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Ester A. Serrão · Lena Kautsky · Susan H. Brawley

Distributional success of the marine seaweed *Fucus vesiculosus* L. in the brackish Baltic Sea correlates with osmotic capabilities of Baltic gametes

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Abstract To understand the unique success of the marine seaweed *Fucus vesiculosus* L. (Phaeophyceae) in the brackish Baltic Sea, the performance of gametes from Baltic [4.1–6.5‰S (Salinity)] and marine populations was studied. Sperm from Baltic *F. vesiculosus* swam with a path velocity of c. 30–110 µm/s and could fertilize eggs in waters of salinities from 4 to 33‰S. In their natural water, Baltic sperm were not negatively phototactic, unlike marine sperm in seawater; this should decrease the sperm:egg concentration at the seafloor and reduce the likelihood of polyspermy. Marine (Iceland, Sweden) sperm in seawater had a path velocity of c. 80–100 µm/s, but performed poorly and could not fertilize eggs in natural or artificial Baltic water ≤6‰S; therefore, Baltic populations have adapted or acclimated to their brackish habitat. Baltic populations appear better adapted to their natural low salinities because, even after culturing Baltic and marine individuals in water from both the Baltic (6.5‰S) and the marine Skagerrak (21‰S), Baltic sperm were in both cases still able to swim and fertilize eggs at lower salinities (4‰S) than marine sperm; fertilization never occurred between marine gametes at 4–6‰S. However, *F. vesiculosus* acclimates to some salinities, since sperm from Baltic and marine males that had been cultured at 21‰S swam better (higher velocity, proportion that were motile and/or linearity) in marine salinities (21–33‰S) than when they were cultured at 6.5‰S. The effects of salinity on sperm motility and fertilization were osmolar rather than due to specific ionic requirements, over the tested range. The osmolalities (< c. 100 mmol/kg) at which fertilization success of Baltic gametes decreases nearly to zero correspond to the osmolality of Baltic water at the northernmost limit of distribution of *F. vesiculosus* in the Baltic Sea. Therefore, the present range of *F. vesiculosus* in the Baltic appears to correspond to the osmotic tolerance of the gametes. Very small natural or anthropogenic increases in ambient osmolality would be likely to cause a substantial expansion of this species into the inner Baltic.

Key words Baltic biogeography · Fertilization ecology · Osmotic stress · Polyspermy · Sperm motility

E. A. Serrão (✉)[1] · S. H. Brawley
Department of Plant Biology and Pathology,
5722 Deering Hall, University of Maine,
Orono ME 04469-5722, USA
fax: (207) 581-2969; e-mail: io20109@maine.maine.edu

Lena Kautsky
Department of Botany, Stockholm University,
S-106 91 Stockholm, Sweden

Permanent address:
[1] Unidade de Ciências e Tecnologias dos Recursos Aquáticos,
Universidade do Algarve, Campus de Gambelas,
P-8000 Faro, Portugal

Introduction

The Baltic Sea is the largest body of brackish water in the world, and its well-known history of large-scale salinity changes makes it an unique model system for ecological and evolutionary studies of adaptation to environmental change. The Baltic Sea had a wide connection to the Atlantic about 7500–3000 BP (the "*Littorina* Sea" period) and appears to have had a rich marine biota (Ignatius et al. 1981). Salinity has decreased since that time due to the narrowing connection to the Atlantic, and this may have caused high rates of evolutionary change (Russell 1985). These rapidly changing conditions have led to the disappearance of most of the Baltic's marine biota, but some species appear to have adapted by selective survival of the more resistant phenotypes, resulting in evolutionary divergence from their North Atlantic counterparts (e.g., Russell 1985, 1988; Rietema 1991). Among the numerous fucoid species present in the North Sea, *Fucus vesiculosus* is the only one that presently occurs over most of the Baltic coast. Belt-forming populations of *F. vesiculosus* occur throughout the Baltic to salinities of c. 4‰S [(Salinity) e.g., Kautsky et al. 1992], with occasional reports of isolated and sparse populations in even lower salinities [i.e., as low as 2‰S (see Wærn 1952)]. The only other species of Fucaceae that occur in

the Baltic Sea are present only to the Bay of Kiel [*F. evanescens* (Schueller and Peters 1994)] or to the island of Gotland [*F. serratus* (Wærn 1952)]. *F. vesiculosus* is the dominant macrophytic seaweed in the Baltic Sea, and it has an essential role in the ecosystem (e.g., Kautsky et al. 1992; Committee 1995).

The reproductive success of dioecious species with external fertilization is dependent on the environmental conditions that the gametes encounter upon release. Reproductive adaptations are thus among the key adaptations to the low salinities of the Baltic that the surviving marine biota, such as *F. vesiculosus*, may have made. Changes in salinity may decrease the success of fertilization by decreasing the viability of the gametes, or they may raise the level of polyspermy, which is lethal to the embryo. Fucoid eggs block the entrance of more than one sperm (i.e., polyspermy) through a rapid depolarization of the egg based on an influx of sodium ions (Brawley 1991), as do many other marine eggs (Jaffe and Gould 1985). In low-sodium seawater, the rate and duration of this depolarization in marine *F. vesiculosus* are slower, and polyspermy increases (Brawley 1987, 1991). In estuarine populations of *F. ceranoides*, fertilization occurs in the high-salinity portion of the tidal cycle, thereby avoiding a potential increase in polyspermy caused by low Na^+ (Brawley 1992). In the Baltic Sea, however, there are populations of *F. vesiculosus* living at very low [Na^+], and, because the Baltic Sea is non-tidal, there are no windows of higher salinity in which fertilization can occur. Despite these conditions, natural polyspermy levels are low (Serrão et al. 1993), and this could be explained by the action of factors that decrease the probability of many sperm fertilizing an egg simultaneously, such as if sperm motility were poor at low salinities. Low salinities do decrease the motility of marine sperm from several species of *Fucus* and decrease fertilization in laboratory experiments (Kniep 1907). Sperm motility may be affected by salinity through (1) osmolality effects, as in many animals (e.g., Morisawa and Suzuki 1980; Morisawa 1994 and references therein) and/or through (2) ionic requirements, since *Fucus* sperm require external sodium and calcium in order to swim (O'Toole and Brownson 1992).

Using *F. vesiculosus* as a model system for studies of the Baltic biota, we have addressed the following questions:

1. How is sperm motility from marine and Baltic populations affected by the range of salinities in which *F. vesiculosus* occurs?
2. How does the success of fertilization in Baltic populations vary within that range of salinities?
3. Do those salinities affect sperm motility and fertilization through effects of osmolality or specific ionic requirements?
4. Are Baltic populations reproductively acclimated or adapted to brackish water?
5. Does brackish water alter sperm motility in ways that reduce the likelihood of polyspermy?
6. Is the present distributional limit of *F. vesiculosus* in the Baltic Sea related to reproductive failure below that salinity?

Our data suggest that Baltic populations are better adapted to reproduce in their brackish habitat than marine populations, and that the present limit of distribution of Baltic *F. vesiculosus* is set by osmotic tolerances of gametes.

Methods

Effects of the natural range of salinities on motility and fertilization

Artificial waters (AWs) were prepared to represent the major inorganic ions of seawater [33‰S: 0.450 M NaCl, 0.030 M $MgCl_2$, 0.016 M $MgSO_4$, 0.009 M $CaCl_2$, 0.010 M KCl, and 0.0025 M $NaHCO_3$ (Brawley 1987)] and brackish water of the central Baltic [6‰S: 0.079 M NaCl, 0.0043 M $MgCl_2$, 0.0049 M $MgSO_4$, 0.0021 M $CaCl_2$, 0.0016 M KCl, and 0.0011 M $NaHCO_3$ (S. Andersson personal communication, based on tables from Remane and Schlieper 1971; Riley and Skirrow 1975)]. Other AWs were prepared by appropriate dilution of 33‰S AW (i.e., 21, 16, 10 and 8‰S) and 6‰S AW (i.e., 4 and 3‰S); use of Baltic AW at 6‰S (with slightly different ionic proportions than 33‰S AW) was necessary because AWs of ≤6‰S prepared from dilution of 33‰S artificial seawater would have concentrations of Ca^{2+} in particular, that would be much lower than in natural Baltic water. An AW of 3‰S was included to evaluate reproductive failure at a salinity lower than the ambient salinity (≈4‰S) at the present limit of continuous populations of *F. vesiculosus* in the Gulf of Bothnia.

Gametes of *F. vesiculosus* are produced in receptacles, which are reproductive structures located at the extremities of the thalli. Receptacles from males release antheridia containing sperm (64 sperm/antheridium), and female receptacles release oogonia containing eggs (8 eggs/oogonium); external fertilization occurs between free sperm and eggs. The sperm have two flagella inserted laterally and oriented in opposite directions (Manton and Clarke 1956). They exhibit chemotaxis towards fucoserraten, the pheromone secreted by the eggs (Müller and Gassmann 1978).

Receptacles of *F. vesiculosus* were collected from two Baltic and two marine populations (Fig. 1) in mid-late July 1993. The Baltic populations were from Askö (≈6.5‰S) and Järnäs (≈4.1‰S), and the marine populations were from Tjärnö (Swedish west coast, ≈15–33‰S, 30‰S at the time of collection) and Reykjavik (Iceland, ≈35‰S). Receptacles were blotted dry and stored at 4°C for 1–2 days (Askö), 3–8 days (Järnäs), 10 days (Iceland) or 3 days (Tjärnö) prior to release. Gametes were released by placing receptacles into the treatment seawaters (14°C; 100 µmol photons m^{-2} s^{-1}) either directly from storage (Baltic gametes) late in the afternoon (which is the natural period of release for Baltic gametes), or after treatment by standard techniques [marine gametes (Quatrano 1980)]. The treatments were AWs of 3, 4, 6, 10, 16, 21 and 33‰S, and each population's natural water was also included as a control, except seawater from Iceland, which was unavailable. Motility of sperm from Iceland was also quantified at 8‰S, to better understand the sharp difference observed between 6 and 10‰S. Three males were used as replicates per treatment. Treatments were monitored for sperm release approximately every 10 min. Maximal sperm motility was observed within about 15 min after sperm were released from antheridia. At this time, sperm were diluted (1:9, sperm suspension: treatment solution) to prevent artifactual increase of salinity or osmolality due to the potential release of solutes by the receptacles, and 1 ml of the diluted suspension was transferred into a phytoplankton chamber (24 mm diameter, 3 ml total capacity). Sperm in the chamber were filmed within 2 min of removal from the culture chamber, using a video-camera (Ikegami Tsushinki Comp. Ltd.) attached to an in-

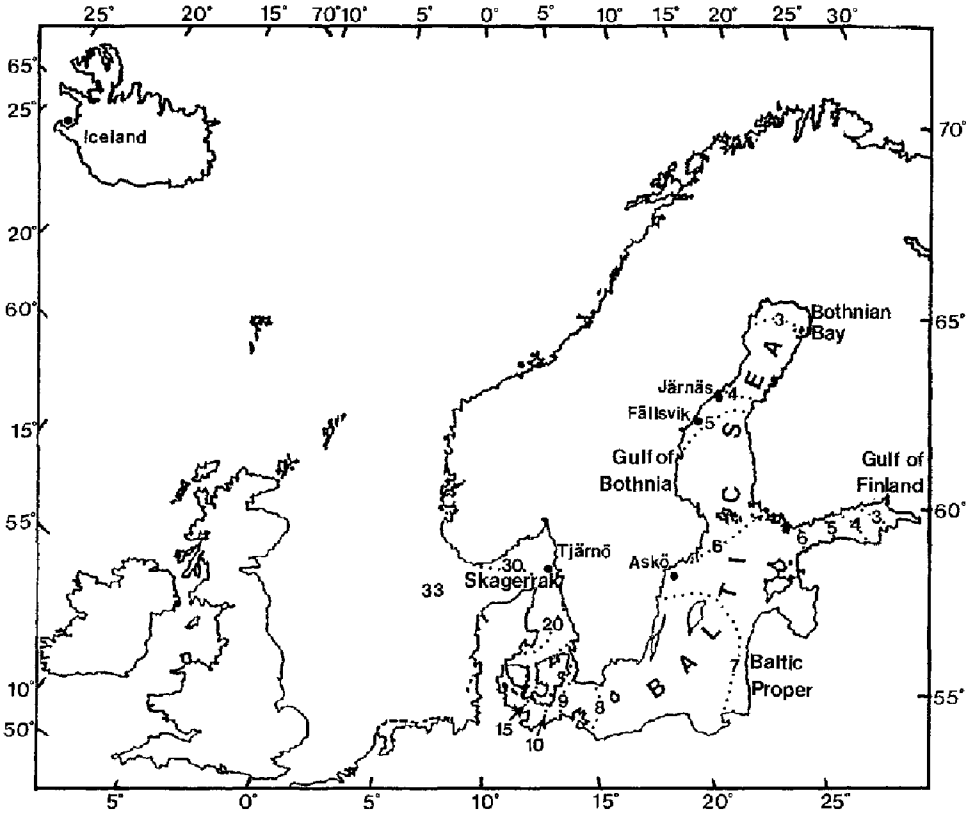

Fig. 1 Map showing sites where receptacles of *Fucus vesiculosus* were collected. The approximate locations of the isohalines (in ‰S) are indicated (after Andersson et al. 1992)

verted microscope (Olympus CK2) and a video recorder (Panasonic NV-FS 200HQ). The images were taken from areas in the middle of the chamber in order to avoid edge artifacts.

Sperm velocity and path linearity were determined from the video-tapes using the Hamilton Thorn internal visual optical system (IITM-IVOS, Hamilton-Thorn Research, Beverly, Mass.), which determines numerous parameters of the motion of cells. The bright-field option was used for all analyses, and for each scan, sperm were tracked for 30 frames at a frame acquisition rate of 30 Hz. Multiple scans were performed per sample, until a minimum of 20 sperm tracks had been analyzed. Each sperm track was checked for accuracy after every scan, using the playback and edit options of the HTM-IVOS. This permitted tracks that did not represent the path of a single sperm (a result sometimes observed at higher sperm densities) to be eliminated from the analysis. Three measures of velocity and one measure of linearity were chosen from the motility parameters measured by the HTM-IVOS system (described in Anonymous 1992). Progressive velocity (VSL) is the linear distance between the beginning and end of a track divided by the elapsed time (see figure in Results). Track speed (VCL) is the sum of the distances between successive positions for a given cell during the acquisition of the track, divided by the elapsed time (VSL<VCL). Path velocity (VAP, velocity of the average path) is the total distance along the average path for each cell divided by the elapsed time, where the average path is defined by the smoothed average position of the center of brightness of the cell (5-point running average); this reduces the effect of lateral head displacement and gives an average cell path velocity. VCL (track speed) is strongly dependent on the frame acquisition rate, since the track length depends on the time between frames. Due to smoothing, VAP is less dependent on frame rate and provides a better estimate of the velocity of a cell's average path; therefore, this is the parameter we discuss in the results. The degree of departure from linear progression was measured by linearity (LIN), which is the ratio between VSL and VCL. Linearity is used here to quantify differences in the swimming behavior of sperm before contacting an egg (when close to an egg, the swimming pattern of the sperm is altered by pheromone, Maier and Müller 1986). The proportion of motile sperm in each treatment water was determined visually rather than with the HTM-IVOS due to the presence of particles of similar size and brightness as the sperm in the video-images. These determinations were performed using a VCR (Panasonic, AG2500) to classify a minimum of 50 sperm per treatment as motile or non-motile; these were chosen using a random numbers table and a grid over the television screen.

Gametes from populations at Askö (in 1993 and 1994) and at Tjärnö (in 1994) were used to study the influence of salinity on the success of fertilization. The treatments were the same ones used in the studies of sperm motility, that is: AWs of 3, 4, 6, 10, 16, 21 and 33‰S, and controls, which were fertilizations in natural water from the Baltic at Askö (6.5‰S) and Järnäs (4.1‰S) for Askö gametes (1993), or from Askö and Tjärnö (30‰S) for gametes from Askö and Tjärnö (1994). Sperm were always released directly into the treatment solutions. In 1993, eggs from Askö were released in natural water from Askö; whereas, in 1994, eggs from Askö and Tjärnö were released directly into the treatment solutions. However, in AWs of 3 and 4‰S, most oogonia from Askö females did not release eggs. Thus, eggs released at 6‰S were transferred to AWs of 3 and 4‰S to perform these treatments. Similarly, most oogonia from Tjärnö females did not release eggs in AWs of 6, 4 and 3‰S; eggs released in AWs of 8 and 10‰S had to be transferred to these salinities. Intact oogonia were removed from eggs by pouring the suspensions through 105 µm nitex (Small Parts, Inc., Miami Lakes, Fla.) into fresh treatment AWs. Fertilizations were performed at a 1000:1 sperm:egg ratio in 3 ml (1993) or 5 ml (1994) of each AW. Two (1993) or three (1994) pairs of males and females were used as replicates for all treatments. Fertilization success was quantified c. 14 h later by staining eggs in a 0.0001% solution of calcofluor white, a fluorescent dye that stains the cell wall; secretion of the cell wall in *Fucus* begins a few minutes after fertilization (Evans et al. 1982; Brawley and Bell 1987). The proportion of fertilized eggs was determined by counting a sample of at least 100 eggs from each replicate.

Hierarchical (nested) three-factor ANOVAs (salinity×population nested within sea, where the factor sea has two levels: marine

or Baltic) were used to test for differences between the means of each dependent variable (path velocity, linearity and proportion that were motile). One-way ANOVA was used to test for salinity effects on success of fertilization. Velocity data were log-transformed and proportions were arcsin transformed to improve homogeneity of variances, as checked by Cochran's test, and normality. Tukey tests were used for all posthoc multiple comparisons of means. For all of the experiments described in this paper, it will only be indicated if P was above or below a 0.05 pre-selected level of significance when referring to the results of one or several tests.

Acclimation experiments

F. vesiculosus was collected in May 1995 from the marine Skagerrak (Tjärnö, ≈15–33‰S, 21‰S at time of collection; stored 1 day at 0°C during transport to Askö) and from the Baltic Sea (Askö, ≈6.5‰S; no storage). Individuals from both populations were cultured together (males and females separated) for 16–18 days in aquaria (2/treatment, 8 l each) with natural water from Tjärnö (21‰S in May 1995) or from Askö (6.5‰S). The eight aquaria were placed in a large flow-through aquarium in a culture chamber (18:6 h light:dark, 16×10^{15} quanta s^{-1} cm^{-2}) to maintain temperatures at ambient levels (c. 7°C) with Askö water during the study. The waters inside the eight aquaria were changed every 6 days. After culture for 16–18 days, receptacles were collected, blotted dry, and placed immediately in the treatment waters: AWs of 1, 2, 3, 4, 5, 6, 7, 8, 10, 16, 21, 33‰S and controls, which were natural waters from Askö (6.5‰S) and Tjärnö (21‰S). AWs of 6 and 33‰S were those described above; intermediate salinities were achieved by diluting 33‰S with 6‰S, and AWs of <6‰S were prepared by dilution of 6‰S. Sperm from three males (i.e., 3 replicates/treatment) were prepared, filmed, and analyzed with the IVOS system as described above. Velocity data were log-transformed to homogeneize variances (as checked with Cochran's test), proportions (proportion of sperm that were motile and linearity) were arc-sin transformed to normalize distributions. Three-way factorial ANOVAs (populationxculturing salinityxtesting salinity) and Tukey tests were used to test for differences between means.

For fertilization assays, sperm were released in each treatment water (as for motility assays); whereas, eggs were released in natural water from Askö and Tjärnö and then transferred to the treatments. Intact oogonia were removed from eggs by pouring the suspension through 105 µm nitex (Small Parts, Inc., Miami Lakes, Fla.) into fresh Askö or Tjärnö waters. Eggs released in Askö water (6.5‰S) were transferred to 1, 2, 3, 4, 5, 6, 7 and 8‰S, and eggs released in Tjärnö water (21‰S) were transferred to all treatments at salinities ≥10‰S. Fertilizations were performed in glass dishes with 5 ml of treatment water at ca. 2000:1 sperm:egg ratio. The proportion of fertilized eggs was determined ca. 10–12 h later, by counting at least 100 eggs per treatment, using calcofluor white (as described above). Data were arc sin transformed before statistical analyses by three-way factorial ANOVA and Tukey tests.

Ionic versus osmolar effects on motility, fertilization and phototaxis

Artificial waters (AWs) were prepared to correspond to the ionic compositions of seawater (≈33‰S) and natural waters in the Baltic Sea at Askö (≈6.5‰S), Fällsvik (≈4.3‰S), and in areas of the Bothnian Bay (≈2.2‰S). Water of 2.2‰S was used because a few individuals of *F. vesiculosus* have been reported to occur in isolated populations that have salinities this low during at least part of the year (see Wærn 1952), although 4‰S is the present limit of continuous populations (e.g., Kautsky et al. 1992). AWs of 33 and 6‰S were prepared as above. AW of 4.3‰S is 0.053 M NaCl, 0.0033 M $MgCl_2$, 0.0030 M $MgSO_4$, 0.0015 M $CaCl_2$, 0.0014 M KCl, and 0.0011 M $NaHCO_3$ (recipe based on direct analysis of a sample of Fällsvik water from July, 1993, by the Environmental Chemistry Laboratory, University of Maine). AW of 2.2‰S is 0.0241 M NaCl, 0.0012 M $MgCl_2$, 0.0018 M $MgSO_4$, 0.0006 M $CaCl_2$, 0.0006 M KCl, and 0.0008 M $NaHCO_3$ (a dilution of the Järnäs recipe, based on direct analysis of a sample from July 1993, by the E.C.L., University of Maine). Other AWs were also prepared with the same concentrations of cations as the AWs of 2.2 and 4.3‰S, but the osmolality of these AWs was increased with *N*-methyl-glucamine chloride (*N*-methyl-glucamine is an impermeant cation, Brawley and Bell 1987) to 180 and 980 mmol/kg (the same osmolality as the 6 and the 33‰S AWs, because the sperm were known to swim well at these salinities). Osmolalities were measured with a vapor pressure osmometer (Wescor 5500, Logan, Ut.), and salinities were measured with a portable induction salinometer (Beckman RS9, Fullerton, Calif.).

Sperm from Fällsvik (≈4.3‰S) were used in this experiment. The eight treatments were artificial waters of salinity and osmolality, respectively: (1) 2.2‰S, 70 mmol/kg, (2) 4.3‰S, 125 mmol/kg, (3) 6.0‰S, 180 mmol/kg, (4) 33.0‰S, 980 mmol/kg, (5) composition of cations as in 2.2‰S, osmolality increased with *N*-methyl-glucamine chloride to 180 mmol/kg, (6) composition of cations as in 2.2‰S, osmolality increased to 980 mmol/kg, (7) same composition of cations as in 4.3‰S, osmolality increased to 180 mmol/kg, (8) same composition of cations as in 4.3‰S, osmolality increased to 980 mmol/kg. Three males were used as replicates. Sperm were released, filmed, and analyzed using the HTM-IVOS system and VCR, as described above.

Fertilizations were performed using gametes from Fällsvik (≈4.3‰S) in the same AWs as for the motility assays. Eggs were released in media of similar osmolality as in the treatments, to avoid potential effects of large osmolality changes. Intact oogonia were removed from eggs with 105 µm nitex before inseminating eggs in 10 ml of seawater, at a 1000:1 sperm:egg ratio. Eggs were stained with calcofluor white (as above) approximately 9 h after fertilization. The proportion of fertilized eggs was determined by counting a sample of at least 100 eggs in each replicate of the treatments.

Different combinations of ionic concentration and osmolality were tested using one-way ANOVA for all four dependent variables (path velocity, linearity, proportion motile, and fertilization). A two-way factorial ANOVA was not used, despite two factors being involved, because some of the orthogonal treatments were not possible (e.g., it is impossible to have the cationic composition of a marine seawater while keeping the osmolality as low as in a seawater of 2.2‰S). Proportions were arc sin transformed, and Tukey tests were used for post hoc comparisons, as described above.

Effects of salinity on sperm phototaxis were studied with Baltic and marine males by releasing sperm at separate times from male receptacles from Askö (≈6.5‰S) and Fällsvik (≈4.3‰S) in their natural waters, and from Maine (USA, ≈32‰S) in their natural water and in AWs of 33‰S, a 9‰S dilution, and an AW with the same ionic composition of the 33‰S AW but with all NaCl replaced by *N*-methyl-glucamine chloride. The water containing sperm was transferred into small beakers and stirred gently to make the initial distribution of sperm homogeneous within the beaker. A unilateral light gradient (fiber optics or fluorescent tubes) was established horizontally across the beakers and changes in the distribution of sperm during the following hour were noted.

Results

Effects of the natural range of salinities on motility and fertilization

Lower salinities reduced the velocity (Fig. 2a) of sperm from *F. vesiculosus* and reduced the proportion of sperm that were motile (Fig. 2b), but the responses differed between populations ($P<0.05$). Sperm from marine (Tjärnö and Iceland) populations of *F. vesiculosus* were unable to

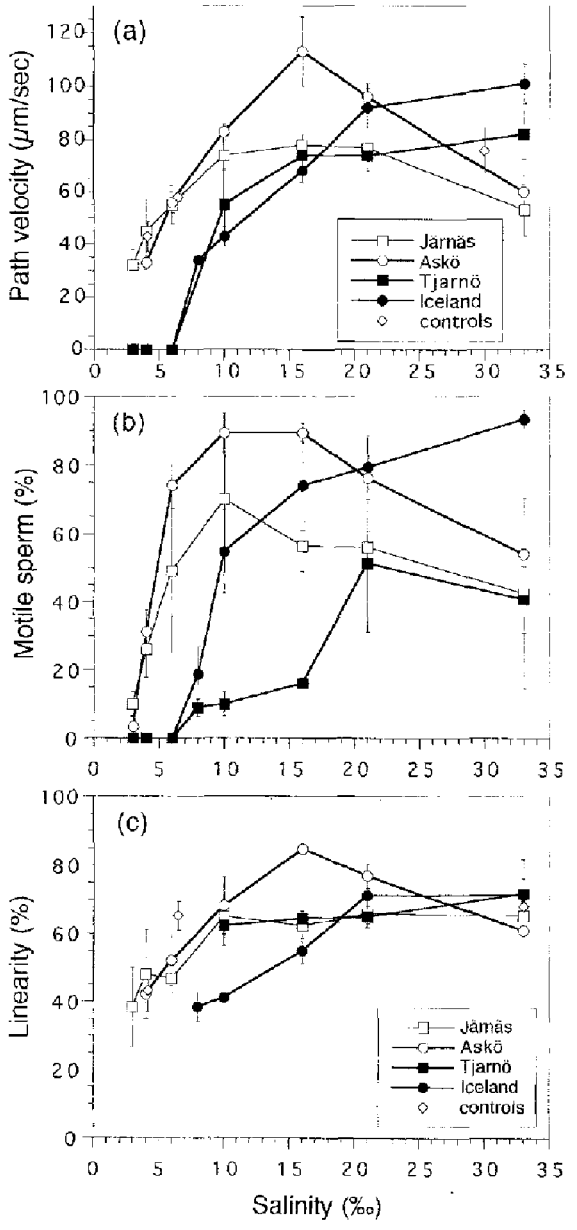

Fig. 2a–c Motility of *F. vesiculosus* sperm from two marine (Iceland and Tjärnö) and two Baltic (Askö and Järnäs) populations in artificial waters of salinities from 3 to 33‰S: **a** velocity of the average path (mean±SE), not determined for Askö sperm at 3‰S because too few sperm were able to swim; **b** proportion of motile sperm (mean±SE); legend for **2b** (not shown) is the same as in **2a** and **2c**; **c** linearity (mean±SE). The *diamonds* represent controls: sperm from Järnäs (≈4.1‰S), Askö (≈6.5‰S), and Tjärnö (≈30‰S, in July 1993) in their natural waters; Icelandic seawater was not available

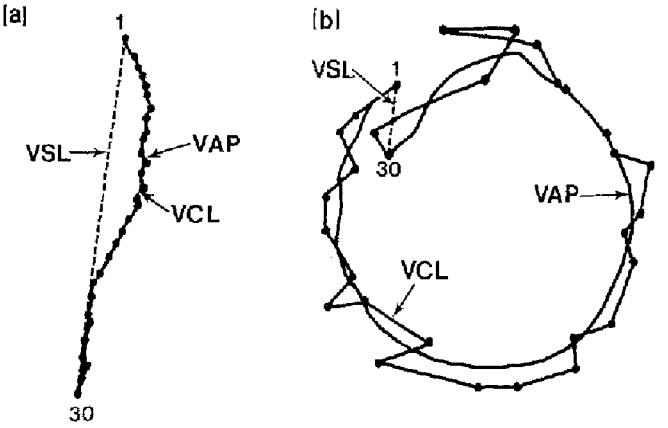

Fig. 3a, b Tracks of sperm from *F. vesiculosus* followed for 1 s at 30 Hz (30 frames per second, the two tracks are at different scales). **a** Track with high linearity, common above 10‰S in all sperm. **b** Track with low linearity (tumbling sperm); often observed at 3–4‰S for Baltic sperm and at 8‰S for marine sperm. For each track, the 30 *dots* represent the positions of the center of brightness of the sperm in each frame; the *lines* represent the path used to calculate each velocity: *VCL* is velocity of the center of brightness, measured from the distances between successive positions; *VAP* is velocity of the average path, where the average path is defined by a 5-point running average; *VSL* is the progressive velocity, measured from the straight line between the initial and final positions of the sperm (detailed explanation in Methods)

swim at Baltic salinities below 6‰S. Path velocity of Icelandic sperm at 8–10‰S was reduced ($P<0.05$) to about half the speed observed in seawater (Fig. 2a). A small proportion (c. 3–10%) of Baltic (Järnäs and Askö) sperm swam at 3‰S (Fig. 2b), although it was not possible to measure average path velocity of Askö sperm at that salinity, because only one of three males released sperm that could swim, and even these were too few for analysis. However, at the salinities predominant in the central Baltic Sea (4–8‰S), Baltic sperm swam well (c. 55 μm/s), especially when compared to sperm from marine populations (Fig. 2a). Indeed, no significant differences were found between the velocities of Baltic sperm in their natural brackish water and in seawater ($P>0.05$). Baltic sperm swam at maximal velocities (c. 80–110 μm/s) at 10–21‰S, that is, at salinities that are intermediate between those of the Baltic Sea and the Atlantic Ocean, and these velocities were equivalent ($P>0.05$) to the velocities of Icelandic and Tjärnö sperm in seawater. The proportion of sperm that were motile was variable from male to male (Fig. 2b), but varied with salinity in a similar pattern as the velocity of the average path, the main difference being the low percentage of sperm from Tjärnö that were motile below 20‰S.

Lower salinities appeared to affect linearity of the sperm path (Fig. 2c) less than velocity or the proportion of sperm that were motile. The effects of salinity on linearity differed between populations ($P<0.05$). The lower the linearity, the higher the departure of the movement of the sperm from a straight line path (see Methods). Thus, if all sperm had perfectly straight paths, linearity would be 100%. Sperm from Askö showed maximum linearity at 16‰S, the same salinity at which they achieved maximal velocity, decreasing ($P<0.05$) towards higher and lower salinities. At and below 6‰S, where only Baltic sperm swam, the linearity of both Järnäs and Askö sperm was lower ($P<0.05$). At 3 and 4‰S, motile sperm often oscillated or tumbled in circles (Fig. 3), instead of swimming in any particular direction. Tumbling was also observed in some marine (Icelandic) sperm at their lower

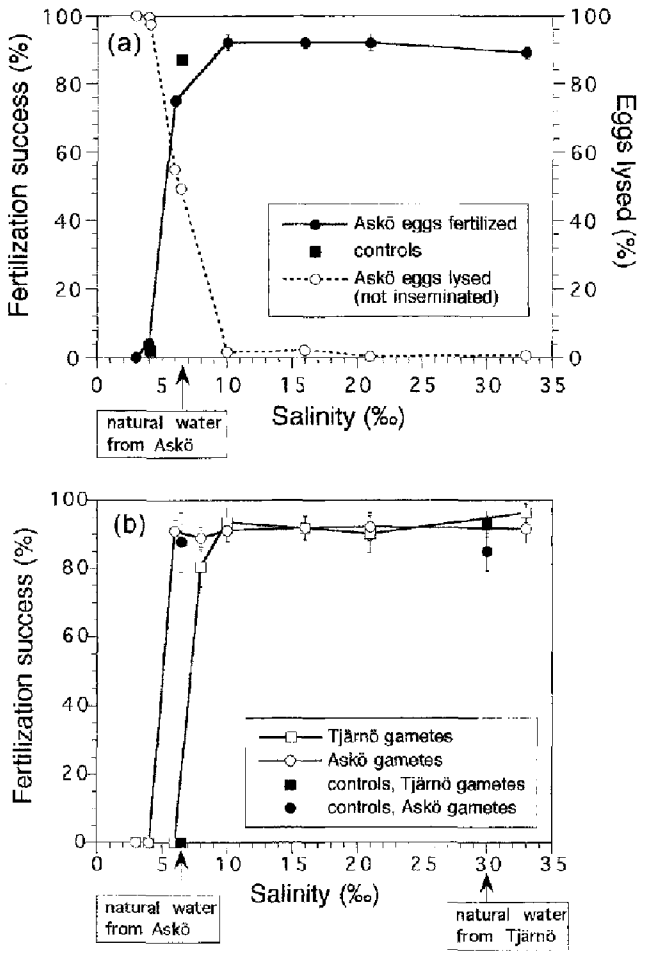

Fig. 4a, b Proportion of fertilized eggs (mean ±SE) of *F. vesiculosus* 14 h after insemination in artificial waters of 3–33‰S. **a** Gametes from Askö (Baltic Sea, ≈6.5‰S). *Square symbols* represent controls: fertilizations in natural waters from the Baltic at Järnäs (≈4.1‰S) and Askö (≈6.5‰S). The proportion of uninseminated eggs from Askö that had lysed after 14 h of exposure to the treatment salinities is also shown. **b** Gametes from Askö (Baltic Sea, ≈6.5‰S) and from Tjärnö (Swedish west coast, ≈15–33‰S). Dark symbols represent controls for Askö (*circles*) and Tjärnö (*squares*): fertilizations in natural waters from the Baltic at Askö (≈6.5‰S) and from the North Sea at Tjärnö (≈30‰S, in June 1994)

limit of motility (8‰S), and their linearity was low at this salinity and 10‰S, increasing (P<0.05) with salinity up to 21‰S, and being similar (P>0.05) at 21–33‰S. The linearity of the paths of sperm from Tjärnö was similar at all salinities between 10 and 33‰S.

Fertilization success (Fig. 4a,b) was affected by salinity (P<0.05). The proportion of eggs from Askö (≈6.5‰S) fertilized at 3, 4‰S and in natural water from Järnäs (≈4.1‰S) was very low or zero, and strongly differed (P<0.05) from the high success of fertilization at all salinities between 6 and 33‰S (Fig. 4a, b). At 3–4‰S, both inseminated and uninseminated eggs from Askö swelled rapidly and most burst (Fig. 4a, data for inseminated eggs not shown). At 6‰S and in natural Askö water (≈6.5‰S), fertilization protected a large proportion of Askö eggs from lysis, which occurred in approximately 50% of uninseminated eggs (Fig. 4a) but in less than 20% of the inseminated eggs (data not shown). Sperm did not fertilize Tjärnö eggs at 3–6.5‰S, whereas fertilization success was high at 8–33‰S (Fig. 4b).

Acclimation experiments

Sperm from Askö males cultured in marine water from the Skagerrak (Tjärnö, 21‰S) did not behave like marine sperm at 4–15‰S (Fig. 5a, b, c); their performance was similar (P>0.05) to that from males cultured in water from Askö (6.5‰S). The acclimation treatments only affected the motility of Baltic sperm at marine (21–33‰S) salinities, in which Baltic sperm swam faster (P<0.05) when cultured in Tjärnö water than when cultured in Askö water (Fig. 5a). Culturing Askö sperm at 21.5‰S also improved (P<0.05) the proportion of sperm that were motile at 21–33‰S (Fig. 5b), whereas, the proportions of motile sperm at all tested salinities below 21‰S were similar (P>0.05) between males cultured in waters from Tjärnö and from Askö.

Culturing marine (Tjärnö, ≈15–33‰S) males in Baltic (Askö, 6.5‰S) water had no effect (P>0.05) on sperm velocity (Fig. 5a) but reduced (P<0.05) the proportion of motile sperm (Fig. 5b) at nearly all salinities and also reduced (P<0.05) the linearity (Fig. 5c) of the sperm paths at marine salinities. Between 21 and 33‰S, most Tjärnö sperm from males cultured in Askö water swam slower than those cultured in Tjärnö water (21‰S), but these differences were not significant; thus, we cannot distinguish if (1) they were caused by chance or (2) if they were real differences but our tests lacked the power to detect them. At the lowest salinity (5‰S) in which at least a few sperm from Tjärnö swam, sperm from males cultured in Tjärnö water swam faster (P<0.05) than from males cultured in Askö water. However, this does not reflect a real difference in velocity; the lower velocity from males cultured in Askö water is due to one of the three replicate males having sperm that were completely immotile at 5‰S, thus lowering the average velocity of this treatment. The sperm from the other two replicates swam at a similar velocity to sperm from males cultured in Tjärnö water. The proportion of sperm from Tjärnö that were motile (Fig. 5b) was higher (P<0.05) for males cultured in Tjärnö water than in Askö water except at 33‰S (P>0.05) and where very few or none of the sperm from Tjärnö swam in any of the treatments, below 6‰S (P>0.05). The low proportion of Tjärnö sperm that were motile when males had been cultured at 6.5‰S may be a consequence of the fact that marine *F. vesiculosus* dies during prolonged periods of culture in low salinity water (see discussion); individuals from Tjärnö cultured in Askö water were starting to decay. Most sperm from Tjärnö males cultured in Tjärnö water swam at 8–21‰S, but a smaller (P<0.05) proportion were motile below 8‰S and at 33‰S (Fig. 5b). The small proportion (c. 55%) of sperm that were able to swim at 33‰S seems surprising for a marine species, but a similar trend for Tjärnö versus Icelandic sperm was found earlier (see Fig. 2b). The linearity (Fig. 5c) of the

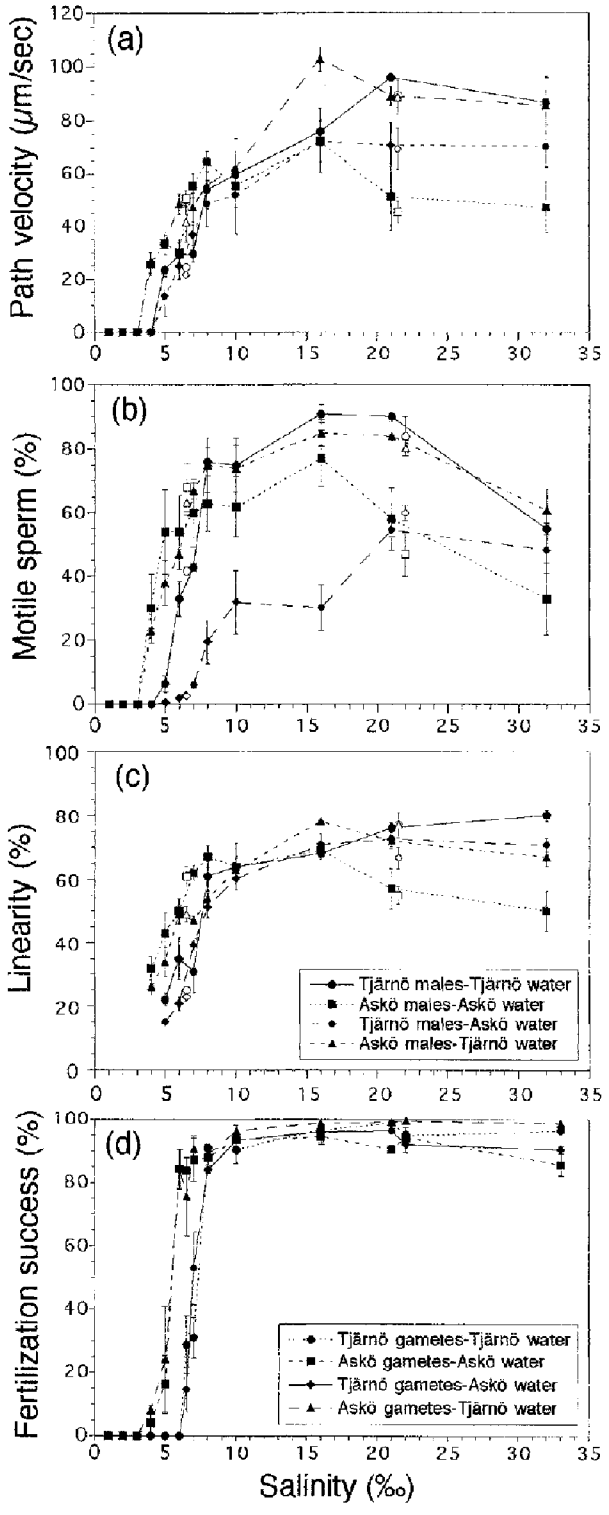

Fig. 5a–c Sperm motility (means±SE) and **d** fertilization success (means±SE) of *F. vesiculosus* from Baltic (Askö) and marine (Tjärnö) individuals cultured in Baltic water from Askö (6.5‰S) and in seawater from Tjärnö (21‰S), in artificial waters of salinities from 1 to 33‰S. **a** velocity of the average path; at 1–5‰S symbols for Askö males cultured in Askö water (*squares*) overlap symbols for Askö males cultured in Tjärnö water (*triangles*); **b** proportion of motile sperm; **c** linearity. Symbol legends for **5a** and **5b** (not shown) are the same as in **5c**. The *open symbols* indicate controls (for the treatment corresponding to symbol's shape) in natural water from the Baltic at Askö (6.5‰S) or in seawater from Tjärnö (21‰S, drawn at 21.5‰S for clarity)

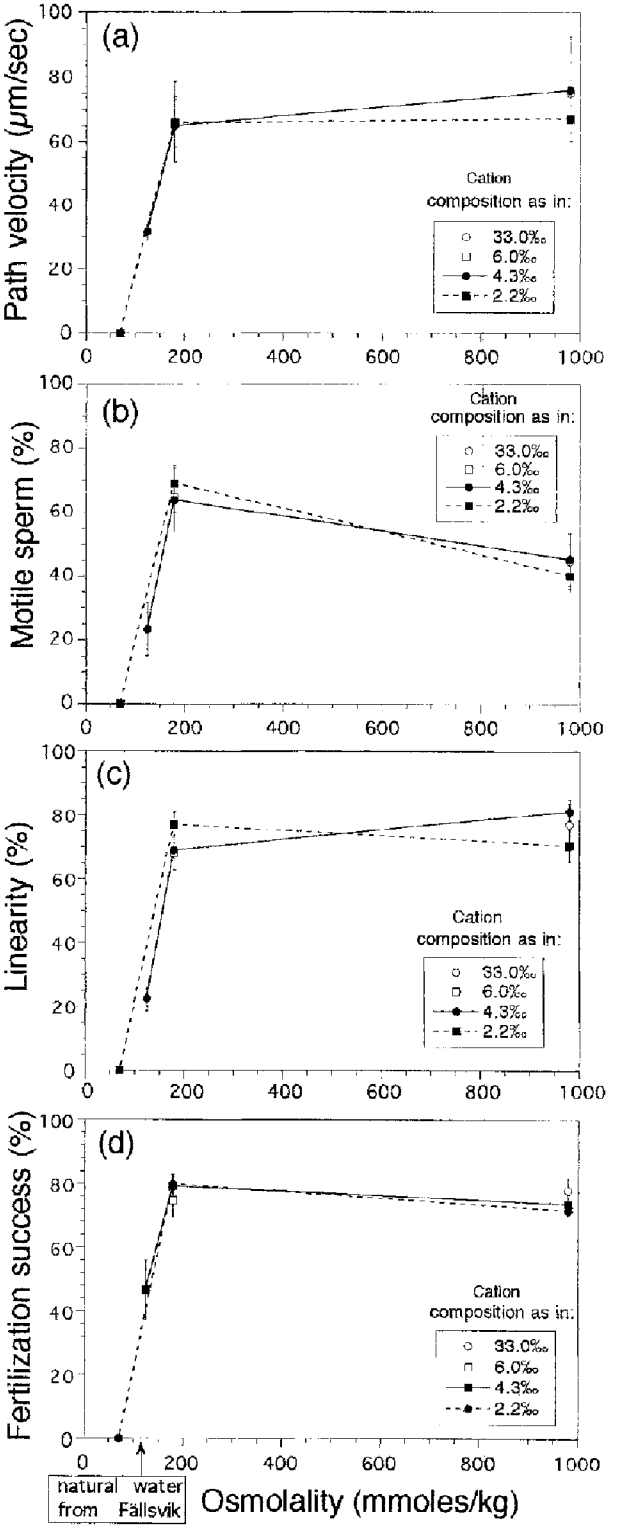

Fig. 6a–c Sperm motility (means±SE) and **d** fertilization success (means±SE) of *F. vesiculosus* from Fällsvik (Baltic Sea, 4.3‰S) in artificial waters with various combinations of salinity and osmolality (listed in Methods). Osmolar supplementation with *N*-methyl-glucamine chloride relieved inhibition by low salinity on sperm motility and fertilization

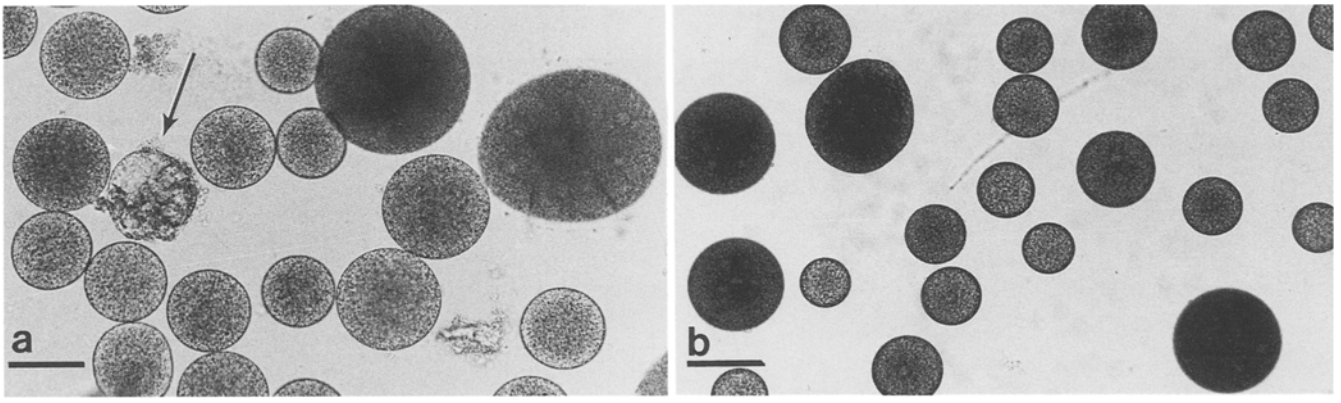

Fig. 7a, b Eggs and oogonia from Baltic *F. vesiculosus* at Fällsvik (≈4.3‰S) immediately after release in artificial waters with osmolalities of **a** 125 mmol/kg (≈osmolality of 4.3‰S water) and ionic composition of 4.3‰S (see Methods) and **b** 980 mmol/kg (≈osmolality of seawater by supplementation with *N*-methyl-glucamine chloride) and ionic composition of 4.3‰S artificial water. Note lysed egg (*arrow*) in **a**. *Bar* = 100 µm

paths was reduced ($P<0.05$) at the highest salinities tested (significant differences at 21.5–33‰S for Tjärnö sperm, 16–33‰S for Askö sperm) when males from either population were cultured in Askö water. Sperm from Askö swam with higher ($P<0.05$) linearity than sperm from Tjärnö at all salinities below 8‰S, independently of the acclimation treatments.

Fertilization success (Fig. 5d) in experiments with Baltic or marine gametes was not affected ($P>0.05$) by the salinity of the water in which individuals had been cultured (6.5 or 21‰S), except at 33‰S, where c. 10–20% fewer eggs were fertilized in experiments with gametes from individuals cultured in Baltic water (6.5‰S). Fertilization with marine (Tjärnö) gametes was only possible above 6‰S, and was high (>80%) at 8–33‰S; whereas, with Baltic (Askö) gametes it was high at 6–33‰S, and at 4‰S, a small proportion of the eggs were fertilized. At salinities ≤6‰S, most unfertilized eggs had burst prior to assay.

Ionic versus osmolar effects on motility, fertilization and phototaxis

Sperm from Fällsvik (≈4.3‰S; 125 mmol/kg) did not swim in AW of 70 mmol/kg osmolality (2.2‰S), but swam well if shed into 2.2‰S AW supplemented to an osmolality of 180 or 980 mmol/kg with *N*-methyl-glucamine chloride (Fig. 6a, b, c). Fällsvik sperm swam at c. 30 µm/s (Fig. 6a) in AW with the same ionic composition as Fällsvik water (≈4.3‰S), but the path velocity increased to c. 70 µm/s if sperm were shed into 4.3‰S water supplemented with *N*-methyl-glucamine chloride to an osmolality of 180 or 980 mmol/kg (i.e., the osmolalities of 6 and 33‰S waters). In these treatments, the velocity of the sperm was similar to that in unamended waters of 6 and 33‰S. Thus, at this range of salinities (2.2–33‰S), sperm velocity was dependent on osmolality ($P<0.05$), not the composition of Na^+, Ca^{2+}, K^+ or Mg^{2+} in the AWs.

Fertilization success (Fig. 6d) was affected by osmotic changes in the media ($P<0.05$). Most eggs from Fällsvik (≈4.3‰S) were fertilized when inseminated in AWs of 180 and 980 mmol/kg (corresponding in osmolality to 6 and 33‰S, respectively). The average levels

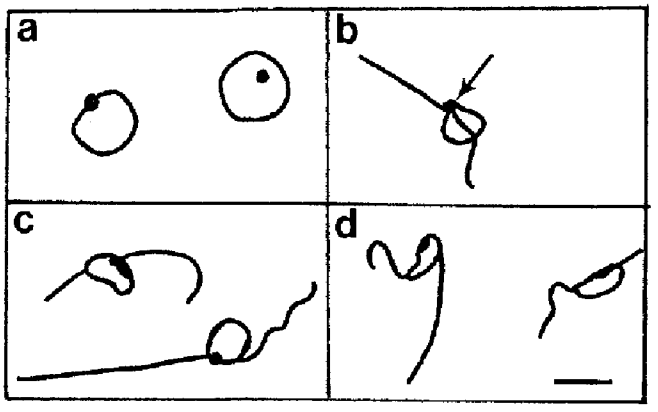

Fig. 8a–d Sperm (line drawings traced from photomicrographs) from Baltic *F. vesiculosus* at Fällsvik (=4.3‰S) immediately after release in artificial waters of **a** 2.2‰S (i.e., 70 mmol/kg), **b** 4.3‰S (i.e., 125 mmol/kg), and **c**, **d** ionic composition of 4.3‰S and osmolality supplemented (see Methods) to **c** 180 mmol/kg (=osmolality of 6‰S water) and **d** 980 mmol/kg (=osmolality of seawater). Portions of the flagella are not shown because they were out of the plane of focus. *Arrow* indicates eyespot. *Bar* = 10 µm

of fertilization did not differ at these osmolalities ($P>0.05$), but the proportion of eggs fertilized at 70 mmol/kg differed from that at 125 mmol/kg and both differed from all other treatments ($P<0.05$). At 4.3‰S (osmolality of 125 mmol/kg), fewer than 50% of the eggs were fertilized. No eggs were fertilized at 2.2‰S. Low osmolalities (70–125 mmol/kg) caused an increase in the volume of both the eggs (Fig. 7) and sperm (Fig. 8), and most eggs that were not fertilized burst at these salinities.

Fällsvik sperm were pear-shaped cells, similar to the shape of marine sperm, at the osmolalities of marine (980 mmol/kg) and Askö (180 mmol/kg) water, but were slightly rounded at their normal salinity (4.3‰S), and lost flagella at 2.2‰S (Fig. 8). The eyespot of Baltic

sperm was abnormally positioned in their natural water, and under these conditions sperm from Baltic (Fällsvik and Askö) populations lacked negative phototaxis in their respective natural waters (4.3‰S for Fällsvik, 6.5‰S for Askö). Marine sperm were not negatively phototactic in 9‰S AW, but swam to the darkest regions of the light gradient when in 33‰S AW or in a low sodium AW with the same osmolality as a 33‰S AW.

Discussion

This study demonstrates adaptive features of populations of a marine species that has had to withstand a recent change of habitat to one that is nearly freshwater. Our results suggest that Baltic gametes of *F. vesiculosus* are partially adapted to their natural low salinities, since sperm have good motility and laboratory fertilization is successful at the salinities in which Baltic populations live, ones in which marine populations perform poorly even after being cultured in Baltic water. However, at most of the salinities in which *F. vesiculosus* lives in the Baltic (c. 4–7‰S), sperm have larger volumes and rounder shapes than marine sperm, and this appears to cause a displacement of the sperm's eyespot; this may account for the lack of negative phototaxis of sperm in brackish water. Acclimation (phenotypic change) may be responsible for the differences between Baltic and marine gametes in salinities approaching seawater, since culturing males from either population in natural waters from the Baltic (6.5‰S) or from the marine Skagerrak (21‰S) respectively decreased or improved sperm motility at c. 21–33‰S. In artificial waters of different osmotic and cationic compositions, sperm motility and fertilization were dependent on osmolality, suggesting that osmolality-dependent effects on sperm and/or eggs may be important in establishing the northernmost limit of *F. vesiculosus* in the Baltic Sea. If reproduction is impaired, a population cannot establish itself, no matter how tolerant to salinity the adults might be, because *F. vesiculosus* does not reproduce parthenogenetically or vegetatively (Brawley and Bell 1987).

Whether *F. vesiculosus* is acclimated or adapted to its Baltic habitat is an important question. The hypothesis that Baltic sperm are adapted (genetically) to swim at lower salinities has not been falsified by our acclimation experiments since Baltic sperm (Askö) were able to swim and fertilize eggs at lower salinities (4–6‰S) than marine sperm (Tjärnö) both when cultured in Askö (6.5‰S) and in Tjärnö (21‰S) water. Performance of sperm from Askö populations at low salinities was unaffected by culturing in Tjärnö water. However, Baltic *Fucus* acclimates to higher salinities; culturing adults in 21‰S improved sperm motility and fertilization success at 21–33‰S, relative to controls cultured at 6.5‰S. We and others (Bäck et al. 1992; our unpublished work) were previously unsuccessful in attempts to culture marine individuals in Baltic water. When we cultured reproductive marine individuals from Tjärnö in 1994 in water from the Baltic at Askö (6.5‰S, 10°C), the reproductive structures decayed after a week, and, shortly thereafter, the entire thallus decayed. We were later (1995) able to keep marine individuals from Tjärnö in culture in Askö water for 2.5 weeks. A significant difference may have been the lower ambient salinity of Tjärnö water (21‰S in 1995 versus 30‰S in 1994) when individuals were collected for the acclimation experiments, and/or the lower culture temperature (c. 7°C) in 1995 compared to 1994. Even under these conditions, the Tjärnö individuals cultured in 1995 had started to decay at the end of the 2.5-week period of culture in Askö water. The 2.5-week-long culture period seems long enough for complete maturation of gametes under acclimation conditions, because peaks of gamete release occur with a weekly or fortnightly periodicity in association with lunar phases (Andersson et al. 1994; Serrão et al., in press).

Some marine species can be partially acclimated to lower salinities. For example, fertilization in sea urchins cultured at 31‰S, was maximal in full strength seawater and decreased abruptly below 15‰S to zero at 10‰S; however, it could be improved at the lower salinities and extended down to 8‰S, if the adults had been acclimated to slightly lower salinity water [27–28‰S (Dinnel et al. 1987; also see Hintz and Lawrence 1994)]. Marine populations of *F. vesiculosus* respond similarly to lower salinity, and may be able to acclimate to lower salinities, since some reproductive characteristics of marine *Fucus* were more similar to those of the Baltic populations during a period when the ambient salinity was seasonally low in spring (21‰S). However, the degree of possible acclimation of Tjärnö males in our experiments was limited: the overall pattern of their performance was still characteristic of marine sperm with slightly better performance at lower salinities (5–6‰S, cf. Tjärnö in Fig. 2a, b, and Tjärnö males cultured in Tjärnö water in Fig. 5a, b). Also, although it seems likely that the ability of some marine (Tjärnö) sperm to swim at 5–6‰S was due to the sperm having matured at naturally lower salinities (21‰S) than in the initial experiment (30‰S), we cannot rule out possible influences of two other variables that differed between the two experiments: ambient temperature and time of collection during the reproductive season. The fact that the motility characteristics of sperm from Tjärnö were consistently intermediate between those of the Icelandic and Baltic populations may also be explained by the location of this marine population near the Baltic Sea, which causes it to be subjected to large seasonal fluctuations in salinity (≈15–33‰S) during the reproductive season and requires the ability to tolerate both high and low salinities. To summarize, our acclimation experiments indicate that (1) the better sperm motility and fertilization success of Baltic gametes at low salinities (c. 4–6‰S) may be an adaptation, and (2) show that acclimation can occur at salinities near marine levels (21–33‰S).

In the study of Kniep (1907) of salinity tolerance of gametes from the Norwegian coast at Bergen, the tolerance increased as *F. spiralis* < *F. serratus* < *F. vesiculo-*

sus. In agreement with these results, both *F. serratus* and *F. vesiculosus* have populations in the brackish Baltic Sea, with that of *F. vesiculosus* being particularly abundant and widespread, whereas *F. spiralis* is absent. The results of Kniep (1907) indicate that, in the absence of acclimation, marine populations of *F. vesiculosus* and *F. serratus* are better adapted than *F. spiralis* to reproduce in brackish habitats. However, the data presented here suggest that Baltic populations of *F. vesiculosus* are adapted to even lower salinities than marine populations. Both our results and those of Kniep (1907) show that at c. 6‰S, sperm from marine *F. vesiculosus* stop swimming, and that at the lower salinities in which motility is possible, sperm may oscillate or tumble in the same place for a short period before becoming completely immotile. Low salinities inhibited the release of eggs from the oogonia, and the salinities at which this effect was observed were also different for marine (at and below 6‰S) and Baltic (at and below 4‰S) populations. The inhibition of release of eggs from oogonia from marine *F. vesiculosus* had already been reported by Kniep (1907).

Other Baltic species also seem to be limited by effects of salinity on sperm motility and fertilization (Solemdal 1967; Westin and Nissling 1991). Minimum salinities needed for successful fertilization of Baltic flounder are lower (6.5‰S) than for marine flounder [11‰S (Solemdal 1967)]. However, for successful fertilization and development of cod eggs, a minimum of 11‰S is needed, which in the Baltic only occurs in a few deep basins (Westin and Nissling 1991).

The effects of salinity in the range of 2.2–33‰S on sperm motility and the success of fertilization in *F. vesiculosus* were osmotic. Sperm of marine *Fucus* require external Na^+ and Ca^{2+} for motility (O'Toole and Brownson 1992); however, we have found that at the salinities in which *Fucus* lives in the Baltic Sea, the concentrations of these ions were not low enough to affect sperm motility, whereas low osmolality clearly did. If natural or anthropogenic changes to the inner Baltic (i.e., Bay of Bothnia, Gulf of Finland) that increased osmolality even slightly were to occur, a range expansion would become likely. It is possible that the sparse isolated populations reported at salinities of 2–4‰S (see Wærn 1952) were made possible by local or temporarily higher ambient osmolality.

Media of low osmolality may decrease the proportion of swimming sperm due to the absence of an osmotic stimulus to initiate motility, as in sperm from some marine animals (e.g., Morisawa and Suzuki 1980; Morisawa 1994 and references therein). However, because velocity and linearity of the sperm paths were also affected, it seems more likely that most sperm were immotile because of damage caused by the increase in intracellular osmotic pressure. In marine *F. vesiculosus*, a hypotonic medium causes a substantial increase in the volume of the sperm (Wright and Reed 1990), and we observed changes in the shape of Baltic sperm at low osmolalities. The response of sperm from *F. vesiculosus* to osmolality was similar to other marine species (e.g., Morisawa and Suzuki 1980) in that sperm swam better above 300–400 mmol/kg (equivalent to 10–14‰S salinity), even for Baltic populations, which may live at osmolalities as low as 100 mmol/kg. However, it is notable that Baltic (but not marine) sperm were still able to swim at the same low osmolalities (i.e., 100–200 mmol/kg) needed to trigger motility of sperm from some freshwater animals (e.g., Morisawa and Suzuki 1980).

Higher polyspermy in marine *F. vesiculosus* results when fertilization occurs in artificial seawaters of normal osmolality but low sodium concentration (Brawley 1987, 1991), because the electrical polyspermy block is sodium-dependent. Additionally, polyspermy increases under all conditions as the sperm:egg ratio is increased (Brawley 1991). Despite the low [Na^+] of Baltic water, natural levels of polyspermy are low (Serrão et al. 1993), and this may be explained by effects of low salinities in decreasing the likelihood of more than one sperm encountering and fusing with an egg simultaneously, before the polyspermy blocks are triggered by the first sperm entry. Slower sperm motility, fewer motile sperm, or an impairment of orientation behaviors that concentrate sperm at the egg surface are such possible effects. We have found good sperm motility in brackish water, but only about half of the sperm that were released were motile and, perhaps most important, these sperm were unresponsive to light gradients. In seawater, the negative phototaxis of sperm is probably an important factor in increasing the sperm:egg ratio at the egg surface, with final contact with the negatively-buoyant egg occurring through a short-range pheromonal attraction, which is operative in Baltic gametes. Additionally, a slow rate of fertilization after sperm are bound to the egg would protect against polyspermy; Wright and Reed (1990) have suggested that volumetric increases caused by low osmolalities could alter the spatial relationships of the gamete receptors in estuarine algae (also see Stafford et al. 1992). Alternatively, or additionally, polyspermy could still be low in the Baltic if a change in the ionic basis of the fast polyspermy block had occurred during adaptation of *F. vesiculosus* to decreasing salinities [e.g., instead of being based on an influx of Na^+, an efflux of Cl^- could occur, as in some freshwater species (Grey et al. 1982; Kobayashi et al. 1994)]. In laboratory experiments we have found variable rates and levels of fertilization in different portions of the reproductive season. However, in optimal periods (early–mid July near Järnäs), sperm reach eggs quickly, and a fertilization potential (i.e., the depolarization that is the fast block against polyspermy) is observed as quickly as that which occurs between a marine egg and sperm (S.H. Brawley, unpublished work). Natural data on sperm:egg ratios are required to confirm the significance of our observations; however, some protection against polyspermy is likely to result from the effects of brackish water on sperm motility described here: (1) lack of negative phototaxis, (2) lower motility than that of marine sperm in seawater and (3) a decreased proportion of sperm that are motile.

The impairment of sperm motility, including loss of flagella, at low osmolalities is sufficient to explain the very low success of fertilization observed at 2–4‰S in the laboratory. However, we have observed that, at salinities below 6‰S, lysis of eggs occurs unless sperm fertilize the eggs soon after their release from oogonia. This suggests that the time available for fertilization to occur in nature following gamete release in the central to northern Baltic is short. By only 2–3 h post-fertilization, zygotes of marine fucoids can tolerate brackish water that kills gametes or younger zygotes (Kniep 1907; Burrows 1964), probably because of the protective effect of the cell wall that forms following fertilization (Kniep 1907). Even the European estuarine fucoid *F. ceranoides* requires tidal windows of high salinity for reproduction (Burrows 1964; Brawley 1992), and their absence in the atidal Baltic is a sufficient explanation for the absence of this fucoid in the Baltic Sea. Populations of *F. vesiculosus* in the Baltic have no such windows of higher salinity during which to reproduce, but fertilization of Baltic eggs in our laboratory experiments was successful (approximately 50–95%) at their ambient salinities. The low or null fertilization success at osmolalities lower than c. 100 mmol/kg suggests that reproduction constrains the distribution of *F. vesiculosus* in the Baltic (Wærn 1952) to its observed northern limit. We conclude that the wide distribution of *F. vesiculosus* in most of the Baltic Sea is possible because its gametes are partially adapted to lower salinities (i.e., osmolalities), allowing fertilization to occur at salinities in which gametes of marine *F. vesiculosus* (and other *Fucus* species) cannot function.

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