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# Polarity of Sperm Entry in the Ascidian Egg

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We have investigated the point of sperm entry in denuded eggs of the ascidian *Phallusia mammillata*. In contrast to what is generally believed, the sperm show a strong tendency to enter the animal hemisphere rather than the vegetal hemisphere. After entry, the sperm nucleus is carried toward the vegetal pole of the egg during the cortical contraction which occurs within a few minutes after fertilization. This polarity of sperm entry is abolished and the entry point is randomized by pretreating the eggs with cytochalasin D. We suggest that cytochalasin may act by randomizing components needed for sperm attachment or fusion, or structures needed for sperm entry. 

## INTRODUCTION

Since Conklin (1905) published his paper on the development of the ascidian *Cynthia (Styela)*, it has generally been believed that sperm tend to enter near the vegetal pole of ascidian eggs (see, e.g., Slack, 1983). However, it subsequently became clear that both animal and vegetal fragments of various ascidian eggs can be fertilized (Dalcq, 1932; Ortolani, 1958; Talevi and Dale, 1986; Bates and Jeffery, 1987). In addition, we have recently observed that the wave of elevated free calcium initiated by the sperm in *Phallusia* usually starts in the animal hemisphere (Speksnijder *et al.*, 1988). These findings suggested that Conklin's claim should be reexamined.

Unfortunately, it is not possible to directly observe the site of sperm entry in the living ascidian egg, because it is surrounded by test cells and a chorion with adhering follicle cells. The sperm entry site could be determined by serial sectioning of fixed material; however, this approach would be tedious and time-consuming. We overcame these difficulties by using eggs of the European ascidian *Phallusia mammillata*. These extremely clear eggs can be easily denuded with trypsin, then monospermically fertilized, and reared to normal tadpole larvae, provided that the embryos are kept from adhering to each other (Zalokar, 1979; Zalokar and Sardet, 1984). By using such denuded *Phallusia* eggs, which were vitally stained with a DNA-specific fluorescent dye and subsequently inseminated, we were able to visualize both the female chromosomes marking the animal pole as well as the nucleus of the sperm that had entered the egg. This enabled us to determine the position of the sperm nucleus relative to the animal pole in whole eggs fixed at various times after insemination.

Our results clearly show that sperm tend to enter the animal hemisphere of the egg and then move toward the vegetal pole during the first few minutes following fertilization.

## MATERIAL AND METHODS

### *Gametes*

Mature eggs and sperm were collected from the gonoducts of ripe *P. mammillata*, which were obtained from the Mediterranean Sea (Bassin de Thau, Sète, France) and the English Channel (Roscoff, France) and held in aquaria at Woods Hole (Massachusetts) or Villefranche-sur-Mer (France) at 18–22°C for several months. The eggs were washed in sea water (SW) and denuded by gentle agitation in 0.1% trypsin in SW for 2 hr at 18–20°C. The denuded eggs were washed extensively in SW and always handled in dishes and pipets coated with dried gelatin/formaldehyde (Zalokar and Sardet, 1984). Such denuded eggs develop into normal tadpoles after insemination, provided they are kept from adhering to each other.

### *Hoechst Staining*

The female chromosomes and male nucleus were visualized by staining with the DNA-specific vital dye Hoechst 33342 according to the method of Hinkey *et al.* (1987). Denuded eggs were incubated for 30 min in a solution of 10 μg/ml Hoechst 33342 (Calbiochem, La Jolla) in SW buffered with 10 m*M* TAPS (tris[hydroxymethyl]methylaminopropane sulfonic acid; Sigma, St. Louis, MO) at pH 8.0, and rapidly rinsed in SW TAPS, pH 8, to eliminate extracellular dye. The eggs were then inseminated with sperm (1 μl dry sperm/1 ml egg sus-

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pension) that had been preactivated by incubation with chorionated eggs in SW TAPS, pH 8, for 30 min (Sardet *et al.*, 1989). This labeling procedure not only stains the female chromosomes marking the animal pole, but it also labels the nucleus of the sperm that has entered the egg, and only that sperm.

From 6–7 sec to 5 min after insemination, aliquots of the egg suspension were rapidly diluted in 500 times their volume of SW TAPS, pH 8, containing 5.6% formaldehyde. After at least 1 hr, the eggs were washed two times in SW TAPS, pH 8, and mounted between a slide and a coverslip. To prevent distortion of the eggs, care was taken not to compress them by using an appropriate spacer. The eggs were subsequently examined with epifluorescence under uv-illumination (excitation filter G365; emission filter LP 420), and the locations of the male and female chromosomes were recorded. In control eggs, which were similarly denuded and inseminated but not fixed, the majority went on to form tadpoles.

In some experiments, the cortical contraction was blocked by treating the denuded eggs with cytochalasin D (Sigma) for 1 hr. Such eggs were first incubated in 2 μg/ml cytochalasin D in SW TAPS, pH 8, for 30 min and then in a solution of 2 μg/ml cytochalasin in SW TAPS to which 10 μg/ml Hoechst 33342 was added. The eggs were subsequently rinsed, inseminated, and fixed as described above, except that 2 μg/ml cytochalasin D was present continuously during these procedures.

### *Determination of the Sperm Entry Point*

The relative position of the female chromosomes marking the animal pole and the sperm nucleus was determined by estimating their angular distance during observation of each individual egg under the fluorescence microscope. This method is valid since both male and female chromosomes remain close to the egg surface for about 20 min after fertilization and because the eggs were not distorted by compression. These angular distances were divided into four categories: 0–45, 45–90, 90–135, and 135–180°. In each experiment, two to four aliquots of egg suspension were fixed at different times after insemination, and for each time point, the number of eggs in each angular category was determined. These numbers were then corrected for differences in surface area between the four angular categories, since the area from 0–45° represents only 15% of the total surface area, whereas that from 45–90° represents 35%. The final data in this paper therefore represent frequencies per unit area.

Each experiment was repeated at least three times; the results of the different experiments were similar and, therefore, the data of the different experiments were pooled. Unless stated otherwise, a total of about 50–200 eggs showing both egg and sperm chromosomes (see Fig. 1) was counted for each time point. The $\chi^2$ test was used to determine the statistical significance of our data.

## RESULTS

Figure 1 shows four typical images obtained in the fluorescence microscope after insemination of Hoechst-loaded *Phallusia* eggs. The female chromosomes, which mark the meiotic site and thus the animal pole of the egg, can be easily distinguished from the small, rod-shaped sperm nucleus. Both female and male chromosomes remain in the egg cortex for about 20 min after fertilization. The angular distance between the two was determined in large batches of eggs fixed at different times after insemination.

Our results are shown in Fig. 2. At 6–7 sec after insemination, the sperm nucleus is found in the animal hemisphere in 80% of the eggs (Fig. 2a). At 11–20 sec, the sperm nucleus is located in the animal hemisphere in 67% of the eggs. By 2 min after insemination, as a wave of cortical contraction moves toward the vegetal pole (Sawada and Osanai, 1981; Sardet *et al.*, 1986, 1989), the sperm nucleus is now present in the vegetal hemisphere in 59% of the eggs. By 5 min, when the contractile wave has reached the vegetal pole, 93% of the eggs have the male nucleus in the vegetal hemisphere. In fact, in 75% of the eggs the sperm nucleus is in the most vegetal part by this time (Fig. 2a). At these four different times, the percentages of eggs in which we could observe both the female chromosomes and the male nucleus were respectively 5, 10, 60, and 60%, indicating that there is some degree of fertilization asynchrony during the first minute or so. However, these percentages really are an underestimate of the percentage of eggs fertilized at these different times, since it often was not possible to see whether a sperm nucleus (or even female pronucleus) was present at the side of the egg facing away from the microscope objective due to background fluorescence and problems of focusing through the entire 140-μm egg. Still, about 90% of the control eggs that had not been fixed after insemination went on to cleave normally, indicating that at least 90% of the eggs were in fact fertilized.

Statistical analysis of the distribution of angles between the sperm nucleus and the female pronucleus using the $\chi^2$ test demonstrated statistical significance at 6–7 sec, 11–20 sec, and 5 min, but not at 2 min ($P \leq 0.01$). This indicates that there is a progressive shift in the distribution of sperm from animal (6–7 sec, 11–20 sec), to random (2 min), and finally to vegetal (5 min). The fact that the percentage of fertilized eggs between 2

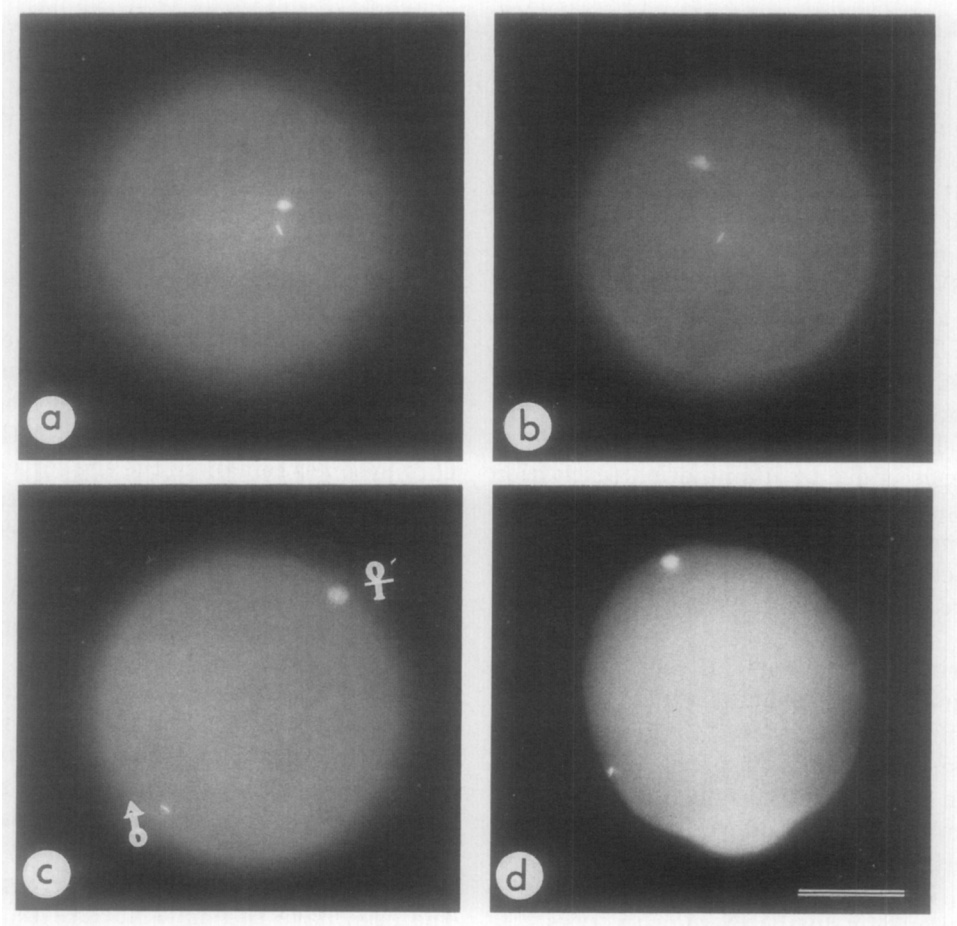


FIG. 1. Staining of female chromosomes and male nucleus with the fluorescent DNA-specific dye Hoechst 33342. The female chromosomes (♀) marking the meiotic site and thus the animal pole of the egg can be easily distinguished from the small rod-shaped sperm nucleus (♂). Bar = 50 μm. (a–c) Eggs fixed 20 sec after insemination. In these eggs the sperm nuclei were estimated to be at about 10, 30, and 140° from the animal pole. (d) Egg fixed at 5 min after insemination; the sperm nucleus is at about 130° from the animal pole. Note the characteristic bulge in the vegetal region.

and 5 min does not further increase, whereas a shift in sperm distribution still occurs, demonstrates that there is actual movement of the sperm nucleus toward the vegetal pole rather than a vegetal shift in entry preference.

Because the cortical contraction that follows fertilization is blocked by cytochalasins, drugs that affect the actin microfilament network (Zalokar, 1974; Sawada and Osanai, 1981), we thought that pretreating the eggs with cytochalasin would inhibit the movement of the sperm nucleus and yield a stable distribution of sperm nuclei peaking in the animal hemisphere. We found, in fact, that incubation with cytochalasin D for 1 hr prior to insemination inhibited the movement of the sperm nucleus toward the vegetal pole. More interestingly, cytochalasin also abolished the preference for entry in the animal hemisphere; the sperm nuclei were found to be randomly distributed among the four parts of the egg from 15 sec up to 5 min after insemination as tested by the $\chi^2$ test (see Fig. 2b). As is also shown in this figure, the nontreated control eggs showed a distribution of sperm nuclei similar to that in the first series of experiments (Fig. 2a), thereby confirming the validity of our finding. Again, the $\chi^2$ test revealed that the nonrandom distributions of sperm in these nontreated control eggs at 25 sec, 3 min, and 5 min are statistically significant ($P \leq 0.01$). At 15 sec such significance was not found, which is most likely due to the small number of observations at this time ($n = 29$).

## DISCUSSION

In this paper, we have investigated the point of sperm entry in the denuded egg of the ascidian *P. mammillata* by using a vital fluorescent dye which visualizes both the meiotic pole and the entering sperm immediately after fertilization. Our results show that 6 to 7 sec after insemination, in most eggs, the sperm nucleus is located

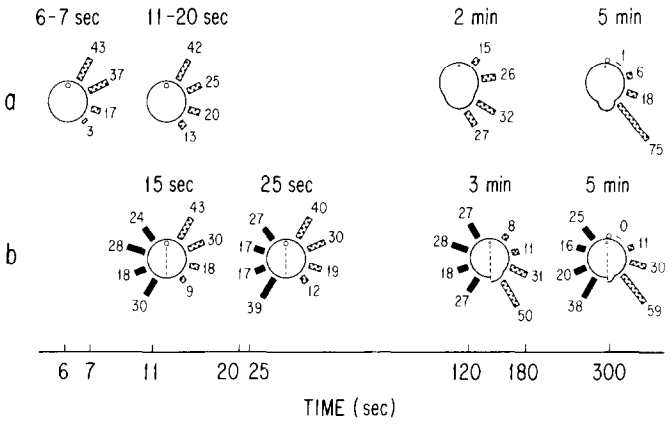


FIG. 2. (a) Distribution of the angles between the sperm nucleus and the female chromosomes at the animal pole at four different times after insemination. The circle at the animal pole (top) of each egg indicates the position of the female chromosomes. The bars on the egg surface indicate the frequencies of angles per unit area (expressed as percentages) between 0–45, 45–90, 90–135, and 135–180° (see Materials and Methods). The numbers of eggs counted at the four times are 57, 214, 133, and 59, respectively. (b) Effects of cytochalasin D on the distributions of the angles between female and male chromosomes at four different times after insemination. Controls are shown at the right side of each egg (stippled bars); cytochalasin effects are shown on the left side (black bars). The numbers of eggs counted at the four times are respectively 29, 92, 106, and 96 for control eggs and 49, 78, 86, and 104 for cytochalasin-treated eggs.

in the animal hemisphere. In eggs fixed 3 to 5 min after insemination, the sperm nucleus in most eggs is found in the most vegetal part. Although we cannot exclude the very remote possibility that these results are due to removal of the chorion, we are confident that our data represent a true polarity in sperm entry sites, since it would be hard to envision how trypsin treatment could create polarity in the egg surface. In addition, development of these denuded eggs proceeds normally. Therefore, we infer from our results that sperm prefer entry in the animal hemisphere and that after entry the sperm nucleus moves toward the vegetal pole.

Most likely, this movement is driven by the cortical contraction which accompanies the first phase of ooplasmic segregation and sweeps from the animal toward the vegetal pole during the first few minutes following fertilization (Sawada and Osanai, 1981; Sardet *et al.*, 1986, 1989). This cortical contraction is known to move a large complex of cortical and surface components, which includes cytoskeletal structures, cortical pigment granules, and mitochondria, as well as test cells, supernumerary sperm, and various artifically attached surface particles (Conklin, 1905; Ortolani, 1955; Sawada and Osanai, 1981, 1985; Jeffery and Meier, 1983; Sardet *et al.*, 1989). It seems very likely that this cortical contraction would also carry the fertilizing sperm toward the vegetal pole after it had entered. In fact, the progressive shift in position of the sperm during the period between 2 and 5 min—when the percentage of fertilized eggs does not increase further—demonstrates that there is actual movement of the sperm nucleus toward the vegetal pole rather than a vegetal shift in entry preference. Supporting evidence for such movement of the sperm nucleus comes from our recent observations on the free calcium wave that follows fertilization (Speksnijder *et al.*, in preparation) which show that (1) in cytochalasin D-treated eggs, in which the cortical contraction is blocked, the sperm aster is first visible at the same location as the starting position of the calcium wave. This shows that the calcium wave starts at the site of sperm entry, as has also been demonstrated in many other species (see, e.g., Gilkey *et al.*, 1978; Busa and Nuccitelli, 1985; Hafner *et al.*, 1988). (2) In nontreated eggs, the wave usually starts in the animal hemisphere, yet the sperm aster is first visible in the vegetal hemisphere (Speksnijder *et al.*, 1988; Speksnijder *et al.*, unpublished). These results can only be explained by an actual movement of the sperm nucleus toward the vegetal pole after entry in the animal hemisphere.

Our observations also explain why it has been long believed that sperm enter near the vegetal pole of ascidian eggs: Conklin fixed *Cynthia* (now *Styela*) eggs several minutes after fertilization. The sperm nucleus he observed near the vegetal pole presumably moved there during the first phase of ooplasmic segregation, mediated by the cortical contraction wave.

The randomization of sperm entry by cytochalasin treatment indicates that an intact actin filament network is required for polarized sperm entry. This actin network could localize components of the plasma membrane—either receptors or fusogenic sites—which mediate attachment or fusion of the sperm with the egg. Another intriguing possibility is the actin-controlled localization of components in the egg cortex which mediate sperm entry *after* attachment and fusion. There is evidence that sperm fusion does not necessarily lead to entry of the fused sperm; sperm have been demonstrated to detach again after fusion to sea urchin oocytes and eggs (Dale and Santella, 1985; McCulloh and Chambers, 1986). This suggests that certain control mechanisms for sperm entry may exist which exert their effect *after* fusion has occurred and are independent of the fusion process itself. In addition, both medaka fish eggs (Yamamoto, 1961) and *Rana* eggs (Goldenberg and Elinson, 1980) are more easily activated by a needle (as well as by sperm) in their animal halves, which suggests that the preference for sperm entry in the animal hemisphere is controlled by a mechanism following sperm fusion. Such a control mechanism

might be at the level of the cortical network of endoplasmic reticulum which is believed to be the main source of calcium for the waves of elevated free calcium that activate deuterostome eggs like *Phallusia* (Jaffe, 1985; Speksnijder *et al.*, 1986). Preliminary data have shown an abundant cortical endoplasmic reticulum containing calcium in the *Phallusia* egg (Sardet *et al.*, 1988; Gualtieri and Sardet, unpublished data). If there were an animal concentration of this network—similar to what has been shown in *Xenopus* eggs (Gardiner and Grey, 1983; Charbonneau and Grey, 1984; Campanella *et al.*, 1984)—then it could play a role in the preference for animal entry of sperm by facilitating in that area the initiation of the calcium wave, which in turn might be a prerequisite for sperm entry after fusion.

In large eggs, entry near the meiotic site shortens the path to nuclear fusion; but it clearly does not serve that function in ascidians. Most likely, the polarity of sperm entry reflects the underlying polar organization of the egg, either of its surface or of its submembranous physiological machinery.

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