Application of staining techniques to improve viability assessment of turbot (*Psetta maxima*) ova

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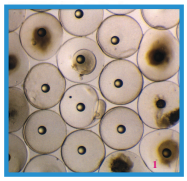
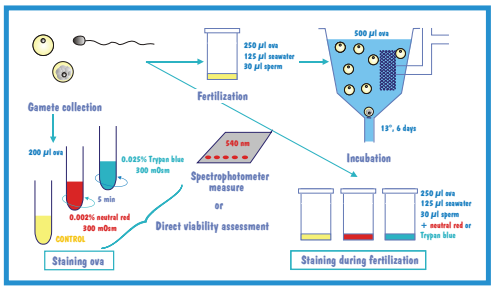
Good pratical criteria simple to use are required for the determination of ova quality. Shields *et al*, 1997 used blastomere morphology to predict the survival potential of fertilized eggs.



Assessment of morphological aspect coupled with staining techniques for mollusc gametes (Valdez Ramirez *et al*, 1999) try to improve the viability prediction in term of fertilization capacity and development of the embryos. This way was applied to the turbot ova : we retain the neutral red vital dye (viable cells become red due to uptake of dye in the lysosomes, while un-viable cells do not) and the Trypan blue exclusion test (dye don't penetrated the viable cells, dead cells are coloured).



Protocol of ova staining and egg quality evaluation by fertilization and hatching tests (*triplicate*).

Ova in routine viability assessment (photo 1) : spherical and translucent ova are viable ; irregular shape and coalescent yolk are un-viable ova.

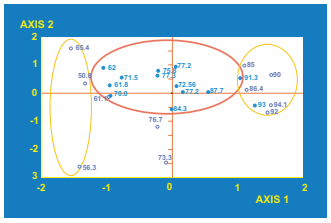
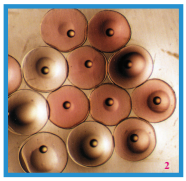
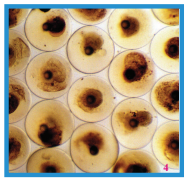


Fig. 1 : Significant differences (●) in ova viability assessment between with or without staining :
- 56 % observations *in the center of the figure*
- viability rates around 70 - 85 %
- overestimation of ova controls/staining = 14% ;
(○) no significant difference.
Each egg batch is represented by co-ordinates of the point calculated by Component principal analysis following axis 1 (86.5 % variability) and axis 2.

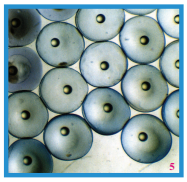


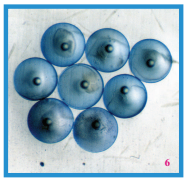


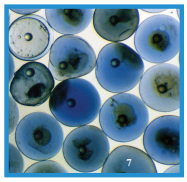
Ova staining in neutral red : red homogeneously coloured ova are viable (ph.2) ; pale ova (ph.2), damaged and coloured ova (ph.3), coalescent yolk, no spherical ova and no stained are un-viable (ph.4).

Fertilized embryos

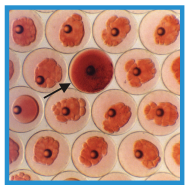
Control (ph.10)







Ova staining in Trypan blue : ova with a peripherical weakly blue ring are viable (ph.5) ; damaged coloured ova (ph.6), no spherical, coalescent yolk intense blue stained are un-viable (ph.7).



Staining in neutral red (ph.11)
immature oocyte (→) ?

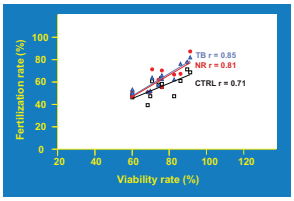
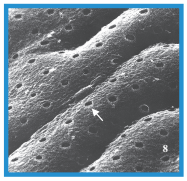
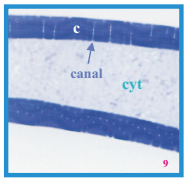


Fig. 2 : Linear regression shows high correlations between viability rates assessed with dyes and fertilization rates (NR r = 0.81, TB r = 0.85 *versus* CTRL r = 0.71). Nevertheless, low correlations were observed with hatching rates (NR r = 0.26, TB r = 0.40, and CTRL r = 0.33) *(no represented)*.



The peripherical weakly blue ring in viable cells was elucidated by the presence of regularly pores ( ) in the chorion observed by SEM (ph.8).
The pores show a conic shape (0.7 µm, up to 0.3 µm, down).



Semi-thin section (1µm) (ph.9) coloured by toluidine blue observed by photonic microscope, shows canals ( ) in the chorion (c) ; cytoplasm (~~cyt~~).

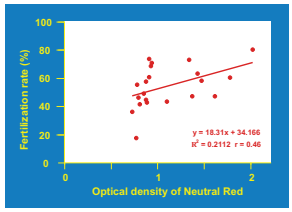
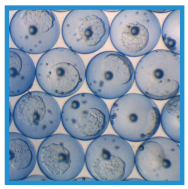


Fig. 3 : Linear regression shows low correlation between optical density of the uptake neutral red by viable ova and fertilization rates (r = 0.46)



Staining in Trypan blue (ph.12)

Staining techniques are:

- rapid, more objective than routine assessment.
- useful to improve viability assessment of ova ; female gamete injuries such as vitelline membrane detachment, coalescent yolk due to overripening or atretic phenomena, and dead ova were more easily observed.
- useful to predict fertilization capacity and also to reveal segmentation of blastomeres after staining by neutral red.
- not 100 % reliable methods to predict larval rearing potential. Results proved that cytological integrity was not the only parameter involved in quality. Biochemical criteria of ova and/or environmental conditions during embryonic development should also explained variable successes among egg batches.

Both Trypan blue and neutral red dyes were valid to check live-dead female gametes; neutral red when several colour intensities were classified, seems to be more informative to describe cell.
Results had concerned pools of live-dead ova that explain in part low relationship between fertilization rates and spectrophotometer measures.
Moreover these miniature tests in multiwell plates were long.
Microscopic evaluation, oocyte per oocyte from sub-samples was preferred to assess cell viability.
It should be interesting to couple the assessment method with image analysis.

References
Shields R.J., N.P. Brown and N.R. Bromage, 1997. Blastomere morphology as a predictive measure of fish egg viability. Aquaculture 155 : 1 - 12.
Valdez Ramirez M. E., M. Le Pennec, G. Dorange, N. Devauchelle and G. Nonnotte, 1999. Assessment of female gamete quality in the Pacific oyster Crassostrea gigas. Invertebrate Reproduction and Development (in press).
Acknowledgements to L.Quemener for statistical analyses assistance and members of GDorange's laboratoty for technical assistance.

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In: Norberg, B., Kjesbu, O.S., Taranger, G.L., Andersson , E. , Stefansson, s.a. (Eds.). Proceedings of the 6th International Symposium on the Reproductive Physiology of Fish, Bergen, July 4-9 1999, p. 435.

Application of staining techniques to improve the viability assessment of turbot (*Psetta maxima*) ova

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Summary:

Staining-dye procedures were tested on the turbot ovule in order to develop a rapid technique for determining ovule viability. Neutral red and Trypan blue dyes provided better assessment of the proportion of viable or dead female gametes when inducing fertilisation.

Introduction:

To predict the survival potential of fertilised eggs, Shields et al, 1997, studied the fish egg blastomere morphology, Valdez Ramirez et al, 1999, evaluated the morphological aspect of mollusc ova coupled with colouring techniques. We tested this approach on turbot ova, using neutral red vital dye and the exclusion test with Trypan blue.

Materials and methods:

200 µl egg sub-samples were observed without colouring, control CTRL, or were coloured by a solution of neutral red NR to 0.002% final concentration, or a solution of Trypan blue TB to 0.02% final concentration, prepared to 300 mOsm. After slow agitation for 5 minutes, the ovule viability was visually evaluated in terms of cell integrity and colouring intensity. To check uptake of the neutral red by viable cells, measurement by microplate reader (to 540 nm) was taken after 3 hours incubation of stained ova in multiwell plates.

In order to correlate the viability assessment with rearing success, eggs were fertilised, and others were incubated. Colouring was also tested at the time of fertilisation to evaluate the segmentation of blastomeres. The egg chorion was examined using SEM scanning electronic microscopy, and semi-thin sections (1µm) of the chorion, coloured with toluidine blue, were observed using photonic microscopy.

Results

A significant difference ($p<0.05$) was observed between coloured and uncoloured conditions in 56% of the egg batches. In routine evaluation, this leads to an average over-estimation of the viability rate by 14 % (ranging from 70-85%). A correlation was obtained between viability and fertilisation rates, which were higher in coloured conditions (NR r=0.81, TB r=0.85) than in the control, (CTRL r=0.71); a lower correlation was observed with hatching rates (ranging from 9.3 to 70.2%), (NR r=0.26, TB r=0.40), and between the spectrophotometric measure of the uptake of red stain in viable cells and fertilisation rates (r=0.46).

The blastomeres were mainly coloured by red stain in developing embryos. Some eggs, probably immature, were intensely coloured, whereas slight colouring by neutral red observed in several cells was linked to the loss of fertilisation. The presence of regular pores and canals in the chorion explained a slightly blue peripheral colouring in viable ovules.

Discussion

The staining procedures are fast and more objective than the current evaluation technique. They are useful in ascertaining fertilisation capability as well as in indicating blastomere segmentation. They are not 100% reliable in predicting larval rearing potential. The results showed that cytological integrity is not the only quality parameter involved. The neutral red stain used to classify intensities of red seems to provide more information than the Trypan blue.

References

Shields, R.J., Brown, N.P. and Bromage, N.R. 1997. Blastomere morphology as a predictive measure of fish egg viability. Aquaculture 155: 1-12.

Valdez Ramirez, M.E., Le Pennec, M., Dorange, G., Devauchelle, N. and Nonnotte, G. 1999. Assessment of female gamete quality in the Pacific oyster Crassostrea gigas. Invertebrate Reproduction and Development, 36, 73-78.

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