

Germ-Cell Warfare in Ascidians: Sperm From One Species Can Interfere With the Fertilization of a Second Species

CHARLES C. LAMBERT*

Department of Biological Science, California State University, Fullerton, California 92834-6850

Ascidians (invertebrate chordates) are very abundant in many marine subtidal areas. They often live in dense multi-species clumps; thus, interspecific competition for space may be intense. Although most noncolonial species are broadcast spawners, their eggs can be fertilized only by sperm of the same species (1). Multiple fertilization is lethal and all animals have evolved blocks to polyspermy. Ascidian eggs block polyspermy by enzymatic (2) and electrical mechanisms (3). Sperm bind to N-acetylglucosamine groups on the vitelline coat (4, 5, 6, 7). Follicle cells surrounding the vitelline coat release N-acetylglucosaminidase during egg activation (8), preventing the binding of all sperm but a few (2). I show here that this interaction is not species-specific; sperm from one species can cause glycosidase release from follicle cells of a second species. Furthermore, once glycosidase release has been induced, the subsequent addition of sperm from the egg-producing species fails to fertilize a substantial proportion of these eggs. This leads to the hypothesis that sperm from one species of ascidian can interfere with fertilization of a second species. While intraspecific sperm competition has been well documented in several taxa (9, 10), this is the first record of sperm competition between species, or interspecific sperm competition.

As previously shown by numerous authors (1), ascidian fertilization is species-specific. Eggs of *Phallusia mammillata*, *Phallusia julinea*, *Ascidia* (= *Phallusia*) *nigra*, and *Ascidia sydneyensis* are induced to undergo cleavage only when fertilized with sperm from the same species (Table 1). In *Phallusia mammillata* eggs, the failure to fertilize is the result of sperm from *Ascidia mentula*, *Ciona intestinalis*,

and *Ascidella aspersa* being unable to penetrate the vitelline coat. Penetration of the egg coverings is also involved in the block to hybridization of *Ascidella aspersa* (11) and *Ascidia malaca* eggs (12, 13). Hybridization is possible after removal of the vitelline coat from eggs of *Ciona intestinalis*, *Phallusia mammillata*, *Ascidella aspersa*, *Ascidia malaca*, and *Ascidia mentula* (14), and *Ciona savignyi* (15). In eggs from *A. sydneyensis*, *A. nigra*, and *Phallusia julinea*, the precise mechanism of the block remains unknown; nevertheless, sperm from these species are unable to fertilize the eggs of other species. However, in all other known cases, failure of hybridization is clearly at the level of the egg coverings, so this is also likely to be true for these species.

While fertilization is species-specific, glycosidase release clearly is not (Table 1). Sperm from several different species of ascidians can all induce glycosidase release; in all cases, heterologous sperm were capable of inducing glycosidase release from each of the four species of eggs used. This nonspecific glycosidase release varied considerably between the different egg and sperm combinations; even sperm from *Herdmania momus* (Order Stolidobranchia) elicited glycosidase release from *Ascidia sydneyensis* (Order Phlebobranchia) eggs. However, sperm from the oyster *Saccostrea cucullata* failed to cause glycosidase release from *A. nigra* eggs (data not shown).

The source of the glycosidase is the follicle cells, because sperm induce glycosidase release in the presence of the drug suramin, which prevents the sperm from penetrating the vitelline coat (8). In addition, homologous sperm added to isolated follicle cells of *Phallusia mammillata* (8) and *Ascidia ceratodes* cause glycosidase release (K. McKinney and C. C. Lambert, unpubl. obs., 1997). The nature of this interaction remains unknown, although phospholipase *c* and tyrosine phosphorylation appear to be involved in the actual

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* Current address: 12001 11th Ave. NW, Seattle, WA 98177. E-mail: clambert@fullerton.edu

Table I

Glycosidase release and fertilization of ascidian eggs fertilized with homologous and heterologous sperm

Eggs	Sperm	% Glycosidase release	Fertilization (cleavage)
<i>Phallusia</i>			
<i>mammillata</i>	<i>P. mammillata</i>	100% (Control)	Yes
<i>P. mammillata</i>	<i>Ascidia mentula</i>	46.92 ± 16.6 (7)	No
<i>P. mammillata</i>	<i>Ascidella aspersa</i>	66.53 ± 8.7 (18)	No
<i>P. mammillata</i>	<i>Ciona intestinalis</i>	63.6 ± 16.1 (6)	No
<i>Ascidia</i> (= <i>Phallusia</i>)			
<i>nigra</i>	<i>Ascidia nigra</i>	100% (Control)	Yes
<i>A. nigra</i>	<i>A. sydneienseis</i>	71.5 ± 20.4 (4)	No
<i>A. nigra</i>	<i>Phallusia julinea</i>	130.25 ± 37.6 (4)	No
<i>Ascidia sydneienseis</i>	<i>A. sydneienseis</i>	100% (Control)	Yes
<i>A. sydneienseis</i>	<i>A. nigra</i>	117.2 ± 29.2 (6)	No
<i>A. sydneienseis</i>	<i>Phallusia julinea</i>	99 ± 45.5 (4)	No
	<i>Herdmania</i>		
<i>A. sydneienseis</i>	<i>momus</i>	112.98 ± 26.6 (4)	No
<i>Phallusia julinea</i>	<i>P. julinea</i>	100% (Control)	Yes
<i>P. julinea</i>	<i>A. sydneienseis</i>	16.1 ± 9.9 (2)	No
<i>P. julinea</i>	<i>Ascidia nigra</i>	26.2 ± 17 (6)	No

Note that although fertilization is strictly species-specific, glycosidase release can be initiated in most species by a variety of heterologous sperm. Glycosidase release is expressed as a percentage of that occurring with homologous fertilization. The indicated values are means ± the standard error of the mean. The numbers in parentheses indicate the number of replicates. Normal fertilization was achieved by using 5 µl of a 1:1000 dilution of dry sperm in each 0.5 ml aliquot of eggs. *N*-acetylglucosaminidase activity was measured using a fluorometric assay as in reference 2.

Phallusia mammillata, *Ascidia mentula*, *Ascidella aspersa*, and *Ciona intestinalis* were collected from floating docks near Brest, France. *Ascidia* (= *Phallusia*) *nigra*, *Ascidia sydneienseis* and *Herdmania momus* were collected from floating docks in Keehi Lagoon and Kewalo Basin, Honolulu, Hawaii, and buoys in Apra Harbor, Guam. *Phallusia julinea* was collected from coral reefs in Apra Harbor, Guam.

release (16). Living sperm have not been observed to bind to isolated follicle cells under the microscope; rather, they bounce into the cells and make them move. If the sperm activates a receptor, it may not have to occupy the receptor continuously in order for the glycosidase release to occur.

Since sperm from non-egg (heterologous) species can induce glycosidase release and since the glycosidase reduces sperm binding, it seemed possible for sperm from one species of ascidian to interfere with the actual fertilization of a second species by its own sperm. Accordingly, eggs of *Ascidia sydneienseis*, *Ascidia nigra*, and *Phallusia julinea* were each exposed to sperm from the other two species, incubated 5 min for the completion of glycosidase release (17), and then re-inseminated with sperm from the egg-producing (homologous) species. In all cases, cleavage was definitely impaired when compared to the controls, which had received only homologous sperm (Table 2). The highest inhibition (44%) occurred in *Ascidia sydneienseis* eggs using

Ascidia nigra as the first sperm; interestingly, the lowest (10%) also occurred in *A. sydneienseis* eggs first inseminated with *Phallusia julinea* sperm. The reciprocal of *A. nigra* eggs exposed first to *A. sydneienseis* sperm also yielded strong inhibition of *A. nigra* fertilization (27%). This is significant because *A. nigra* and *A. sydneienseis* co-occur in dense aggregations on floats in Keehi Lagoon in Honolulu, Hawaii, and on harbor buoys in Apra Harbor, Guam. *Phallusia julinea* is not evident in Hawaiian harbors, and in Guam it is never a member of the fouling community but always found in reef crevices at the bottom of the bay. Thus it is not likely that eggs of *A. nigra* or *A. sydneienseis* would contact *P. julinea* sperm to an appreciable degree in nature.

In the laboratory, sperm of *A. sydneienseis* and *A. nigra* can clearly interfere with the fertilization of each other's eggs. Does such an interference occur in nature? Direct evidence is lacking on this point, but in Keehi Lagoon and Kewalo Basin in Honolulu *A. nigra* is much more abundant than *A. sydneienseis*. Sperm of *A. nigra* interfere much more (44%) with the fertilization of *A. sydneienseis* eggs than the reverse (27%). I speculate that at least a portion of this unequal abundance is the result of fertilization interference. Ideally, for this to occur both species would have to spawn at about the same time. Although there is considerable information on the spawning of several ascidian species (18, 19, 20), there are apparently no studies on the spawning of any ascidian in the family Ascidiidae, which includes both of these species. However, ascidian sperm are capable of fertilizing eggs 12 h or more after dilution (21), and sperm attraction to eggs is not species-specific (22); consequently spawning does not have to be synchronous for interspecific sperm competition to occur.

These experiments support the hypothesis that foreign sperm can cause a failure in the ability of eggs to be

Table II

Fertilization interference between ascidian species

Egg	First sperm	Second sperm	% Inhibition of cleavage (n)
<i>Ascidia</i>			
<i>sydneienseis</i>	<i>Ascidia nigra</i>	<i>A. sydneienseis</i>	44 ± 13.7 (5)
<i>A. sydneienseis</i>	<i>Phallusia julinea</i>	<i>A. sydneienseis</i>	10 ± 5 (2)
<i>Ascidia nigra</i>	<i>A. sydneienseis</i>	<i>A. nigra</i>	27.7 ± 3.8 (16)
<i>A. nigra</i>	<i>Phallusia julinea</i>	<i>A. nigra</i>	33.5 ± 9.6 (2)
<i>Phallusia julinea</i>	<i>A. sydneienseis</i>	<i>P. julinea</i>	27 ± 6.0 (2)
<i>P. julinea</i>	<i>A. nigra</i>	<i>P. julinea</i>	20 ± 6.0 (2)

In these experiments 2 ml of eggs were first fertilized with 10 ml of a 1:1000 dilution of heterologous sperm, then 5 min later with 10 ml of a 1:1000 dilution of homologous sperm. When the control cells reached the 2-cell stage they were fixed in 1% formaldehyde and the percentage of 2-cell embryos was counted in at least 100 eggs. The mean percentage inhibition of cleavage relative to the controls is indicated ± the standard error of the mean; (n) = number of replicates.

fertilized by their own species' sperm. Inhibition of fertilization success in one species by another could enhance the sperm producer's overall reproductive success relative to its competitors. Since space is a limiting resource in the fouling community, reduction of competition by fertilization interference would definitely enhance the success of the interfering species in occupying space (23). Fertilization in the sea is chancy and less than 100% fertilization is the norm (24). These experiments were carried out with sperm concentrations of the homologous species high enough that over 90% of the eggs were routinely fertilized in the controls. It is very likely that reducing the homologous sperm concentration to one that would produce a more realistic level of control fertilization—perhaps 50%—would yield much higher values of heterologous sperm interference. Indeed, nonhomologous sperm can cause the release of at least as much glycosidase activity as homologous sperm in several cases, yet the greatest inhibition is only 44%. Using a lower concentration of homologous sperm might result in a higher level of inhibition. However, even the modest levels of fertilization interference demonstrated here in these preliminary studies could reduce competition for space. The hypothesis of *interspecific* sperm competition is supported by these experiments. This could be an important factor in the distribution and abundance of many broadcast-spawning organisms, including both plants and animals, because all organisms have blocks to polyspermy, many of which involve sperm-induced enzyme release (25). Although fertilization leading to cleavage is species-specific, until now there have been no studies on the specificity of the polyspermy block. The more well-known form of sperm competition involves animals with internal fertilization in which sperm from several males compete to fertilize eggs of the same species (9, 10). This *intraspecific* sperm competition also occurs in certain colonial ascidians with internal fertilization (26). Thus ascidians reveal both intra- and interspecific modes of sperm competition.

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Literature Cited

1. **Reverberi, G. 1971.** Ascidians. Pp. 507–550 in *Experimental Embryology of Marine and Fresh-water Invertebrates*, G. Reverberi, ed. North-Holland, Amsterdam.
2. **Lambert, C. C. 1989.** Ascidian eggs release glycosidase activity at fertilization that aids in the block against polyspermy. *Development* **105**: 415–420.
3. **Goudeau, H., Y. Depresle, A. Rosa, and M. Goudeau. 1994.** Evidence by a voltage clamp study of an electrically mediated block to polyspermy in the egg of the ascidian *Phallusia mammillata*. *Dev. Biol.* **166**: 489–501.
4. **Lambert, C. C. 1986.** Fertilization-induced modification of chorion N-acetylglucosamine groups blocks polyspermy in ascidian eggs. *Dev. Biol.* **116**: 168–175.
5. **Godknecht, A. J., and T. G. Honegger. 1995.** Specific inhibition of sperm β -N-acetylglucosaminidase by the synthetic inhibitor N-acetylglucosaminono-1,5-lactone O-(phenylcarbamoyl)oxime inhibits fertilization in the ascidian, *Phallusia mammillata*. *Dev. Growth Differ.* **37**: 183–189.
6. **Honegger, T. G. 1986.** Fertilization in ascidians: Studies on the egg envelope, sperm and gamete interactions in *Phallusia mammillata*. *Dev. Biol.* **118**: 118–128.
7. **Hoshi, M., S. Takizawa, and N. Hirohashi. 1994.** Glycosidases, proteases and ascidian fertilization. *Semin. Dev. Biol.* **5**: 201–208.
8. **Lambert, C. C., H. Goudeau, C. Franchet, G. Lambert, and M. Goudeau. 1997.** Ascidian eggs block polyspermy by two independent mechanisms, one at the egg plasma membrane, the other involving the follicle cells. *Mol. Reprod. Dev.* **48**: 137–143.
9. **Parker, G. A. 1984.** Sperm competition and the evolution of animal mating strategies. Pp. 1–60 in *Sperm Competition and the Evolution of Animal Mating Systems*, R. L. Smith, ed. Academic Press, Orlando, FL.
10. **Roldan, E. R. S., M. Gomendio, and A. D. Vialto. 1992.** The evolution of eutherian spermatozoa and underlying selective forces: female selection and sperm competition. *Biol. Rev.* **67**: 551–593.
11. **Villa, L., and E. Patricolo. 1992.** Ascidian interspecific fertilization. I. Preliminary data on the involvement of the follicle cell layer. *Eur. Arch. Biol.* **103**: 25–30.
12. **Patricolo, E., and L. Villa. 1992.** Ascidian interspecific fertilization. II. A study of the external egg coating in hybrid crosses. *Anim. Biol.* **1**: 9–15.
13. **Patricolo, E., and L. Villa. 1995.** Ascidian interspecific fertilization. III. Ultrastructural investigations of sperm-egg interaction. *Eur. J. Morph.* **33**: 433–442.
14. **Minganti, A. 1959.** Lo sviluppo embrionale ed il comportamento dei cromosomi in ibrid tra cinque specie di Ascidie. *Acta Embryol. Morphol. Exp.* **2**: 269–301.
15. **Byrd, J., and C. C. Lambert. 2000.** Mechanism of the block to hybridization and selfing between the sympatric ascidians *Ciona intestinalis* and *Ciona savignyi*. *Mol. Reprod. Dev.* **55**: 109–116.
16. **Robert, L. K., L. M. Lucio, C. A. Goode, K. McKinney, and C. C. Lambert. 1999.** Activation of a follicle cell surface phospholipase by a tyrosine kinase dependent pathway is an essential event in ascidian fertilization. *Mol. Reprod. Dev.* **54**: 69–75.
17. **McDougall, A., C. Sardet, and C. C. Lambert. 1995.** Different calcium-dependent pathways control fertilization-triggered glycosidase release and the cortical contraction in ascidian eggs. *Zygote* **3**: 251–258.
18. **Lambert, C. C., and C. L. Brandt. 1967.** The effect of light on the spawning of *Ciona intestinalis*. *Biol. Bull.* **132**: 222–228.
19. **Lambert, G., C. C. Lambert, and D. P. Abbott. 1981.** *Corella* species in the American Pacific Northwest: distinction of *C. inflata* from *C. willmeriana* based upon morphology, reproduction and genetics. *Can. J. Zool.* **59**: 1493–1504.
20. **West, A. B., and C. C. Lambert. 1976.** Control of spawning in the

- tunicate *Styela plicata* by variations in the natural light regime. *J. Exp. Zool.* **195**: 263–270.
21. **Bolton, T. F., and J. N. Havenhand. 1996.** Chemical mediation of sperm activity and longevity in the solitary ascidians *Ciona intestinalis* and *Ascidella aspersa*. *Biol. Bull.* **190**: 329–335.
22. **Yoshida, M., K. Inaba, and M. Morisawa. 1993.** Sperm chemotaxis during the process of fertilization in the ascidians *Ciona savignyi* and *Ciona intestinalis*. *Dev. Biol.* **157**: 497–506.
23. **Branch, G. M. 1984.** Competition between marine organisms: ecological and evolutionary implications. *Oceanogr. Mar. Biol. Annu. Rev.* **22**: 429–593.
24. **Levitan, D. R., and C. Petersen. 1995.** Sperm limitation in the sea. *Trends Ecol. Evol.* **10**: 228–231.
25. **Jaffe, L. A., and M. Gould. 1985.** Polyspermy preventing mechanisms. Pp. 223–250 in *Biology of Fertilization*, Vol 3, C. B. Metz and A. Monroy, eds. Academic Press, Orlando, FL.
26. **Yund, P. O. 1998.** The effect of sperm competition on male gain curves in a colonial marine invertebrate. *Ecology* **79**: 328–339.