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Stage selection and restricted oviposition period improves cryopreservation of dipteran embryos ☆

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ABSTRACT

Embryos of two dipteran species (*Musca domestica* and *Lucilia sericata*) were assessed for an effective sampling time that would result in the highest post-cryopreservation hatch rate, with a primary goal to define species-specific egg collection periods and the effects of manual stage selection on post cryopreservation yield. The effects of the time taken to collect eggs on, (a) the proportion of embryos reaching a specific developmental stage between 17 and 20 h of development, and (b) the post-cryopreservation hatch rate were assessed. Permeabilization treatment applied at any stage of embryonic development did not significantly reduce embryo viability. Eggs collected over longer durations significantly reduced the number of embryos available in a specific developmental stage amenable to cryopreservation. Hatch percentage after cryopreservation of the embryos of *M. domestica* collected over a 60 min period was 10.7 ± 8.7% compared to 31 ± 5% for the eggs collected for just 15 min. Similarly, percent hatch in *L. sericata* resulted in 17.0 ± 3.9 and <2% for 15 and 60 min samples, respectively. Significantly higher hatching rates were obtained for cryopreservation after manual selection of specific embryonic developmental stages from the dechorionated samples. Post-cryopreservation hatching rate for stage-selected *M. domestica* embryos was 86.5 ± 5.5% compared to 33.3 ± 4.5% for embryos staged only by an overall visual confirmation. In the case of *L. sericata*, the hatching percentage was 79.0 ± 11.1 for stage-selected embryos compared to 17.0 ± 3.9% without individual selection.

Introduction

Since the 1990s, optimized cryopreservation protocols have been reported for numerous dipteran insects of economic and scientific importance [5]. These include, *Drosophila melanogaster* [7,19], *Musca domestica* [20], *Culicoides sonorensis* [11], *Cochliomyia hominivorox* [6], *Ceratitis capitata* [13], *Anastrepha ludens* [12] and *Lucilia sericata* [15]. The technique used for the above dipterans was also reported to be successful for embryos of the lepidopteran, *Pectinophora gossypiella* [14]. The development of cryopreservation protocols for insects even on a small scale is becoming more important as insects of economic importance are being increasingly used in research including genetic manipulation [1]. This results in numerous strains of the same species that are required to be continuously reared in the laboratory costing both time and labor [18]. Embryonic cryostorage is an alternative to continuous rearing that could cut costs as well as preserve the germplasm in a form that can be easily revived [5,9].

Leopold and Rinehart [5] reviewed the various challenges that hinder, widespread adoption of insect embryo cryopreservation. Most of these challenges relate to obtaining sufficient numbers of high quality embryos that are developmentally synchronized from small colonies of laboratory maintained insects. The problem is further exacerbated by the fact that unlike the mammalian embryos that are amenable to cryopreservation in many of their early developmental stages (exception: porcine embryos [10]), the dipteran embryos can only be cryopreserved at a precise developmental stage during late organogenesis [5,6,12]. This necessitates the collection of embryos that are developing uniformly. Visual estimates during prior studies on *L. sericata* indicated that a shorter duration of egg collection could result in more uniformly developing embryos [15].

Two dipteran models were used in this study: the common green bottle fly, *L. sericata* and the common housefly, *M. domestica*. The embryos of both species develop at very similar rates. And

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with one exception, the process of preparing the embryos for cryopreservation is also very comparable. However, the permeabilization procedure was adjusted due to the higher sensitivity of the embryos of *L. sericata* and *M. domestica* to 2-propanol and hexanes, respectively [15,20]. Both of the above studies also describe the process of determination of the developmental stage for cryopreservation by identifying specific developmental markers. These markers include: the structure of the embryonic midgut, the cephalic furrow and the appearance of the mandibles and cuticle. However, these markers can often be difficult to identify when there are variations in the overall developmental rates of the embryo samples of different species.

Several strategies have been employed in dipteran embryo cryopreservation to increase post-embryonic viability since the early reports of dipteran cryopreservation [7,20]. These include, optimized embryo collection time, and stage selection by monitoring for the appearance or disappearance of specific embryonic markers [5,6,12,15] and handpicking specific developmental stages for cryopreservation which was first reported in cryopreservation studies with *A. ludens* [12]. The present study was designed to compare the effectiveness of three approaches to increase the proportion of viable embryos after cryopreservation by, (1) assessing if the length of egg collection period can affect the efficiency of a cryopreservation protocol, (2) analyzing the efficiency of cryopreserving selected embryos of three different manually sorted developmental stages, and, (3) evaluating if the duration of egg collection can affect manual stage selection of the embryos.

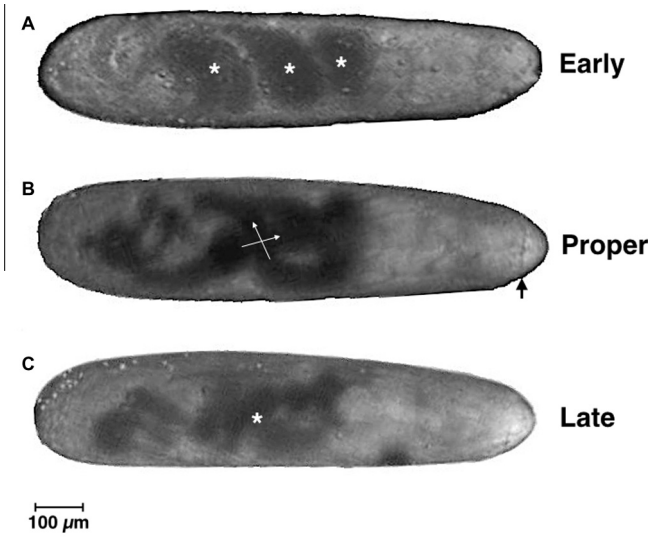

Fig. 1. Image crops A, B and C represent the three stages of development in *Lucilia sericata* that were manually selected for the study. For the purpose of cryopreservation, they are identified as early (A), proper (B) and late (C) stage embryos. When incubated at 19 °C after oviposition, stage 'early' (A) occurs approximately after 17 h of incubation and development. This stage is characterized by the presence of 3 or 4 coils of yolk filled gut marked with an asterisk. Stage 'proper' (B) are mostly seen after 18 h of development and is characterized by the helix (thin arrows) and the maxillary-mandibular furrow (thick arrow). 'Late' stage (C) embryos were observed after 18.5 h of development and show a distinct refractive cuticle and, beaded and dispersed mid-gut (asterisk) and hind-gut. *M. domestica* embryos are not depicted due to their similarity to *L. sericata* embryos.

Materials and methods

Embryo collection and maintenance

Embryos of *M. domestica* Linn. and *L. sericata* (Meigen) were obtained from colonies reared at the USDA-ARS-Biosciences Research Laboratory facility in Fargo, North Dakota, USA. The embryos were incubated at 19 ± 0.1 °C (Eco Therm in30, Torrey Pines Scientific, Solana Beach, California, USA) on a moist filter paper in a Petri dish for up to 20 h prior to processing.

Timed embryo collection and stage selection

To obtain embryos of definitive age, the houseflies were permitted to oviposit for 10, 15, 20, 30, 40, 60 min in containers of spent housefly larval diet. The blowflies were offered a piece of warm cattle liver to oviposit. Embryos were removed from the medium using a moist paintbrush and incubated as described above until further studies.

Specific embryonic stages designated as early (E), proper (P) and late (L) were utilized in cryopreservation experiments (Fig. 1). The stage designation was based on the extent of development of the alimentary system (specifically, the midgut and the hindgut) as well as the development of the cephalic and abdominal characteristics [6,12,15]. Early ('E'-stage) embryos possess a large tri-saccular yolk filled mid-gut. Stage P or 'proper' embryos are characterized by bi-helical midgut and thin tubular hindgut. The late stage embryos ('L'-stage) in both species exhibit translucent cuticle and meager mid-gut yolk content. The embryonic stages selected for cryopreservation were examined immediately after permeabilization while in the Schneider's insect cell culture medium (Cat. No. S-9895; Sigma–Aldrich; containing calcium chloride and sodium bicarbonate) to minimize possible osmotic shock. The embryos not in the exact developmental stage to be tested were sacrificed while the embryos in the stages E, P or L were separated and transferred to fresh cell culture medium using a hard bristle brush trimmed to a single hair. The embryos were processed immediately or permitted to develop further.

Pre-cryopreservation treatments

Prior to cryopreservation the embryos were dechorionated and permeabilized as previously described for *M. domestica* and *L. sericata* [15,20]. During dechorionation the embryos were rinsed in 25% sodium hypochlorite (Cat. No. 425054; Sigma–Aldrich, St. Louis, MO, USA) for one minute or until all the embryos float and desist from sinking. This event signifies dissolution of the chorion and the exposure of the hydrophobic wax layer. Permeabilization procedure involved de-wetting the embryos in 2-propanol followed by removal of the wax layer enveloping the vitelline membrane using either hexane in the case of *L. sericata* and heptane for *M. domestica*. The embryos were then placed in Schneider's insect cell culture medium until further processing.

Cryopreservation and recovery

M. domestica and *L. sericata* embryos were cryopreserved as per the previously published protocol [15,20]. Embryos were then vitrified in liquid nitrogen while attached to the surface of a polycarbonate membrane (Whatman/GE Life Sciences, USA – Cat. No. 110614). The process was a two-step procedure with an initial one minute relaxation in the vapor phase of the liquid nitrogen followed by quenching in liquid nitrogen. This was done to avoid freeze-fracture damages during the quenching process [3,12,16,17]. The embryos were then stored in liquid nitrogen for at least 24 h before recovery. To thaw and recover the embryos, the polycarbonate membrane supporting the embryos was re-suspended in the vapor phase above the liquid nitrogen for one minute or until all traces of liquid nitrogen evaporated from the membrane. This took approximately one minute. Thereafter the membrane was quickly immersed into a solution of 0.5 M tre-