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Permeabilization of *Cochliomyia hominivorax* (Diptera: Calliphoridae) Embryos

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ABSTRACT Embryos of the primary screwworm, *Cochliomyia hominivorax* (Coquerel), were successfully permeabilized for use in subsequent cryopreservation studies. Mortality was greater for eggs incubated for <5 h before treatment. The mean survival of embryos to first instars was 55.7, 61.1, and 62.6% when the embryos were incubated for 5, 5.5, and 6 h before treatment, respectively. The survival to the pupal and adult stages was low. An improved media for culturing the embryos during and immediately after treatment needs to be devised and the procedure for rearing the larval stages also needs to be altered to improve survival for emerging adults.

KEY WORDS primary screwworm, cryopreservation, survival, pupa, adult

THE LARVAE OF the primary screwworm, *Cochliomyia hominivorax* (Coquerel), are obligate parasites of warm-blooded animals, including humans. Eggs are deposited close to wounds. Upon hatching, the first instars penetrate the tissues of the host and cause primary myiasis. Larval feeding increases the size of the wound and if left untreated, multiple infestations can lead to death. This insect is the most destructive medical and veterinary insect pest in Central and South America. Eradication of the fly with the sterile insect technique (SIT) has been successful in the United States (Krafsur et al. 1987), Mexico, and the northern part of Central America (Gavin and Wyss 1996). An outbreak of *C. hominivorax* in Libya, where the parasite was accidentally introduced (El Azazy 1989, Gabaj et al. 1989), was also successfully eradicated by the SIT (FAO 1992).

The success of the SIT depends on production of large numbers of sterile flies to be released. However, production strains tend to lose their field competitiveness when reared under artificial conditions for extended periods (Spates and Hightower 1970, Bush et al. 1976), and new strains must be developed frequently (Crystal and Whitten 1976). To reduce the need for frequent primary screwworm strain development, Bram (1993) suggested the use of cryopreservation techniques. Development of a cryopreservation technique could eliminate the need to maintain numerous genetic strains and to avoid the side effects

of continuous rearing such as contamination, mutations, genetic drift or selection (Mazur et al. 1992, 1992b).

Because of the importance of *Drosophila melanogaster* Meigen and *Musca domestica* L. as model organisms (Strong-Gunderson and Leopold 1989, St. Johnston and Nusslein-Volhard 1992), numerous strains are maintained and high quality specimens are in demand. To avoid laborious and costly insect rearing procedures, cryopreservation of the embryos of these two species and of certain other dipteran livestock pests has been studied (Heacox et al. 1985; Mazur et al. 1992b; Steponkus and Caldwell 1993; Leopold 1993, 2000; Leopold and Atkinson 1999). To avoid intracellular ice formation and chilling injury of the embryo, water must be removed and cryoprotectant introduced. The dipteran egg has an outer chorion and a waxy vitelline membrane that act as barriers to the influx of cryoprotectant. Cryopreservation therefore depends on permeabilization of these barriers. Successful permeabilization procedures in a cryopreservation protocol, have been reported for *Drosophila* and *M. domestica* (Heacox et al. 1985, Strecker et al. 1994, Steponkus and Caldwell 1993, Schreuders et al. 1996). However, no procedure is available for primary screwworm embryos.

Thus, our objective was to develop a procedure to permeabilize primary screwworm embryos and maintain viability to enable future studies on the cryopreservation of colonized strains.

Materials and Methods

Embryos. Eggs were collected from the Costa Rica 92 strain on ≈ 10 g of ground beef placed on the lid of a 100-ml plastic beaker. The lid was placed on the beaker containing hot water (45–50°C). Two drops of

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putrid blood drippings from ground beef were added as an attractant. The egg container was placed in a cage containing 7- to 10-d-old adults and removed after 30 min. Egg masses were immediately transferred to three petri dishes with two sheets of grade 1 qualitative (90 mm diameter), filter paper moistened with 4 ml distilled water, and incubated in 37°C for 5, 5.5, and 6 h, respectively. The timing for the age of the embryos began 15 min before the containers were removed from cages.

After incubation, ≈ 100 mg of eggs were transferred to the permeabilization basket constructed of 100-mesh stainless steel screen bottom PVC pipe (25.4 mm diameter by 27 mm high). Remaining eggs were stored in a -70°C ultralow freezer for assessment of embryonic development.

Permeabilization. Approximately 15 ml of each chemical solution or medium was placed in 30- or 50-ml glass beakers. Chemicals were replaced for each batch of eggs. The permeabilization procedure consisted of the following four steps: (1) The eggs were separated from the egg mass by placing them in a permeabilization basket and agitating in a 1% NaOH solution for 5 min with the aid of a glass medicine dropper. This was followed by a 2-min rinse with distilled water. To assess control hatchability, a sample of 100 eggs was collected with a sable hair brush, placed in 100-mm petri dishes with moistened filter paper. (2) With the remaining eggs, the chorions were removed by agitating the embryos with a Pasteur pipette in a 50% solution of Clorox bleach (Clorox, Oakland, CA) (2.6% sodium hypochlorite) for 1 min, followed by a 3-min rinse in distilled water. (3) To remove the aqueous layer on the surface of the embryos, they were immersed in isopropanol and agitated with a Pasteur pipette for 30 s. The isopropanol was allowed to evaporate for 3 min before, (4) the embryos were immersed in hexane for 30 s in the same manner as in step 3, and the hexane was allowed to evaporate for 30 s. The basket was transferred to a beaker containing Schneider's insect cell culture media (Sigma, St. Louis, MO) for 10 min. The embryos were rinsed and agitated with a glass medicine dropper as in step 1. After treatments 2–4, samples of eggs were placed in a 30-mm disposable petri dish that contained 1 ml of Schneider's insect cell culture media. The petri dishes containing embryos were placed in a humid chamber that consisted of a 100-mm glass petri dish with moistened filter paper and incubated at 37°C for 18 h. The proportions of hatched, embryonated, and not hatched eggs were recorded to monitor viability.

Permeabilization was assessed by placing a sample of the embryos in either a 1 M sucrose solution or in 1% rhodamine B in Schneider's media for 10 min. With the sucrose solution, the embryos were visually examined and the elapsed time for shrinkage to a flattened condition noted. When using the dye, the embryos were rinsed with distilled water to remove superfluous stain. Stained embryos were then categorized as ruby red, pink, or white, which corresponded

Table 1. The mean percentage hatch after treatment of three ages of embryos with 1% sodium hydroxide, 2.6% sodium hypochlorite, isopropanol, and hexane

Age, h	Mean % hatch \pm SE			
	Control	NaOH/NaClO	Isopropanol	Hexane
5	70.4 \pm 3.5a	67.1 \pm 3.5ab	66.9 \pm 5.5ab	55.7 \pm 5.3b
5.5	68.2 \pm 3.1a	62.0 \pm 1.6a	65.7 \pm 3.0a	61.1 \pm 3.2a
6	65.4 \pm 3.3a	63.8 \pm 3.1a	65.8 \pm 1.2a	62.6 \pm 1.4a

Means in the same row followed by a different letter are significantly different at $\alpha = 0.05$.

to fully permeabilized, partly permeabilized, or unpermeabilized, respectively.

Embryos and larvae were examined under a Zeiss Stemi-2000C stereo microscope (Carl Zeiss, Thornwood, NY) to record survival, permeabilization success, and stage of development. Each trial was carried out in five replicates. Data were analyzed as a randomized complete block design by analysis of variance (ANOVA) (SAS Institute 1992). For statistical purposes, the chemicals to which the embryo samples were exposed were the treatments and the ages of the embryos were the blocks.

Survival of Permeabilized Eggs to Pupae and Adults. The ability of treated embryos to survive to the pupal and adult stage was examined. Eggs were collected as before and incubated at 37°C for 3, 5, and 7 h. The eggs were permeabilized and a sample was taken after each step as described above. The embryos were incubated at 37°C for 18–20 h. Active, hatched, larvae were counted using a stereomicroscope. The newly hatched larvae were transferred to 237-ml paper ice cream cups that contained 50 ml of gelled larval media (Harris et al. 1985). The cups were covered with plastic lids that had air holes and placed in an incubator at 38°C for 48 h. At the end of 2 d the lids were removed and 50 ml of larval media were added. The cups were placed in a 34°C incubator for 24 h. An additional 100 ml of larval media was added, the cups were placed in pans containing sawdust and then placed in a 30°C incubator for 6 d. The larvae crawled from the cups and pupated in the sawdust. The sawdust was removed from the pupae by sifting through a 7 mesh U.S. Standard Testing Sieve (W. S. Tyler, Mentor, OH). The pupae were counted and weighed, placed in paper ice cream containers with plastic lids that had air holes, and held at 25°C for 6 d. The number of adults that emerged was counted.

Results

Permeabilization. The mean percentage of viable embryos after dechoriation, treatment with isopropanol, and treatment with hexane for embryos aged 5, 5.5, and 6 h is given in Table 1. Hexane significantly reduced hatching of the 5-h-old but not on the 5.5- or 6-h-old embryos.

The embryos became dehydrated after <1 min exposure to 1 M sucrose, as indicated by shrinkage of the embryo. This showed that the vitelline membrane was

Table 2. The mean (\pm SE) percentage of embryos that show permeabilization using the rhodamine B stain as an indicator

Age, h	Ruby red	Pink	White
5	96.1 \pm 2.4	3.9 \pm 2.4	0
5.5	99.6 \pm 0.2	0.4 \pm 0.2	0
6	96.8 \pm 2.3	3.2 \pm 2.3	0

Ruby Red, permeabilized; Pink, partially permeabilized; White, Unpermeabilized.

permeable to the efflux of water. All embryos exposed to the rhodamine B absorbed it, which was also indicative of successful permeabilization of the vitelline membrane to allow permeation of molecules of at least M.W. 480. An average of 96.1, 99.6, and 98.8% of the embryos aged 5, 5.5, and 6 h, respectively, were fully permeabilized as assessed by the rhodamine dye absorption method (Table 2).

Survival of Permeabilized Embryos to Pupae and Adults. Some of the permeabilized embryos were capable of completing development and emerging as adults (Table 3). Of the ages treated, the 3-h-old embryos have the lowest survival to all stages after being permeabilized. The survival to the first instar was similar between treatments for 5- and 7-h-old embryos. The survival to the pupal and adult stages was also similar between treatments for the 7-h-old embryos, but survival to the pupal and adult stages was lower for permeabilized 5-h-old embryos. The maximum survival to adult was 17.7, 13.6, and 0.7% for 7-, 5-, and 3-h-old embryos, respectively.

Discussion

There are two major challenges to developing a successful procedure for the permeabilization of muscid and calliphorid embryos. The first is the determination of the proper embryological stage at which permeabilization can be accomplished with adequate survival of the treated individuals. The removal of the chorion and the waxy layer of the vitelline membrane exposes the embryo to desiccation and substances in its environment. Application of alkanes has a greater affect on the viability on young *Drosophila* embryos than embryos at a later stage in embryogenesis (Lynch et al. 1988). To achieve successful cryopreservation, the most resistant embryonic age to treatment needs

to be determined (Lynch et al. 1988, Mazur et al. 1992a, Steponkus and Caldwell 1993, Strecker et al. 1994). With *Drosophila*, this stage occurs during dorsal closure but before the cuticle is formed (Mazur et al. 1992b). Thus, the proper age represents a relatively narrow window within the process of embryogenesis. Because of variability in development rates within the species and incubation equipment, this developmental window must be identified not only by the number of hours an embryo has been incubated at a specified constant temperature, but also by the morphological characteristics of the embryo. Through preliminary studies we determined that embryos younger than 5 h were sensitive to hexane and were not near the dorsal closure stage. We also found that embryos 7 h or older were resistant to treatment and produced good viability. Yet, they were rejected because development was too advanced to allow adequate permeation of a cryoprotectant. Therefore, we chose to test 5-, 5.5-, and 6-h-old embryos.

The second challenge is to determine the use of the proper chemicals that can successfully permeabilize the embryo with minimal detrimental effects on survival. Screwworm eggs are laid in masses. Unlike *Drosophila*, the egg mass must be separated for the eggs to be sufficiently exposed to the chemicals used in their permeabilization. When agitated in a 1% sodium hydroxide solution, the egg mass becomes completely separated in 5 min. The resulting egg hatch is 60–70%. Isopropanol removes water from the surface of the embryo before treatment with the hydrophobic hexane. Steponkus and Caldwell (1993) emphasized the need to leave an adequate amount of isopropanol on *Drosophila* eggs when immersing in the alkane. Air-drying for 30–120 s resulted in poor permeabilization. Mazur et al. (1992a) reported high permeabilization but poor survival when *Drosophila* eggs were transferred directly from isopropanol to the alkanes. When the eggs were allowed to air dry for 2 min, survival and permeability were both acceptable. During preliminary studies with screwworm embryos, survival was good after treatment with sodium hydroxide, sodium hypochlorite, and isopropanol, but declined when the embryos were exposed to hexane. An attempt to permeabilize the embryos without the use of isopropanol was without success. Lengthening exposure to hexane in the absence of isopropanol was also tried without

Table 3. The mean (\pm SE) percentage survival of eggs to first instar, pupae and adult after dechoriation, treatment with isopropanol, and permeabilization

Treatment	Age, h	Survival of eggs to		
		First Instar	Pupa	Adult
Dechorionated	3	50.59 \pm 6.13	17.28 \pm 5.63	12.93 \pm 4.89
Dechorionated	5	50.57 \pm 4.45	23.88 \pm 11.34	17.50 \pm 10.30
Dechorionated	7	59.44 \pm 8.16	15.30 \pm 7.22	12.88 \pm 6.73
Post-Isopropanol	3	67.50 \pm 8.74	17.05 \pm 7.47	12.13 \pm 5.85
Post-Isopropanol	5	56.04 \pm 6.55	18.42 \pm 5.99	14.55 \pm 7.54
Post-Isopropanol	7	65.26 \pm 6.18	23.73 \pm 6.66	20.72 \pm 7.11
Permeabilized	3	7.12 \pm 3.17	0.61 \pm 0.33	0.54 \pm 0.10
Permeabilized	5	50.73 \pm 6.60	9.96 \pm 3.60	6.83 \pm 2.82
Permeabilized	7	63.28 \pm 9.75	20.18 \pm 6.62	12.63 \pm 6.19

success. Because isopropanol was found to have little effect on survival by itself, the isopropanol was allowed to evaporate for various lengths of time (30 s to 4 min) to reduce the damaging effect of the residual alcohol/hexane interaction. Air-drying for 3 min gave the best and most consistent results. This method produced excellent permeability (96–99%) to the rhodamine B and the embryos also had acceptable survival to hatching (55–62%). When placed in a 1.0-M sucrose solution, osmotic contraction was observed in <1 min. Osmotic contraction was not completed for 5–10 min in *Drosophila* embryos (Lynch et al. 1989, Steponkus and Caldwell 1993). This difference in permeability between the species may be caused by differences in the lipids forming the barrier on the surface of the vitelline membrane and the size and number of pores penetrating the vitelline membrane (Leopold and Nelson 1997). The embryos treated by this method should be suitable for the passing of substances (e.g., cryoprotectants) having molecular weights that are ≤ 480 .

The survival of the embryos after each step of the permeabilization process was >50% for 5- and 7-h-old embryos, but 3-h-old embryos were susceptible to hexane. The survival past the first instar was variable regardless of age of embryo or treatment. The 7-h-old embryos had better survival to the adult stage than did the embryos treated as 3- or 5-h-old. The 7-h-old embryos had begun to develop a cuticle and they were therefore somewhat resistant to the effects of the alkane. It is difficult to make conclusions about the effect of permeabilization on survival because of this variability. It has been observed that untreated eggs in small numbers also show this variability. This indicates that novel methods in rearing need to be developed to obtain sufficient survival of larvae from permeabilized embryos. Normally, a small piece of ground beef is placed near the eggs as a source of nutrition for newly hatched larvae. For permeabilized eggs, an adjustment in the cell culture media may be needed to supply the newly hatched first instars with their required nutrients. In the course of these studies we observed that the larvae show a retarded development. Thus, an adjustment in larval collection and in the rearing schedule may be required to supply the larvae with fresh media when they need it and before they become weakened by a posthatching delay in active feeding. The length of time they are exposed to higher rearing temperatures may need to be increased to accelerate their development or to adequately simulate conditions within a wound site.

The method we describe produces excellent permeability and survival. Successful absorption of rhodamine B dye indicates that most normally penetrating cryoprotectants can be loaded into the fly embryos. Although our major objective was to develop a method to permeabilize the screwworm embryo to load cryoprotectants, this method can be used to test the effect of other compounds (e.g., insecticides, hormones) on the development and survival of the embryo. Embryos held at 37°C for 5 h have been shown to have good survival after permeabilization.

The age at which the embryos are permeabilized will depend on the experiment. For cryopreservation the age is ≈ 6 h.

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