Protocol for fertilization tests in Ciona intestinalis

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1. All specimens were of standardized size (6-7 cm), maturation status level 3-4, and as far as possible exempt of epiphytes.

2. Stressed animals require at least one week to recover after delivery to the laboratory (ASSEMBLE-JRA1-Protocol-03.00) after which they can be used for research purposes.

Eggs and sperm are removed separately from the gonoducts:

3. *Eggs:* Make a longitudinal incision through the test, on the dorsal side starting just above the atrial siphon. Eggs are collected from the gonoduct with a Pasteur pipette (PP) and transferred into a Petri dish containing filtered seawater. Eggs should have a short time to rest (5 - 10 min) before insemination to facilitate the expansion of the chorion (follicle cells), which makes the eggs float and thus improves fertilization.
4. *Sperm:* Collect "dry" by introducing a PP into the spermiduct or sucking up drops coming from the broken spermiduct. Dilute sperm in seawater just before being added to the eggs to ensure maximal motility during insemination (one drop of very dry sperm in 50 ml of seawater).

Fertilization:

5. Mix sperm and oocytes from 2 or 3 individuals and then perform the fertilization of mixed oocytes with the mix of sperm. Gentle agitation helps the mixing of gametes.
6. Dishes should be kept at a temperature in the range of 18 - 20 °C, to bring about development within 18 hours.
7. Water should be changed 1 h after insemination, in order to remove surplus sperm.
8. Check the mix after two or three hours to establish if the first one or two cell divisions have taken place. Failure at this point should be scored as 'infertile' and represents serious problem with egg or sperm quality.

Development:

9. Check the developing larvae/embryos 15-18 hours later when larva should be hatching. At this point it should be obvious if development has proceeded normally or not. Miss-developed, arrested embryos (see notes) should be scored as 'failed development'.

Animals: Healthy adults collected from natural population.

Apparatus: Scalpels, Pasteur pipettes, Petri dish, filtered seawater.

Parameters: T° 18

Additional information:

1. Avoid excess of sperm, which sticks to the follicular cells of the eggs and endangers insemination.

2. Sperm and eggs are usually collected from different specimens to obtain cross fertilization.

3. Mutations Note that some failures of embryonic development are due to genetic problems (mutations) during the recombination event (quality of the sperm and eggs OK). This problem can be verified by counting the number of normal vs. miss-developed larvae. If the ratio is 3:1 or some such similar proportion (Mendelian ratio) it may be safely assumed that this is not a problem of sperm or egg quality.

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