# Sperm activation in *Balanus balanoides* (Crustacea: Cirripedia)

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*Summary.* Ammonium ions alone will activate both vesicula and inseminated spermatozoa of *Balanus balanoides*. Maximum activity, however, occurs when inseminated spermatozoa are treated with the blood and oviducal gland fluid of *B. balanoides*.

*Balanus balanoides* (L.) is a littoral boreo-artic hermaphroditic barnacle. In North Wales, cross-fertilization takes place during November and early December; the spermatozoa are stored in the vesiculae seminales (vesicula spermatozoa[2]) for up to 2 months prior to this period. Earlier workers have reported the relative inactivity of these vesicula spermatozoa in *B. balanoides*[2,3]. During cross-fertilization the penis of the 'acting male' searches for the opercular opening of an 'acting female' and transfers spermatozoa into the mantle cavity of the recipient. Observations during November and December 1976, suggested that more than 1 insemination (up to 8) was needed before oviposition could commence[4]. Newly inseminated spermatozoa which occur as discrete gelatinous masses within the mantle cavity of the recipient, are inactive, but just prior to and during oviposition they are said to become active[5]. Barnes et al.[2] believe that spermatozoan activity increases after passage through the penis, whereas Walley et al.[3] have shown that activity of inseminated spermatozoa is initiated by the clear fluid from the oviducal glands, small glands situated at the terminations of the oviducts[6]. The results in the present paper show how both vesicula and inseminated spermatozoa from *B. balanoides* can be activated by ammonium ions.

*Material and methods.* The early experiments in October and November were performed on vesicula spermatozoa because cross-fertilization had not yet occurred in animals at the top of the shore[7]. The morphology and some of the ultrastructure of vesicula spermatozoa in this species has already been described[2,3,8]. Vesicula spermatozoa were virtually inactive in sea water and remained inactive when chelating agents such as ethylene-diamine-tetra-acetic acid, glycine or L-histidine were added. Addition of metal cations ($Na^+$, $Zn^{++}$, $Ca^{++}$, $Mg^{++}$ and $Li^+$) to the sea water, or sugars (fructose, sucrose) in various concentrations also had no effect. There was slight activity in potassium chloride or urea solutions (0.01 M) made up in sea water, but much greater activity occurred with ammonia in sea water and with ammonium salt solutions. Ammonium ions were used because it is known that *B. balanoides* produces ammonia as an excretory product (White, personal communication) which is released into the mantle cavity, where it could have an effect on the inseminated spermatozoa. A stock solution of ammonia in sea water was prepared by adding 1 ml of 0.88 ammonia per l to filtered, UV-irradiated sea water. The stock solution was diluted, the pH measured and the ammonia solutions tested on vesicula spermatozoa. The ammonium salts [$NH_4Cl$ and $(NH_4)_2SO_4$] were made up in sea water and similarly tested.

*Results and discussion.* Table 1 shows that ammonium ions stimulate the spermatozoa into activity over a wide concentration range. Although the level of activity was not high (< 20% sperm active), it was significant compared with the sea water controls (< 1% sperm active). Induced activity took approximately 2 min to begin and the mean swimming velocity of activated vesicula spermatozoa at 12 °C was 119 μm/sec (range 57–182 μm/sec). Later experiments conducted in November and December were carried out on the inseminated spermatozoa. Barnacles taken from various levels on the shore during this period were closely observed in aquaria in the laboratory and when an insemination was completed the recipient was removed carefully from the substrate and dissected to reveal the discrete mass of inseminated spermatozoa. Masses collected in this way were transferred into fresh sea water and small portions viewed under the microscope

Table 1. Activation of vesicula spermatozoa by ammonium ion solutions

| | μg N $ml^{-1}$ | pH in sea water | Activity |
|---|---|---|---|
| Ammonia | 204 | 9.9 | + |
| | 41 | 9.0 | + |
| | 20 | 8.7 | + |
| | 10 | 8.5 | — |
| $NH_4Cl$ | 1400 | 7.0 | + |
| | 140 | 7.6 | + |
| | 70 | 7.7 | + |
| | 35 | 7.7 | — |
| $(NH_4)_2SO_4$ | 2800 | 6.8 | — |
| | 280 | 7.5 | + |
| | 140 | 7.6 | + |
| | 70 | 7.8 | — |

+, Active; —, inactive.

Table 2. Activation of inseminated spermatozoa by ammonium ion solutions

| | μg N $ml^{-1}$ | pH in sea water | Activity |
|---|---|---|---|
| Ammonia | 37 | 9.0 | + |
| | 18 | 8.9 | ++ |
| | 9 | 8.5 | ++ |
| | 5 | 8.4 | + |
| | 4 | 8.0 | — |
| $NH_4Cl$ | 1400 | 7.0 | — |
| | 140 | 7.6 | + |
| | 14 | 7.9 | + |
| | 3.5 | 7.9 | + |
| $(NH_4)_2SO_4$ | 280 | 7.5 | — |
| | 140 | 7.6 | + |
| | 28 | 7.9 | + |
| | 5.6 | 7.9 | + |

++, Highly active; +, active; —, inactive.

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3 L. J. Walley, F. White and K. M. Brander, J. mar. biol. Ass. U.K. *5*, 489 (1971).
4 D. J. Clegg, Rep. Challenger Soc. *3* (IX), 18 (1957).
5 H. Barnes and D. J. Crisp, J. mar. biol. Ass. U.K. *35*, 631 (1956).
6 L. J. Walley, J. mar. biol. Ass. U.K. *45*, 115 (1965).
7 D. J. Crisp, Oikos *10*, 275 (1959).
8 E. A. Munn and H. Barnes, J. exp. mar. Biol. Ecol. *4*, 261 (1970).

Table 3. List of blood samples and plasma which initiated activity in inseminated spermatozoa

| Source | | pH | μg ammonium-N $ml^{-1}$ |
|---|---|---|---|
| Barnacles | | | |
| *Balanus balanoides* | Blood | – | – |
| *B. crenatus* | Blood | – | – |
| *Chthamalus montagui* | Blood | – | – |
| *Elminius modestus* | Blood | – | – |
| *B. hameri* | Plasma | 7.4 | 3.1–5.8* |
| Crabs | | | |
| *Carcinus maenas* | Plasma | 7.6 | 6.4–7.8* |
| *Cancer pagurus* | Plasma | 7.7 | 9.0–14.2* |
| Bivalves | | | |
| *Mytilus edulis* | Blood | – | – |
| *Pecten maximus* | Blood | – | – |
| Fish | | | |
| Dab (*Limanda limanda*) | Plasma | 6.9 | 3.2–4.1* |
| Bird | | | |
| Quail (*Coturnix coturnix japonica*) | Plasma | 8.2 | 3.2* |
| Human | Plasma | 7.4 | 2.8–8.0 (normal range for human plasma) |

* Determined by Conway diffusion method[8].

for signs of activity. Inactive inseminated spermatozoa only were used in the tests (table 2).

The results show that inseminated spermatozoa are more susceptible to ammonium ions than vesicula spermatozoa, since activity is initiated at lower levels of ammonium-N. Inseminated spermatozoa kept in sea water for 3–4 h remained inactive, so passage through the penis does not initiate activity[2]; at the end of this time the inseminated spermatozoa could no longer be induced to become active and were presumed to be dead.

Fluid collected by micropipette from the oviducal glands did not activate vesicula spermatozoa but readily activated inseminated spermatozoa. An instant 'explosion' of activity was observed when a portion of the inseminated spermatozoan mass was placed in a drop of oviducal gland fluid. Such activated spermatozoa had a mean speed of 159 μm/sec (range 105–200 μm/sec) at 12 °C. The epithelium lining each of the oviducal glands produces an elastic sac into which the eggs pass at oviposition[6]. When an 'acting female' is in a receptive state the oviducal glands are invariably swollen with fluid. How this fluid arises is not known at present, but a clue to its origin lies in the fact that the blood of *B. balanoides* initiates high activity in inseminated spermatozoa. The epithelium lining the oviducal glands at the time of insemination is reduced in size and the underlying basement membrane is very thin[6], so filtration of the blood across the lining could give rise to the oviducal gland fluid.

The discovery that the blood of *B. balanoides* activates inseminated spermatozoa stimulated the testing of the blood of other animals (table 3). Although all the samples tested initiated activity in inseminated spermatozoa, the blood of *Cancer pagurus* was the most effective. None of the blood samples activated vesicula spermatozoa. The high levels of activity initiated by blood could not be mimicked by the ammonium ion solutions alone, even when adjusted to blood pH values. It is concluded that a further factor must be present in the blood (and oviducal gland fluid).

As only a small amount of blood can be collected from *B. balanoides*, the blood from either *B. hameri* or *Cancer* was used in the further experiments. An ultrafiltrate of *B. hameri* plasma (Amicon UM 20 membrane, which according to the makers has a mol. wt cut-off at 20,000) was still active and initiated high activity in inseminated spermatozoa. Boiling the filtrate for several min so that its ammonium-N level becomes negligible completely destroys the activity. In a more detailed experiment plasma from *Cancer* was boiled for different lengths of time and subsampled for bioassay. The activity progressively decreased as the ammonium-N level fell from 9.0 μg $ml^{-1}$ to 2.8 μg $ml^{-1}$.

Although the evidence is partially circumstantial, there is reason to believe that the activation of inseminated *B. balanoides* spermatozoa is initiated, in part, by ammonium ions; a further factor normally present in the blood (and oviducal gland fluid) is necessary for maximum activity. Oviducal gland fluid is undoubtedly the activating fluid and is released just prior to oviposition[9]. The initiation of activity of the spermatozoa is synchronized for once activated the gametes remain so for only 5–6 min. Oviducal gland fluid also appears to cause the progressive breakdown of the gelled mass of inseminated spermatozoa; breakdown may be the result of enzymic and/or pH effects, or it may simply be that the spermatozoa once activated are able to swim clear of the mass. This is important because it means that there is continuous release of activated spermatozoa throughout the oviposition period, so ensuring successful fertilization.

9 E. J. Conway, in: Microdiffusion analysis and volumetric error. Crosby Lockwood and Son Ltd, London 1950.

## Resolution of small $G_1$ and $G_2$ populations of L1210 leukemia by flow microfluorometric analysis aided by velocity sedimentation[1]

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*Summary.* L1210 leukemic cells grown in vitro were subjected to kinetic analysis using a flow microfluorometer. A single broad peak was found for the DNA content distribution if unfractionated cells were used; prior fractionation using lg velocity sedimentation allowed the separation of small peaks with smaller ($G_1$) and larger ($G_2$) DNA contents from the dominant S phase peak with intermediate DNA content.

Initial kinetic analysis of our in vitro L1210 leukemia line using flow microfluorometric techniques failed to demonstrate the typical double peaked DNA content distribution seen for other L1210 leukemias, usually grown in vivo[4]. One explanation for our result was that a preponderance of cells in the S-phase of cell cycle could mask the identification of smaller numbers of cells in the $G_1$ and $G_2$ phases of cycle. The validity of this explanation is reported here.