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# Definition of fusion medium and electric parameters for efficient zygote electrofusion in the Pacific oyster (*Crassostrea gigas*)

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**Abstract**

Cell electrofusion has been widely used in the induction of tetraploidy in mammals, but little attention has been paid in molluscs. This work pursued the establishment of fusion medium (ionic vs. non-ionic) and electric parameters in the electrofusion of Pacific oyster zygotes (prior to the completion of the first mitotic division), minimizing all deleterious effects possible to D-larval stage. The tested combinations of electric field intensity ($Vcm^{-1}$) and number of square DC pulses applied (for 50 $\mu$s each) were (Voltage × N° pulses): 400 × 1, 400 × 2, 400 × 3 and 600 × 1, 600 × 2, 600 × 3.

When pulses were applied for first time, it was determined that an ionic fusion medium (microfiltered seawater) offered better conditions than the non-ionic fusion media previously used (0.6 M sucrose or 0.6 M mannitol) in terms of embryo survival and lysis rates. In this fusion medium, two different combinations of electric parameters (3 square DC pulses of 400 $Vcm^{-1}$ for 50 $\mu$s each at 26 °C and 1 square DC pulse of 600 $Vcm^{-1}$ for 50 $\mu$s at 26 °C) offered the best technical results of fusion (57 and 79% respectively) and survival until D-larva (44 and 41% respectively). In conclusion, these electric parameters could be established, using seawater as electrofusion medium, for further approaches to evaluate individual ploidy and survival beyond spat.


*Keywords:* Cell electrofusion; Zygote; Embryo; Pacific oyster; *Crassostrea gigas*

## 1. Introduction

Triploid oysters are economically interesting for aquaculture because of reduced gamete output (functional reproductive sterility) and improved meat quality and growth [1]. To achieve this objective, different procedures have been assayed with low and variable efficiencies: Cytochalasin B treatments have been perfected over time so that success rate in producing triploids was about 90%, but there has been concern over repercussions from the public [2] because this product is not only expensive but also carcinogenic [3]. Other procedures commonly used are thermal and pressure shocks, but they present drawbacks in terms of equipment and handling [3]. Whatever the case, the triploid induction efficiency must be as high as possible to homogenize commercial stocks, although to date the only available method that achieves efficacies of 100% triploid production is mating tetraploid and diploid oys-

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ters [4–5]. As a result, the obtainment of tetraploid oysters is of evident commercial interest for triploid seed production [6–7], and different approaches to tetraploidy induction have been used [8], with the inhibition of polar body 1 in eggs from triploids being the most effective option [9].

In mammals, the obtainment of tetraploid embryos by electrofusion of the first two blastomeres has been widely used [2,10–12]. This cell electrofusion methodology [13] is based on the formation of reversible pores in the cell plasma membrane by a determined electrical field stimulus (and usually applied in a non-ionic media). In accordance with this, and when two or more cells are in contact (surface contact must be perpendicular to electrical field lines), the induced reversible pores allow both cytoplasmic interchange and cytoskeleton interconnection, finally resulting in a single cell in which the nucleus is also the result of fusion of the cell nuclei.

This method reaches high fusion rates (90%) [11] and thus becomes the most efficient established method of tetraploid obtainment in mammals. From these observed results, the major advantage of electrofusion in oysters would be the direct obtainment of tetraploid specimens from diploids, unlike other processes which require more previous steps, such as the obtainment of triploids or the required use of chemicals such as cytochalasin B [9]. However, the extrapolation of this technique to molluscs has only been assayed in some detail by Cadoret [14] in oyster and mussel. Unfortunately, results obtained from that work did not clearly validate the electrofusion technique in relation to the parameters and conditions employed. In this respect, one of the difficulties that arose was the definition of a non-ionic electrofusion medium with an osmolarity compatible with embryo survival. The non-ionic medium used in electrofusion (based on high density sugar solutions) was at an osmolarity hypotonic to the oyster embryos (750 mOsm instead of 1130 mOsm), compromising embryo survival and also causing flotation of the embryos. This made embryo handling difficult in the electrofusion chamber.

Although non-conductor media are the most common, perhaps because they allow dielectrophoresis while ionic media cause electrohydrolysis and warming phenomena [13], said work did not raise the possibility of using ionic media, which are successfully used in mammalian fusion experiments [12].

In molluscs, cleavage speed of first cell cleavages is high at 26 °C [15–16]. Moreover, after the immediate observation of the 2-cell embryo, the second mitotic division was already taking place. This forced us to apply the electric fusion pulse a few moments before the cytokinesis completion of the first mitotic division [14].

On this basis, the present work aimed to evaluate different media and electrical parameters for two nuclei zygote electrofusion. In this way, the oyster embryos were used prior to their completion of the first cell cleavage and results were assessed by monitoring the percentage of fused embryos and their survival until the D-larval stage.

## 2. Materials and methods

### 2.1. Oyster source

The experiments were carried out in the middle of the breeding period (from July to September) of the oyster *Crassostrea gigas* in our climatic area.

Oysters were provided weekly by ACUIMA S.L. (Burriana, Comunidad Valenciana, Spain). They were transported to our lab, located at 60 Km from the company, where they were carefully cleaned and stored "dry" in a refrigerator at 4–5 °C until use. Oysters remained refrigerated for a maximum of 5 days.

### 2.2. Obtaining of gametes

Gametes were obtained as recommended by the FAO [5]. Briefly, oysters were opened by removing the flatter shell valve and after validating adequate gonad maturation [17], the gonad was biopsied with two sterile hypodermic needles. Gametes were removed by exerting gentle suction with a sterile Pasteur pipette inserted beneath the overlying gonad epithelium under the maximum possible sterility conditions.

Males were only selected when sperm had a minimum of 70% motility with a high degree of lineal motility. Motility was estimated visually at 200× magnification and was expressed as the percent of cells actively moving in a forward direction. Sperm vibrating in place were not considered to be motile [1]. On the other hand, the selected females had viable eggs (around 40–60 $\mu$m diameter) [18] and rounded out (activated) completely some minutes after the first contact in seawater, thus observing the germinal vesicle breakdown at 100× magnification [1,19–20]. In both males and females, the cleanliness of samples obtained was also a selection criterion.

For each daily experimental session, the lowest number of oysters used to minimize individual variability was two males and two females [1,21].