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Technical note

Setting tools for the early assessment of the quality of thawed Pacific oyster (*Crassostrea gigas*) D-larvae

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Abstract

Parameters used to assess the survival of larvae after cryopreservation generally misestimate the damages that prevent larval development. The objectives of the present study were to 1) define the reliability of the survival rate, assessed at 2 and 7 days post fertilization, to estimate Pacific oyster larval quality after thawing, and 2) select complementary tools allowing an early and reliable estimation of their quality. Oyster larvae were reared for 25 h after fertilization at 19 °C and cryopreserved at early D-stage. Then, thawed larvae were incubated in 2-L beakers. At 2 days post fertilization, the survival rate of thawed Pacific oyster larvae was lower than that of fresh larvae for only one experiment (Experiment 3) among the four identical experiments carried out in this work (Experiments 1–4). By contrast, the survival of thawed larvae, as assessed 7 days after fertilization, was lower than that of fresh larvae for the four experiments. These results confirm that the quality of thawed larvae is lower than that of fresh larvae and that the survival rate, estimated 2 days *post* fertilization, is not adapted to a reliable estimation of the subsequent development ability of thawed larvae. Then, complementary parameters were tested at 2 days: the movement characteristics (Experiments 1 and 2) and the morphologic features (Experiments 3 and 4) of thawed larvae. Compared to values observed on fresh larvae, the percentage of thawed motile larvae was different for only one experiment (Experiment 2) of the two. Compared to control, a reduced Average Path Velocity (VAP) of larvae (determined at the D-larval stage using a CASA-Computer Assisted Sperm Analysis-system) was observed after thawing for both experiments (Experiments 1 and 2), suggesting the ability of larval movement velocity to assess the decrease of the quality of thawed oyster larvae. Using an ASMA (Automated Sperm Morphology Analysis) device, a lower area of thawed larvae was observed, compared to control and for the two experiments (Experiments 3 and 4). By contrast, the Crofton perimeter of thawed larvae was lower than that of control larvae for only one experiment (Experiment 3) and no significant difference of circularity between fresh and thawed larvae was recorded for Experiments 3 and 4. In conclusion, changes in the movement velocity (assessed by CASA) and in the area (measured by ASMA) of D-larvae allow an early and reliable estimation of the quality of thawed Pacific oyster larvae.
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Keywords: ASMA; CASA; *Crassostrea gigas*; Cryopreservation; D-larvae

1. Introduction

Since the first report of the survival of mammalian embryos frozen to −196 °C [1], a huge number of domestic and laboratory animals have been produced using embryo cryopreservation. In aquatic animals, while sperm cryopreservation methods are well established [2,3], the preservation of embryos is still far from being achieved. Fish embryos are large, reducing the water and cryoprotectant exchanges, are composed of different compartments differing in permeability properties, and have a high water content [4]. As a consequence, fish embryo cryopreservation has not yet

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been successful, as was reported for sea bream (*Sparus aurata*) [5] or Japanese flounder (*Paralichthys olivaceus*) [6].

In molluscs, the problem is different compared to fish species because oocytes generally have a smaller diameter and a lower yolk content, as reported in Pacific oyster [7]. Furthermore, embryos show a holoblastic cleavage, more favorable to cryoprotectant penetration. The cryopreservation of embryos has been investigated in a few molluscs, such as blue mussel (*Mytilus galloprovincialis*) [8], Eastern oyster (*Crassostrea virginica*) [9] and pearl oyster (*Pinctada fucata martensii*) [10]. Moreover, embryo cryopreservation studies have mainly focused on Pacific oyster (*Crassostrea gigas*), because of its commercial importance worldwide [11]. The scientific literature was reviewed by Robles, et al. [4] and Paniagua-Chavez, et al. [9], underlying the problem of embryo survival estimation after cryopreservation. The survival of thawed Pacific oyster embryos was generally estimated by counting the number of motile trochophores (the first stage of larvae development which occurs a few hours after oocyte fertilization) or D-larvae (the fully shelled stage observed from 20 h *post* fertilization) [9,12,13]. However, the percentage of swimming Pacific oyster larvae measured just after thawing on 9 to 21 h *post* fertilization larvae, ranged from 20 to 40% while only one larva succeeded to settle after 29 days rearing [13]. Furthermore, 85% of blue mussel larvae showed active swimming 3 h after thawing while only half of these larvae had microalgae in their stomach, compared to 80% for the control group [8]. Both observations suggest that the percentage of motile larvae is not sufficiently integrative and misestimates the damages of thawed larvae or thawed embryos.

Considering this discrepancy, complementary analysis have been developed to precisely estimate embryo or larva quality after thawing, including morphologic appearance of thawed embryos observed under an optical or an electron microscope [14], fluorescence microscopy [15] and a subjective estimation of movement velocity [13]. Image analysis is a major advance in cell movement study, providing a rapid and objective measurement of cell movement velocity [16]. A new, freely accessible Computer Assisted Sperm Analysis (CASA) software system has been developed for ImageJ, and was primarily designed for fish sperm movement analysis [17]. Motility is considered as a fine parameter to estimate the quality of thawed spermatozoa, because it integrates both morphologic integrity and cell functionality [18]. A CASA system was used to quantify the decreased quality of thawed sperm in black-lip pearl oyster compared to fresh cells (*Pinctada margaritifera*) [19]. Concerning embryos or larvae, a CASA system was only used to assess the movement velocity of marine snail (*Crepidula fornicata*) larvae [20]. Then, an ASMA (Automated Sperm Morphology Analysis) device was successfully used to study the effects of cryopreservation on morphologic characteristics of spermatozoa [21].

The aim of the present work was to 1) define the reliability of the survival rate (assessed at 2 and 7 days post fertilization) used to estimate Pacific oyster larval quality after thawing, and 2) select complementary tools allowing an early and reliable estimation of the quality of thawed Pacific oyster larvae in comparison with fresh larvae.

2. Materials and methods

2.1. *Defining the reliability of the survival rate*

Oyster larvae were obtained from three different females according to Song, et al. [22] and incubated in 2-L beakers (100 000 larvae l^{-1}). Twenty-five h *post* fertilization at 19 °C, early D-larvae were pooled, filtered at 20 μm and transferred in 20-mL seawater. Larvae were cryopreserved, according to the protocol reported by Tervit, et al. [23] for oocyte cryopreservation: briefly, the pools of larvae were diluted in cryoprotectant (10% ethylene glycol with 1% PVP and 200 mM trehalose in Milli-Q water, dilution rate: 1:1) and transferred in 0.5-mL straws (IMV, France; 15 000 larvae in each straw). Straws were placed into a program freezer (Kryo10, Planer) which was programmed to cool at 1 °C min^{-1} from 0 to −10 °C (hold for 5 min at −10 °C) and at 0.3°C min^{-1} from −10 to −35 °C at which temperature the straws were plunged inliquid nitrogen. Then, straws were thawed in a water bath (37 °C, 10 s) and transferred in 2-L seawater.

Thawed larvae were incubated in 2-L beakers (triplicate) using non-limiting density conditions (from 50 000 to 150 000/beaker), according to a standardized protocol [24]. The survival rate of fresh and thawed D-larvae was estimated after 2 days of culture at 19 °C. Then, D-larvae were transferred in 5 L flow-through culture system (triplicate) and reared using non-limiting density conditions (from 10 000 to 100 000 in 5 L tank) according to Rico-Villa, et al. [25]. Larvae survival rate was estimated after 7 days. Four identical experiments (1–4) were carried out using the same protocol but different pools of larvae.

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