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Effects of Salinity on Spawning and Early Development of the Tube-Building Polychaete *Hydroides elegans* in Hong Kong: Not Just the Sperm's Fault?

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Abstract. Ambient salinities drop dramatically during monsoon season in Hong Kong coastal waters, posing a number of problems for externally fertilizing species like the polychaete *Hydroides elegans.* In this study, we investigated (1) whether adults would retain their gametes when external salinity dropped to levels too low to support fertilization and development, and (2) whether failure of development at low salinity reflects a failure of fertilization or a failure of fertilized eggs to cleave. Adults released eggs and sperm in the laboratory even at the lowest salinity tested, a practical salinity (*S*) of 5, and yet very few eggs cleaved at salinities below about 22. By mixing gametes at high salinity and then transferring the fertilized eggs to low-salinity seawater, we found that salinities below about 22 reduced the percentage of fertilized eggs that cleaved. Similarly, mixing gametes at salinities as low as 15 and then transferring the eggs to full-strength seawater ($S = 30$) rescued a substantial number of eggs, many more of which cleaved after their transfer to the higher salinity. The results suggest that failure of early development at low salinity in this species in large part reflects an inability of newly fertilized eggs to complete meiosis and cleave, rather than simply a failure of fertilization.

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Note: In this paper, salinity values from the literature are presented as ppt (parts per thousand) if that notation was used in the original report; ppt values are equivalent to practical salinity values (*i.e.,* salinity expressed on the practical salinity scale).

Introduction

Thorson's (1950) influential review, in which he speculated that most free-spawned eggs probably become fertilized in the wild because of high coordination in the spawning activities of neighboring males and females, has inspired many interesting studies on the ecology of fertilization. Most of this literature has focused on the influence on fertilization success of adult density and spatial distribution (Pennington, 1985; Levitan, 1991, 1996; Atkinson and Yund, 1996; Coma and Lasker, 1997; Phillippi *et al.,* 2004), spawning synchrony (*e.g.,* Marshall, 2002), gamete longevity (Levitan *et al.,* 1991, 1992; Bolton and Havenhand, 1996; Meidel and Yund, 2001; Johnson and Yund, 2003), egg size (Podolsky and Strathmann, 1996; Robertson, 1996; Farley and Levitan, 2001; Jantzen *et al.,* 2001; Podolsky, 2001; Williams and Bentley, 2002; Styan *et al.,* 2005), chemical attractants (Miller, 1977, 1978; Bolton and Havenhand, 1996; Riffell *et al.,* 2002), shear forces (*e.g.,* Denny *et al.,* 2002), or water depth (Babcock *et al.,* 2000).

Much less is known about the influence of salinity on spawning and fertilization success. Just (1929, 1930a, b) found that unfertilized eggs of some echinoids and polychaetes could tolerate and recover from exposure to very low salinities, and could in fact cleave at salinities as low as about 7–10 ppt, but that the eggs became more susceptible to low salinities immediately after fertilization. Working with the estuarine nereid *Hedistes* (as *Nereis) diversicolor,* Smith (1964) further established that eggs were most sensitive to low salinities immediately after fertilization, when the polar bodies are extruded and cleav-

age begins. Greater sensitivity to salinity extremes during cleavage than at fertilization was also seen for the asteroid *Luidia clathrata* by Hintz and Lawrence (1994). According to Fujiya (1970), eggs of the oyster *Crassostrea gigas* can be fertilized at salinities that do not permit development, but no experimental details or results were given. These experiments suggest that in areas of fluctuating salinities, salinity might be important for fertilization success and subsequent early development.

The tube-building polychaete *Hydroides elegans* Haswell is a common and important fouling organism in the coastal waters of Hong Kong and other subtropical and tropical regions (Qiu and Qian, 1997). In Hong Kong coastal waters, ambient salinities drop considerably during June, July, and August, due to the heavy rains characterizing monsoon season; surface salinity can drop in some areas to as low as a practical salinity (S) of 10 after a heavy rain, and under some conditions surface salinity can remain at nearly 0 for several hours (Qian, unpubl. data). Recruitment of *H. elegans* also drops to its lowest level of the year during this period (Qiu and Qian, 1997; Thiyagarajan and Qian, 2003; Thiyagarajan *et al.,* 2003; Dahms *et al.,* 2004). Qiu and Qian (1997) showed that larvae of *H. elegans* could develop successfully at salinities of 25 ppt and above, but not at salinities of 20 ppt and below. This poses an interesting problem for adults of this species, which release their gametes into the surrounding seawater for fertilization: will adults avoid shedding gametes when the salinity is too low to support fertilization and early development?

In this study, we investigated whether there was a critical salinity below which adults of *H. elegans* would not release their gametes, and if so, whether this critical salinity corresponded to salinities allowing successful fertilization and cleavage. We also examined whether eggs and sperm were equally susceptible to the effects of low salinity, and whether failure to cleave—a common measure of fertilization success in annelids and molluscs that do not show a conspicuous fertilization membrane (*e.g.,* Fong *et al.,* 1995; Qiu and Qian, 1997; Ushakova and Sarantchova, 2004)—at low salinity was due to a failure of fertilization or an inhibition of cleavage. We also tested an anecdotal suggestion that mature worms could be induced to release gametes by keeping adults in air for a few hours.

Materials and Methods

Source of adults

Tubes of *Hydroides elegans* were collected from a mixture of bryozoan (*Bugula neritina*) and *H. elegans* colonies attached to ropes hanging about 0.1–0.5 m below the surface from fish rafts at a commercial fish farm at Yung Shue O, near Sai Kung (22°19′N, 114°16′E), Hong Kong, and either used immediately (for spawning studies, see below) or transported in a cooler to the laboratory at Hong Kong University of Science and Technology (HKUST) within 2 h of collection. Animals were then held in flowing seawater at ambient salinity at the HKUST Coastal Marine Laboratory for up to 2 d before being used in experiments. Animals with their tubes were collected on 10 June 2004 for one series of experiments and again the following year (24 June 2005) for a second series of experiments. Animals were also collected on 14 December 2004 for a second study of spawning activity at low salinity. Ambient surface salinity at the fish farm was 29 on 10 June 2004; 32 on 14 December 2004; and 31 on 24 June 2005. Finally, animals were collected in June 2006 for several additional control experiments (*e.g.,* to determine whether low-salinity shock might itself cause unfertilized eggs to cleave).

Influence of low salinity on gamete release

Experiments were conducted in June and December of 2004. In June 2004, individual worm tubes were carefully removed, without damage, from the substrate at the fish farm, placed into individual 5-ml screw-cap vials without seawater, and brought back to the laboratory at HKUST within 2 h. In the laboratory, we added seawater (4 ml) at one of five salinities ranging from 5 to 30, and checked for gamete release after 10 min, 2 h, and 3 h. Salinities were reduced to the desired levels by diluting full-strength seawater (about $S = 34$) with double-distilled (DI) water. Dilution with DI had a negligible effect on seawater pH over the range 10–35 ppt, but it reduced pH from about 8.0 to about 7.75 at a salinity of 5 (Qian, unpubl. data). Twenty replicate worm tubes were included for each tested salinity. The following day we opened all of the tubes from which no gametes were released to determine which ones were empty; those tubes were excluded from the data set. The experiment was repeated 6 months later using worms collected from the same location. For that experiment, water of reduced salinity was added either following a 2-h exposure of the worm tubes to air (30 worm tubes used per tested salinity) or without such prior air exposure (20 worm tubes used per tested salinity), to see whether exposure to air increased the incidence of spawning. Control salinity was 34. At the end of the study we again determined the number of worm tubes that contained no worms; empty tubes were excluded from analysis. The actual numbers of replicates (tubes containing living worms) used in each experiment are presented with the data.

Obtaining gametes for fertilization studies

Gametes were obtained using standard procedures (Bryan *et al.,* 1997; Qiu and Qian, 1997; Qian and Pechenik, 1998), in June 2004 and June 2005. Briefly, individual tubes were carefully separated from their neighbors, placed in separate small petri dishes, and carefully broken apart with forceps. Gametes were then generally released by mature individuals

within several minutes and were collected by pipette. Eggs were immediately transferred into about 30 ml of seawater at a salinity of 30, while sperm were kept undiluted in small beakers until use. Sperm were then diluted with about 10–20 ml of filtered seawater. For each experiment, eggs were obtained from 5–8 females, and sperm were obtained from 4–6 males.

The effect of low salinity on fertilization and early development

The experiment was conducted at room temperature (about 24 ± 1 °C) in June 2004. Gametes were obtained as described above. Fifteen milliliters of 0.22-μm-filtered seawater of the desired salinities (5, 10, 15, 18, 20, 22, 24, and 30) was then poured into 30-ml glass beakers, with three replicates per treatment. Seawater was oxygenated by violent shaking before it was distributed among the glass beakers. Into each beaker we then dispensed 100 μl of sperm suspension and 250 μl of egg suspension. Each beaker typically ended up containing thousands of eggs. Because fertilization success in this species is near maximal over a remarkably wide range of sperm concentrations (approximately 10^5–10^8 cells ml^{-1}—even at 10^3 sperm ml^{-1} at least 60% of eggs are fertilized) (Pechenik and Qian, 1998), we made no attempt to regulate the specific sperm concentrations that were used in each experiment, other than keeping them within an estimated range of approximately 10^5–10^7 cells ml^{-1}. The same sperm concentration was used in all replicates and for all treatments within each experiment.

Three additional beakers of eggs in seawater at $S = 30$ served as controls; no sperm were added to these beakers so that we would be able to tell whether eggs had been contaminated by sperm before the experiment started. Eggs were subsampled after 2.5 h, with about 50–150 eggs per subsample, and examined for cleavage at a magnification of 100× using a compound microscope. In two pilot studies (data not shown), no eggs cleaved within 30 min of fertilization at room temperature and a salinity of 30, about 50%–65% of eggs cleaved within 1 h of fertilization, and more than 80% of eggs were at the 8-cell stage or beyond 45 min later. These data agree with those published previously by Qiu and Qian, 1997.

In these studies, the normality of cleavage was not assessed, only whether or not cleavage occurred.

Determining the causes of cleavage failure

These experiments were conducted in June 2004 and June 2005. Failure of eggs to cleave at low salinity could reflect either lack of fertilization or a direct effect of low salinity on cleavage. To determine the potential effects of low salinity on cleavage *per se,* 150 μl of sperm suspension and 250 μl of egg suspension were mixed together in 15 ml of seawater as described above, at a salinity of 30. After allowing 5 min for fertilization to occur after sperm were added, eggs were gently removed from the suspension by using a 30-μm-mesh Nitex filter. The eggs were then thoroughly rinsed free of sperm by agitating the mesh filter through four beakers of filtered seawater ($S = 30$) and transferred to seawater at salinities of 15, 18, or 20. Eggs maintained at full-strength seawater (controls) were treated the same way, except that they were transferred back into full-strength seawater. Three replicates were included per treatment, with many hundreds of eggs per replicate.

After about 2 h at room temperature, percent cleavage was determined for two replicates of each treatment as described earlier; remaining samples were covered with Parafilm to prevent evaporation and stored in a refrigerator overnight to slow development; embryos are much harder to count once they start to swim. At room temperature, embryos commonly begin swimming about 3 h after fertilization (unpubl. data). Percent cleavage in the refrigerated samples was determined the following morning. Refrigerated samples showed the same trends as unrefrigerated samples, and the embryos looked no different than those maintained at room temperature and examined earlier.

To determine whether at least the early events of fertilization could be accomplished at low salinity, gametes were mixed together in 15 ml of seawater at salinities of either 15, 18, or 20, and left for 5 min to allow fertilization to occur. Eggs in three replicates at each salinity were then transferred to full-strength seawater ($S = 30$) after excess sperm were thoroughly rinsed off as previously described, to document the actual percentage of eggs fertilized. Eggs in another three replicates at each salinity were rinsed free of excess sperm and transferred to new containers at the same initial salinities. Three replicates were included per treatment. After 2 h, percent cleavage was determined for one replicate per treatment; the remaining samples were stored overnight in a refrigerator to slow development and were examined for cleavage the following day as described earlier. These experiments were conducted both in June 2004 (Series I) and June 2005 (Series II).

For every experiment, two beakers of eggs (no sperm added) were checked the following day to be sure that the eggs used in these experiments had not been inadvertently fertilized by neighboring males while the eggs were being harvested.

A follow-up experiment was conducted in June 2006 to help distinguish between effects of low salinity on the later stages of fertilization and effects on cleavage. Eggs from five females were mixed with sperm from four males at a salinity of 30. Eggs were subsampled after 5 min, 15 min, and 30 min; retained on a 25-μm-mesh filter and thoroughly rinsed with filtered seawater; and then transferred to seawater with a salinity of 18. Additional subsamples of eggs were transferred after 20 min to other beakers of seawater at 30 to

serve as controls. We used three replicates per treatment, with many hundreds of eggs per replicate. Eggs from all treatments were subsampled 3 h after fertilization and fixed in 10% formalin for examination later that day, when percent cleavage was determined.

An additional follow-up experiment was conducted in June 2006 to test the effectiveness of our sperm-removal technique. A 100-μl sample of sperm suspension (approximately 10^8 cells ml^{-1}) was added to each of three beakers containing 30 ml of seawater ($S = 30$). Egg suspension was then added to each beaker in a tube with a mesh filter bottom as in previous experiments, and the filters were rinsed free of sperm as described above, using filtered seawater. We then squirted seawater through each filter into empty beakers, so that any remnant sperm, but no eggs, would be washed into the beakers. We then added egg suspension and seawater to each beaker. Eggs were maintained at room temperature and checked for cleavage 3 h and 24 h later.

Relative sensitivity of sperm and eggs to low-salinity seawater

The relative sensitivity of sperm and eggs to low salinity was examined in one experiment (June 2005). Small glass beakers (30-ml capacity) of seawater were set up at salinities of 15, 18, and 30 (control), with three replicates per treatment and 15 ml of seawater per beaker. To each beaker we then added either 150 μl of sperm suspension or 250 μl of egg suspension. Five minutes later we added the missing gamete to each beaker and mixed the gametes together using a pipette. About 5 min later we rinsed off excess sperm and transferred the eggs to full-strength seawater ($S = 30$) to monitor development. After about 2.5 h, percent cleavage was determined for one replicate per treatment; remaining beakers were covered in Parafilm to prevent evaporation and stored at 4 °C overnight. Eggs were subsampled within 24 h (at least 50 eggs per beaker) and examined for evidence of cleavage at 100× using a compound microscope.

As in previous experiments, two beakers contained eggs to which no sperm were added. Those eggs were also checked the following day to be sure that the eggs used in the experiments had not been inadvertently fertilized by neighboring males while the eggs were being collected.

Data analysis

Percentage data were arcsin transformed before analysis (Sokal *et al.,* 1993) and then analyzed by one-way analysis of variance (ANOVA). When significant differences among means were found, the source of the differences was determined using either Bonferonni or Dunnett's multiple comparisons tests or Tukey's HSD test, for selected comparisons. When appropriate, means were compared using Student's *t* tests.

Results

Effect of salinity on gamete release

In the experiment conducted in June 2004, both males and females of *Hydroides elegans* released gametes at all salinities tested, including 5 (Fig. 1). No worms released gametes into the surrounding water during the first 10 min after the worms were transferred to seawater, suggesting that adults did not release gametes during their exposure to air, but only after their return to seawater. About 29% of worm tubes collected in June 2004 were empty, and provided no data. When this experiment was repeated 6 months later, in December 2004, adults again released gametes at salinities as low as 5, and this was true whether the adults had previously been exposed to air (Fig. 2a) or not (Fig. 2b). Only 6% of the worm tubes that we collected in December 2004 were empty, so that final sample sizes were larger in the second study.

Effects of salinity on fertilization success and cleavage

Exposure of unfertilized eggs to low salinities did not in itself induce cleavage (data not shown). Control experiments verified that our rinsing technique effectively removed all sperm from the mesh filters; no eggs cleaved when mixed with the final rinse water from the mesh filters.

Eggs that were exposed to sperm at salinities below 22 in June 2004 generally failed to cleave, and no eggs cleaved when gametes were mixed at 5 or 10 (Fig. 3). Salinity had

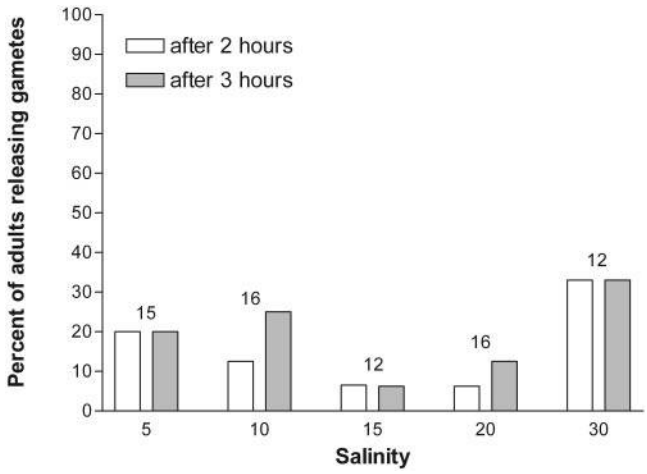

Figure 1. The influence of salinity on the release of gametes by adult worms, *Hydroides elegans,* June 2004. Worm tubes were collected from substrates in the field and exposed to air for 2 h before being placed in screw-capped vials of seawater at the designated salinities, with one worm tube per vial. Number of worms tested at each salinity, after accounting for empty worm tubes, is shown above each pair of bars.

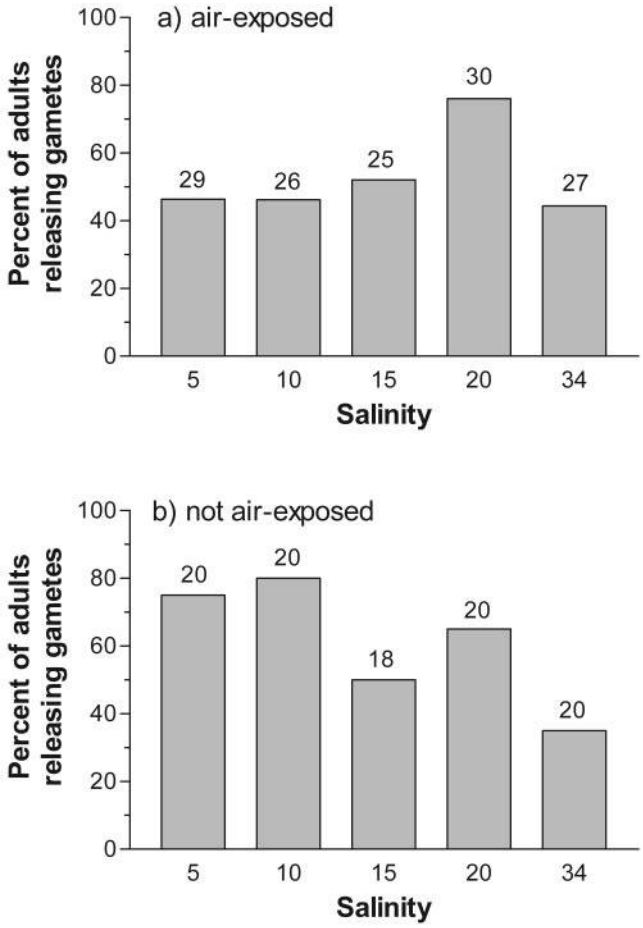

Figure 2. The influence of salinity on the release of gametes by adult worms, *Hydroides elegans,* December 2004. This experiment was similar to that summarized in Fig. 1, except that in one experiment (b) worms were not exposed to air for 2 h before being immersed in seawater of the designated salinities. Number of worms tested at each salinity, after accounting for empty worm tubes, is shown above each pair of bars.

no significant effect ($P > 0.10$) on the percentage of eggs cleaving over the range of 22–30 (Fig. 3). Cleavage failure was not caused by manipulation of the eggs during transfers between salinities, since eggs in control salinity (30) were manipulated in the same way and yet developed normally in all experiments.

A substantial proportion of eggs that were exposed to sperm at full-strength salinity (30) failed to cleave when transferred to lower salinities; in particular, fewer than 15% of eggs cleaved, on average, after their transfer to salinities of 18 or lower (Fig. 4a). In the June 2006 experiment, results were the same whether eggs were transferred to the lower salinity (18) after spending 5 min, 15 min, or 30 min at the higher salinity (30) (Fig. 5). The amount of time spent at the high salinity before eggs were transferred to the low salinity had no significant effect on subsequent percent cleavage at 3 h (Tukey HSD, $P > 0.10$). Many of the uncleaved eggs displayed polar bodies.

When eggs were mixed with sperm at low salinity (15 or 18) and then transferred to full-strength salinity (30), a fairly high percentage of eggs that had been at the lower salinities cleaved (compare the last two columns of Fig. 4b with the last two columns of Fig. 4a; the difference between the means of the two treatments at salinities of 18 was significant: $P < 0.05$). The same effect was also seen clearly in a second experiment, in which eggs were mixed with sperm at salinities of 20 for either 2.5 or 5 min and then transferred to full-strength seawater (Fig. 6): more than 6 times as many eggs cleaved ($P < 0.05$) if they were transferred to a salinity of 30 after 5 min than if they were left at 20 (Fig. 6, compare columns 1 and 3). The same effect was again seen clearly in our June 2005 experiments, in which eggs were either exposed to sperm at salinities of 15, 18, or 20 and left to develop at those low salinities for 24 h, or exposed to sperm at those low salinities for 5 min and then transferred to full-strength seawater for the next 24 h (Fig. 7). In every case, a dramatically higher number of eggs cleaved if they were transferred to the high salinity after having been exposed to sperm at the low salinities, indicating that low salinity affected the ability of eggs to cleave even after the eggs had been successfully bound with sperm. Many of the uncleaved eggs displayed polar bodies.

Relative sensitivity of sperm and eggs to low-salinity water

It made little difference to cleavage success ($P > 0.10$) whether eggs were first exposed to the low salinity, with sperm added 5 min later, or whether sperm were first

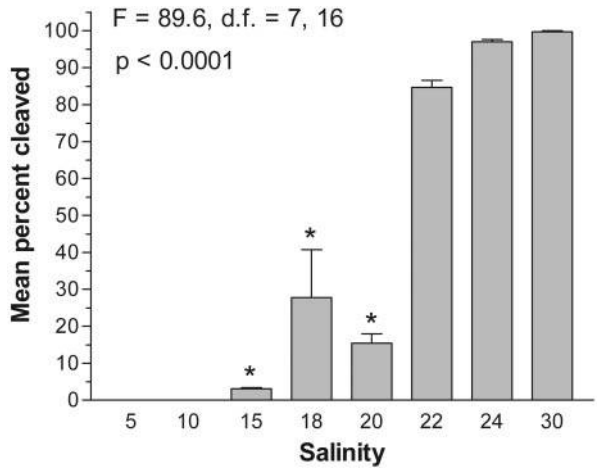

Figure 3. The effect of salinity on fertilization and cleavage of *Hydroides elegans.* Eggs and sperm were mixed together simultaneously in June 2004. Eggs were left to develop at the salinities at which they were exposed to sperm, at about 24 °C. Each bar is the mean of three replicates, with about 50–150 eggs being examined for each replicate. Error bars represent one standard deviation above the mean. *signifies means that are significantly different ($P < 0.01$) from the control mean (Dunnett's multiple comparisons test).

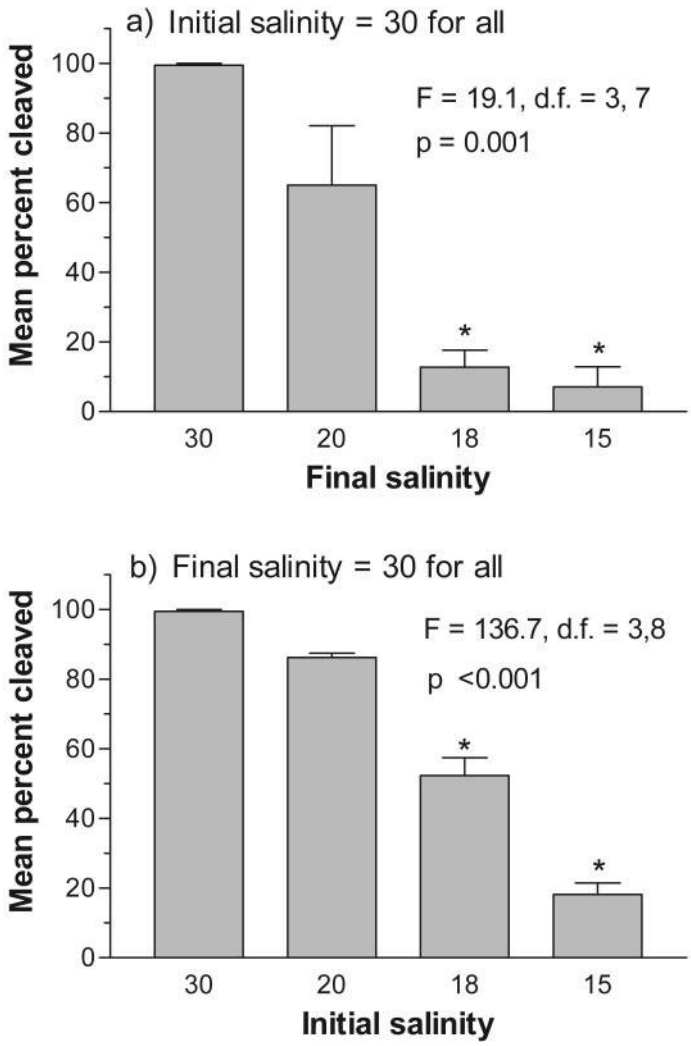

Figure 4. The influence of low salinity on fertilization and cleavage in *Hydroides elegans.* In (a), gametes were mixed for 5 min at full salinity ($S = 30$). Eggs were then rinsed free of sperm and transferred to the different salinities shown to monitor development. In (b), gametes were mixed for 5 min at one of the five salinities shown. Eggs were then rinsed free of sperm and transferred to full-strength seawater to monitor development. Each bar is the mean of three replicates, with at least 50 eggs being examined for each replicate. Error bars represent one standard deviation above the mean. *indicates means that are significantly different ($P < 0.01$) from the control means.

exposed to the low salinity, with eggs added 5 min later (Fig. 8). The percentage of eggs cleaving after exposure to sperm at salinities of either 15 or 18 was significantly less ($P < 0.05$) than the percentage cleaving at 30.

Discussion

This study confirms prior evidence that the newly fertilized eggs and embryos of *Hydroides elegans* are generally unable to develop at practical salinities (S) of 20 or lower (Qiu and Qian, 1997), and adds the information that the cut-off salinity below which substantial cleavage does not occur lies between 20 and 22 (Fig. 3). The effect is probably due to changes in osmotic concentration: when seawater was diluted with double-distilled water in the laboratory, pH remained between about 8.0 and 8.1 over the range of salinity 10–35 (Qian, unpubl. data). Also, maximum dissolved oxygen concentration increases as salinity is reduced (Harvey, 1969), so that dissolved oxygen should in fact be more readily available at lower salinities than at higher salinities. Since these were short-term studies and the water was well-oxygenated at the start, reduced oxygen concentration is not likely to have limited development.

A salinity of 22 ppt was also the approximate cutoff for normal development for the polychaete *Nereis virens* in the White Sea (Ushakova and Sarantchova, 2004). In that study, about 90% of eggs mixed with sperm were able to cleave

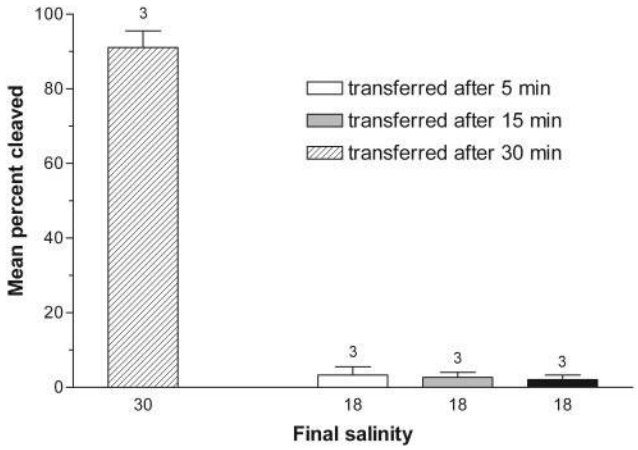

Figure 5. The influence of low salinity on fertilization and cleavage in *Hydroides elegans.* In June 2006, gametes were allowed to interact at full salinity ($S = 30$) for either 5, 15, or 30 min before the eggs were washed and transferred to seawater with a salinity of of 18. Bars represent mean percent cleavage after 3 h, based on three replicates of at least 150 eggs each.

and develop to the trochophore stage at 22 ppt, but only about 55% of eggs were able to do so at the next lowest salinity tested, 18 ppt.

Surprisingly, adults of *H. elegans* released their gametes in the laboratory at salinities far below those that allow development (22–25, this paper; and Qiu and Qian, 1997), and did so as readily at low salinity as at high salinity, even

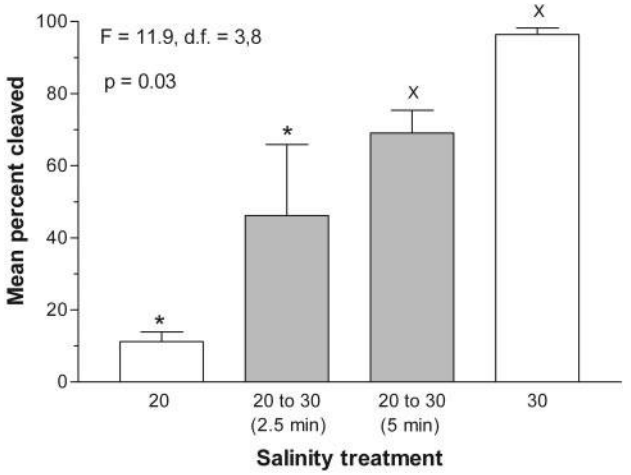

Figure 6. Failure of *Hydroides elegans* to develop at a salinity of 20 is largely due to a failure to cleave, not a failure of fertilization. Gametes were mixed at the low salinity, rinsed free of sperm after either 2.5 or 5 min, and then transferred to full-strength seawater ($S = 30$) to monitor development over the next several hours. Each bar is the mean of three replicates, with at least 50 eggs being examined for each replicate. Error bars represent one standard deviation above the mean. *indicates means that differ significantly ($P < 0.05$) from the mean obtained at a salinity of 30. X indicates means that differ significantly ($P < 0.05$) from the mean obtained at $S = 20$.

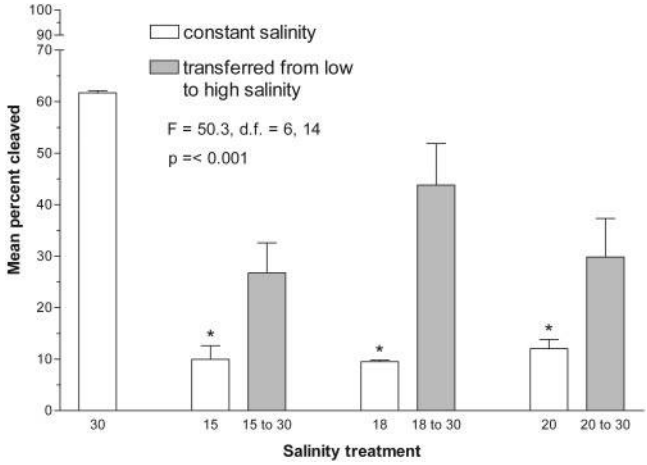

Figure 7. The relative importance of low salinity on the processes of fertilization and cleavage tested at three salinities. Gametes were mixed for 5 min at a low salinity (15, 18, or 20) in June 2005. Eggs were then either left to develop at that low salinity (after removing excess sperm), or rinsed free of sperm and transferred to full-strength seawater to monitor development. Each bar is the mean of three replicates, based upon the examination of approximately 100–300 eggs from each replicate. Error bars represent one standard deviation above the mean. *indicates means that differ significantly ($P < 0.05$), for comparisons of cleavage at fixed salinity *versus* after transfer to the higher salinity.

at the lowest salinity tested (5, Figs. 1, 2). The brackish-water bivalve *Corbicula japonica* also released gametes at salinities that did not support its development (Baba *et al.,* 1999). This unexpected behavior might be a factor limiting the distribution of these animals in estuaries and shallow-water coastal areas. In contrast, the oyster *Crassostrea gigas* apparently spawns only at salinities above 27 ppt, well above the lowest salinity that permits successful fertilization

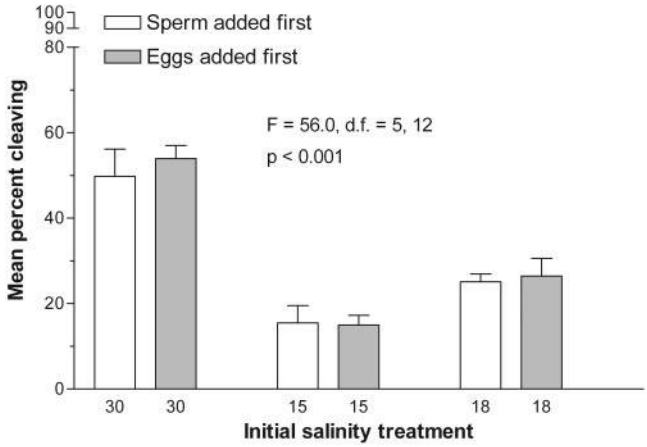

Figure 8. The relative sensitivity of sperm and eggs to low salinity. One type of gamete was added 5 min after the first was added, in June 2005. Each bar represents the mean of three replicates, with approximately 100–200 eggs inspected per replicate. Error bars show one standard deviation above the mean.

and embryonic development (summarized by Fujiya, 1970; as cited by Kinne, 1971, p. 951). Similarly, the zebra mussel, *Dreissena polymorpha,* withheld its gametes at some tested salinities (Fong *et al.,* 1995). Whether or not *H. elegans* spawns naturally at low salinities in the field remains to be determined.

For animals that do not display a fertilization membrane, such as polychaetes, bivalves, and gastropods, fertilization success is sometimes assessed by the ability of eggs to cleave (*e.g.,* Fong *et al.,* 1995; Qiu and Qian, 1997; Ushakova and Sarantchova, 2004). Developmental failure at low salinity is often thought to reflect a failure of the early steps of fertilization, possibly due to substantial reductions in sperm motility—sperm are generally thought of as the weaker gamete (D. Epel, Stanford University, pers. comm.; Fong *et al.,* 1995; Griffin *et al.,* 1998). In keeping with this idea, decreased sperm mobility at elevated salinity is thought to account for decreased fertilization of Pacific herring eggs (Griffin *et al.,* 1998). In contrast, the eggs of at least some polychaete species are remarkably resistant to the effects of low salinity: for example, the eggs of *Nereis limbata* from Woods Hole, Massachusetts, could withstand at least 1 h at 33%–40% seawater (roughly 10–12 ppt salinity) without losing the ability to become fertilized and cleave normally when returned to full-strength seawater (Just, 1930a). On the other hand, Greenwood and Bennett (1981) found that the eggs of sea urchins were much more sensitive than the sperm to low salinities. Similarly, oyster sperm (species name not given) can apparently remain active at salinities as low as 5 ppt, although embryonic development is successful only at salinities above 14 ppt (Clark, 1935; as cited by Kinne, 1971, p. 951).

In our experiments with *H. elegans,* there was no evidence that one gamete was any more sensitive to low salinity than the other: cleavage success was numerically and statistically equivalent ($P > 0.10$) whether eggs were exposed to low salinity for 5 min before sperm were added or sperm were exposed to low salinity for 5 min before eggs were added (Fig. 8). Either the gametes were equally sensitive to salinity reduction under the conditions of our experiment, or one of the gametes—and *only* one of them—was affected instantaneously to the full degree by low salinity while the other gamete was essentially unaffected, which seems less likely. The results are consistent with the hypothesis that low salinity affects cleavage in this species.

Nevertheless, our data do suggest that sperm function was compromised at low salinity: even when eggs were transferred from low salinity to high salinity after being exposed to sperm at low salinity, fertilization success was usually lower than it was under control conditions, when gametes were mixed at full salinity and allowed to develop at that same high salinity (see, for example, Fig. 7). In addition, the greater fertilization success seen at a salinity of 20 if sperm were kept in contact with eggs for 5 min rather than 2.5 min (Fig. 6) suggests that sperm motility was somewhat compromised in those experiments. Further studies in which gametes are allowed to interact for different lengths of time over a wide range of salinities might be instructive; longer times to complete fertilization at lower salinities would provide evidence of a functional effect on sperm motility. The effect of low salinity on sperm mobility could also be quantified directly (*e.g.,* Pacey *et al.,* 1994; Fong *et al.,* 1995; Griffin *et al.,* 1998; Au *et al.,* 2001). These data might be difficult to interpret, however, since gametes in our experiments were thoroughly mixed at the start of each experiment, which would bring eggs and sperm into contact even if the sperm were incapable of swimming.

Our data for *H. elegans,* in agreement with those of Smith (1964) for the polychaete *Nereis diversicolor,* also suggest that failure of eggs to develop at low salinity largely reflects an inability of fertilized eggs to complete meiosis and cleave, rather than simply an inability of eggs to become fertilized at those low salinities: substantial numbers of eggs were able to cleave if they were transferred to full-salinity seawater after being mixed with sperm at low salinity, even when gametes were mixed at salinities as low as 15 (Fig. 7). This result would only be possible if sperm had been able to successfully bind to many of the eggs at low salinity before the eggs were transferred to the higher salinity. Also, the percentage of eggs cleaving was severely reduced when eggs were exposed to sperm at full-strength seawater and then transferred—even 30 min later—to water of low salinity in the absence of additional sperm (Figs. 4a, 5).

Remarkably, this work is one of only a few studies that begin to separate out, for marine invertebrates, the effects of low salinity on fertilization *per se* and on subsequent cleavage. What key processes are being affected when gametes come into contact at low salinity? Fertilization itself involves a number of steps (Alberts *et al.,* 2002): activation of newly released sperm (*e.g.,* Pacey *et al.,* 1994); penetration of sperm through outer egg coverings and binding of sperm to the egg surface; injection of the genetic material from the sperm into the egg; completion of maternal meiosis; movement of the pronuclei toward each other; fusion of the male pronucleus with that of the egg to form a diploid zygote; and activation of the egg's metabolism just prior to cleavage (Gilbert, 2003).

Our data indicate clearly that cleavage failure at low salinity is not due to a failure of sperm to become activated or to bind at the egg surface, since many eggs rinsed free of excess sperm at low salinity developed normally after they were transferred to water of high salinity (Figs. 4b, 6, 7); at least the early events of fertilization had obviously occurred. It remains to be determined whether all the later steps of fertilization can be completed at low salinities, through maternal meiosis, pronuclear fusion, and egg activation. Such studies will require direct examination of nuclear events (Cherr *et al.,* 1990). The fact that most eggs failed to

cleave after transfer to seawater at a salinity of 18 even after having spent 30 min in the presence of sperm at salinity 30 certainly suggests that low salinity suppresses the ability of fertilized eggs to cleave in this species. This does not mean that low salinity might not also have a detrimental effect on the late stages of fertilization, such as pronuclear fusion. Additional studies are required to determine precisely when pronuclear fusion occurs in this species. For the sea urchin *Paracentrotus lividus,* pronuclear fusion takes place within 30 min (Moubax *et al.,* 2001).

How low salinity might affect the ability of eggs to cleave also remains to be studied. The mechanisms of mitosis are still somewhat uncertain, but seem to involve the interactive effects of many different enzymes operating within the cell (Sharp *et al.,* 2000a). Reduced osmotic pressure might affect the ability of the mitotic spindles to self-assemble, or it could directly affect the functioning of the microtubule-based protein motors whose complex interactions apparently move the spindles apart (Sharp *et al.,* 2000b). Alternatively, cytokinesis itself might be affected (Gilbert, 2003).

Surprisingly few studies have considered the role of salinity in determining fertilization success for marine invertebrates. A search on the combined terms "fertilization" and "salinity" in the ISI Web of Knowledge in May 2005 returned only a few references on marine invertebrates; most published studies on this topic have focused on fish, plants, and seaweeds. Among macroalgae, for example, Steen (2004) found that fertilization in *Sargassum muticum* was impaired at salinities of 15 ppt and below, even though oogonial extrusion occurred normally at salinities as low as 10 ppt, and germlings survived at salinities as low as 5 ppt; the tentative conclusion was that sperm were especially sensitive to low salinities. We suggest that salinity fluctuation may play an important role in controlling reproductive cycles and mediating successful fertilization and early development for coastal organisms that fertilize externally, and that the topic is worthy of further investigation. In addition, by potentially uncoupling key steps from each other during the processes of fertilization or mitosis (or both) low-salinity treatments coupled with ultrastructural and molecular investigations might shed further light on the details of how key events of fertilization and cleavage are controlled.

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Literature Cited

Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter. 2002. *Molecular Biology of the Cell,* 4th ed. Garland Publishing, New York.

Atkinson, O. S., and P. O. Yund. 1996. The effect of variation in population density on male fertilization success in a colonial ascidian. *J. Exp. Mar. Biol. Ecol.* **195:** 111–123.

Au, D. W. T., C. Y. Lee, K. L. Chan, and R. S. S. Wu. 2001. Reproductive impairment of sea urchins upon chronic exposure to cadmium. Part I: Effects on gamete quality. *Environ. Pollut.* **111:**1–9.

Baba, K., M. Tada, T. Kawajiri, and Y. Kuwahara. 1999. Effects of temperature and salinity on spawning of the brackish water bivalve *Corbicula japonica* in Lake Abashiri, Hokkaido, Japan. *Mar. Ecol. Prog. Ser.* **180:** 213–221.

Babcock, R., E. Franke, and N. Barr. 2000. Does spawning depth affect fertilization rates? Experimental data from the sea star *Coscinasterias muricata. Mar. Freshw. Res.* **51:** 55–61.

Bolton, T. F., and J. N. Havenhand. 1996. Chemical mediation of sperm activity and longevity in the solitary ascidians *Ciona intestinalis* and *Ascidiella aspersa. Biol. Bull.* **190:** 329–335.

Bryan, P. J., P.-Y Qian, J. L. Kreider, and F.-S. Chia. 1997. Induction of larval settlement and metamorphosis by pharmacological and conspecific associated compounds in the serpulid polychaete *Hydroides elegans. Mar. Ecol. Prog. Ser.* **146:** 81–90.

Cherr, G. N., J. Shoffner-McGee, and J. M. Shenker. 1990. Methods for assessing fertilization and embryonic/larval development in toxicity tests using the California mussel (*Mytilus californianus*). *Environ. Toxicol. Chem.* **9:** 1137–1145.

Clark, A. E. 1935. The effects of temperature and salinity on the early development of the oyster. *Prog. Rep. Atl. Biol. Stn.* **16:** 10. (Cited in Kinne, 1971.)

Coma, R., and H. R. Lasker. 1997. Effects of spatial distribution and reproductive biology on *in situ* fertilization rates of a broadcast-spawning invertebrate. *Biol. Bull.* **193:** 20–29.

Dahms, H.-U., J. Tao, and P.-Y. Qian. 2004. Adrenoceptor compounds prevent the settlement of marine invertebrate larvae: *Balanus amphitrite* (Cirripedia), *Bugula neritina* (Bryozoa) and *Hydroides elegans* (Polychaeta). *Biofouling* **20:** 313–321.

Denny, M. W., E. K. Nelson, and K. S. Mead. 2002. Revised estimates of the effects of turbulence on fertilization in the purple sea urchin, *Strongylocentrotus purpuratus. Biol. Bull.* **203:** 275–277.

Farley, G. S., and D. R. Levitan. 2001. The role of jelly coats in sperm-egg encounters, fertilization success, and selection on egg size in broadcast spawners. *Am. Nat.* **157:** 626–636.

Fong, P. P., K. Kyozuka, J. Duncan, S. Rynkowski, D. Mekasha, and J. L. Ram. 1995. The effect of salinity and temperature on spawning and fertilization in the zebra mussel *Dreissena polymorpha* (Pallas) from North America. *Biol. Bull.* **189:** 320–329.

Fujiya, M. 1970. Oyster farming in Japan. *Helgol. Wiss. Meeresunters.* **20:** 464–479. (Cited in Kinne, 1971.)

Gilbert, S. F. 2003. *Developmental Biology,* 7th ed. Sinauer, Sunderland, MA.

Greenwood, P. J., and T. Bennett. 1981. Some effects of temperature-salinity combinations on the early development of the sea urchin *Parechinus angulosus* (Leske). Fertilization. *J. Exp. Mar. Biol. Ecol.* **51:** 119–132.

Griffin, F. J., M. C. Pillai, C. A. Vines, J. Kääriä, T. Hibbard-Robbins, R. Yanagimachi, and G. N. Cherr. 1998. Effects of salinity on sperm motility, fertilization and development in the Pacific herring, *Clupea pallasi. Biol. Bull.* **194:** 25–35.

Harvey, H. W. 1969. *The Chemistry and Fertility of Sea Waters.* Cambridge University Press, London. P. 184.

Hintz, J. L., and J. M. Lawrence. 1994. Acclimation of gametes to

reduced salinity prior to spawning in *Luidia clathrata* (Echinodermata: Asteroidea). *Mar. Biol.* **120:** 443– 446.

ISI Web of Knowledge. [Online database]. Thomson Scientific, Philadelphia, PA. [cited May 2005]. Available from: http://www.isiwebofknowledge.com/. Subscription required.

Jantzen, T. M., R. de Nys, and J. N. Havenhand. 2001. Fertilization success and the effects of sperm chemoattractants on effective egg size in marine invertebrates. *Mar. Biol.* **138:** 1153–1161.

Johnson, S. L., and P. O. Yund. 2004. Remarkable longevity of dilute sperm in a free-spawning colonial ascidian. *Biol. Bull.* **206:** 144–151.

Just, E. E. 1929. Hydration and dehydration in the living cell. II. Fertilization of eggs of *Arbacia* in dilute sea-water. *Biol. Bull.* **57:** 443–448.

Just, E. E. 1930a. Hydration and dehydration in the living cell. III. The fertilization capacity of *Nereis* eggs after exposure to hypotonic seawater. *Protoplasma* **10:** 24–32.

Just, E. E. 1930b. Hydration and dehydration in the living cell. IV. Fertilization and development of *Nereis* eggs in dilute sea-water. *Protoplasma* **10:** 33–40.

Kinne, O. 1971. *Marine Ecology,* Vol. I, *Environmental Factors.* Wiley-Interscience, New York. P. 951.

Levitan, D. R. 1991. Influence of body size and population density on fertilization success and reproductive output in a free-spawning invertebrate. *Biol. Bull.* **181:** 261– 268.

Levitan, D. R. 1996. Predicting optimal and unique egg sizes in free-spawning marine invertebrates. *Am. Nat.* **148:** 174–188.

Levitan, D. R., M. A. Sewell, and F.-S. Chia. 1991. Kinetics of fertilization in the sea urchin *Strongylocentrotus franciscanus:* interaction of gamete dilution, age, and contact time. *Biol. Bull.* **181:** 371–378.

Levitan, D. R., M. A. Sewell, and F.-S. Chia. 1992. How distribution and abundance influence fertilization success in the sea urchin *Strongylocentrotus franciscanus. Ecology* **73:** 248–254.

Marshall, D. J. 2002. *In situ* measures of spawning synchrony and fertilization success in an intertidal, free-spawning invertebrate. *Mar. Ecol. Prog. Ser.* **236:** 1113–1119.

Meidel, S. K., and P. O. Yund. 2001. Egg longevity and time-integrated fertilization in a temperate sea urchin (*Strongylocentrotus droebachiensis*). *Biol. Bull.* **201:** 84–94.

Miller, R. L. 1977. Chemotactic behavior of the sperm of chitons (Mollusca: Polyplacophora). *J. Exp. Zool.* **202:** 203–211.

Miller, R. L. 1978. Site-specific sperm agglutination and the timed release of a sperm chemo-attractant by the egg of the leptomedusan, *Orthopyxis caliculara. J. Exp. Zool.* **205:** 385–392.

Moubax, I., N. Bontemps-Subielos, B. Banaigs, G. Combaut, P. J.-P. Girard, and D. Pesando. 2001. Structure-activity relationship for bromoindole carbaldehydes: effects on the sea urchin embryo cell cycle. *Environ. Toxicol. Chem.* **20:** 589–596.

Pacey, A. A., J. C. Cosson, and M. G. Benthley. 1994. The acquisition of forward motility in the spermatozoa of the polychaete *Arenicola marina. J. Exp. Biol.* **195:** 259–280.

Pechenik, J. A., and P.-Y. Qian. 1998. Onset and maintenance of metamorphic competence in the marine polychaete *Hydroides elegans* Haswell in response to three chemical cues. *J. Exp. Mar. Biol. Ecol.* **226:** 51–74.

Pennington, J. T. 1985. The ecology of fertilization of echinoid eggs: the consequences of sperm dilution, adult aggregation, and synchronous spawning. *Biol. Bull.* **169:** 417– 430.

Phillippi, A., E. Hamann, and P. O. Yund. 2004. Fertilization in an egg-brooding colonial ascidian does not vary with population density. *Biol. Bull.* **206:** 152–160.

Podolsky, R. D. 2001. Evolution of egg target size: an analysis of selection on correlated characters. *Evolution* **55:** 2470–2478.

Podolsky, R. D., and R. R. Strathmann. 1996. Evolution of egg size in free-spawners: consequences of the fertilization-fecundity trade-off. *Am. Nat.* **148:** 160–173.

Qian, P.-Y., and J. A. Pechenik. 1998. Effects of larval starvation and delayed metamorphosis on juvenile survival and growth of the tube-dwelling polychaete *Hydroides elegans* (Haswell). *J. Exp. Mar. Biol. Ecol.* **227:** 169–185.

Qiu, J.-W., and P.-Y Qian. 1997. Combined effects of salinity, temperature, and food on early development of the polychaete *Hydroides elegans. Mar. Ecol. Prog. Ser.* **152:** 79–88.

Riffell, J. A., P. J. Krug, and R. K. Zimmer. 2002. Fertilization in the sea: the chemical identity of an abalone sperm attractant. *J. Exp. Biol.* **205:** 1439–1450.

Robertson, D. R. 1996. Egg size in relation to fertilization dynamics in free-spawning tropical reef fishes. *Oecologia* **108:** 95–104.

Sharp, D. J., H. M. Brown, M. Kwon, G. C. Rogers, G. Holland, and J. M. Scholey. 2000a. Functional coordination of three mitotic motors in *Drosophila* embryos. *Mol. Biol. Cell* **11:** 241–253.

Sharp, D. J., G. C. Rogers, and J. M. Scholey. 2000b. Microtubule motors in mitosis. *Nature* **407:** 41–47.

Smith, R. I. 1964. On the early development of *Nereis diversicolor* in different salinities. *J. Morphol.* **114:** 437–464.

Sokal, F. J. Rohlf, and R. R. Sokal. 1993. *Biometry,* 3rd ed. W. H. Freeman, New York.

Steen, H. 2004. Effects of reduced salinity on reproduction and germling development in *Sargassum muticum* (Phaeophyceae, Fucales). *Eur. J. Phycol.* **39:** 293–299.

Styan, C. A., M. Byrne, and E. Franke. 2005. Evolution of egg size and fertilisation efficiency in sea stars: large eggs are not fertilised more readily than small eggs in the genus *Patiriella* (Echinodermata: Asteroidea). *Mar. Biol.* **147:** 235–242.

Thiyagarajan, V., and P.-Y. Qian. 2003. Effect of temperature, salinity and delayed attachment on development of the solitary ascidian *Styela plicata* (Lesueur). *J. Exp. Mar. Biol. Ecol.*. **290:** 133–146.

Thiyagarajan, V., T. Harder, and P.-Y. Qian. 2003. Combined effects of temperature and salinity on larval development and attachment of the subtidal barnacle *Balanus trigonus* Darwin. *J. Exp. Mar. Biol. Ecol.* **287:** 223–236.

Thorson, G. 1950. Reproductive and larval ecology of marine bottom invertebrates. *Biol. Rev.* **25:** 1–45.

Ushakova, O. O., and O. L. Sarantchova. 2004. The influence of salinity on fertilization and larval development of *Nereis virens* (Polychaeta, Nereidae) from the White Sea. *J. Exp. Mar. Biol. Ecol.* **301:** 129–139.

Williams, M. E., and M. G. Bentley. 2002. Fertilization success in marine invertebrates: the influence of gamete age. *Biol. Bull.* **202:** 34–42.