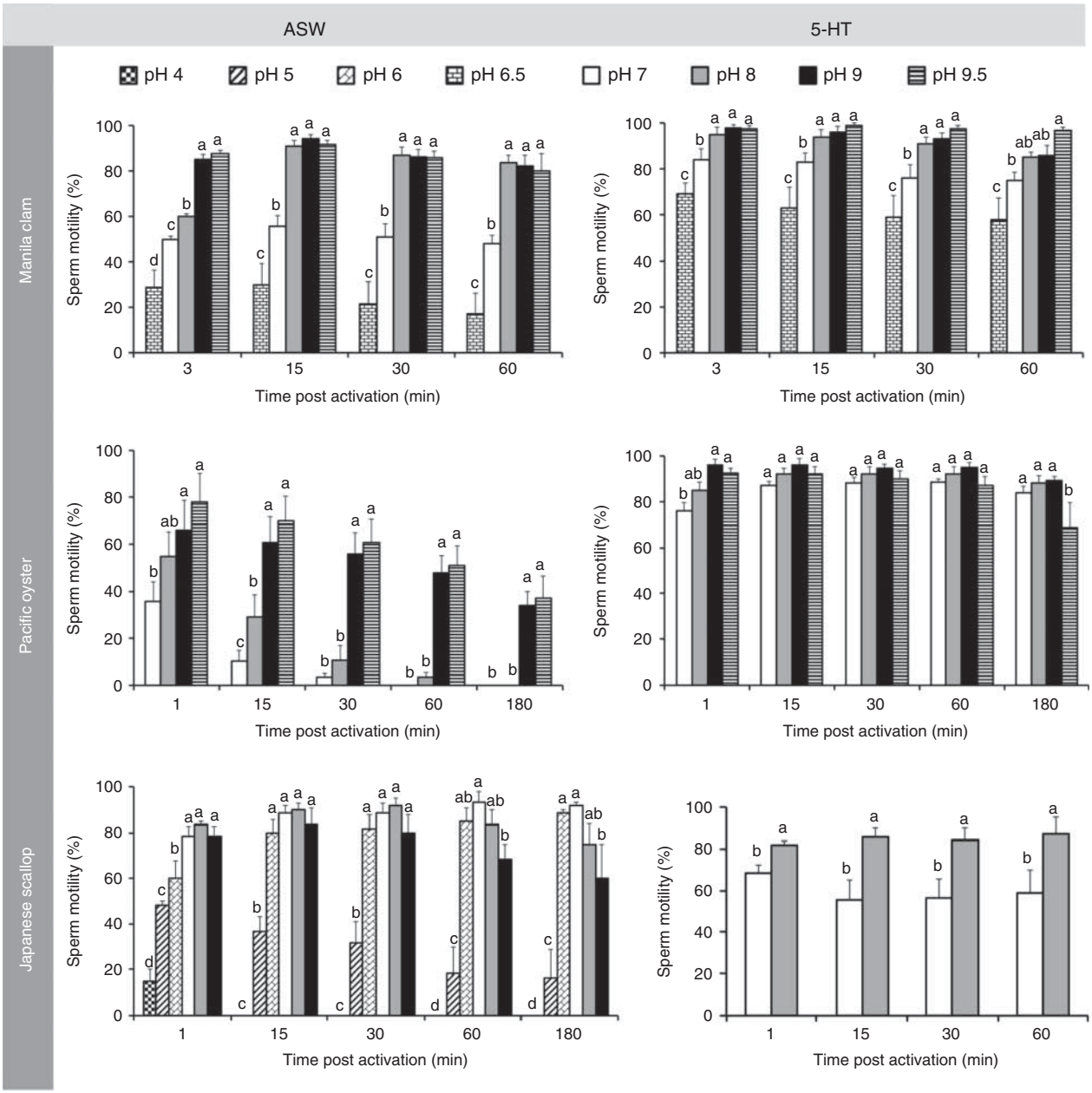

Figure 2 Differences in sperm motility after activation in artificial seawater (ASW) or 5-hydroxytriptamine creatinine sulfate (5-HT) at different pH values in Manila clam (*Ruditapes philippinarum*, 10^{-5} M 5-HT, $n=5$), Pacific oyster (*Crassostrea gigas*, 10^{-5} M 5-HT, $n=5$), and Japanese scallop (*Patinopecten yessoensis*, 10^{-3} M 5-HT, $n=3$). 5-HT induced initiation of sperm motility in Manila clam and Pacific oyster, which was suppressed at pH≤7. At the same time post activation, values with different letters are significantly different ($P<0.05$).

Sperm velocity was lower in ASW or 5-HT at pH 7 (testicular condition) compared to that of pH 8 (ASW condition) ($P<0.05$).

These data suggest that acidic $[pH]_e$ plays an inhibitory role for the initiation of sperm motility in marine bivalve mollusks. As $[pH]_e$ contributes to $[pH]_i$ (Christen *et al.* 1982), a further experiment was performed where intracellular pH ($[pH]_i$) was increased by addition of NH_3 to ASW or ASW containing 5-HT to investigate the effects of $[pH]_i$ on sperm motility initiation. Sperm motility in Manila clam was triggered in ASW containing ≥2 mM NH_3 ($P<0.05$, Fig. 3A). In both ASW and 5-HT, sperm motility was fully triggered in the presence of 2 mM NH_3 at 1 min post activation. Note that while sperm motility was not triggered in ASW ($P<0.001$, Fig. 3B), it was triggered in 5-HT at 4 min post activation ($P<0.01$, Fig. 3C). In Japanese scallop, sperm motility in ASW containing nigericin (20 μM), an exchanger for $[K^+]_i$ and $[H^+]_e$, was triggered at pH 8.3, while >40% motility was recorded in ASW, pH 4.5

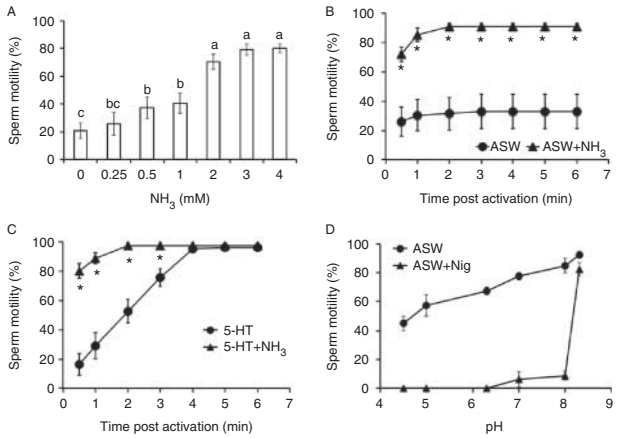

Figure 3 Effects of ammonia and nigericin on sperm motility in marine bivalve mollusks. Manila clam sperm motility was triggered in ASW containing ≥2 mM NH_3 (A, values with different letters are significantly different, $P<0.05$, $n=11$). In the presence of 0.0, 0.25, 0.5, 1, 2, 3, and 4 mM NH_3, pH was measured at 8.2, 8.4, 8.5, 8.8, 9.0, 9.0, and 9.3 respectively. (B) In Manila clam, sperm motility was higher in ASW containing 2 mM NH_3 than in ASW alone (*$P<0.01$, $n=9$). (C) Initiation of Manila clam sperm motility was accelerated by addition of 2 mM NH_3 to ASW containing 10^{-5} M 5-hydroxytriptamine creatinine sulfate (5-HT) (*$P<0.01$, $n=5$). (D) In Japanese scallop (*Patinopecten yessoensis*), sperm motility was >40% at pH 4.5, increased by increasing pH of ASW, while it was only triggered at pH 8.3 when 20 μM nigericin was added to ASW ($n=3$). To test nigericin, ASW contains 0.4% ethanol.

($P<0.01$, Fig. 3D). These results show a requirement for $[pH]_i$ to be alkaline to trigger the initiation of sperm motility in marine bivalve mollusks.

Effects of $[K^+]_e$ on sperm motility and velocity

As higher concentrations of K^+ were observed in testicular fluid compared with ASW, the following experiments were conducted at pH 7 (testicular pH) and pH 8.2 (ASW pH) to investigate the effects of $[K^+]_e$ on sperm motility initiation (Fig. 4). In both ASW and 5-HT, pH 7, Japanese scallop sperm motility was decreased at 0 mM and at ≥40 mM K^+ ($P<0.05$). In 5-HT, sperm motility did not differ between 5-HT and 10 mM K^+. In ASW pH 7, sperm motility was higher at 10 mM K^+ than at 0 or ≥40 mM K^+ at 5–60 min post activation ($P<0.05$). In ASW pH 7, sperm velocity was decreased at 0–150 mM K^+ at 1–60 min post activation ($P<0.05$). In 5-HT pH 7, sperm velocity was decreased in the presence of 0 mM or ≥40 mM K^+ at 1–60 min post activation ($P<0.05$). Compared with 10 mM K^+, sperm velocity was decreased at ≥40 mM K^+ at 5–60 min post activation ($P<0.05$).

At pH 8.2, sperm motility in Japanese scallop was initiated in ASW, pH 8.2, at 0 mM K^+ and decreased at 60 min post activation ($P<0.05$, Fig. 4). Japanese scallop sperm motility was decreased in 5-HT at 0 mM K^+ ($P<0.05$, Fig. 4). Japanese scallop sperm motility and velocity were decreased in ASW or 5-HT, pH 8.2, at ≥50 mM and at >20 mM K^+ respectively ($P<0.05$, data are not shown for velocity). In Pacific oyster, sperm motility was initiated at 0 mM K^+ ASW, pH 8.2, but it was decreased at 0 mM K^+5-HT, pH 8.2, at 120 min post activation ($P<0.05$, Supplementary Figure 2, see section on supplementary data given at the end of this article).

These data show that $[K^+]_e$ has an inhibitory effect on sperm motility and suggests the involvement of K^+ channels in initiation of sperm motility. In the next experiment, Japanese scallop sperm motility was suppressed in both ASW and 5-HT, pH 8.2, containing 2.5, 5, and 10 mM 4-AP at 120, 120, and 60 min post activation respectively ($P<0.05$, Fig. 5). Sperm velocity was decreased in ASW and 5-HT, pH 8.2, containing 5 and 1 mM 4-AP respectively ($P<0.05$, Fig. 5). As 4-AP is a voltage-dependent K^+ channel blocker, these data suggest a requirement for K^+ efflux to trigger the initiation of sperm motility.

Effects of $[Ca^{2+}]_e$ on sperm motility and velocity

The effects of $[Ca^{2+}]_e$ on sperm motility and velocity were studied using EGTA (Fig. 6). In Pacific oyster, sperm motility was decreased in ASW or 5-HT containing 1 mM EGTA and suppressed at ≥5 mM EGTA. In Japanese scallop, sperm motility was decreased in ASW or 5-HT containing 2.5–5 mM EGTA and suppressed at 10 mM EGTA. In Manila clam, initiation of sperm motility was suppressed in ASW or 5-HT containing 5 and 10 mM EGTA.

In the presence of 1 mM EGTA, Japanese scallop sperm velocity was lower than that in ASW at 1 min and in 5-HT at 1–5 min post activation ($P<0.05$, Fig. 6). In ASW and 5-HT, sperm velocity was decreased at ≥2.5 mM EGTA at all time points post activation ($P<0.05$, Fig. 6).

These results show a requirement for external Ca^{2+} to trigger initiation of sperm motility and suggest the involvement of Ca^{2+} channels in the initiation of sperm motility. In the next experiment, we observed decrease in Japanese scallop sperm motility following activation in ASW containing mibefradil (50 μM), verapamil (200 μM), nifedipine (200 μM) at 1, 120, and 15 min post activation respectively ($P<0.01$, Fig. 7 and Supplementary Figure 3, see section on supplementary data given at the end of this article). In 5-HT, decrease in sperm motility was observed in mibefradil, verapamil, and nifedipine at 1, 1, and 5 min post activation respectively ($P<0.01$). Japanese scallop sperm velocity was decreased in ASW and 5-HT containing mibefradil, verapamil, and nifedipine at 1 min post activation ($P<0.01$, Fig. 7 and Supplementary Figure 4, see section on supplementary data given at the end of this article). These data show that both

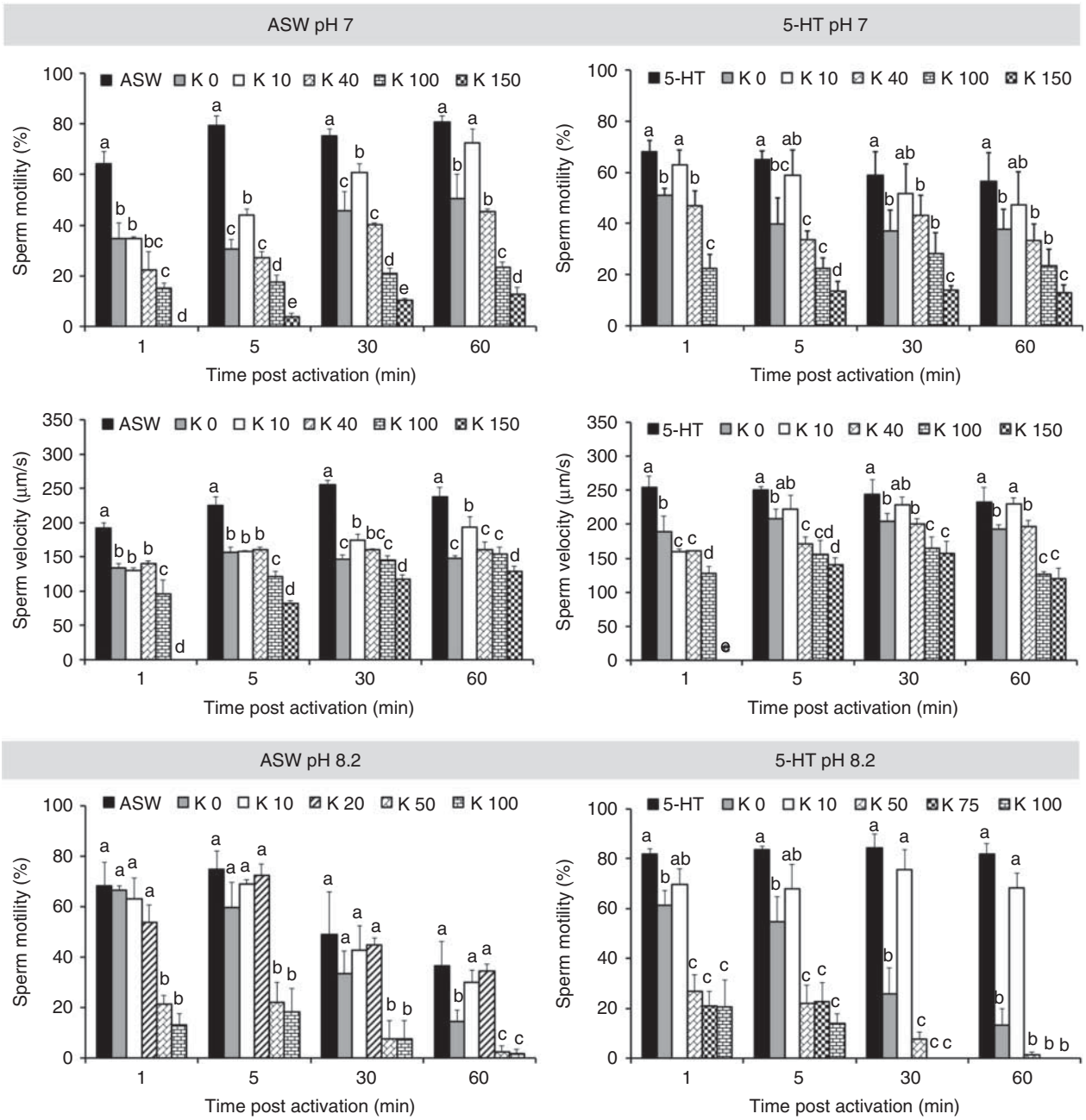

Figure 4 Sperm motility and velocity in Japanese scallop (*Patinopecten yessoensis*) were suppressed and decreased by extracellular potassium (K^+) in artificial seawater (ASW) or 5-hydroxytriptamine creatinine sulfate (5-HT) at pH 7 or 8.1±0.1. Different concentrations of K^+ were added to K^+-free ASW with or without 5-HT (10^{-3} M). At the same time post activation, values with different letters are significantly different ($P<0.05$).

L-type and T-type Ca^{2+} channels are involved in the initiation of sperm motility.

Further examinations of Japanese scallop and Pacific oyster showed that sperm motility was completely suppressed in Ca^{2+}-free ASW or 5-HT at pH 7. Considering the presence of Ca^{2+} in the testicular fluid, Ca^{2+} could not be an inhibitory factor to maintain sperm in the quiescent state in the testis.

Effects of $[Na^+]_e$ on sperm motility and velocity

As Na^+ was identified as a predominant ion in testicular fluid, an experiment was performed to investigate whether a Na^+-rich activation medium could trigger initiation of sperm motility. In all species, sperm motility was not triggered in Na^+-rich activation medium (500 mM NaCl, 20 mM Tris, pH 8.0±0.1) containing 5-HT or Ca^{2+} 10 mM. In Na^+-rich activation medium

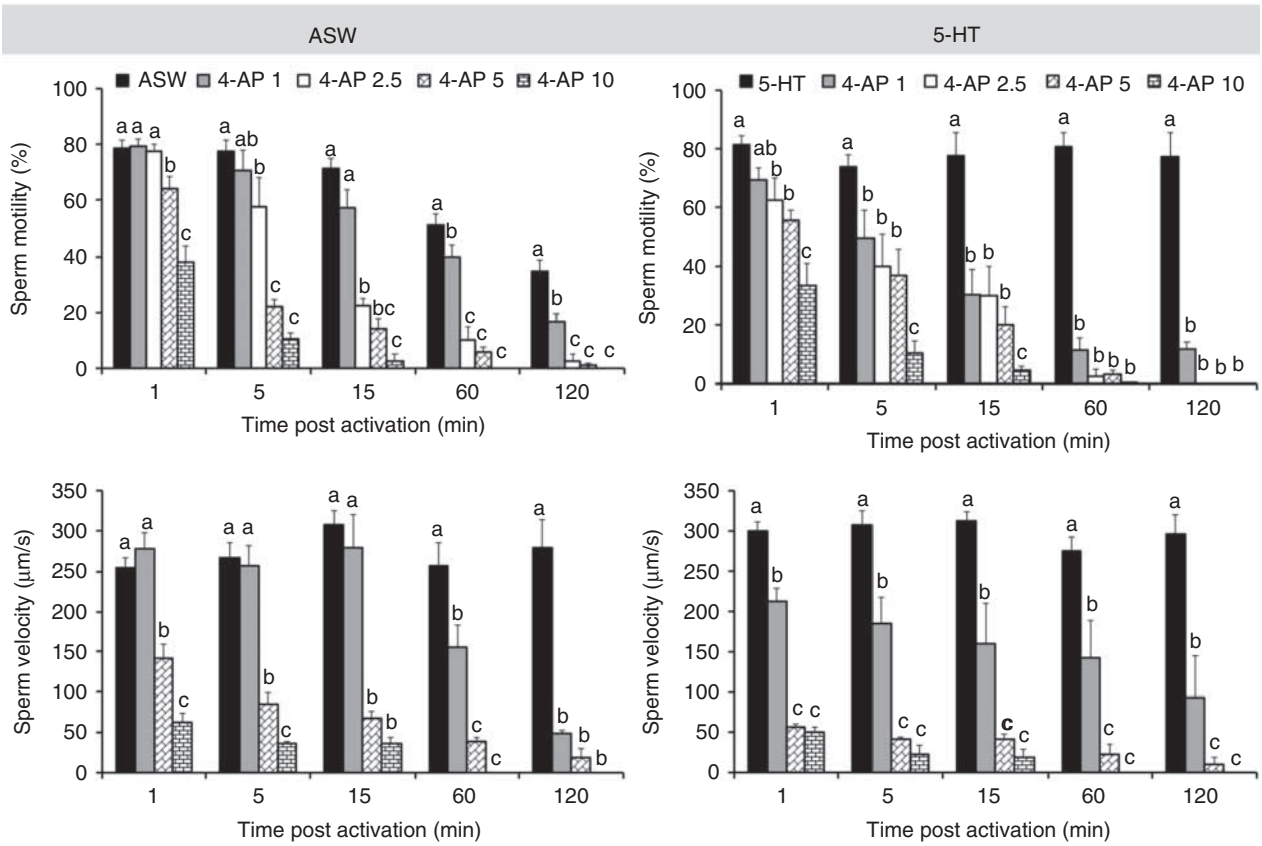

Figure 5 Japanese scallop (*Patinopecten yessoensis*) sperm motility ($n=7$) and velocity ($n=4$) were decreased in ASW or 5-hydroxytriptamine creatinine sulfate (10^{-3} M 5-HT) containing 1–10 mM 4-aminopyridine (4-AP, a K^+ channel blocker) in a dose-dependent manner. At the same time post activation, values with different letters are significantly different ($P<0.05$).

containing 10 mM Ca^{2+} and 20 μM Ca^{2+} ionophore A23187, sperm motility was measured <10% at 1 min and decreased to 0% at 5 min post activation. These experiments show that a Na^+-rich activation medium could not trigger sperm motility even in the presence of Ca^{2+}.

In Manila clam, sperm motility was triggered in ASW (similar to results shown in Fig. 1), while it was not triggered in Na^+-free ASW (0% motility). Sperm motility in Japanese scallop and Pacific oyster was decreased in Na^+-free ASW compared with ASW (Fig. 8). In Japanese scallop, initiation of sperm motility was not triggered in Na^+-free 5-HT. Interestingly, Pacific oyster sperm motility was triggered in Na^+-free 5-HT; however, the percentage of motile sperm was lower than in 5-HT. In Japanese scallop, the addition of 20 μM nigericin or Ca^{2+} ionophore A23187 to Na^+-free 5-HT or Na^+-free ASW did not trigger initiation of sperm motility. These data show that that $[Na^+]_e$ is essential for triggering sperm motility in marine bivalve mollusks.

As 5-HT could induce initiation of sperm motility in Pacific oyster following activation in Na^+-free 5-HT, the effects of differences in $[Ca^{2+}]_e$ were investigated. Sperm motility was decreased in Na^+-free ASW at 1 mM EGTA (Fig. 8). In 5-HT, sperm motility was triggered in Na^+-free 5-HT containing 1–5 mM EGTA but lower than in 5-HT and suppressed in 10 mM EGTA. Flunarizine (50 μM), a Na^+/Ca^{2+} channel blocker, decreased sperm motility (Fig. 7) and velocity (Supplementary Figure 4) in ASW and 5-HT, suggesting involvement of a Na^+/Ca^{2+} exchanger in sperm signaling to trigger initiation of motility.

Further experiments on Japanese scallop and Pacific oyster showed that sperm motility was not triggered in Na^+-free ASW or Na^+-free 5-HT under testicular conditions (pH 7). Considering the presence of Na^+ in the testicular fluid, Na^+ is not an inhibitory factor in maintaining sperm in the quiescent state in the testis.

Intracellular Ca^{2+} oscillations following sperm motility initiation

Changes in $[Ca^{2+}]_i$ were examined in Manila clam sperm following activation in ASW or 5-HT each containing EGTA (10 mM) or mibefradil (50 μM). In 5-HT, $[Ca^{2+}]_i$ was increased and Ca^{2+} oscillations were observed in a time-dependent manner (Fig. 9). Note that 5-HT triggers initiation of sperm motility in Manila clam

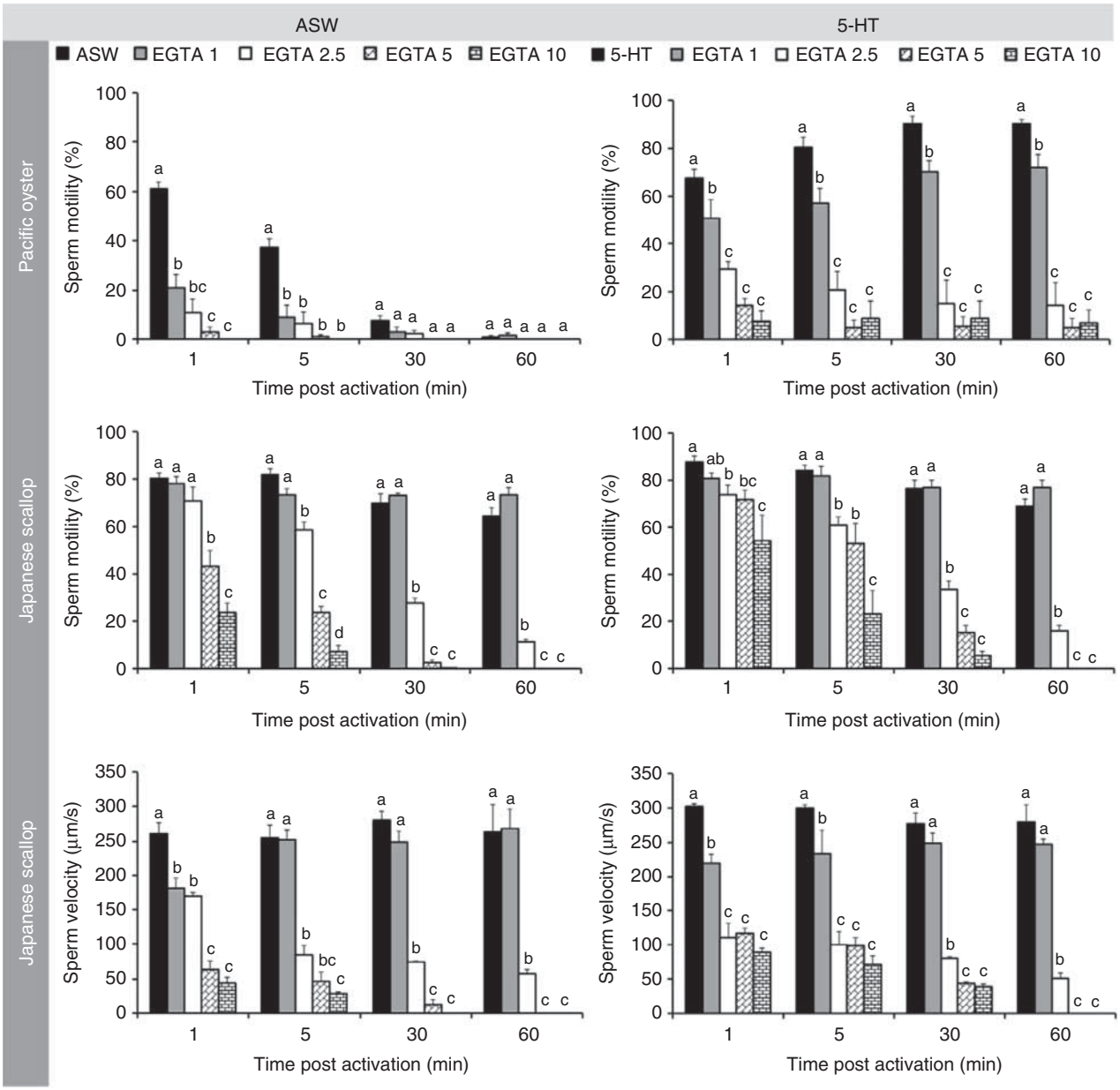

Figure 6 Effects of extracellular calcium (Ca^{2+}) on sperm motility and velocity in Pacific oyster (*Crassostrea gigas*, $n=7$) and Japanese scallop (*Patinopecten yessoensis*, $n=5$). EGTA at 1–10 mM was added to artificial seawater (ASW) or 5-hydroxytriptamine creatinine sulfate (10^{-5} and 10^{-3} M 5-HT in Pacific oyster and Japanese scallop respectively) to eliminate extracellular Ca^{2+}. Free $[Ca^{2+}]_e$ concentrations were 8.3×10^{-3}, 6.8×10^{-3}, 4.3×10^{-3}, and 1.1×10^{-7} M in the presence of 1, 2.5, 5, and 10 mM EGTA respectively. At each time post activation, values with different letters are significantly different ($P<0.05$).

in a time-dependent manner, usually 3–5 min post activation (Fig. 1). In ASW, $[Ca^{2+}]_i$ rise and Ca^{2+} oscillations were comparable to those in the presence of 5-HT. In ASW, $[Ca^{2+}]_i$ changes were shown within 3–4 min post activation, because Manila clam sperm motility is not initiated at this time and usually occurs within 15–30 min post activation (Fig. 1). No $[Ca^{2+}]_i$ rise or Ca^{2+} oscillations were observed in either ASW or 5-HT containing EGTA. In the presence of mibefradil, Ca^{2+} oscillations was observed, but $[Ca^{2+}]_i$ rise was constant in ASW and low in 5-HT throughout the activation period.

Effects of Ca^{2+}/calmodulin antagonist (W-7) on sperm motility

As a requirement for external Ca^{2+} and increases in $[Ca^{2+}]_i$ were observed, an experiment was performed to study the potential involvement of calmodulin (CaM) axonemal beating in the initiation of sperm motility.

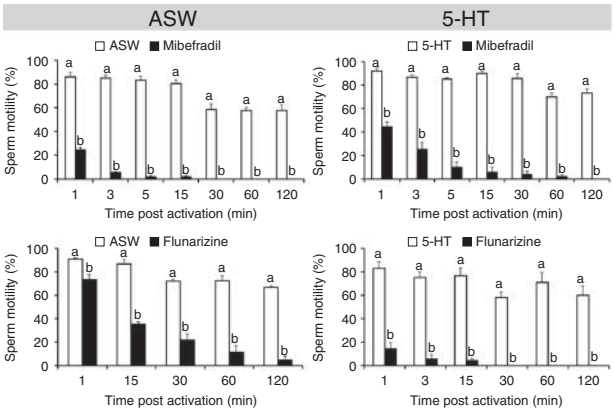

Figure 7 Japanese scallop (*Patinopecten yessoensis*) sperm motility was decreased in artificial seawater (ASW) or 5-hydroxytriptamine creatinine sulfate (10^{-3} M 5-HT) containing mibefradil, a Ca^{2+} channel blocker (50 μM, $n=5$) or containing flunarizine, a Na^{+}/Ca^{2+} exchange channel blocker (50 μM, $n=3$). To test flunarizine, ASW and 5-HT contains 0.2% DMSO. At each time post activation, values with different letters are significantly different ($P<0.01$).

In control, Manila clam and Pacific oyster sperm motility were initiated in ASW or 5-HT (as shown in Fig. 1), while it was completely suppressed after adding 100 or 200 μM W-7 (an inhibitor for CaM activated phosphodiesterase) to the ASW or 5-HT.

Effects of osmolality on sperm motility

To investigate the potency of osmolality to trigger sperm motility, the initiation of sperm motility was examined in non-ionic activation medium. In all species, sperm motility was not triggered in sucrose-rich activation medium (1000 mM sucrose, 20 mM Tris, pH 8.0±0.1) containing 5-HT or Ca^{2+} (10 mM). In Japanese scallop, addition of 20 μM Ca^{2+} ionophore A23187 to sucrose-rich activation medium with or without 5-HT and 10 mM Ca^{2+} did not trigger initiation of sperm motility. This demonstrates that osmolality is not a key signal for the initiation of sperm motility even in the presence of $[Ca^{2+}]_e$.

Discussion

The details of reproductive biology are largely unknown in mollusks, the second largest animal phylum with almost 100 000 species, of which 15 000 are bivalves (Barnes *et al.* 1993). This is the first report that demonstrates K^+ and 5-HT are key inhibitory and stimulatory determinants for the initiation of sperm motility in marine bivalve mollusks. The results presented here demonstrate that the initiation of sperm motility is an osmotic-independent mechanism and requires K^+ efflux and Ca^{2+} influx through voltage-dependent K^+ and Ca^{2+} channels respectively. Alkaline $[pH]_e$ and $[Na^+]_e$ are also essential for sperm signaling. The stimulatory effects of 5-HT were associated with ionic fluxes, which led to an increase in $[Ca^{2+}]_i$ and $[pH]_i$ to induce sperm motility.

Inter-species differences in the initiation of sperm motility were observed following activation in ASW. In contrast to Manila clam, sperm motility in Pacific oyster and Japanese scallop was immediately triggered in ASW, which is consistent with the findings of previous studies (Faure *et al.* 1994, Dong *et al.* 2002). In general, sperm motility was not fully activated, or immediately decreased, after activation in ASW (Fig. 1). Interestingly, both sperm motility and velocity were increased in the presence of 5-HT. The stimulatory concentrations for the initiation of sperm motility were determined to be $\geq 10^{-6}$, $\geq 10^{-7}$, and $\geq 10^{-3}$ 5-HT in Manila clam, Pacific oyster, and Japanese scallop respectively. In addition, a time-dependent stimulatory effect of 5-HT on sperm motility was observed, which is consistent with time-dependent stimulatory effects of 5-HT on oocyte maturation in marine bivalve mollusks (Osada *et al.* 1998). It is therefore considered here that 5-HT triggers sperm motility through a receptor-mediated pathway. In mammals, 5-HT enhances sperm hyperactivation in golden hamster (*Mesocricetus auratus* Waterhouse) and increases human sperm velocity (Fujinoki 2011, Jiménez-Trejo *et al.* 2012). This is further supported by the facts that i) the molecular structure of the motility apparatus (the axoneme) is highly conserved in cilia and flagella (Inaba 2011); ii) most identified 5-HT receptors are similar, belonging to the G-coupled receptor family (Tanabe *et al.* 2010, Pytliak *et al.* 2011); and iii) the molecular structure of 5-HT is also conserved (Walther & Bader 2003).

In ASW, the optimal pH to trigger initiation of sperm motility was shown to be pH >7 for Japanese scallop, pH >8 for Pacific oyster, and pH >9 for Manila clam (Fig. 2). If 5-HT was added to ASW, the optimal pH initiation of sperm motility was determined as pH >8 for all species (Fig. 2). We observed high interindividual variations in the initiation of sperm motility in Manila clam following activation in ASW pH 8. In one experiment (Fig. 2), sperm motility was measured about 60% at 3 min post activation, while it was about 30% at the same time in another experiment (Fig. 3).

Inhibition and stimulation of sperm motility by decreasing and increasing $[pH]_e$ of ASW respectively (Figs 2 and 3) are consistent with the hypothesis that an increase in $[pH]_e$ raises the $[pH]_i$ required for dynein ATPase activity (Christen *et al.* 1982, Boitano & Omoto 1991, Nakajima *et al.* 2005). However, $[pH]_e$ is not a key determinant to maintain sperm in the quiescent state in the testis because i) the $[pH]_e$ at which sperm motility was suppressed is much lower than the pH of testicular fluid (Faure *et al.* 1994, Demoy-Schneider *et al.* 2012, present study) and ii) 5-HT induced sperm motility in Manila clam and Pacific oyster under acidic conditions, which is consistent with stimulation of germinal vesicle breakdown (GVBD) in the oocytes by 5-HT at similar pH

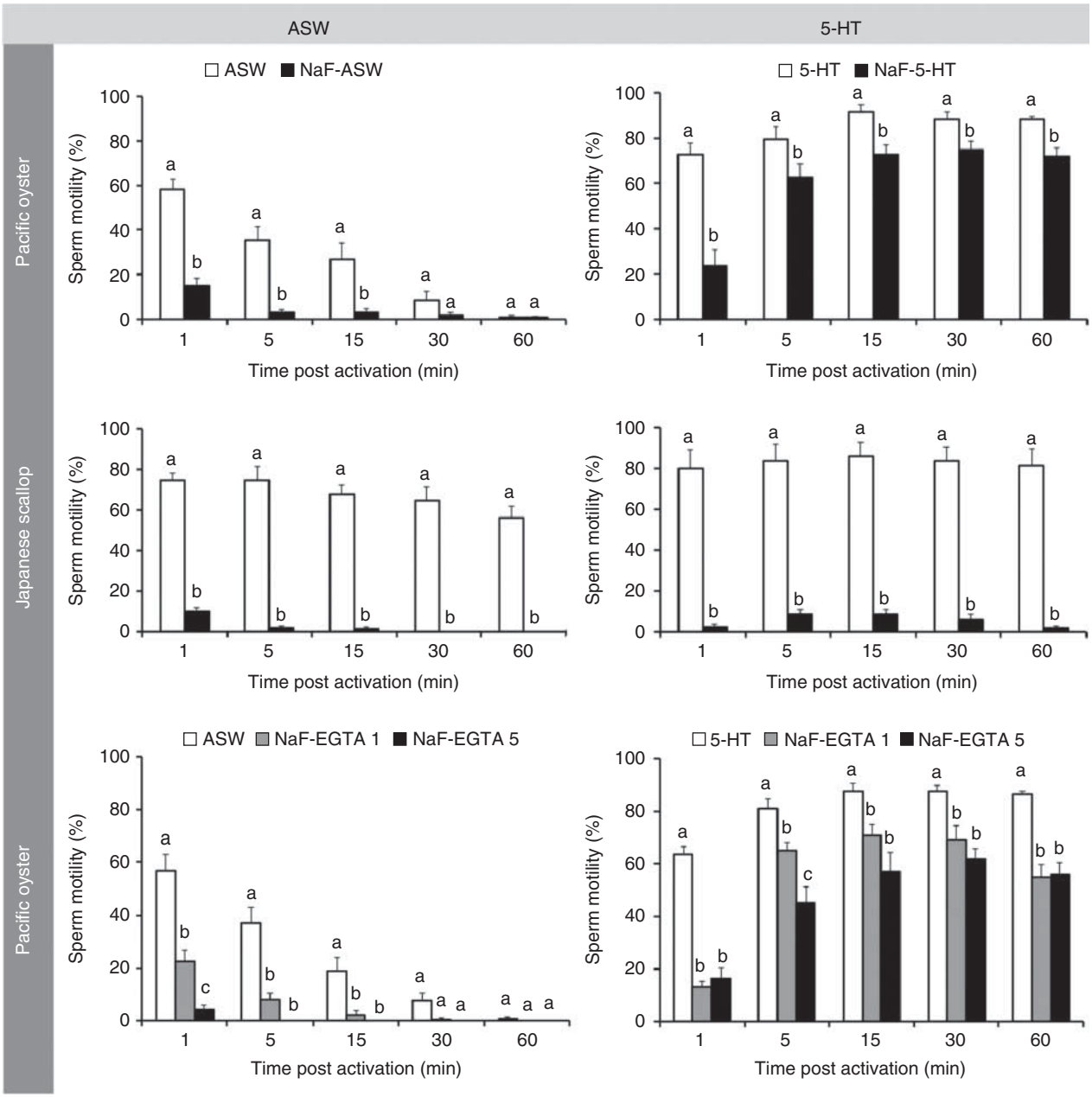

Figure 8 Sperm activation in the Pacific oyster (*Crassostrea gigas*) ($n=8$) and Japanese scallop (*Patinopecten yessoensis*) ($n=4$) was suppressed in Na^+-free (Na-F) artificial seawater (ASW), but it was triggered in Na^+-free 5-hydroxytriptamine creatinine sulfate (10^{-5} and 10^{-3} M 5-HT in Pacific oyster and Japanese scallop respectively). In Pacific oyster, sperm motility was triggered in Na^+-free 5-HT containing 1–5 mM EGTA and completely suppressed at 10 mM EGTA. At each time post activation, values with different letters are significantly different ($P<0.05$).

(Osanai 1985). In 5-HT containing NH_3, sperm motility was triggered immediately (Fig. 3C), suggesting that $[pH]_i$ is not the primary target for 5-HT to induce sperm motility initiation.

To characterize the function of $[pH]_i$, initiation of sperm motility was examined in the presence of nigericin, the $[K^+]_i/[H^+]_e$ exchanger. $[pH]_i$ is equivalent to $[pH]_e$ in the presence of nigericin, if $[K^+]_e$ is equivalent to $[K^+]_i$ (Thomas *et al.* 1979). In the presence of nigericin, $[H^+]$ efflux occurs in sperm following activation in a solution with $[pH]_e$ higher than $[pH]_i$ (Takai & Morisawa 1995). Our results showed that sperm motility was only triggered at pH 8.3 in both ASW and 5-HT containing nigericin (Fig. 3D; data not shown for 5-HT), suggesting that the increase in $[pH]_i$ required for the initiation of sperm motility might be regulated by K^+ efflux.

We identified high K^+ concentrations in testicular fluid compared with ASW. Once sperm motility was activated in ASW or 5-HT, either motility or velocity was

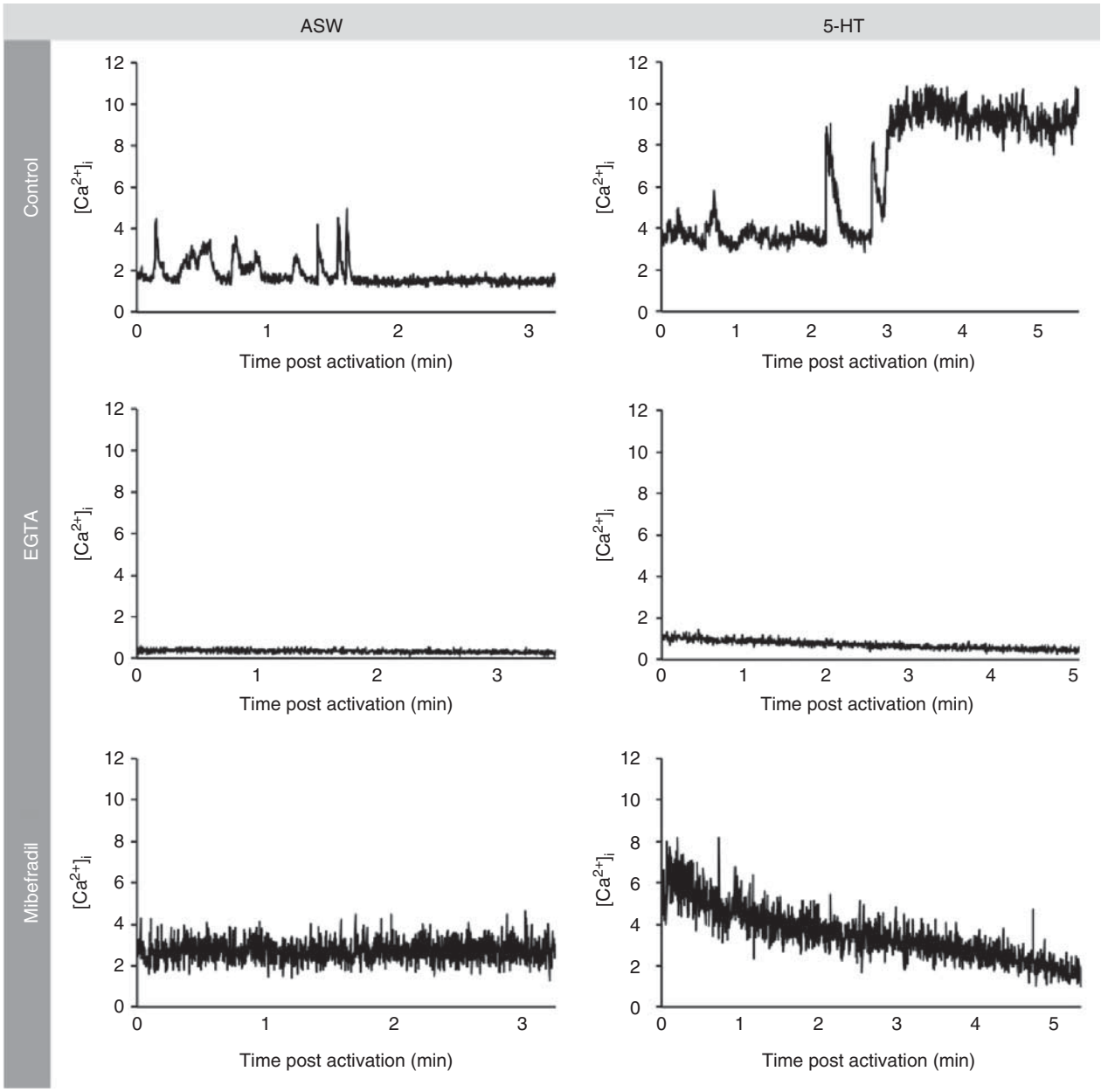

Figure 9 Change in intracellular Ca^{2+} concentration of Manila clam (*Ruditapes philippinarum*) sperm. Sperm was diluted in ASW containing EGTA (10 mM) or mibefradil (50 μM) at ratio 1:2 (v/v) and then suspended in loading mix containing Fluo-8H AM at ratio 1:1 (v/v). Dye-loaded sperm was diluted with ASW or 5-HT containing same concentrations of EGTA or mibefradil and loaded into the observation chamber for Ca^{2+} imaging. Fluorescence intensity of transient increase (F) was normalized against the baseline fluorescence intensity (F_0) and results were shown as $(F–F_0)/F_0$. 5-HT or ASW containing EGTA or mibefradil was applied to chamber within 5–10 s from starting of record for each experiment.

decreased by increasing $[K^+]_e$ to the value similar to that of testicular fluid (Fig. 4). These results provide strong evidence for involvement of K^+ to maintain sperm in the quiescent state in testis and suggest that K^+ efflux through a voltage-dependent K^+ channel is essential for initiation of sperm motility because 4-AP inhibited sperm motility initiation (Fig. 5). Similarly, high K^+ has been identified in seminal fluid of mammals, human, fish, ascidian, and sea urchin and its efflux is required for the initiation of sperm motility (Rothschild 1948, Jones 1978, Johnson *et al*. 1983, Morisawa *et al*. 1983 Izumi *et al*. 1999, Krasznai *et al*. 2000). In addition, sperm velocity was decreased when K^+ efflux was suppressed by 4-AP or $[K^+]_e$ was increased. It is our understanding that K^+ effects on sperm velocity are probably mediated by factors regulating axonemal beating such as $[pH]_i$ and $[Ca^{2+}]_i$. In general, sperm motility was initiated at 0 mM K^+, but lower than ASW or 5-HT containing 10 mM K^+.

It is likely that the presence of 10 mM K^+ is important to create an optimal condition for initiation and maintenance of sperm motility in marine bivalve mollusks similar to that of sea urchin (*Strongylocentrotus purpuratus* Stimpson) (Christen *et al*. 1986) (see above paragraph).

Similar to the $[Ca^{2+}]_e$ requirement for GVBD induction (Osanai & Kuraishi 1988, Kadam *et al*. 1990, Tanabe *et al*. 2006), $[Ca^{2+}]_e$ is also essential for initiation of sperm motility in marine bivalve mollusks because sperm motility was suppressed in the presence of EGTA (Fig. 6). Surprisingly, we observed that sperm motility in Pacific oyster and Japanese scallop was higher in 5-HT compared with ASW, each containing similar concentrations of EGTA. These interesting results suggest that sperm may require lower $[Ca^{2+}]_e$ to trigger initiation of motility in 5-HT compared with ASW. The observed decrease in both sperm motility and velocity following activation in ASW or 5-HT containing mibefradil, verapamil, and nifedipine (Fig. 7, Supplementary Figures 3 and 4) suggests that Ca^{2+} influx to trigger initiation of sperm motility is mediated by both T-type and L-type voltage-dependent Ca^{2+} channels. However, the inhibitory effects of mibefradil on sperm motility initiation were higher than those of nifedipine and verapamil. Ca^{2+} channels also regulate Ca^{2+} influx for the initiation of sperm motility in sea urchin, ascidian, fish, and mammals (Darszon *et al*. 1999, 2011, Inaba 2003, Morisawa & Yoshida 2005).

It has been suggested that progesterone and sperm activation peptides are involved in the regulation of Ca^{2+} influx required for the initiation of sperm motility in sea urchin, ascidian, and mammals (Yoshida *et al*. 1994, Wood *et al*. 2003, Harper *et al*. 2004, Strünker *et al*. 2011). In Manila clam, changes in $[Ca^{2+}]_i$ were investigated following sperm activation in 5-HT or ASW, each containing EGTA or mibefradil, to understand the mechanisms by which Ca^{2+} regulates the initiation of sperm motility (Fig. 9). $[Ca^{2+}]_i$ rise and oscillations were observed in 5-HT comparable with that of ASW, which is consistent with induction of sperm motility in 5-HT compared with ASW (Fig. 1). The increase in $[Ca^{2+}]_i$ was completely suppressed in 5-HT and ASW containing EGTA. In the presence of mibefradil, $[Ca^{2+}]_i$ rise was higher in 5-HT than in ASW and decreased during the activation period. These data strongly suggest that initiation of sperm motility induced by 5-HT could be related to 5-HT-dependent $[Ca^{2+}]_i$ rise and oscillations and confirms the essential roles of $[Ca^{2+}]_e$ stores. Similarly, the Ca^{2+} rise required for GVBD induction has been suppressed in the oocytes of a marine bivalve (*Hiatella flaccida*), Pacific oyster, and Manila clam following incubation in Ca^{2+}-free ASW or 5-HT (Deguchi & Osanai 1995, Leclerc *et al*. 2000, Tanabe *et al*. 2006). $[Ca^{2+}]_i$ plays key role in the regulation of flagellar beating (Darszon *et al*. 2011). Ca^{2+}-dependent or CaM protein phosphatase(s) are found in flagella of sea urchin, ascidian, fish, and mammals including human sperm (Nomura *et al*. 2004, Marín-Briggiler *et al*. 2005, Mizuno *et al*. 2009). Similarly, sperm motility in marine bivalve mollusks was suppressed in the presence of CaM protein phosphodiesterase antagonist. It seems that $[Ca^{2+}]_i$ is not primary factor because the addition of Ca^{2+} ionophore A23187 to sucrose- or Na^+-rich activation medium each containing 5-HT or 10 mM Ca^{2+} did not trigger sperm motility. In mammals (Schmidt & Kamp 2004) and marine fish (Oda & Morisawa 1993), an artificial increase in $[Ca^{2+}]_i$ induces sperm hyperactivation or motility.

Sperm motility was decreased following activation in Na^+-free ASW in all species (Fig. 8). This suggests a prerequisite of $[Na^+]_e$ for initiation of sperm motility in marine bivalve mollusks, similar to sea urchin sperm in which influx of Na^+ is essential for triggering sperm motility (Darszon *et al*. 1999, Inaba 2003). Surprisingly, 5-HT could induce Pacific oyster sperm motility in a Na^+-free medium. For better understanding of the mechanism, we studied initiation of sperm activation in Na^+-free ASW or 5-HT containing EGTA (Fig. 8) and examined effects of flunarizine, a potent inhibitor for Na^+/Ca^{2+} exchange (Fig. 7), because it has been shown that $[Na^+]_e$ contributes to regulate $[Ca^{2+}]_i$ mediated by Na^+/Ca^{2+} exchange (Vines *et al*. 2002, Shiba *et al*. 2006). In the presence of EGTA, sperm motility was suppressed in Na^+-free ASW. In the presence of 1–5 mM EGTA, sperm motility was triggered in Na^+-free 5-HT and suppressed at 10 mM EGTA. It is likely that 5-HT-dependent sperm motility in Pacific oyster requires lower $[Ca^{2+}]_e$ compared with ASW when $[Na^+]_e$ is

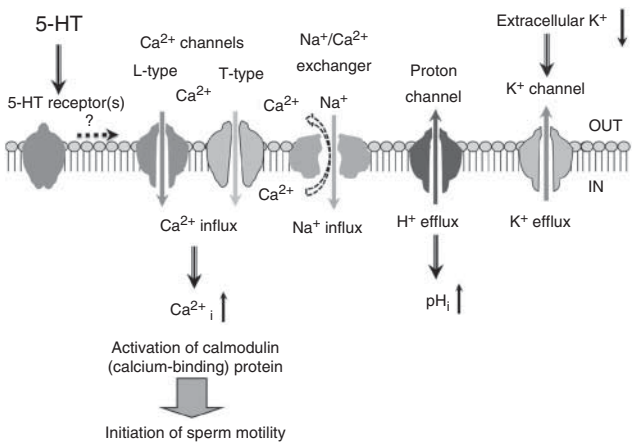

Figure 10 Ionic fluxes required for the initiation of sperm motility in marine bivalve mollusks. The stimulatory effect of serotonin (5-HT) on the initiation of sperm motility was associated with potassium (K^+) efflux, proton (H^+) efflux, and calcium (Ca^{2+}) influx through a voltage-dependent K^+ channel, a proton channel and both L-type and T-type voltage-dependent Ca^{2+} respectively. A sodium (Na^+)/Ca^{2+} exchanger regulates Na^+ influx to control intracellular Ca^{2+} during motility period. These steps stimulate Ca^{2+}-dependent or calcium–calmodulin (CaM) protein phosphatase(s) in the flagellum for the initiation of sperm motility.

eliminated. Moreover, Japanese scallop sperm motility was not initiated in both ASW and 5-HT in the presence of flunarizine. These data suggest that $[Na^+]_e$ is involved in the initiation of sperm motility mediated by regulation of $[Ca^{2+}]_i$ through Na^+/Ca^{2+} exchanger.

In conclusion, this study shows that K^+ suppresses and 5-HT stimulates the initiation of sperm motility in marine bivalve mollusks. The 5-HT-dependent and osmolality-independent initiation of sperm motility in marine bivalve mollusks was associated with a K^+ efflux and Ca^{2+} influx via voltage-dependent ion channels under alkaline conditions. A subsequent increase in $[Ca^{2+}]_i$ triggers CaM-dependent flagellar beating. In this context, $[Na^+]_e$ is also important for the initiation of sperm motility, probably via regulation of Ca^{2+} exchange (Fig. 10). Future studies should investigate whether 5-HT is involved in regulation of the K^+ efflux and Ca^{2+} influx required for the initiation of sperm motility.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-13-0418.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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