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RESEARCH ARTICLE

Autonomous changes in the swimming direction of sperm in the gastropod *Strombus luhuanus*

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ABSTRACT

The sperm of the gastropod *Strombus luhuanus* show dimorphism. The eusperm have a nucleus and fertilize the egg, whereas the other type of sperm, parasperm, are anucleate and are thought to assist fertilization. Here we report the autonomous changes in the swimming pattern of *S. luhuanus* eusperm. In artificial seawater, the eusperm collected from *S. luhuanus* sperm ducts formed sperm bundles and initially swam backward with asymmetric flagellar waveforms to detach from the bundles. One hour later, the sperm began to swim forward and in a circle. After an additional 1 h incubation, the sperm swam straight, with a change in the flagellar waveforms from asymmetric to symmetric. Spontaneous backward swimming with symmetric waveforms was also observed. The eusperm stored in the female seminal receptacle were motile and showed forward symmetric swimming with spontaneous backward swimming, which appeared necessary for detachment from the wall of receptacle. All of these motility changes were observed in the absence of parasperm, suggesting that these changes autonomously occur in eusperm. Our waveform analysis of these swimming patterns revealed that only the swimming with symmetric waveform showed reverse propagation of the flagellar waveforms. Both types of backward swimming were diminished in Ca^{2+}-free seawater and in seawater containing Ni^{2+}, indicating the regulation of swimming direction by Ca^{2+}-dependent signal transduction.

KEY WORDS: Flagellar motility, Sperm dimorphism, Snail reproduction, Internal fertilization, Sperm storage, Ca^{2+} signalling

INTRODUCTION

Changes in the swimming patterns of sperm are commonly seen in animals and plants and are considered to be necessary for efficient fertilization (Cosson, 2010; Inaba, 2003; Morisawa, 1994). Generally, spermatozoa are immotile in the testes and sperm ducts but become motile after spawning into an external or female environment. The factors that regulate the initiation and activation of sperm motility include both physical factors such as osmolality and temperature and chemical factors such as specific ions and egg- or genital tract-derived substances. These factors trigger ion influx/efflux and changes in the membrane potential and intracellular Ca^{2+} concentration, leading to the activation of protein kinase and eventually to the modulation of axonemal dyneins (Darszon et al., 1999; Inaba, 2011). However, a transient increase of the intracellular Ca^{2+} concentration induced by chemoattractants caused changes in flagellar asymmetry, resulting in chemotaxis toward the eggs (Kaupp et al., 2008; Shiba et al., 2008; Yoshida and Yoshida, 2011; Mizuno et al., 2012).

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Animals with internal fertilization exhibit a variety of changes in sperm motility. For example, the motility of mammalian spermatozoa is changed remarkably after a couple of hours of incubation with a solution containing calcium and bicarbonate. This state of motility is called hyperactivation, which is characterized by high amplitude and asymmetric flagellar waveforms, and is required for penetration of the zona pellucida (Suarez, 2008a; Yanagimachi, 1970). In contrast to motility activation, the suppression of sperm motility has also been shown during sperm storage in special female organs called spermatheca or seminal receptacles (Neubaum and Wolfner, 1998). The variety of sperm swimming patterns reflects the diversity of fertilization and reproductive strategies. However, the molecular mechanisms underlying the changes in sperm swimming patterns are incompletely understood.

It is of particular interest that some sperm from internal fertilizers change their moving direction from forward to backward. The backward swimming of sperm has been described in the snails *Turritella communi* (Ishijima et al., 1999) and *Littorina sitkana* (Buckland-Nicks and Chia, 1981), the fruit flies *Ceratitis capitata*, *Dacus oleae*, *Dacus dorsalis* (Baccetti et al., 1989) and *Drosophila melanogaster* (Köttgen et al., 2011; Yang et al., 2011; Yang and Lu, 2011), the myzostomid worm *Myzostomum cirriferum* (Ishijima et al., 1994) and the planarian *Triclad turbellarians* (Ishida et al., 1991). Although the intracellular Ca^{2+} concentration has been shown to be important (Baccetti et al., 1989; Ishijima et al., 1994), the physiological meaning and the mechanisms of backward swimming and the reverse of flagellar propagation have not yet been unveiled.

Sperm dimorphism is reported in various animals such as fish, insect and snails (Buckland-Nicks, 1998; Hayakawa, 2007). Prosobranch snails exhibit some of the most complex and bizarre forms of parasperm (Buckland-Nicks, 1998; Nishiwaki, 1964). Despite the past focus on the structural features of snail parasperm, the motility of eusperm or parasperm has not been studied extensively except for a few cases (Higginson and Pitnick, 2011; Ishijima et al., 1999).

Here we report on the motility of the sperm of *Strombus luhuanus* Linnaeus 1758, a marine snail commonly found on the sand of shallow sandy coasts. *Strombus luhuanus* performs internal fertilization by copulation (Kuwamura et al., 1983). The dimorphism of *Strombus* spermatozoa was described more than a century ago (Brock, 1887; Reinke, 1912). The parasperm of *Strombus* are unique in having an undulating membrane for motility (Koike and Nishiwaki, 1980; Reinke, 1912). The eusperm show typical morphology with a head and a tail, but the axoneme in the midpiece is helically surrounded by mitochondria (Koike and Nishiwaki, 1980; Casse et al., 1994), like that in mammals.

To identify the molecular signaling for the flagellar movement of *S. luhuanus* sperm in light of the reproductive strategy, we recorded the motility under a microscope with a high-speed camera. During the course of this study, we observed dramatic changes in the swimming pattern of *S. luhuanus* sperm after they were incubated

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in diluted seawater. We further examined the role of parasperm and calcium signals in the changes of eusperm motility.

RESULTS

Changes in swimming patterns in *S. luhuanus* eusperm

The semen from the sperm ducts of male *S. luhuanus* contained both anucleate parasperm and eusperm. The ratio of the number of parasperm to that of eusperm was 1:300; concentrations of sperm in the semen were $1.00\times10^4\pm0.38\times10^4$ cells μl^{-1} for parasperm and $324.18\times10^4 \pm 86.16\times10^4$ cells μl^{-1} for eusperm (N=4; Fig. 1A). The eusperm, approximately 50 µm long, presented the typical head–tail appearance (Fig. 1B). The parasperm (~100 µm long and 10 µm wide) were larger than the eusperm and had characteristic granules (~5 µm in diameter) in their cytosol (Fig. 1C). The parasperm were motile with two undulating membranes. Just after the dilution of semen with artificial seawater (ASW), the eusperm formed sperm bundles consisting of up to ~70 eusperm bound by head-to-head adhesion (Fig. 1D).

The eusperm underwent striking motility changes after their dilution in ASW. After 5 min of incubation, most of the eusperm were released from the sperm bundle and started to swim individually (Fig. 2A; see supplementary material Movie 1). The sperm swam backward with highly asymmetric flagellar waveforms (Fig. 2E). This swimming pattern was composed of alternating flagellar bends of small forward propulsion and large backward propulsion. As a whole, sperm showed propulsive wave movement, so as to move backward. After 30 min, the eusperm changed the direction of movement from backward to forward, but their flagellar waveforms were still asymmetric (Fig. 2B,F). Incubation for up to 2 h induced changes in flagellar bending from asymmetric to symmetric waveforms and the sperm showed straight swimming (Fig. 2C,G; see supplementary material Movie 2). Backward swimming with symmetric waveforms and low amplitude appeared occasionally after 60 min of incubation (Fig. 2D,H). The backward swimming occurred spontaneously and reverted back to forward swimming.

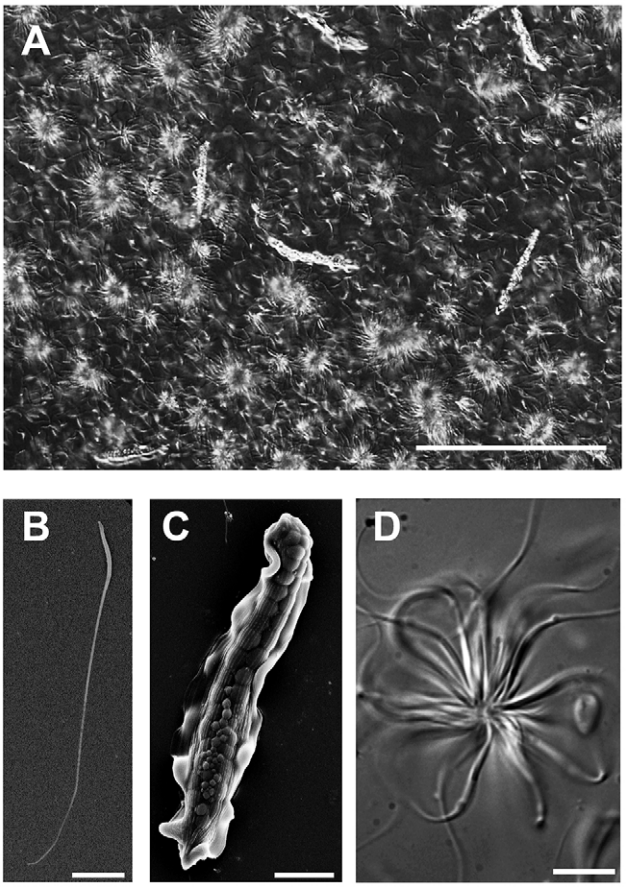

Fig. 1. Sperm dimorphism in *Strombus luhuanus*. (A) Differential interference contrast microscopic image of semen just after dilution with artificial seawater (ASW). (B,C) Scanning electron microscope images of the eusperm (B) and parasperm (C). (D) Magnified image of sperm bundle. Scale bars, 200 µm (A), 10 µm (B–D).

The motility of *S. luhuanus* eusperm that appeared during incubation in ASW was categorized into four patterns according to swimming direction and flagellar asymmetry: backward swimming with an asymmetric waveform (backward + asymmetric; supplementary material Movie 3), forward swimming with an asymmetric waveform (forward + asymmetric; supplementary material Movie 4), forward swimming with a symmetric waveform (forward + symmetric; supplementary material Movie 5), and backward swimming with a symmetric waveform (backward + symmetric; supplementary material Movie 6). To characterize each swimming pattern, we analyzed the flagellar waveforms in detail.

We used Bohboh software to automatically track the flagellar waveforms and calculated the flagellar curvature values and plotted them against the time and distance from the base of the flagellum (Fig. 3A). From fitting to a sine curve, we obtained the flagellar beat frequency, the maximum curvature, the amplitude of the curvature, and the wavelength and velocity of bend propagation (Fig. 3B, Table 1). The swimming patterns backward + asymmetric and forward + asymmetric showed large maximum curvatures, high amplitudes and high beat frequencies. Less than one-half of the backward + asymmetric flagellum came into focus, indicating that the movement is helical (Fig. 2E; see also Fig. 3A).

In contrast, the maximum curvature and the amplitude were low in the forward + symmetric and backward + symmetric patterns. Notably, the sperm exhibiting the backward + symmetric pattern showed very low beat frequency and swimming velocity. The direction of bend propagation in backward + symmetric movement was distinct from other three movement patterns: the bend was propagated from the tip to base of the flagellum (Fig. 3B, Table 1). The amplitude of flagellar waveform was attenuated from the tip to base in the backward + symmetric sperm (Fig. 4), clearly demonstrating the distinct position of bend initiation in this case.

These four swimming patterns with different swimming direction and flagellar symmetry transited from backward + asymmetric, forward + asymmetric, and then to forward + symmetric during incubation in ASW (Fig. 5). Backward + symmetric swimming appeared in 5–10% of the total number of sperm after 60 min, when sperm started to move with symmetric waveforms.

Motility changes of eusperm proceed autonomously, without parasperm

Several hypotheses for the functions of parasperm have been set forth, but most have not been supported by experimental data (see Buckland-Nicks, 1998; Hayakawa, 2007). To determine whether the dramatic changes in the swimming patterns of eusperm depend on the presence of parasperm, we prepared sperm suspensions without parasperm and examined the motility changes after their incubation in ASW (Fig. 6). All four types of swimming pattern were observed during incubation in ASW without parasperm. No significant differences were observed between eusperm with parasperm and those without parasperm in the percentage of each type of motility or the timing for the appearance of each swimming pattern.

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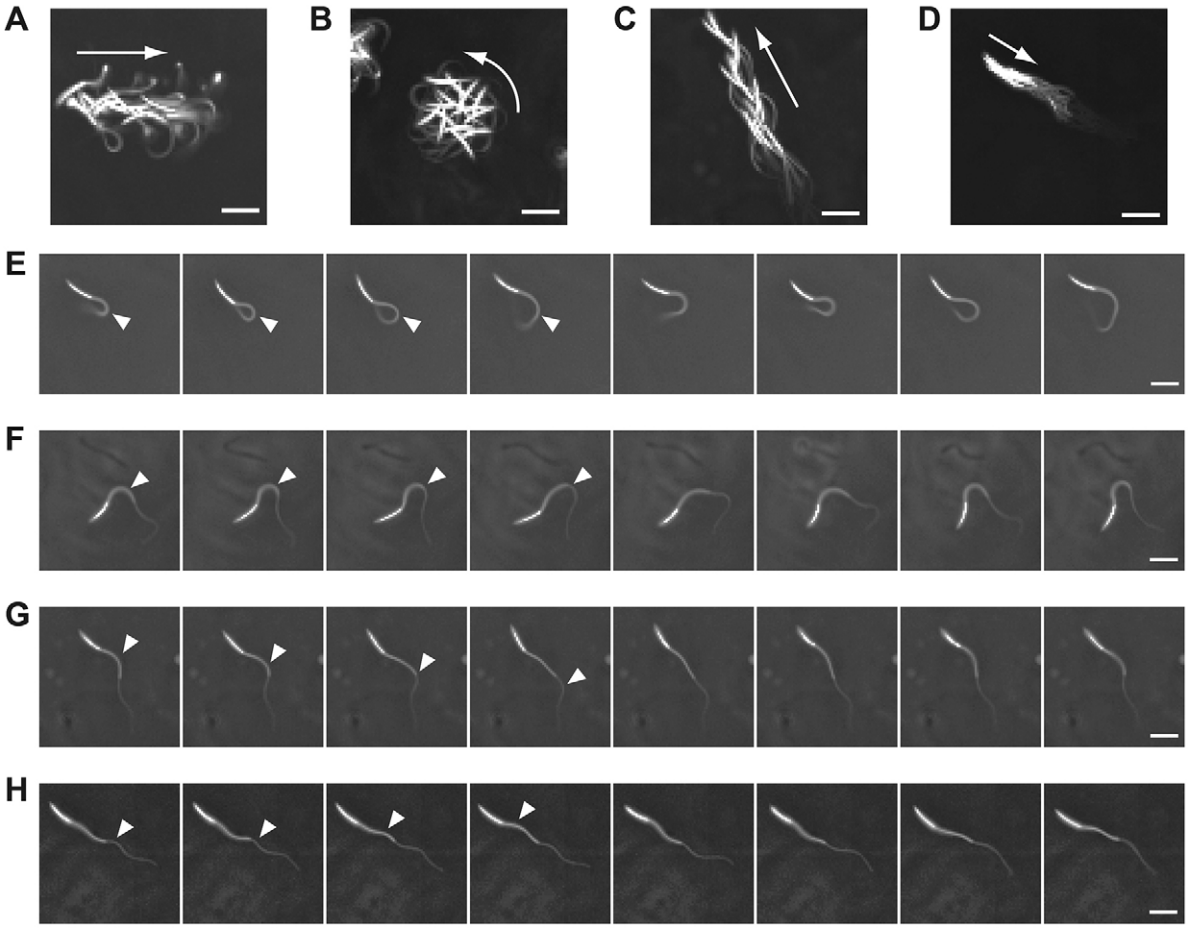

Fig. 2. The four typical swimming patterns observed in *S. luhuanus* eusperm. Phase contrast images of backward swimming with an asymmetric waveform (A,E), forward swimming with a symmetric waveform (B,F), forward swimming with a symmetric waveform (C,G) and backward swimming with a symmetric waveform (D,H). (A–D) Superimposed photographs from 14 images with 15 ms intervals. Arrows indicate the direction of movement. (E–H) Sequential single images at 3.3 ms (E–G) and 33 ms (H) intervals corresponding to the parts of A–D. Arrowheads indicate a flagellar bend. Note the direction of bend propagation. Scale bars, 10 µm.

Swimming pattern of the eusperm in female seminal receptacle

To examine whether the dramatic changes in swimming patterns occur in the normal reproductive process of *S. luhuanus*, we observed the swimming behavior of the eusperm in the female seminal receptacle, where the sperm are stored after copulation. Nearly 90% of these sperm swam forward with symmetric flagellar waveforms (Fig. 7A). Spontaneous backward swimming with symmetric waveforms was also observed. These *in vitro* changes in eusperm movements to forward + symmetric and backward + symmetric in seawater reflect physiological changes in the female. Our histological study revealed that the eusperm were tightly bound to the wall of the seminal receptacle by the head (Fig. 7B–D), as observed in other prosobranchs (Fretter, 1941; Roth, 1960; Giusti and Selmi, 1985). As this implied that straight or backward swimming might have a role in penetrating into the seminal receptacle or in detaching from its wall, respectively, we dissected the seminal receptacle from a mature female, prepared a fragment of its wall and observed the movement of sperm. We found that sperm took off from the wall of the seminal receptacle using backward swimming (Fig. 7E; supplementary material Movie 7). After backward swimming for a while, the sperm changed the bend propagation from base to tip and started forward swimming. It should be noted that some sperm showed a highly asymmetric waveform after backward swimming and changed the moving direction.

Involvement of Ca^{2+} in regulating swimming pattern

To test the requirement of changes in sperm movement for Ca^{2+}, we examined the effects of Ca^{2+}-depleted seawater, an inhibitor of the Ca^{2+} channel and a Ca^{2+} ionophore (Figs 8, 9). In Ca^{2+}-free seawater (CFSW), the percentage of backward + asymmetric movement was remarkably decreased and backward + symmetric movement was rarely observed, suggesting that Ca^{2+} is an important factor in the regulation of the direction of movement (Fig. 8A). Interestingly, Ni^{2+}, a potent Ca^{2+} channel blocker, inhibited not only the backward movement but also the detachment of sperm bundles (Fig. 8A).

In the ASW, the sperm bundles were usually dissociated within 3 min with asymmetric backward swimming. However, the eusperm could not swim backward and remained undetached from bundles for more than 30 min in the presence of Ni^{2+}. In the CFSW, however, the sperm bundles were soon dissociated as in ASW, although they showed no backward swimming (Fig. 8B). Ni^{2+} showed no inhibitory effect in CFSW (data not shown). These results suggest that sperm bundles are maintained by Ca^{2+}-dependent adhesion and that the backward swimming induced by a Ca^{2+} influx via the Ca^{2+} channel promotes the dissociation of the sperm bundles.

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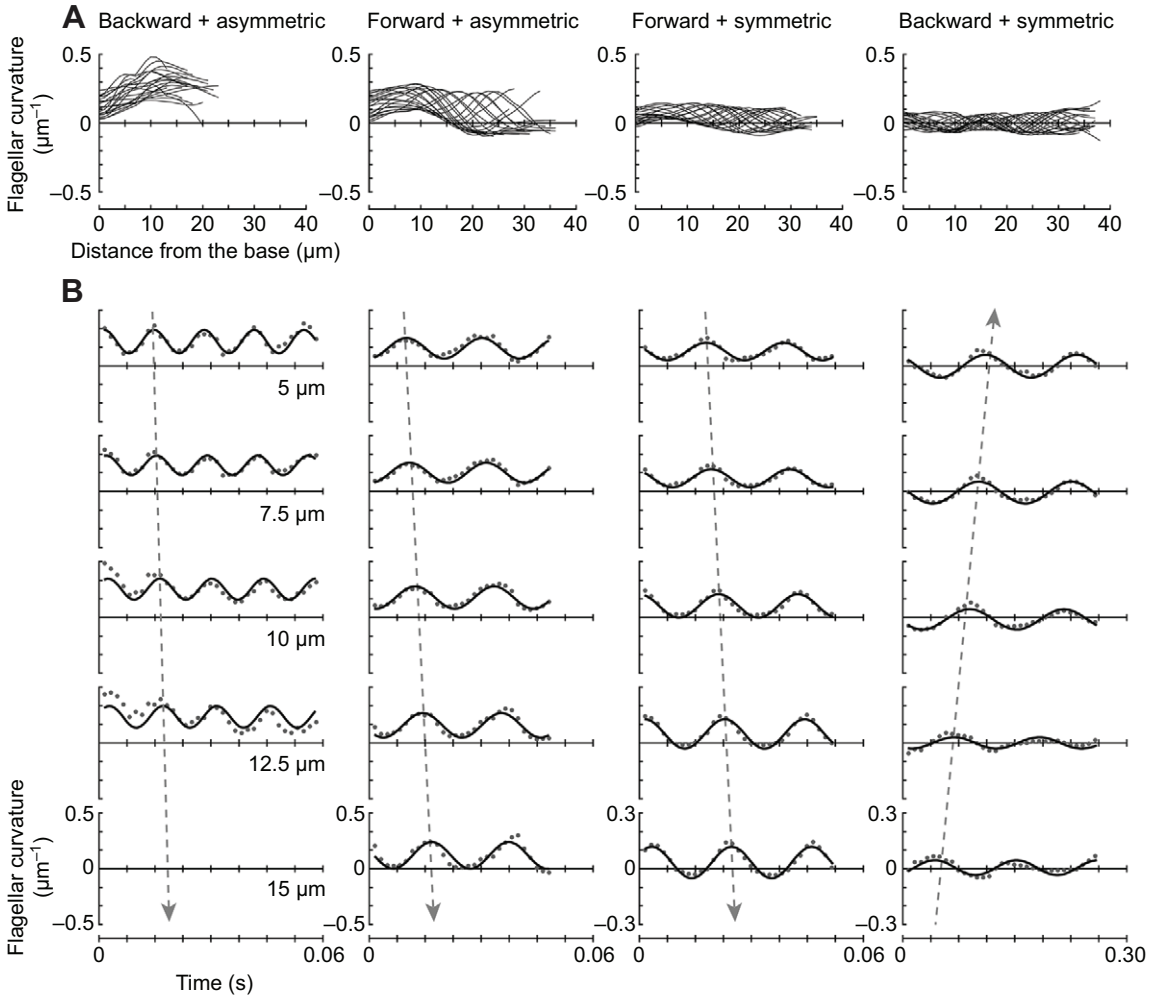

Fig. 3. Analysis of flagellar bending in four swimming patterns of *S. luhuanus* eusperm. (A) Flagellar curvature is plotted against the distance from the base of the flagellum. Data from 30 waveforms are overwritten. (B) Flagellar curvatures at 5, 7.5, 10, 12.5 and 15 μm from the base are plotted against time (gray circles) with regression lines (bold lines). Dashed arrows indicate the direction of bend propagation.

To investigate the role of intracellular Ca^{2+} concentration in sperm swimming, we induced artificial Ca^{2+} influx using the Ca^{2+} ionophore A23187 (Fig. 9). Sperm treated with 10 μmol l^{-1} A23187 in ASW showed swimming with asymmetric flagellar waveforms at 3 min after dilution, but they quickly entered into forward swimming with symmetric flagellar waveforms within 5 min of dilution (Fig. 9A). Sperm in ASW containing 0.1 mmol l^{-1} Ca^{2+} (LCSW) showed a similar response as that observed with A23187, although the forward + asymmetric pattern was continuously observed up to 30 min after dilution (Fig. 9B). In the presence of A23187, the eusperm could not maintain motility for a long time, and most sperm became immotile in approximately 30 min. This suggests that influx of excess Ca^{2+} induces a skip of the asymmetric waveforms and quick transit to the swimming patterns with symmetric waveforms (Fig. 9A). Treatment of sperm with A23187 in CFSW containing 10 mmol l^{-1} EGTA also resulted in the induction of a quick transit to forward swimming with symmetric flagellar waveforms, although the backward + asymmetric pattern was rarely observed at 3 min after dilution (Fig. 9C). This suggests that Ca^{2+} is necessary for inducing backward + asymmetric swimming.

Table 1. Sperm flagellar bending in four swimming patterns in *Strombus luhuanus* eusperm

	Backward + asymmetric	Forward + asymmetric	Forward + symmetric	Backward + symmetric
Bend propagation	Base to tip	Base to tip	Base to tip	Tip to base
Swimming velocity (μm s^{-1})	125.9±62.1	89.05±46.71	72.6±30.1	13.47±7.32
Beat frequency (Hz)	72.2±8.9	73.56±17.11	40.17±6.98	4.65±1.14
Maximum curvature (μm^{-1})	0.312±0.009	0.304±0.035	0.130±0.012	0.065±0.014
Amplitude (μm)	0.078±0.005	0.088±0.002	0.055±0.006	0.058±0.016
Propagation velocity (μm s^{-1})	2688±1288	2103±571	1101±227	90±23
Wavelength (μm)	37.05±17.09	28.44±2.33	27.34±1.78	20.0±6.29

Values are means ± s.d.; *N*=3–5. Sperm were suspended in artificial seawater. Flagellar bending was analyzed from 30 successive waveforms. Individual variables were computed using Eqn 1 (see Materials and methods); the curvature was determined at 7.5 μm from the base. The amplitude was obtained from the curvature.

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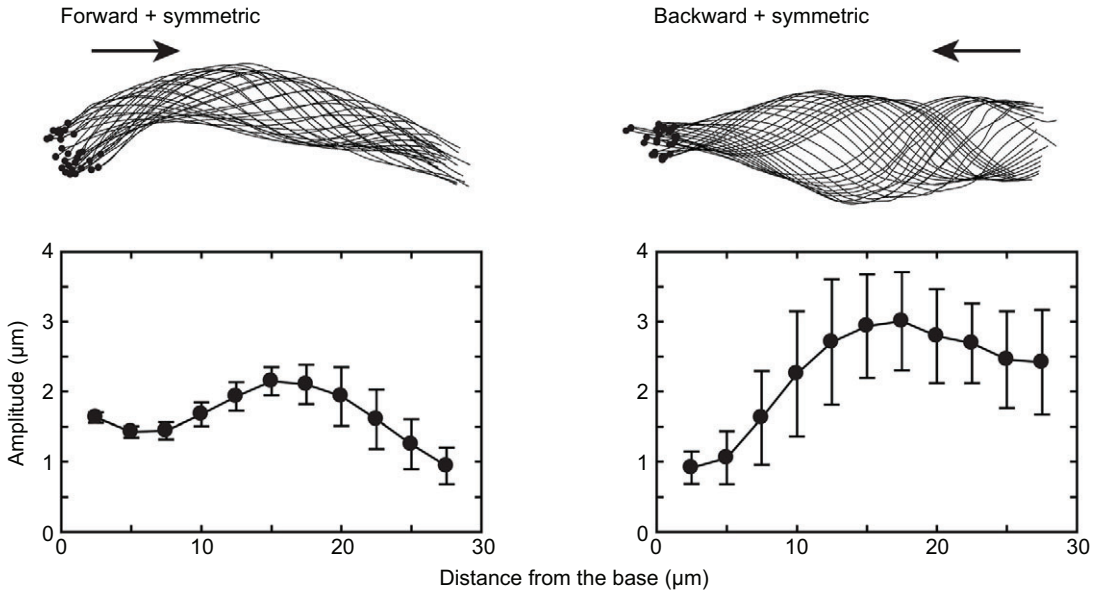

Fig. 4. Changes in the direction of bend propagation of *S. luhuanus* eusperm. Data of flagellar bending pattern in the forward swimming with a symmetric waveform (forward + symmetric) (left) and the backward swimming with a symmetric waveform (backward + symmetric) (right) are shown. Upper and lower figures show overwritten flagellar waveforms and the calculated amplitudes against the distance from the base of flagellum, respectively. Thirty bending forms were chosen every 1.66 ms (forward + symmetric) or 8.33 ms (backward + symmetric). Arrows indicate the direction of bend propagation. Values are means ± s.d.; *N*=4 (forward + symmetric) and 3 (backward + symmetric).

DISCUSSION

The snail *S. luhuanus* is dioecious and performs internal fertilization by copulation. It is thought that the *S. luhuanus* sperm ejaculated into the female's body move to the female seminal receptacle and wait for ovulation, as in other gastropods (Fretter, 1941). Here we observed that *S. luhuanus* eusperm changed their swimming direction and flagellar waveforms depending on the time of incubation in ASW. These changes suggest that the *S. luhuanus* eusperm modulate the swimming to fit their physiological circumstances in the female body. In the seminal ducts, up to ~70 eusperm are attached to each other at the tips of their heads and form a sperm bundle. Just after dilution in seawater, the eusperm swim backward with asymmetric waveforms. We suspect that this backward swimming is required for the detachment of individual eusperm from the sperm bundle (Fig. 10).

The eusperm in ASW then start to swim forward with asymmetric waveforms, and eventually they swim straight with symmetric waveforms. In the female seminal receptacle, the eusperm showed straight swimming with symmetric waveforms, suggesting that forward swimming is necessary to reach the female seminal receptacle (Fig. 10).

The eusperm in the female seminal receptacle showed spontaneous backward swimming with symmetric waveforms. Our results suggest that the eusperm swim backward to detach from the wall of the receptacle and swim to the oviduct for fertilization with ovulated eggs (Fig. 10), because in our observations by tissue section and by scanning electron microscopy, many eusperm were attached to the wall of the receptacle. In fact, we could observe sperm detaching from the wall of fragmented seminal receptacle with backward swimming (Fig. 7E). The rate of the backward swimming was not high in this experiment (Fig. 3).

We could not exclude the possibility that the detachment of sperm from the receptacle with backward swimming was an artificial induction caused by dissection or fragmentation. It is conceivable, however, that there is a cue to stimulate backward swimming for the detachment at ovulation. Several factors that induce sperm release

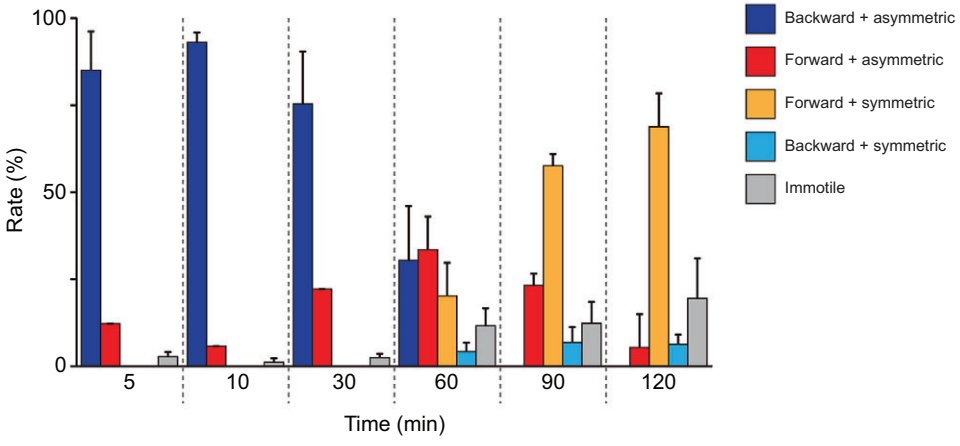

Fig. 5. Changes in swimming patterns of *S. luhuanus* eusperm after dilution in ASW. The number of eusperm showing each pattern (distinguished by the colors of the columns) was counted from recorded movies at each time after dilution. Values are means ± s.e.m.; *N*=5.

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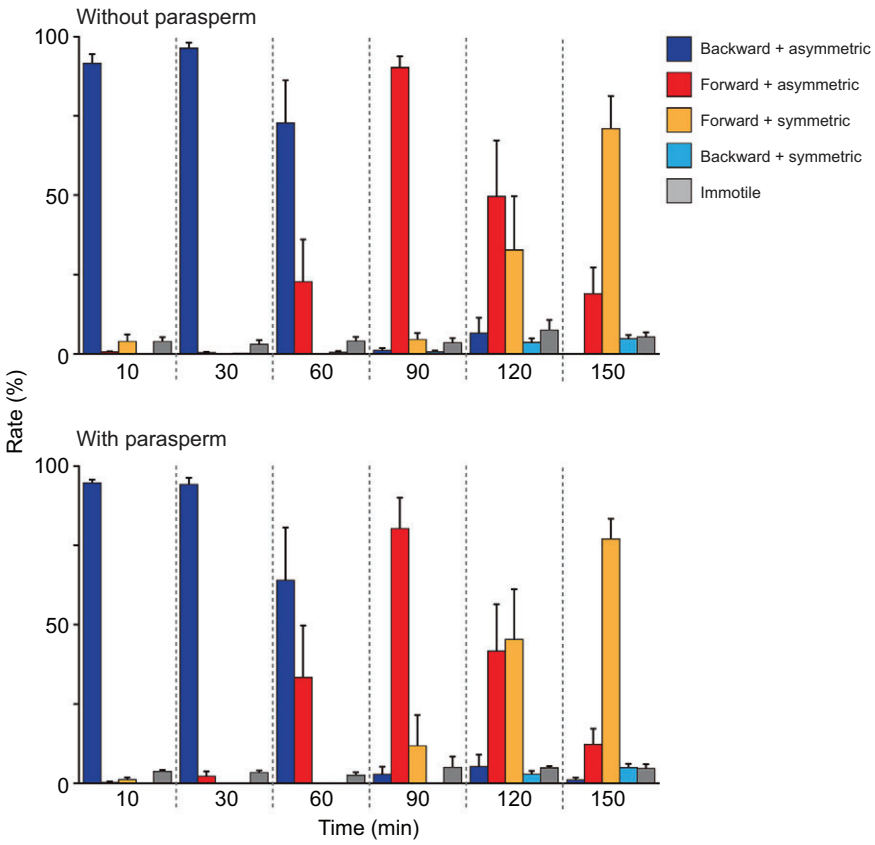

Fig. 6. Changes in swimming patterns of *S. luhuanus* eusperm with and without parasperm. Semen was centrifuged to prepare eusperm with or without parasperm. The number of eusperm showing each pattern (distinguished by the colors of the columns) was counted from recorded movies at each time after dilution. Values are means ± s.e.m.; *N*=3. The eusperm concentrations during incubation were 300.26 and 346.16 cells μl^{-1} for samples without parasperm and with parasperm, respectively. Parasperm in the sample with parasperm was 19.62 cells μl^{-1}.

from a storage organ have been proposed in *Drosophila* (Neubaum and Wolfner, 1999; Schnakenberg et al., 2012) and mammals (Ignotz et al., 2001). Intriguingly, the interaction of the mammalian sperm head with the oviductal epithelium is associated with the state of capacitation (Suarez, 2008b). For example, heparin, a known inducer of capacitation in bull sperm (Parrish et al., 1988), enhances the release of bull sperm attached to the oviductal epithelium (Bosch et al., 2001).

During capacitation, mammalian sperm show changes in the swimming pattern called hyperactivation. Hyperactivated sperm exhibit changes in flagellar waveforms such as amplitude and asymmetry in order to swim efficiently in highly viscous conditions (Ishijima et al., 2002; Mohri et al., 2012; Suarez and Ho, 2003). It should be noted that only hyperactivated sperm detached from the epithelium (Demott and Suarez, 1992). We observed here that *S. luhuanus* eusperm undergo autonomous and marked motility changes and that sperm in the seminal receptacle showed symmetric forward swimming with the occasional occurrence of backward swimming. Taken together, our findings indicate that the release of sperm from a storage organ may accompany signaling from the female organs to change the sperm's motility state, as a general aspect of internal fertilization.

Ca^{2+} is one of the important factors in cell signaling pathways. In *S. luhuanus* eusperm, Ca^{2+} appears to have multiple roles to modulate the sperm function (Figs 8, 9). First, Ca^{2+} is required for the maintenance of the sperm bundle, because the sperm bundles were dissociated in CFSW but not in the presence of a Ca^{2+} channel blocker (Fig. 8). It is not yet known how the sperm heads are bound to each other. Our results suggest that some Ca^{2+}-dependent cell adhesion molecules are involved in the attachment of the eusperm heads. Ca^{2+} influx by a Ca^{2+} channel may facilitate the dissociation of sperm heads by inducing backward swimming.

Second, Ca^{2+} is necessary to induce both asymmetric and symmetric backward swimming (Fig. 8A, Fig. 9). For backward swimming, this is consistent with the previous observation that backward swimming of sperm is induced by an increase in the intracellular Ca^{2+} concentration in the marine snail *Turritella communis* (Ishijima et al., 1999). In the present study, however, the *S. luhuanus* eusperm showed forward swimming with asymmetric waveforms in CFSW after dissociation from the sperm bundle. This does not agree with the fact that flagellar asymmetry of sperm is induced by an increase in the intracellular Ca^{2+} concentration (Brokaw, 1979). Moreover, a strong Ca^{2+} influx to the eusperm by A23187 at an early stage after dilution accelerated the change in the flagellar waveform from asymmetric to symmetric (Fig. 9). Some mechanisms to alter the axonemal response to Ca^{2+} should thus be taken into account.

The influx of Ca^{2+} is known to trigger Ca^{2+}-dependent signaling for the modulation of sperm motility, such as the activation of adenylyl cyclase and the cAMP-dependent phosphorylation of axonemal proteins (Inaba, 2003; Darszon et al., 2006). Thus, an artificial influx of Ca^{2+} may trigger a signal to modulate the function of axonemes necessary to perform symmetric flagellar bending.

In this study, we found that the *S. luhuanus* eusperm swim backward in two distinct ways. One is the backward swimming with highly asymmetric flagellar waveforms (backward + asymmetric) observed immediately after incubation in ASW, and the other is with low amplitude and symmetric waveforms observed at later stages of incubation. In the former case, the flagellar bend propagates from the base to the distal end, for forward swimming. Their flagellar beating is not planar and only the basal part of flagellum is in focus under a microscope. The very sharp bend at the base reverses the orientation of the sperm head to the direction of bend propagation in the distal-most part of the flagellum. Therefore, the findings could

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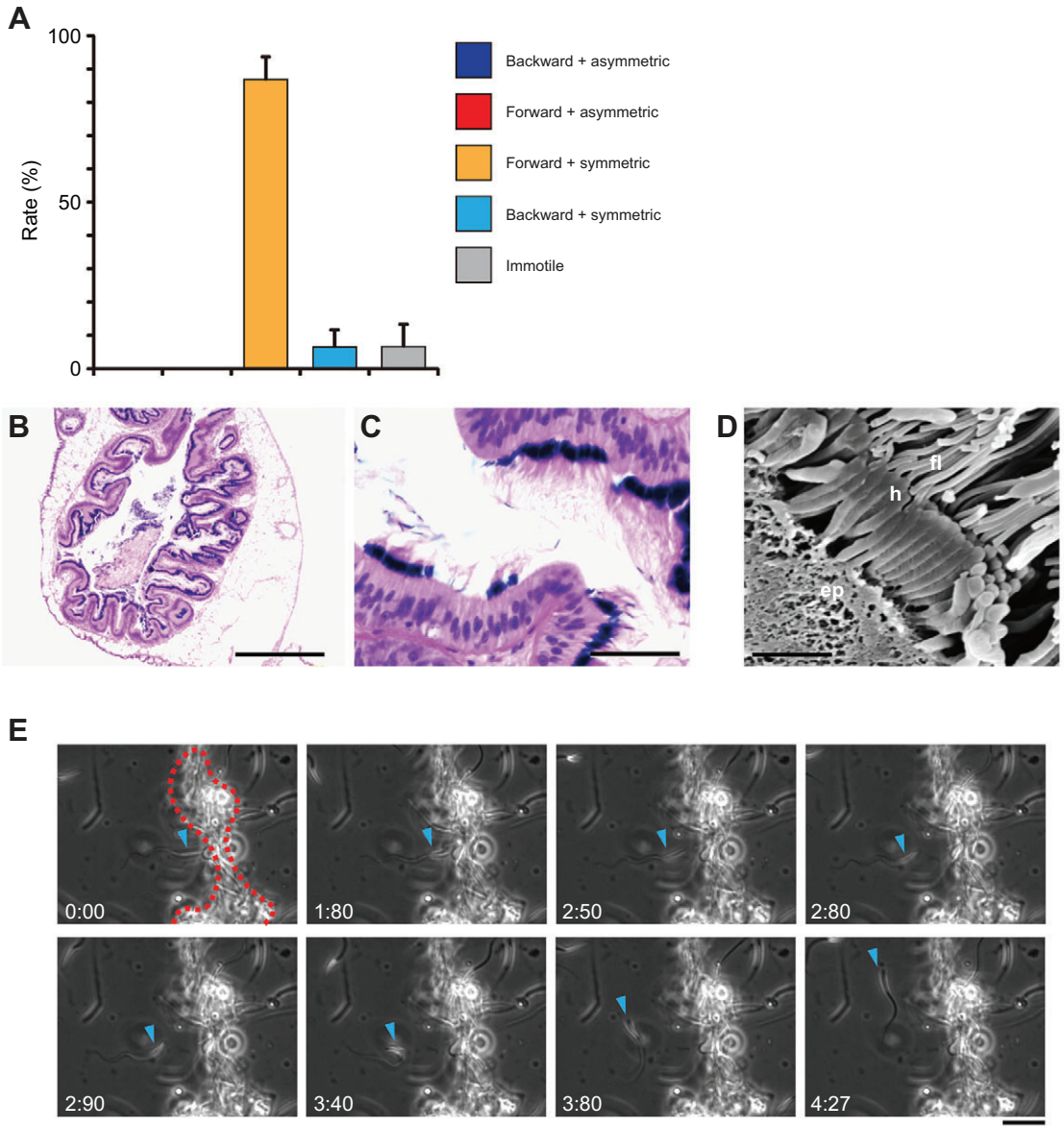

Fig. 7. The *S. luhuanus* eusperm in female seminal receptacle. (A) Swimming patterns of eusperm collected from seminal receptacles. Values are means ± s.e.m.; *N*=3. (B,C) Histological sections of seminal receptacle stained with hematoxylin and eosin. (D) Scanning electron microscopic image. fl, flagella; h, head; ep, epithelium. (E) Sequential images of sperm detached from the wall of fragmented seminal receptacle. The numbers in each image represent the time of the recorded frame (s). Dashed line shows fragmented seminal receptacle. Sperm with backward swimming are indicated by blue arrowheads. Scale bars, 500 µm (B), 50 µm (C), 5 µm (D) and 20 µm (E).

be simply interpreted as indicating that the bend propagation of the distal part of the flagellum works as a propulsive force, pulling the sperm head backward.

It is interesting that the flagellar bending in the other backward swimming pattern (backward + symmetric) propagates from the distal end to the base of the flagellum, directly generating a backward propulsive force (Fig. 2H, Fig. 3). The molecular motor, dynein in the sperm axoneme, is the minus-end-directed motor, which slides an adjacent microtubule in the plus-end direction and generates the bend propagation in the base-to-tip direction (Woolley, 2010; Inaba, 2011). A reversal of the swimming direction has also been reported in other snails, flies and myzostomid worms (Holwill and McGregor, 1976; Buckland-Nicks and Chia, 1981; Baccetti et al., 1989; Ishijima et al., 1994). This phenomenon has been explained by several possible mechanisms, such as the presence of two oscillation centers (Buckland-Nicks and Chia, 1981) and chirality of the flagellar wave (Ishijima et al., 1999).

It is worth noting that sperm flagella showing backward swimming are surrounded by accessory structures, such as the accessory microtubules and mitochondria in *Drosophila* (Kiefer, 1966; Köttgen et al., 2011). *Strombus* sperm axonemes are also surrounded by mitochondria along the most proximal part of the flagella and by arranged glycogen granules at the distal part of the flagella (Koike and Nishiwaki, 1980; Casse et al., 1994). These structures might anchor the axonemes and are responsible for initiating the reverse propagation of flagellar bends in place of basal bodies.

In addition, Ca^{2+} is a key factor controlling the conversion of forward to backward bend propagation (Figs 8, 9) (Ishijima et al., 1999), although it does not change the direction of microtubule

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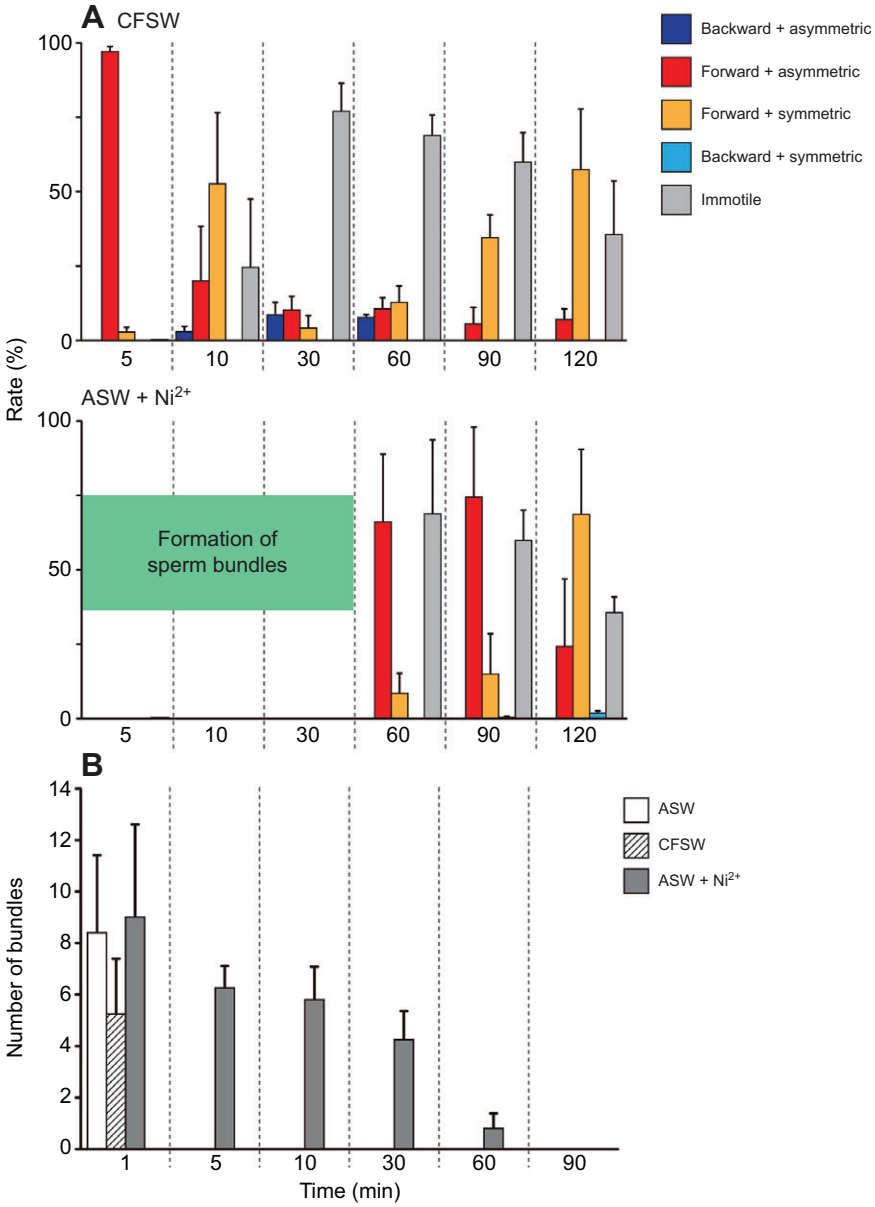

Fig. 8. Regulation of sperm bundle and swimming patterns of the *S. luhuanus* eusperm by Ca^{2+}. (A) Semen was diluted into Ca^{2+}-free SW (CFSW, upper) or ASW containing 5 mmol l^{-1} $NiCl_2$ (lower). The number of eusperm showing each pattern (distinguished by the colors of the columns) was counted from recorded movies at each time after dilution. Note that sperm bundles remained undissociated for 30 min after dilution in Ni^{2+}-containing ASW. Values are means ± s.e.m.; *N*=3–4. (B) Inhibition of sperm bundle dissociation by Ni^{2+}. Semen was diluted in ASW, Ca^{2+}-free SW or ASW containing 5 mmol l^{-1} $NiCl_2$ and the numbers of sperm bundles were counted at each time after dilution. Values are means ± s.e.m.; *N*=4–5.

sliding by dyneins (Mizuno et al., 2012). Mutual alternation of the rotational direction of sperm flagella was proposed to account for the change in the direction of bend propagation. In this model, Ca^{2+} appears to change the sense of flagellar waves (Ishijima et al., 1999). Thus Ca^{2+} may modulate the anchoring structures around the axonemes to switch the point of initiation for bend propagation to the distal end of the flagellum. The parameters necessary for the flagellar bend propagation of backward + symmetric sperm, i.e. beat frequency, maximum curvature and propagation velocity, are much lower compared with those of forward + symmetric sperm (Fig. 3, Table 1). This may be related to the differences in energy production systems between the proximal and distal parts of the flagellum.

One intriguing feature of fertilization in *S. luhuanus* is that they have two distinct types of sperm. Morphological studies of *S. luhuanus* sperm have been carried out since the 19th century (Brock, 1887; Koike and Nishiwaki, 1980; Nishiwaki, 1964; Reinke, 1912), but the physiological aspects of eusperm and parasperm in the process of fertilization are still not well understood. The parasperm have no nucleus, and therefore it is thought that they have a supportive role in fertilization. Because gastropods show high diversity in the morphology of parasperm, several functions of parasperm have been proposed, including transport or aid in the capacitation of eusperm, enhancement of fertilization success in the presence of sperm competition, protection from spermicidal environments, nutrient supply, and control of the sex ratio (Higginson and Pitnick, 2011). However, most of these ideas lack experimental evidence.

In gastropods such as *Fusitriton* or *Littorina*, many of the eusperm are attached to the giant parasperm for transport in female organs (Buckland-Nicks, 1998; Fretter, 1953; Healy and Jamieson, 1993). However, we did not observe any interactions between eusperm and parasperm in *S. luhuanus*. Our observation of eusperm motility also demonstrated that the parasperm do not contribute to the autonomous motility changes of the eusperm. Because we have not observed the detailed changes in the motility of eusperm before and after sperm storage in seminal receptacles, it is necessary to establish an experimental system to trace the behavior and motility of eusperm in female organs. In addition, in light of the concept of ‘sperm competition’, it would be very intriguing to test whether parasperm interfere with the function of eusperm from multiple males.

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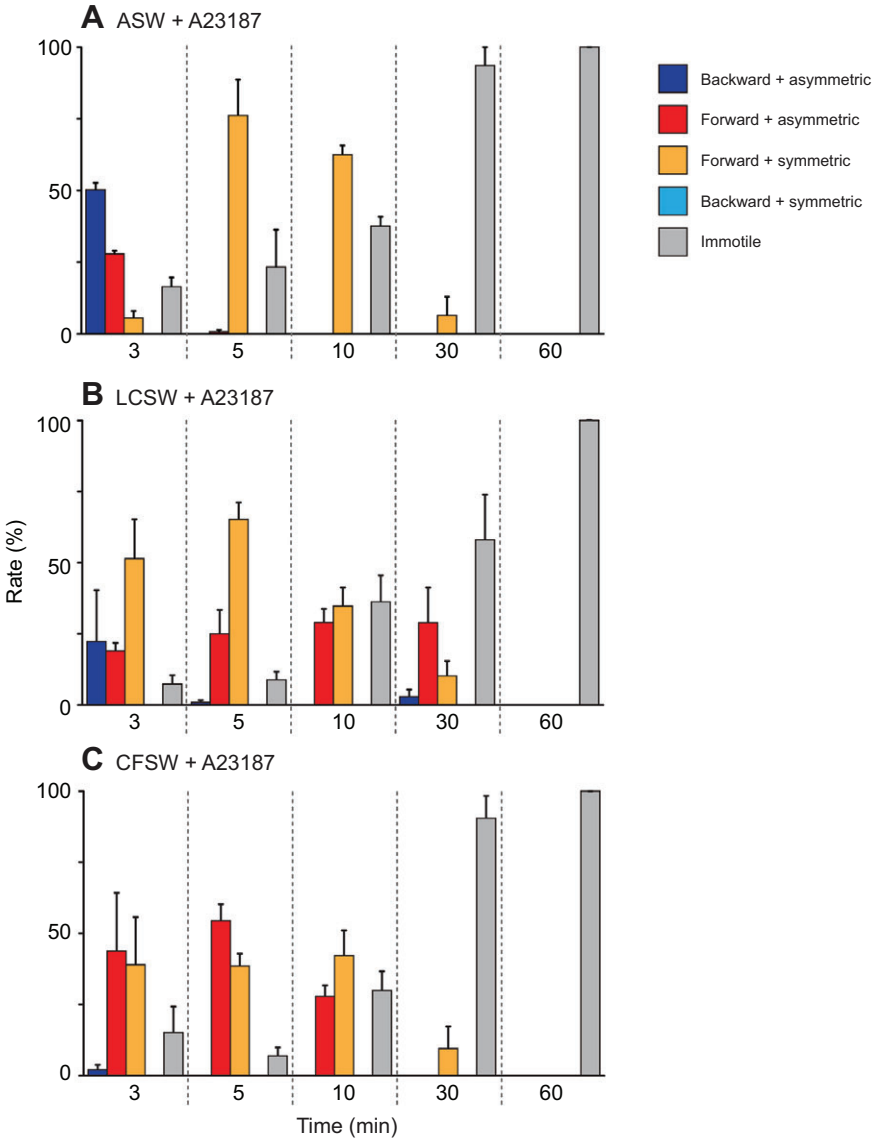

Fig. 9. Effect of extracellular Ca^{2+} concentration and Ca^{2+} ionophore A23187 on the patterns of sperm movements. (A) In the presence of 10 µmol l^{-1} A23187 in ASW, sperm swimming with asymmetric waveform were rarely only seen at 3 min after dilution. Sperm started to show forward movement soon after dilution and then became immotile after 60 min. (B) Swimming patterns of sperm in the presence of A23187 in ASW with 0.1 mmol l^{-1} Ca^{2+} (low-calcium seawater, LCSW). (C) Swimming patterns of sperm in the presence of A23187 in CFSW (10 mmol l^{-1} EGTA). Values are means ± s.e.m.; *N*=3.

MATERIALS AND METHODS

Materials and solutions

Strombus luhuanus were collected at Nabeta Bay near the Shimoda Marine Research Center, University of Tsukuba, Japan, from May to September. Semen was obtained by micropipette from the seminal duct of males or by dissection from the female seminal receptacle after the shell was removed, and then stored on ice. Artificial seawater (ASW) consisted of (in mmol l^{-1}): 460.3 NaCl, 10.11 KCl, 9.18 $CaCl_2$, 35.91 $MgCl_2$, 17.49 $MgSO_4$, 0.1 EDTA and 10 Hepes-NaOH (pH 8.2). To prepare low-Ca^{2+} seawater (LCSW), we replaced the 9.18 mmol l^{-1} $CaCl_2$ with 0.1 mmol l^{-1} $CaCl_2$. To prepare Ca^{2+}-free seawater (CFSW), we replaced the 460.3 mmol l^{-1} NaCl and the 9.18 mmol l^{-1} $CaCl_2$ with 474.07 mmol l^{-1} NaCl and 10 mmol l^{-1} EGTA, respectively. The Ca^{2+} ionophore A23187 was purchased from Sigma-Aldrich (St Louis, MO, USA).

Scanning electron microscopy

Sperm were fixed in 2.5% glutaraldehyde in 0.45 mol l^{-1} sucrose, 0.1 mol l^{-1} sodium cacodylate (pH 7.4) at 4°C for 1 h. Fixed samples were washed three

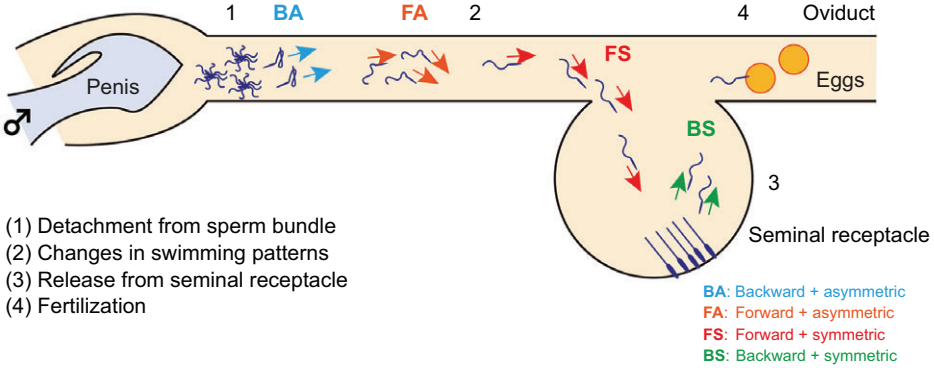

Fig. 10. A schematic model of the motility changes during internal fertilization in the *S. luhuanus* eusperm. Just after ejaculation of sperm into the female reproductive tract, the eusperm swim backward with the asymmetric waveform to detach from sperm bundle (1). Then, sperm change the swimming pattern from forward to backward to progress into the oviduct and attach the wall of seminal receptacle (2). The eusperm are released from the seminal receptacle by the backward swimming with symmetric waveform (3) when the eggs are ovulated for fertilization (4).

times with 0.1 mol l^{-1} sodium cacodylate (pH 7.4) and dehydrated using an ethanol series. Seminal receptacles were fixed in Bouin's solution, dehydrated, embedded in paraffin wax, and serially sectioned at a thickness of 8 µm. Sperm and sections of seminal receptacle were subjected to drying by a freeze drier (JFD-320, JEOL, Tokyo), coated with Au by an ion sputter gun, and observed with a scanning electron microscope (NeoSope JCM-5000, JEOL).

Analysis of sperm motility

Semen was suspended 2000-fold with ASW, CFSW or ASW containing 5 mmol l^{-1} $NiCl_2$ or 10 µmol l^{-1} A23187 and incubated at room temperature. To obtain eusperm free from parasperm, the suspension was centrifuged with a hand-driven centrifuge for 30 s to sediment the parasperm, and the supernatant was used for the motility analysis. The pellet containing both eusperm and parasperm was resuspended in ASW and used as 'eusperm with parasperm' for the comparative analysis of motility with parasperm-free eusperm.

The sperm suspension was placed on a glass slide coated with 1% BSA to avoid adhesion of sperm to the glass. Images were recorded through a phase contrast microscope (BX51, Olympus, Tokyo) with a 10× or 20× objective (UPlan FLN, Olympus) connected to a high-speed CCD camera (HAS220, Ditect, Tokyo) at 50 or 600 frames s^{-1}. Sperm swimming patterns were categorized into four patterns according to swimming direction and flagellar asymmetry: backward swimming with an asymmetric waveform (backward + asymmetric), forward swimming with an asymmetric waveform (forward + asymmetric), forward swimming with a symmetric waveform (forward + symmetric), and backward swimming with a symmetric waveform (backward + symmetric) (see Fig. 2). The rate of each pattern was obtained by counting all spermatozoa in the recording image for 10 s.

Sperm flagellar bending was analyzed by Bohboh software (Bohbohsoft, Tokyo). Individual images of sperm flagella were tracked automatically, and the flagellar curvature was calculated based on the method of Baba and Mogami (Baba and Mogami, 1985). On the basis of the description of the flagellar bending waves as 'sign-generated' (Hiramoto and Baba, 1978), the flagellar curvature at a certain distance from the base was plotted against time and fitted by using the following equation:

$$\gamma(s,t) = \gamma_a(s)\cos\{[S_\gamma(s) - 2\pi ft]\} + \gamma_b(s), \quad (1)$$

where s is the distance from the base; t is time; γ_a is the amplitude of curvature; γ_b is a constant representing mean curvature; and S_γ is phase angle.

Obtained parameters such as beat frequency, amplitude of curvature and wavelength were used to evaluate the flagellar bending. To obtain the 'amplitude' of flagellar waveforms in swimming sperm, the angular direction was used to determine the central axis of wave at s (Hiramoto and Baba, 1978) so that the sinusoidal oscillation of flagellum around that axis gave the 'amplitude'.

Preparation of paraffin-embedded tissue sections

For light microscopic observation, seminal receptacles were collected by dissection and fixed in Bouin's solution at room temperature overnight. Samples were dehydrated in an ethanol series and embedded in paraffin wax. Eight-micrometer-thick sections were serially cut, deparaffinized, rehydrated, and stained with hematoxylin and eosin.

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Competing interests

The authors declare no competing financial interests.

Author contributions

K.S. and K.I. contributed to conception and the design of experiments; K.S. carried out motility experiments; D.S. carried out morphological analysis; K.S. and K.I. contributed to interpretation of the findings; and K.S. and K.I. contributed to drafting and revising the article.

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Supplementary material

Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.095398/-/DC1

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