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THE ACQUISITION OF FORWARD MOTILITY IN THE SPERMATOZOA OF THE POLYCHAETE *ARENICOLA MARINA*

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Summary

Sperm activation in the polychaete annelid *Arenicola marina* was investigated using video microscopy following the *in vitro* and *in vivo* manipulation of gametes. Careful observation of spermatozoa obtained from spawning animals indicated that they were immotile immediately after their release from the gonopores, but that they subsequently became motile following dilution in sea water. It was determined that under the pH conditions of the coelomic cavity (pH 7.3) sperm motility was suppressed, whereas upon dilution in sea water buffered at pH 8.2, motility was triggered. It is hypothesised that sperm activation, under normal conditions, occurs in two stages. The first results in the liberation of free spermatozoa from sperm morulae, allowing them to be spawned; this process is stimulated by an endocrine factor and occurs *in vivo* during normal spawning. The second involves the switching on of the sperm flagellar apparatus, which occurs when spermatozoa are subjected to change in extracellular pH associated with their dilution in sea water. Pharmacological agents such as ammonium chloride and quinacrine are shown to stimulate the breakdown of sperm morulae and the acquisition of sperm motility. Motile spermatozoa of *A. marina*, in artificial sea water buffered at pH 8.2, can remain motile for over 5 h, have a beat frequency of approximately 50 Hz and have average path velocities of between 100 and 120 μm s^{-1}. Motile spermatozoa under these conditions can also display the phenomenon of intermittent swimming.

Introduction

In almost all animal species investigated, it is usual for spermatozoa to become motile either during, or immediately following, their release from storage within the male. In species utilising external fertilisation, such as many echinoderms (Trimmer and Vacquier, 1986), some species of fish (Scheuring, 1924; Stoss, 1983) and amphibians (Hardy and Dent, 1986), spermatozoa become motile once they are diluted into the

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surrounding water column at spawning. Only in a very few documented cases do spermatozoa remain immotile following release; most notable among these are the herring *Clupea* sp. (Yanagimachi, 1957) and the horseshoe crab *Limulus polyphemus* (Clapper and Brown, 1980). In these species, spermatozoa become motile only after interaction with specific chemical substances derived from the egg. Sperm motility prior to the release of sperm from storage within the male is unknown in any species utilizing external fertilisation and has only been recorded for one known species, the rabbit, which utilises internal fertilisation (Turner and Reich, 1985).

The sperm biology of the polychaete *Arenicola marina* has recently been the subject of considerable interest. This species uses external fertilisation and, in some locations, neighbouring individuals spawn in a highly synchronous manner (Howie, 1984). It has been suggested that such synchrony may indicate the existence of a close interaction between environmental factors and the endocrine system (Bentley and Pacey, 1992). Experiments have identified an appropriate endocrine link and it has been shown that a factor released from the prostomium, sperm maturation factor (SMF), can induce spawning in sexually mature males (Pacey and Bentley, 1992*b*). Sperm maturation factor has been putatively identified as the 20-carbon polyunsaturated fatty acid 8,11,14-eicosatrienoic acid (Bentley *et al.* 1990).

Prior to spawning, fully differentiated *A. marina* spermatozoa are retained within the coelomic cavity where they are bathed in coelomic fluid. Spermatozoa are not stored within discrete gonads but, as in almost all polychaetes (see Sawada, 1984), they undergo most of their differentiation within the coelomic cavity. In addition, when they are fully differentiated, spermatozoa are not found as individual cells, but are present in disc-like clusters in which several hundred spermatozoa are cytoplasmically connected to each other. These syncitia are known as sperm 'morulae' and they result from the incomplete cytokinesis which occurs during spermatogonial and meiotic divisions (Bentley and Pacey, 1989; Pacey and Bentley, 1992*a*). When present in sperm morulae, individual spermatozoa are immotile and they are not capable of fertilisation even if they are removed from the coelomic cavity and incubated with mature oocytes (Meijer, 1979). It has been shown that, prior to spawning, it is necessary for the sperm morulae to disrupt and release individual spermatozoa, since only free spermatozoa are able to pass through the gonoducts (Howie, 1961*a*,*b*, 1962). The breakdown of sperm morulae, to release individual spermatozoa, has been likened to a sperm maturation step, since neither spawning nor fertilisation will occur without it.

Experiments investigating the role of SMF have shown that one of its functions is to stimulate the breakdown of sperm morulae, and an ultrastructural investigation of this process has been carried out (Bentley and Pacey, 1989; Pacey and Bentley, 1992*a*). Such studies have been greatly assisted by the development of an *in vitro* bioassay for SMF (Bentley, 1985). In this assay, a small amount of coelomic fluid from a sexually mature male specimen of *A. marina* (containing spermatozoa present in sperm morulae) is diluted in filtered sea water (1:100) and incubated at 14 °C for up to 1 h. Under these conditions, spermatozoa remain bound within morulae, and the morulae remain quiescent unless they are also incubated with an appropriate dose of a specific stimulator of sperm morulae breakdown, such as SMF. Other pharmacological agents, such as

quinacrine and ammonium chloride, are also capable of stimulating sperm morulae breakdown under these conditions (Pacey and Bentley, 1993). Because spermatozoa released from morulae *in vitro* (by the action of SMF or other agonists) exhibit good forward motility, it has been inferred that this must also occur *in vivo*, in the coelomic cavity, prior to spawning (see Pacey and Bentley, 1991). If this were the case, however, then the situation in *Arenicola marina* would be clearly unlike that occurring in almost all animals species investigated. The experiments described in this paper were therefore carried out in order (1) to establish more clearly the point at which spermatozoa become fully motile, and (2) to suggest a possible mechanism for the stimulation of forward motility in this species.

Materials and methods

Collection, transportation and maintenance of animals

Sexually mature specimens of the lugworm *Arenicola marina* (L.) (Annelida: Polychaeta) were collected from a number of sites around the British coastline. The specific details of collection methods and times of collections have been described elsewhere (see Pacey and Bentley, 1992*b*). Following collection, the animals were maintained at the Gatty Marine Laboratory (St Andrews, UK) in a constant-temperature room at 10 °C. They were held individually in clean plastic containers containing approximately 150 ml of sea water, which was replaced daily. Within 7 days of collection, the animals were transported by air to the Station Zoologique (Villefranche-sur-mer, France), where they were maintained under similar conditions. The only detail worthy of note is that the local sea water at Villefranche was diluted to give a comparable osmolality to that of their place of origin (900–1000 mosmol kg^{-1}) and was filtered through a 0.44 μm pore diameter filter. Under these conditions, animals appeared healthy and often could be used for experiments for several weeks.

Induction of spawning

Spawning was induced in sexually mature individuals by an intra-coelomic injection of an appropriate dose of either SMF (prepared from the homogenate of the prostomium from another sexually mature animal) or 8,11,14-eicosatrienoic acid. Specific details of both procedures can be found in Pacey and Bentley (1992*b*).

Preparation of cell-free coelomic fluid

Germ cells and coelomocytes were removed from coelomic fluid by centrifugation. Samples of coelomic fluid (containing spermatozoa present in sperm morulae) were removed from animals using a 1 ml disposable plastic syringe fitted with a 25 gauge needle; this procedure has been described previously (see Pacey and Bentley, 1993). Samples, ranging in volume from 500 μl to 1 ml, were then subject to a two-step centrifugation regime. In step 1, the sample was centrifuged at 500 $\boldsymbol{g}$ for 10 min, after which the top 90 % of supernatant was subject to further centrifugation at 15 000 $\boldsymbol{g}$ for 30 min. Following the second centrifugation step, a small sample (10 μl) of the supernatant was examined microscopically to confirm that all cells and cell debris had

been removed. If the coelomic fluid was not required for experiments immediately, samples were frozen and stored at −20 °C.

Activation of spermatozoa in vitro

Sperm morulae breakdown was stimulated *in vitro* using the bioassay described by Bentley (1985) (see also Pacey and Bentley, 1993). In some experiments, artificial sea water (ASW) was used in preference to filtered natural sea water. Batches for use in experiments were made up as and when required using the formulae of Cavanaugh (1956) in mmol l^{-1}: 420 NaCl; 9.0 KCl; 10 $CaCl_2 \cdot 2H_2O$; 24.5 $MgCl_2 \cdot 6H_2O$; 25.5 $MgSO_4 \cdot 7H_2O$; 2.15 $NaHCO_3$. The pH of ASW was buffered to the required value using 20 mmol l^{-1} Tris–HCl and this was checked routinely prior to experiments. Stock solutions of quinacrine (1 mmol l^{-1}) and ammonium chloride (100 mmol l^{-1}) were prepared in ASW at a concentration 10-fold greater than that required in the final incubation. A stock solution of 8,11,14-eicosatrienoic acid (10 mmol l^{-1}) was prepared in methanol and stored at −20 °C. For use in experiments, samples of fatty acid were diluted at least 100-fold in ASW so that the solvent concentration was less than 1 % by volume. All doses were those that, in previous experiments, were typical mid-range doses shown to elicit a suitable response (see Bentley *et al.* 1990; Pacey and Bentley, 1993).

Measurement of sperm motility

Samples (20 μl) collected during spawning experiments, or during *in vitro* incubations, were placed on a microscope slide as an open drop with no coverslip and were observed under stroboscopic illumination (Strobex Chadwick-Helmuth), using an Olympus BH2 microscope fitted with a Leitz 0.8–0.95 dark-field condenser and Zeiss ×16-0.35 and ×25-0.45 Plan objectives. In all experiments, apart from those in which flagellar beat frequency of the motile spermatozoa was determined, the frequency of the illuminator was set at a constant 50 Hz. In those experiments where the flagellar beat frequency of sperm samples was determined, the frequency of strobeoscopic illumination was adjusted to match that of the beating flagellum so that it appeared to be immobilised and bent (Brokaw, 1986). At least 30 measurements were made on each sample to provide a mean beat frequency for the whole sperm population.

In all experiments, spermatozoa swimming in close contact with the microscope slide were observed and videotaped. Under some experimental conditions, in particular those involving the fatty acid, the movement of spermatozoa was interrupted by their collisions with fatty acid micelles. In order accurately to determine the motion variables of spermatozoa under these conditions, it was, therefore, necessary to observe sperm motion without the influence of the micelles. Since micelles had a tendency to sink, spermatozoa swimming at the surface of the fluid drop were videotaped in experiments involving fatty acids.

Estimates of sperm motion variables, including the percentage of motile spermatozoa, were made by retrospective analysis of videotape recordings. A recording of at least 30 s in duration was made for each sample analysed using a Panasonic CCD (F-10) video camera (frame rate frequency 50 Hz) and a Panasonic super VHS video recorder. Subsequent analysis of the video tapes was carried out as follows.

Values for percentage motility, velocity of the averaged path (VAP) and curvature of path were measured in one of two ways. At low sperm densities (1–5 spermatozoa per ×25 field) the videotape recording was subjected to image analysis using the 'trace' option of a Hamamatsu DVS-3000 image processing and measuring system. Using this function, successive video frames, collected over a defined period, can be overlaid into a single image. From these reconstructed images, the position of the sperm head centroid was traced from the video screen onto acetate sheets for manual analysis. At higher sperm densities (above five spermatozoa per ×25 field), it was impossible to use the image processing system since the reconstructed sperm tracks tended to blend into each other, making it difficult to determine accurately the movement of a single spermatozoon. In this instance, therefore, the reconstruction of spermatozoa tracks was carried out for individual sperm, by drawing the position of the sperm head centroid, in successive frames, directly onto acetate sheets during a frame-by-frame advance of the videotape. 2 s of sperm motion (100 frames) were analysed by this method.

From the acetate sheets (derived by either method), details of the sperm movement variables were obtained. Values for the velocity of the averaged path (VAP) were measured by drawing a line of best fit through the successive positions of the sperm head centroid; this was carried out by eye. Actual distances covered were then measured in millimetres using a map measurer, which was run along the smoothed track from the first to the last position of the head centroid. These values were then converted to micrometres by calibrating the system with reference to a calibrated micrometre slide seen through the same objectives and traced onto an acetate sheet from the video screen. The values for distance covered by the spermatozoa could then be expressed in micrometres following the application of a suitable correction factor. Values for the curvature of sperm trajectory were determined by placing a series of circles, of known diameter, over the line of best fit and selecting the one that most accurately described the path taken by the spermatozoon; the value of the curvature was expressed as the diameter of this circle.

Percentage motility data were obtained by the analysis of single frames of videotape; spermatozoa displaying straight axonemes were counted as immotile, whilst spermatozoa with axonemes bent into a pattern characteristic of sinusoidal beating were classified as motile. Using this method, it was possible to include as motile those spermatozoa that were 'stuck' to the glass slide but in which the flagellum was still beating. Since these spermatozoa generally became stuck by their heads, they had a tendency to rotate about the axis of their head but gained no net movement in space and so would have been excluded from any analysis of motility that considered only net forward movement. Slow-moving spermatozoa were counted as motile unless they failed to move a distance equivalent to half the length of their head and tail combined (approximately 25 μm) during a 2 s period.

Demembranation–reactivation procedure

The demembranation and subsequent reactivation of spermatozoa were carried out in a similar manner to that described by Brokaw (1986) for sea urchin spermatozoa. Briefly, from a stock solution of motile spermatozoa (in sea water at pH 8.2), spermatozoa were diluted 20-fold into a demembranation solution of composition: 0.15 mol l^{-1} potassium

acetate; 1.0 mmol l^{-1} dithiothreitol (DTT); 1.0 mmol l^{-1} EDTA; 0.04 % Triton X-100; and 2.0 mmol l^{-1} Tris–HCl buffer at pH 8.2. After 30 s, a 1 μl sample was diluted 20-fold into a reactivating solution containing 0.15 mol l^{-1} potassium acetate, 1.0 mmol l^{-1} DTT, 1.0 mmol l^{-1} MgATP and 20 mmol l^{-1} Tris–HCl at pH 8.2. Spermatozoa were then observed and details of the flagellar beat pattern and beat frequency recorded.

Statistical analyses

Where applicable, values for the mean and standard error of the mean were calculated for each of the motility variables measured in this study. Statistical differences between treatments were identified using analysis of variance (ANOVA) and, where differences were found, these were further explored by post-testing using a Bonferroni *t*-test.

Results

Characteristics of motile spermatozoa

Motile spermatozoa of the polychaete *Arenicola marina* swim with a planar, sinusoidal, flagellar waveform and follow paths such that when they enter the vicinity of a glass surface they swim in circular trajectories parallel to it. Fig. 1 shows a series of images of a single spermatozoon, swimming in sea water at pH 8.2, and illustrates the typical appearance of the flagellar waveform during steady-state beating. The images are a photographic record taken from freeze-frame video images over a 0.24 s period of sperm motion; the video recording was made at 50 Hz and a still photograph of every fourth video frame (Fig. 1A–E) is shown with a time interval between images of 0.06 s.

Table 1. *The motility characteristics of spermatozoa 1 h after their release from three individuals following spawning under laboratory conditions*

Number	Treatment	Percentage motility	VAP (μm s^{-1})	Diameter of sperm trajectory (μm)	Beat frequency (Hz)
1	SMF	94.7	102.3±5.5	85.2±11.0	51.9±6.8
2	8,11,14-eicosatrienoic acid	96.5	117.0±7.7	83.3±13.7	55.4±4.8
3	Spontaneous	98.9	119.9±8.8	114.4±17.4	54.6±3.6

Spawning was induced by an intra-coelomic injection of an appropriate dose of sperm maturation factor (SMF) (animal number 1) or 8,11,14-eicosatrienoic acid (animal number 2); spawning in animal number 3 occurred spontaneously, presumably following the release of endogenous SMF.

Spermatozoa were maintained in sea water (pH 8.2) and at a constant temperature of 14 °C. Observations of sperm motion were made at room temperature by observing the spermatozoa in a 20 μl sample placed on a microscope slide.

All values quoted, for each variable measured in each individual, were calculated on the basis of observations made on at least 30 spermatozoa. Data shown are means ± S.E.M. ($N \geqslant 30$).

Specific details regarding the calculation of VAP (μm s^{-1}), diameter of sperm trajectory (μm) and beat frequency (Hz) are outlined in Materials and methods.

No significant differences between any of the variables measured were found between the different treatments.

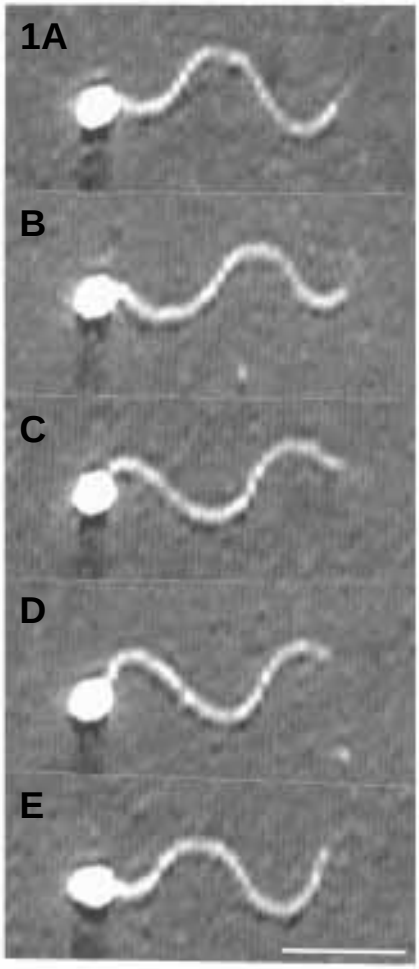

Fig. 1. A series of images taken from a video recording of a single motile spermatozoa from the lugworm *Arenicola marina*. The sequence shows the typical flagellar waveforms obtained during steady-state beating of a spermatozoon swimming in sea water. The images were recorded using dark field microscopy combined with stroboscopic illumination. The time interval between successive images (A–E) is 0.06 s and therefore the entire sequence covers a period of 0.24 s of sperm motion. Scale bar, 20 μm.

Fig. 2 shows the trajectory of a typical spermatozoon, recorded by the Hamamatsu image-processing system under similar conditions; the spermatozoon follows a curved trajectory (approximate diameter 110 μm) and the VAP is estimated at approximately 125 μm s^{-1}.

From detailed observations of the movement of many spermatozoa, following spawning in three individuals (Table 1), mean values for percentage motility, VAP, diameter of sperm trajectory and flagellar beat frequency were determined. Spawning animals were maintained at a temperature of 14 ˚C, and subsequent microscopic observations were made

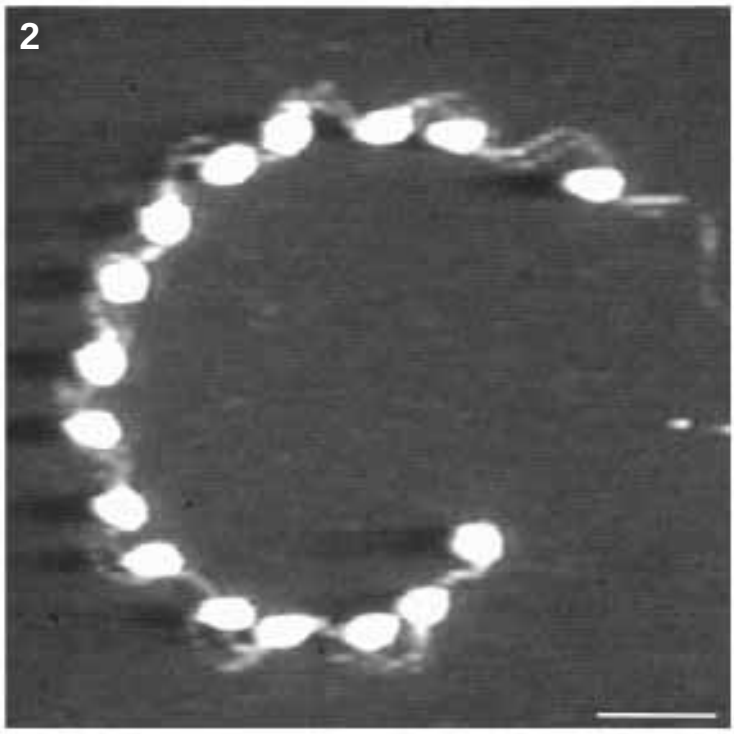

Fig. 2. Combined video images of a single spermatozoon swimming in sea water. Video images were combined using the trace function of the Hamamatsu image-processing system and show a typical sperm trajectory over a 2 s period. From the figure, the velocity of the averaged path is estimated as being 125 μm s^{-1}, with the spermatozoon ascribing a circle approximately 110 μm in diameter. Scale bar, 25 μm.

at room temperature (approximately 20 °C) 1 h after spawning had commenced. Under these conditions, spermatozoa spawned into sea water were observed to have a VAP of 100–120 μm s^{-1} and such spermatozoa had a tendency to follow curved paths so that, in ASW at an extracellular calcium concentration of 10 mmol l^{-1}, the degree of flagellar beat asymmetry caused motile spermatozoa to describe circles with an average diameter of 85–115 μm. The measured beat frequency of such spermatozoa was 50–60 Hz; this was, however, intermittent since the motile spermatozoa of *Arenicola marina* display a phenomenon known as intermittent swimming. Specific details of this type of behaviour will be described elsewhere but, in brief, it occurs when normal flagellar beating is interrupted and the sperm flagellum becomes rigid and assumes a characteristic shape in which the sperm head is bent acutely to one side, forming a configuration that resembles a walking cane. These quiescent periods, when there is no propagation of the flagellar wave, generally last for less than 1 s. Because they are characterised by a distinct shape and are, therefore, readily identifiable, and because they result in only a temporary loss of forward motility, spermatozoa displaying this behaviour were counted as motile for the purposes of estimating percentage motility.

Following spawning and dilution of spermatozoa in ASW (buffered at pH 8.2), over 94 % of spermatozoa were motile 1 h after spawning (Table 1), with characteristics similar to those of freshly spawned spermatozoa. At a constant temperature of 14 °C, it was possible for spermatozoa to remain motile for over 5 h, although this time varied somewhat between individuals. During this period, sperm motility variables (VAP, diameter of sperm trajectory and flagellar beat frequency) did not alter significantly (data not shown). It is also clear that the motility variables of spermatozoa from individuals in which spawning was induced in different ways (by exposure to SMF, 8,11,14-eicosatrienoic acid or following spontaneous spawning; Table 1) did not differ significantly from each other.

In a series of simple experiments, motile spermatozoa (at pH 8.2) were demembranated by dilution in a medium containing the non-ionising detergent Triton X-100. Subsequent dilution in a reactivating medium containing MgATP re-initiated sperm motility in these models. The motile characteristics of such spermatozoa were similar to those described for live spermatozoa, and by varying the ATP concentration in the reactivating solution up to 2 mmol l^{-1}, the theoretical maximum beat frequency (V_{max}) was calculated as being 48 Hz; a value similar to that observed in live (membrane intact) spermatozoa. Other motility characteristics of these sperm models were also very similar to those observed in 'live' (those with an intact membrane) spermatozoa. Although the absolute velocity and asymmetry of the preparations were more homogeneous between spermatozoa under fixed reactivation conditions, these variables were more dependent upon the concentration of extracellular calcium present during the reactivation step. These data are not shown, since the purpose of the present observations was to determine the ability of sperm models to reactivate, rather than to describe how the axoneme responds to specific ionic or biochemical variables. One important detail, however, is that it was possible to reactivate a demembranated axoneme irrespective of the method by which motility had been stimulated (i.e. following natural or induced spawning and spontaneous or induced sperm morula breakdown *in vitro*).

Trigger for motility acquisition

During the spawning of three individuals, the motile status of live spermatozoa was observed at different steps of the spawning process. Following induction of spawning, sperm samples were collected at two points: (1) immediately following the release of spermatozoa from the gonopores and prior to significant dilution of the spermatozoa by the surrounding sea water, and (2) after the release of spermatozoa and their subsequent dilution in sea water.

Microscopic observation of these samples indicated that spermatozoa collected immediately following their release from the gonopores were not associated in 'morulae' (cytoplasmic connections between neighbouring spermatozoa had been broken) and were immotile. Only after dilution of the released spermatozoa was motility stimulated; dilution in sea water under these experimental conditions was assisted by the 'thrashing' movements of the spawning animal. The motility characteristics of the spermatozoa released were identical to those described previously.

To investigate whether the acquisition of motility occurred as a result of removing

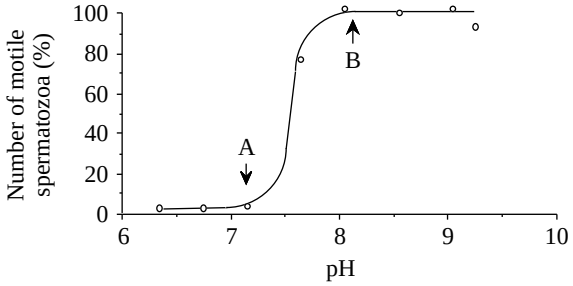

Fig. 3. An experiment showing effect of extracellular pH on the acquisition of forward motility by the spermatozoa of *Arenicola marina*. Immotile spermatozoa were collected immediately following emission from the gonopores of a spawning animal and were diluted 1:100 in artificial sea water buffered at a range of pH values from 6.3 to 9.2. Arrow A indicates the mean pH value for coelomic fluid (pH 7.3±0.13; *N*=5); arrow B indicates the pH of natural filtered sea water (pH 8.2).

spermatozoa from the influence of 'motility inhibiting' factors within the coelomic fluid, or whether it was a consequence of the change in environment concurrent with dilution in sea water, additional experiments were performed. The results of one such experiment, to investigate the role of the pH changes that occur when spermatozoa are released from the environment of the coelomic fluid (pH 7.28±0.13; *N*=5) and are diluted in sea water (pH 8.2), are shown in Fig. 3. In this experiment, spermatozoa from a spawning animal were collected immediately following their emission from the gonopores, but prior to any significant dilution. Observation of these spermatozoa confirmed that motility had not yet been stimulated. Samples (10 μl) of sperm suspension were then diluted 1:100 into sea water, buffered at a range of pH values from 6.3 to 9.2. The results in Fig. 3 clearly show that following dilution of spermatozoa at pH values below 7.2, they remain immotile; whereas at pH values above 7.6, motility is triggered. The effect is clearly 'all or nothing' and, above the threshold value, over 90 % of spermatozoa exhibit good forward motility. It is clear that the threshold pH value required to trigger sperm motility lies somewhere between that of coelomic fluid (arrow A, Fig. 3) and that of sea water (arrow B, Fig. 3).

Additional experiments, carried out *in vitro*, confirm the importance of extracellular pH in the triggering of sperm motility. The results from these are shown in Tables 2 and 3. Table 2 shows that when samples of spermatozoa, removed from the coelomic cavity (and therefore present as sperm morulae), were incubated in two pH regimes (either pH 7.3 or 8.2) for 1 h, in the presence of an appropriate dose of 8,11,14-eicosatrienoic acid, only those spermatozoa incubated at pH 8.2 were released from morulae and displayed forward motility (treatment 2). Under the same conditions but at pH 7.3, spermatozoa were liberated from morulae but displayed no forward motility (treatment 3). If the spermatozoa from treatment 3 were lightly centrifuged (500 ***g*** for 5 min) and the resulting pellet resuspended in ASW at pH 8.2 (step 2), then, within a few seconds, motility with normal swimming characteristics was triggered in 90–100 % of cells. As controls, during step 2 all spermatozoa from other treatments were resuspended in their initial supernatants; sperm motility was largely similar to that observed in step 1 (see, for

Table 2. *The effect of extracellular pH on the acquisition of forward motility following the incubation of sperm morulae with 8,11,14-eicosatrienoic acid*

	Step 1			Step 2		
Treatment	pH	Concentration of fatty acid (mol l^{-1})	Percentage motility	pH	Concentration of fatty acid (mol l^{-1})	Percentage motility
1	8.2	0	*	8.2	0	*
2	8.2	5×10^{-5}	96.9	8.2	5×10^{-5}	98.7
3	7.3	5×10^{-5}	0	8.2	5×10^{-5}	97.5
4	7.3	5×10^{-5}	0	7.3	5×10^{-5}	0

The incubation occurs in two stages: in the first (step 1), only sperm morulae incubated with fatty acid at pH 8.2 (treatment 2) release spermatozoa that subsequently display forward motility; in incubations carried out at pH 7.3, spermatozoa remain immotile. Motility can, however, be triggered if these spermatozoa are transferred to new medium (step 2) buffered at pH 8.2 (treatment 3); spermatozoa transferred to new medium at pH 7.3 (treatment 4) remain immotile. The transfer of spermatozoa between media was carried out by lightly centrifuging the spermatozoa (500 ***g*** for 5 min) and resuspending the resulting sperm pellet in the appropriate medium. Light centrifugation had little effect on the motility of spermatozoa, as shown by treatment 2, in which transfer of already motile sperm to medium of the same original composition (pH 8.2; 5×10^{-5} mol l^{-1} fatty acid) did not alter significantly the value for percentage motility.

An asterisk signifies that no sperm morulae breakdown occurred in the absence of an appropriate dose of fatty acid (treatment 1).

Values for percentage motility were calculated as described in the legend to Table 1.

Table 3. *The effect of extracellular pH on the acquisition of forward motility following the spontaneous breakdown of sperm morulae after dilution in sea water*

	Step 1			Step 2		
Treatment	pH	Concentration of coelomic fluid (%)	Percentage motility	pH	Concentration of coelomic fluid (%)	Percentage motility
1	8.2	100	*	8.2	100	*
2	8.2	1	73.9	8.2	1	65.8
3	7.3	1	0	8.2	1	72.1
4	7.3	1	0	7.3	1	0

The protocol for the experiment is the same as that described in the legend to Table 2.

Spontaneous breakdown of sperm morulae under these conditions occurs only in some (usually older or unhealthy) individuals; specific details of the mechanism are unknown. Under these conditions, as in the experiment described in Table 2, only spermatozoa diluted into sea water buffered at pH 8.2 display forward motility (treatment 2); sperm diluted at pH 7.3 require transfer to pH 8.2 (step 2) in order to trigger motility (treatment 3).

An asterisk signifies little breakdown of sperm morulae in undiluted coelomic fluid (treatment 1).

example, treatment 2). Sperm morulae incubated in the absence of fatty acid at pH 8.2 (treatment 1) did not break down and release individual spermatozoa, either prior to, or following, the centrifugation step; therefore, no forward motility was observed.

In some instances, however, sperm morulae, once removed from the influence of coelomic fluid, appeared to undergo spontaneous breakdown when diluted in sea water. This often occurred in individuals that were close to the time of natural spawning and that had been held under laboratory conditions (at 10 °C, see Materials and methods) for a long period. Using spermatozoa from such an individual, a second series of experiments, identical in design to that described above (in Table 2), was carried out. However, since sperm morulae breakdown occurred spontaneously, there was no requirement for spermatozoa to be incubated with fatty acid (or other agents) during step 1. The results of a typical experiment are shown in Table 3. In step 1 of the experiment, spermatozoa were diluted 1:100 in ASW buffered at the two pH regimes on either side of the threshold pH necessary to trigger motility. After 1 h, only sperm morulae that had been both diluted and incubated at pH 8.2 had fully broken down (spontaneously) and given rise to motile spermatozoa (treatment 2, Table 3). If incubated at pH 7.3, sperm morulae breakdown occurred but the spermatozoa released were not motile. Only following centrifugation and resuspension of these spermatozoa at an appropriate pH (treatment 3, step 2) was full forward motility observed. Sperm morulae held in undiluted coelomic fluid (treatment 1) showed no evidence of breakdown to free spermatozoa.

Reversibility of the pH-dependent stimulation of forward motility

The ability of motile spermatozoa to swim under conditions in which they had previously been held immotile was determined in an additional experiment. A sample of motile spermatozoa (in ASW at pH 8.2) was split into three and, following centrifugation, the resulting sperm pellets were resuspended in one of the following media: (1) coelomic fluid (with a measured pH of 7.3), (2) ASW at pH 7.3, or (3) ASW at pH 8.2. The

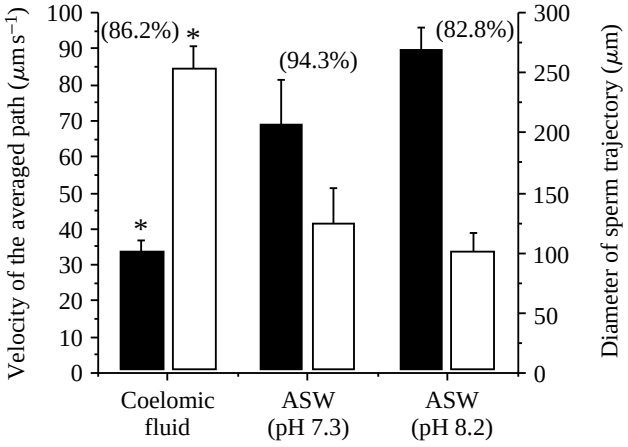

Fig. 4. Mean velocity of the averaged path (μm s^{-1}) (filled bars) and mean diameter of sperm trajectory (μm) (open bars) for spermatozoa transferred from ASW (pH 8.2) into coelomic fluid (pH 7.3), ASW at pH 7.3, or ASW at pH 8.2. Values are mean ± S.E.M. (N=30). Figures in parentheses indicate the percentage motility for each treatment. An asterisk denotes a statistically significant difference with respect to other treatment groups (P<0.001).

subsequent values for percentage motility, mean VAP and mean diameter of sperm trajectory were measured and the results are shown in Fig. 4.

In all instances, the percentage motility was not significantly affected by a change in the composition of the medium (Fig. 4): motile spermatozoa transferred into either coelomic fluid or ASW at a pH value identical to that of coelomic fluid remained fully motile. This result tends to rule out the possibility of specific motility-inhibiting factors present within coelomic fluid. It also suggests that, because spermatozoa remain motile when transferred into coelomic fluid or ASW at pH 7.3, the pH-dependent stimulation of motility is not easily reversed. In addition, following the transfer of spermatozoa to more acidic pH conditions, there was also a subsequent change in their behaviour, both in terms of their VAP and in terms of the diameter of sperm trajectory. Motile spermatozoa, transferred into coelomic fluid (pH 7.3), had a significantly lower mean VAP and showed an increase in mean diameter of trajectory ($P<0.001$). In other words, spermatozoa transferred into coelomic fluid had a tendency to swim more slowly and in straighter trajectories than did spermatozoa retained in ASW, at either pH value. The mean diameter of sperm trajectory for spermatozoa transferred into coelomic fluid was 254.9±14.99 μm; this was, over the area covered by a ×25 objective through which these observations were made, almost equivalent to a straight line. Spermatozoa transferred to ASW at a pH of 7.3 (equivalent to that of coelomic fluid) also had a tendency to display lower values for mean VAP as well as increased values for the mean diameter of sperm trajectory, although these values were not statistically different from those observed for spermatozoa maintained at pH 8.2.

The effect of changes in the extracellular pH on sperm behaviour, over a much wider range of pH values than that shown in Fig. 4, was further investigated in an experiment the results of which are shown in Fig. 5. Motile spermatozoa (obtained following SMF-induced spawning and subsequently maintained at pH 8.2) were diluted into ASW buffered at pH values ranging from 6.0 to 9.5. At each pH value, spermatozoa were allowed to equilibrate at the new conditions for 5 min, after which sperm motion characteristics were videotaped. From Fig. 5, it can be seen that alterations in the extracellular pH can significantly alter the behaviour of motile spermatozoa: at more acidic pH values, spermatozoa followed slower straighter trajectories, whereas at more alkaline pH values, they swam in small circular trajectories (approximately 20 μm in diameter). Sperm velocity was also influenced by extracellular pH, although the change was less acute than that observed for the trajectories. As the extracellular pH increased from 6.0 to 8.0, sperm velocity increased steadily, although at pH values of 9.0 and above sperm velocity was markedly reduced. This change is concurrent with a high degree of flagellar waveform asymmetry, leading to trajectories of very small diameter. Fig. 6 shows typical trajectories of spermatozoa under four of the pH conditions shown in Fig. 5 and further illustrates the nature of these changes upon the asymmetry of the flagellar beat characteristics. In addition, the results of these experiments further support the inference that the pH-dependent acquisition of forward motility is not easily reversed.

The action of pharmacological agents

In a further experiment, sperm morulae were incubated with a dose of ammonium chloride (10 mmol l^{-1}) or quinacrine (100 μmol l^{-1}) sufficient to stimulate their

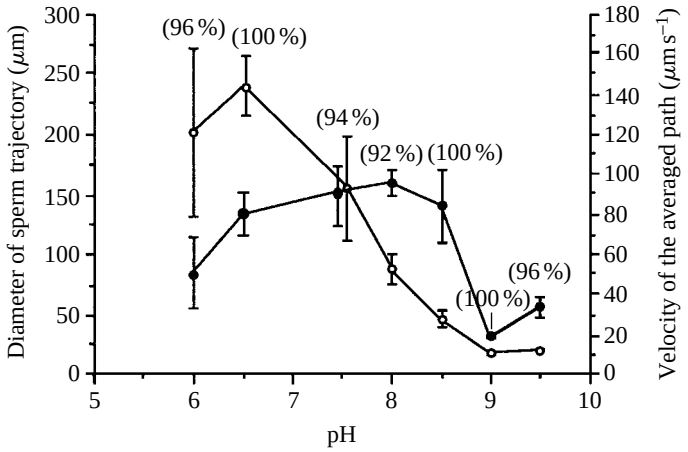

Fig. 5. Mean velocity of the averaged path ($\mu m\,s^{-1}$) (filled circles) and mean diameter of sperm trajectory (μm) (open circles) of spermatozoa transferred from ASW (pH 8.2) to ASW at a variety of pH values between pH 6.0 and 9.5. Figures in parentheses indicate the percentage motility at each pH value. The data are derived from detailed analysis of sperm trajectories from a minimum of 20 cells at each pH value. Bars show S.E.M.

breakdown. Incubations were made at pH 7.3 and 8.2, since previous experiments identified these values as being either side of the threshold for acquisition of forward motility. At both these pH values, ammonium chloride and quinacrine stimulated both the breakdown of sperm morulae and the acquisition of forward motility in individual spermatozoa. In the presence of the pharmacological agents under these conditions, the processes of sperm morula breakdown and the acquisition of forward motility in the released spermatozoa could not be separated.

The behaviour of motile spermatozoa under these conditions was also examined following analysis of videotapes. Photographs showing typical sperm trajectories following incubation with these substances, under the two pH conditions, are shown in Fig. 7; quantitative data derived from a detailed analysis of these videotapes are also summarised in Table 4. The data show that at pH 7.3, the mean values for VAP were significantly higher ($P<0.02$) than those measured in spermatozoa incubated at pH 8.2, although there were no significant differences with respect to the diameter of sperm trajectories ($P>0.05$). Also of note is that, at a given pH value, the mean values for VAP and the diameter of sperm trajectories for spermatozoa incubated with either substance were not significantly different from each other ($P>0.05$), although there was a tendency for spermatozoa incubated in quinacrine to swim in trajectories of greater curvature than those observed in ammonium chloride (cf. Fig. 7A,B and C,D). In addition, at pH 8.2, the mean values for VAP of spermatozoa incubated with ammonium chloride or quinacrine were significantly lower than those measured in spermatozoa following spawning ($P<0.01$), although this latter observation was made on the spermatozoa from a different individual and may not, therefore, be entirely comparable. Following spawning and

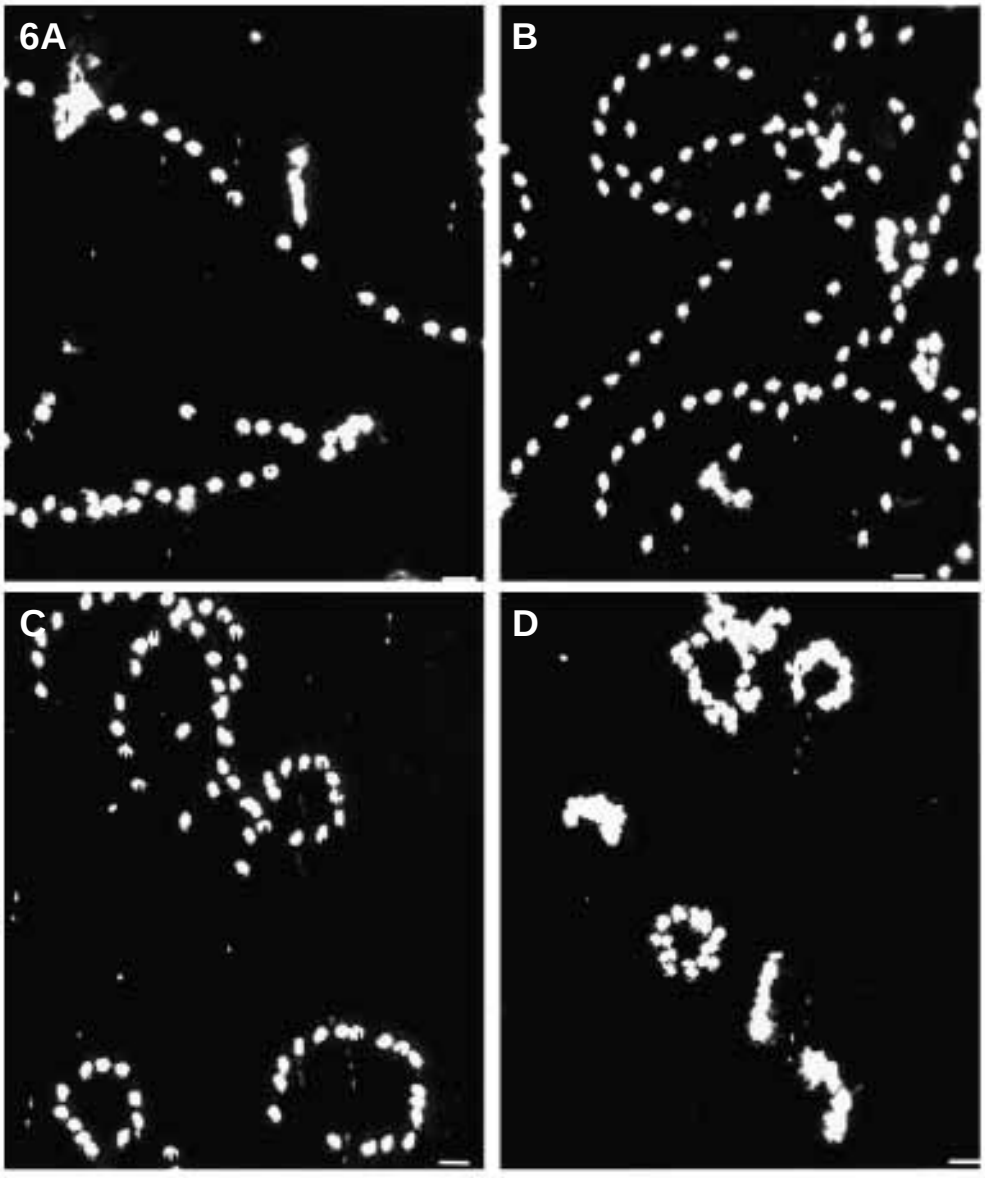

Fig. 6. Photographs showing the typical trajectories followed by spermatozoa incubated under the pH conditions shown in Fig. 5. The photographs were taken 5 min following the introduction of spermatozoa to each of the pH conditions: (A) pH 6.5, (B) pH 7.5, (C) pH 8.5 and (D) pH 9.5. Each figure shows typical trajectories followed by spermatozoa under each condition; the time interval between successive positions of the sperm head is 0.02 s, and 2 s of sperm motion are shown in each case. Scale bars, 10 μm.

dilution of spermatozoa at pH 7.3, forward motility was not stimulated (see above), so no comparisons at this pH value can be made.

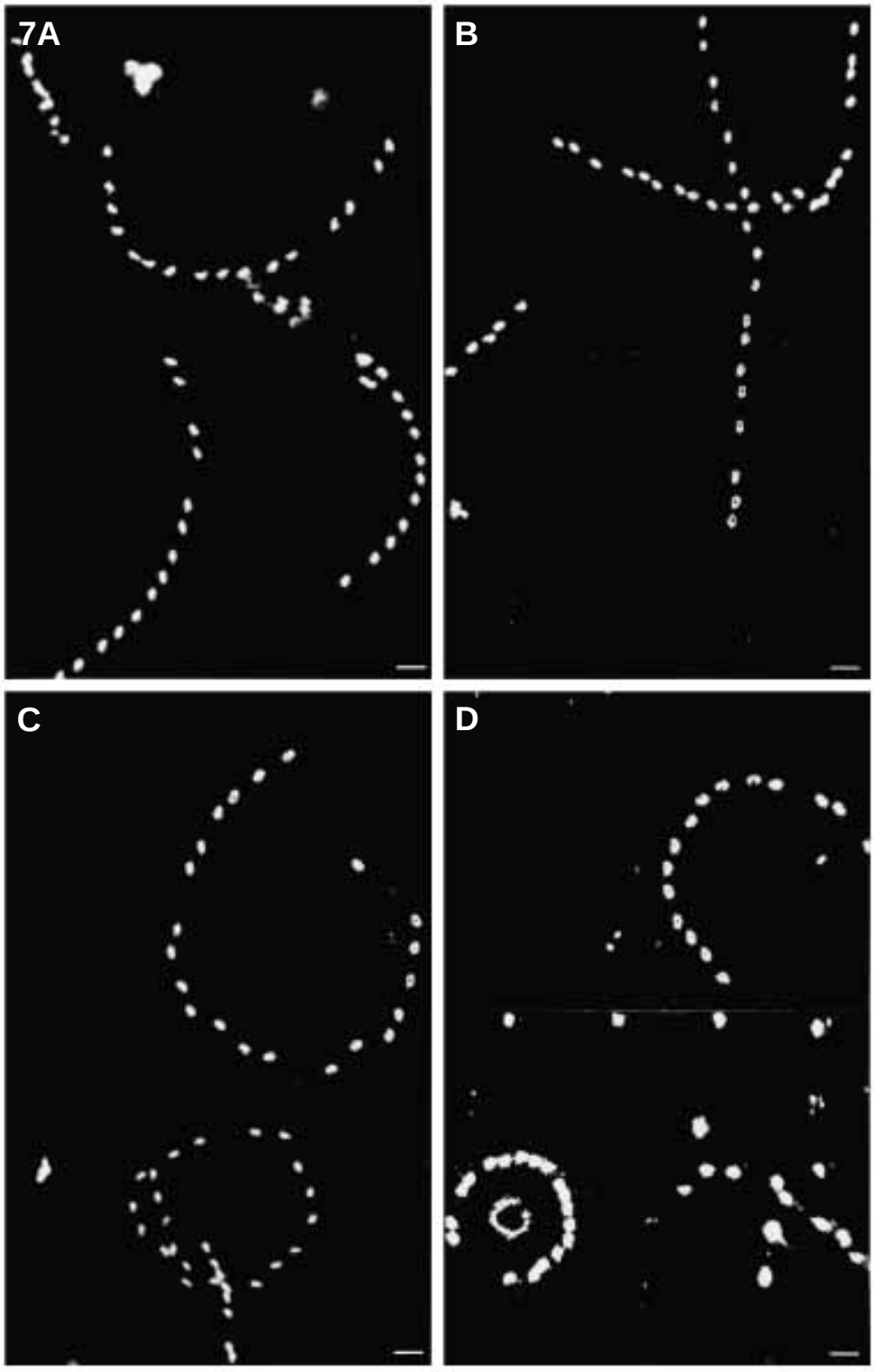
7A
B
C
D

Fig. 7. Photographs showing typical trajectories followed by spermatozoa incubated with the pharmacological agents of ammonium chloride (10 mmol l^{-1}) and quinacrine (100 μmol l^{-1}) under the two pH conditions shown in Table 4: (A) ammonium chloride at pH 7.3; (B) ammonium chloride at pH 8.2; (C) quinacrine at pH 7.3; (D) quinacrine at pH 8.2. In each case, 2 s of sperm motion are shown, with the time interval between successive positions of the sperm head being 0.02 s. Each photograph shows a single video frame to illustrate typical sperm motion, with the exception of D, which is a composite of two separate video frames. Scale bars, 10 μm.

Discussion

Comprehensive studies concerning the mechanisms responsible for the stimulation and control of sperm motility have been carried out in only a limited number of invertebrate groups. In marine invertebrates, such studies have been restricted to the tunicates (Brokaw, 1984, 1987), several species of molluscs (for example, Kadam and Koide, 1990) and crustaceans (Clapper and Brown, 1980). It is, however, within the Echinodermata that significant advances have been made, with most attention focusing on the sea urchins. Through the sea urchin model, we now understand in some detail the specific mechanisms involved in the control of motility acquisition (see Trimmer and Vacquier, 1987, for a review), energy production and metabolite channelling (Tombes and Shapiro, 1985; Quest and Shapiro, 1991), as well as the factors involved in axonemal regulation (Brokaw, 1987) and many biophysical aspects of sperm movement (for reviews, see Gibbons, 1982, 1989).

In view of the phylogenetic position of the echinoderms within the deuterostomes, it is of interest to compare the known mechanisms of sperm activation in this group with that of a protostome group such as the polychaetes (see Willmer, 1990, for a detailed discussion of phylogenetic relationships). In addition, the ecological importance of species such as *A. marina*, and the fact that their commercial value has increased the

Table 4. *Motility characteristics of spermatozoa following incubation with the quinacrine and ammonium chloride at two pH values*

	pH 7.3		pH 8.2	
Treatment	VAP (μm s^{-1})	Diameter of Sperm trajectory (μm)	VAP (μm s^{-1})	Diameter of sperm trajectory (μm)
Quinacrine	94.6±0.83*	103.5±11.10	75.4±6.05	96.0±23.16
Ammonium chloride	108.9±8.8*	136.9±15.60	73.7±7.70	141.0±22.50
Spawning	–	–	116.7±7.4†	111.4±14.60†

Following incubation with both quinacrine (100 μmol l^{-1}) or ammonium chloride (10 mmol l^{-1}), sperm motility is triggered at both pH 7.3 and pH 8.2. Sperm motility following spawning and dilution of sperm at pH 7.3 is not triggered (see Fig. 3).

Data shown are means ± S.E.M. for at least 30 measurements made under each condition.

*Indicates a significant difference from the same treatment at a different extracellular pH (P<0.02).

†Indicates a significant difference from other treatments (P<0.01).

VAP, velocity of the averaged path of sperm motility.

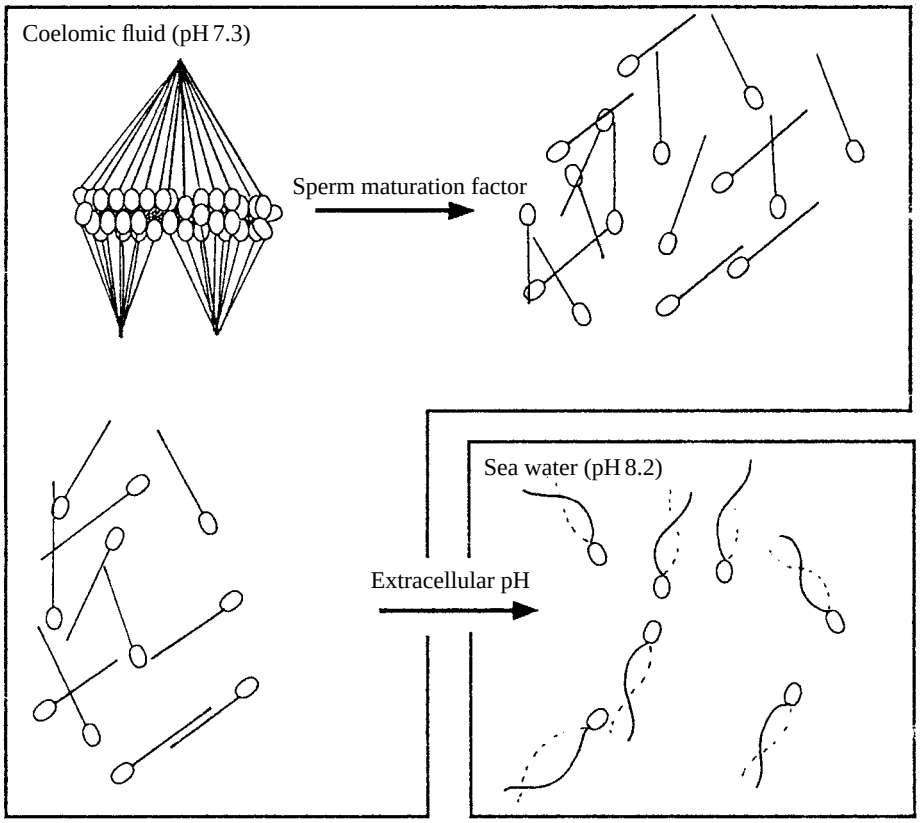

Fig. 8. Schematic diagram of the suggested hypothesis for the two-step mechanism of sperm activation in *Arenicola marina*. The first step involves the sperm maturation factor (SMF)-induced breakdown of sperm morulae; the second step involves the stimulation of flagellar beating by a change in extracellular pH as the spermatozoa are diluted in sea water. The diagram is not to scale.

interest in their potential aquaculture, mean that studies of the sperm activation mechanisms of species such as *A. marina* are becoming of interest in their own right.

It is clear that the mechanisms controlling sperm activation in *A. marina* are more complex than those in many other invertebrates that employ external fertilisation, including sea urchins. As outlined in the Introduction, the spermatozoa of *A. marina* develop within a syncitium, the ‘sperm morula’, and spermatozoa must first be liberated from this before being released from the body cavity (for a review, see Bentley and Pacey, 1992). The experiments detailed in this paper clearly show that the processes of sperm morula breakdown and acquisition of sperm motility can be separated under conditions that mimic those encountered *in vivo* during natural spawning. The endocrine action of SMF serves to release spermatozoa from morulae; these spermatozoa then acquire forward motility following dilution in sea water upon release. A schematic diagram outlining this hypothesis is shown in Fig. 8.

Although the hypothesis shown in Fig. 8 clearly establishes a two-stage dissociation–activation mechanism ultimately resulting in the release of spermatozoa and stimulation of flagellar beating, it is appropriate to question whether this rather simple experimental model accurately reflects the more complex spawning mechanism *in situ*. During natural spawning, for example, the animals remain within their burrows in the sand with the spermatozoa released from the gonopores being ejected from the burrow (possibly assisted by muscular contractions of the spawning animal) to lie at the surface of the beach sediment; such sperm pools (illustrated in Fig. 2 of Bentley and Pacey, 1992) are subsequently disrupted by the incoming tide. It is clearly of interest to establish the precise moment of sperm motility acquisition under such conditions since, if the spermatozoa present in sperm pools are immotile until they are mixed with the water from the incoming tide, then this may represent an adaptive advantage not seen in other externally fertilising species, such as sea urchins, which release spermatozoa directly into the water column. In this respect, the sperm biology of other species which live in a similar biotope and share a similar spawning mechanism (such as *Nepthys hombergii*; see Bentley *et al.* 1984) also require a detailed investigation.

In the case of *A. marina*, the release of spermatozoa from the relatively acidic environment of the coelom appears to be a central event in the stimulation of flagellar beating necessary for the acquisition of forward motility. In sea urchins, a similar correlation has been observed and it has been shown that Na^+/H^+ countermovements (Christen *et al.* 1982, 1983; Lee *et al.* 1983), which stimulate an increase in intracellular pH (pHi), accompany dilution. It is thought that this may be sufficient to stimulate motility directly by bringing the pHi of sperm to within the optimal range for flagellar dyenin ATPase activity (pH 8.0), thus inducing axonemal function (Christen *et al.* 1982). From the experiments outlined in the present paper it is not possible to conclude that a change in intracellular pH is important for the acquisition of sperm motility, since changes in extracellular pH are not necessarily reflected by changes in intracellular pH. It is therefore clear that specific experiments are necessary to establish whether such mechanisms are utilised in the spermatozoa of *A. marina*. It is, however, interesting to note that a pharmacological agent as simple as ammonium chloride, known to stimulate an increase in the pHi of many cell types (see Roos and Boron, 1981; Thomas, 1984), is able to stimulate both the breakdown of sperm morulae and the acquisition of forward motility. Also, the fact that demembranated sperm models can be reactivated by the addition of MgATP, with no requirement for other factors such as cyclic AMP, suggests that the sperm activation mechanism of *A. marina* may be relatively simple, like that employed in sea urchin spermatozoa.

The ability of ammonium chloride (and quinacrine) to stimulate sperm morulae breakdown has been reported elsewhere (Bentley, 1986; Pacey and Bentley, 1993). It is interesting to note from the experiments described in the present paper that both agents can induce a breakdown of sperm morulae and stimulate sperm motility at pH 7.3; a condition where *in vivo*, spermatozoa usually remain immotile even when they have been exposed to SMF and spermatozoa have been released from sperm morulae. In addition, the action of these substances does not appear to follow the mechanism proposed in Fig. 8: the acquisition of sperm motility cannot be separated from the step that stimulates

the breakdown of sperm morulae. It is possible that a rise in pHi, stimulated by either ammonium chloride or quinacrine, is responsible for the induction of flagellar beating in individual spermatozoa still in the morulae and that this results in the mechanical disruption of morulae through the forces generated from beating flagella rather than through the action of these substances *via* a mechanism common to the action of SMF. It has been noted previously that the kinetics of sperm morula breakdown, as stimulated by these agents, appear to differ from those observed with more natural agonists such as SMF (Pacey and Bentley, 1993): the action of quinacrine and ammonium chloride is much more rapid than that of SMF.

Details concerning the precise action of SMF, and how it functions to stimulate the breakdown of sperm morulae, are at present unavailable, but probable mechanisms are being investigated. It is apparent, however, that the sperm maturation step, and the endocrine involvement of SMF, distinguishes *A. marina* from most other invertebrate groups. The tendency for spermatozoa to develop within syncitia is a feature almost exclusive to the Annelida, although it is far from universal in this class. Probable functions for such structures have been suggested and include providing a sink for residual cytoplasm during the division of spermatogonia and helping to synchronise the development of individual spermatozoa within the syncitium (Olive, 1983; Sawada, 1984). In spite of this and phylogenetic differences (outlined above), it is clear that there may be much common ground between the sperm activation mechanisms of *A. marina* and that of the sea urchin model system. As stated previously (Clapper and Brown, 1980), since most flagellated spermatozoa are based around the same axonemal structure, there may be common mechanisms to initiate and control the motility of spermatozoa in diverse animal groups (for a review see Gagnon, 1991). Studies need to be carried out in many different phyla in order to establish whether this is so.

Ultrastructural studies (Bentley and Pacey, 1989; Pacey and Bentley, 1992*a*) have shown that the spermatozoa of *A. marina* and sea urchin share many morphological features: the sperm type of both conforms to the primitive pattern outlined by Franzén (1956). In addition, the description of sperm movement outlined here indicates similarities in behaviour of motile spermatozoa from different species. When swimming in sea water, motile spermatozoa of *A. marina* display a phenomenon known as intermittent swimming, which has also been described in the spermatozoa of several species of sea urchins (Gibbons, 1980; Gibbons and Gibbons, 1980) as well as in the tunicate *Ciona intestinalis* (Brokaw, 1987). A detailed analysis of this behaviour has been carried out and will form the basis of a subsequent report (Pacey *et al.* 1994).

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