Permeability Barriers to Embryo Cryopreservation of *Pectinophora gossypiella* (Lepidoptera: Gelechiidae)

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**ABSTRACT** The aim of this study was to develop a method to cryopreserve the embryos of the pink bollworm moth, *Pectinophora gossypiella* (Saunders). Previously developed dipteran cryopreservation protocols were not directly adaptable to use with the embryos of this lepidopteran species. Physiochemical and electron microscope observations revealed substantial differences in the structure of the chorion, wax layer, and vitelline membrane complex when comparing the cryopreservable embryonic stages of *P. gossypiella* and dipteran embryos. Thus, the initial steps dealing with dechorionation and permeabilization were ineffective and had to be altered. Exposure to the sodium hypochlorite-based chorion removal step decreased *P. gossypiella* embryo viability to a very low level. Survival increased and permeability was evident when an alkane wash was used as the first step in the procedure. After the alkane treatment with a surfactant yielded the maximum exchange of cryoprotectant with water as evidenced by a significant lowering of the supercooling point of the cryoprotectant-loaded embryos. The remainder of the cryopreservation and storage recovery protocol for *P. gossypiella* was similar to those developed for dipteran embryos. Survival of recovered, hatched embryos to adulthood was ≈7%.

**KEY WORDS** *Pectinophora gossypiella*, pink bollworm moth, cryopreservation, embryos, permeabilization

The pink bollworm moth, *Pectinophora gossypiella* (Saunders), is a major pest affecting cotton production, cost United States producers >$47 million each year in crop losses and insect control [10]. Sterile pink bollworm moths have been released in the central valley of California as part of a control program for the past 30 yr. Recent efforts have been made to increase the effectiveness and reduce costs of the release program. Using transgenic technology, numerous strains have been produced and screened for a conditional lethal trait that results in only male insects. However, the cost of rearing multiple strains and the high probability of loss of strains remain a concern. This study was initiated to develop a cryopreservation protocol for storing pink bollworm embryos to aid in research and to provide a cryobank to back-up the mass-rearing program of the released strain.

Numerous studies over the past decade conducted in our laboratory on diverse dipteran species (*Musca domestica* [Wang et al. 1999], *Cochinymyia hominivorax* [Leopold et al. 2001], *Anastrepha suspensa* [Wang et al. 2001], *Ceratitis capitata* [Rajamohan et al. 2003], *Anastrepha ludens* [Rajamohan and Leopold 2007], *Lucilia sericata* [unpublished data]) have shown that the principle factors that govern the successful formulation of a cryopreservation procedure include, the developmental stage of the embryo, permeability of the egg shell-membrane complex, and the volume of the embryo. However, the principal impediment to developing a successful procedure, as pointed out by Steponkus et al. (1990), is the lipid or wax barrier located on the surface of the vitelline membrane and below the chorion. This layer plays a major role in water-proofing and desiccation resistance of insect eggs (Beament 1946a).

Cryobiological preservation at the temperature of liquid nitrogen is a technique that is dependent on replacement of cytoplasmic water with cryoprotective agents (CPA) to protect the cell, tissue, or organism against freezing damage (Mazur 1970). To reanimate the cell, tissue, or organism, the replacement process is reversed by removing the cryoprotectant and replacing it with water. However, for biological systems that are resistant to dehydration, impermeable, or both, to cryoprotectant entry, the development of cryopreservation methodology is challenging.

There have been three previous studies aimed at developing a cryopreservation procedure for moth embryos. Luo et al. (2006) tested six cryoprotectants in an effort to preserve *Spodoptera exigua* (Hübner) in liquid nitrogen. Although they reported hatching of larvae, none of the larvae survived. Roveri et al. (2008) cryopreserving the embryos of the wax moth *Galleria mellonella* (L.) reported a hatch percentage of 1.6 ± 0.5% immediately after retrieving from liquid nitrogen. Cosi et al. (2010), cognizant of the above

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lower hatch percentage in *G. mellonella*, reported on further assessments using Tween 80 and sodium hydroxide. However, they noted that the modified treatments did not improve the rate of permeation of the cryoprotectant required for higher postcryopreservation survival.

Thus, the studies reported here on pink bollworm embryo cryopreservation centered on solving problems related to two aspects: determination of the correct treatment stage and permeabilizing egg membranes. As with earlier studies on dipterans, we found that identifying the late-stage embryo of the pink bollworm most tolerant to the vitrification procedure and implementing the exchange of cellular water with a CPA among the most critical issues in developing a cryopreservation protocol for this insect.

### Methods and Materials

**Insects.** Pink bollworm eggs were obtained from The Center for Plant Health Science and Technology, USDA–APHIS–PPQ, Phoenix, AZ. The eggs were collected 30 min after females oviposited on waxed, brown paper. The eggs, on the paper, were transported via overnight mail in an insulated container at a temperature ranging between 18–24°C for not >24 h. Upon arrival, they were incubated at 20°C at 50% relative humidity (RH) for 48, 55, 72, and 96 h, before they were processed to determine the stage of development most tolerant to cryopreservation.

**Dechorionation Testing.** Moistened embryos on the waxed paper were agitated in 25% Clorox for 90, 120, 150, and 180 s. The embryos were dislodged from the paper by further agitating them while held under tap water. Embryos were rinsed in running cold tap water for an additional 60 s.

**Permeabilization Testing.** After treatment with the commercial bleach solution, the embryos were rinsed with 2-propanol (99.9% chromatography grade, Sigma-Aldrich, St. Louis, MO) for precisely 20 s in a stender dish, before being dried with compressed air. The dry embryos were agitated in a stender dish containing hexanes (98%, Burdick and Jackson) for 40 s (2 × 20 s rinses with fresh hexanes) keeping them always submerged. The embryos were quickly removed, air-dried, and dislodged from the mesh using a gentle fine spray of water from a spray bottle. The well-spread embryos were kept until further treatment by floating them on Schneider’s insect cell culture medium (Sigma-Aldrich, St. Louis, MO).

**Reversal of Dechorionation or Permeabilization.** Reversing the typical dechorionation or permeabilization procedure was initiated because removal of the chorion was ineffective and there was evidence that a hydrophobic layer existed on the surface of the chorion. Thus, 7 by 7 mm pieces of paper covered with embryos were submerged in hexane for 40 s, as described above. The embryos were then dried with a stream of compressed air and rinsed in 25% Clorox for 1.5 min. During the rinsing process, embryos were agitated off the paper, transferred into a mesh basket, and rinsed in running tap water for 1 min. The embryos remained floating on Schneider’s insect cell culture medium until further treatment was initiated.

A detergent solution (7% [wt:vol] p-tert-octylphenox-yloxy polyethylene alcohol) was also tested for efficacy in permeabilization, using three different treatments: 1) rinsing the embryos for 2 min in detergent followed by rinsing in running tap water for 1 min; 2) 2 min of detergent rinsing followed by 1 min in running tap water and then dechorionation in 25% Clorox for 1.5 min; 3) 2 min detergent treatment followed by a 20 s rinse in 2-propanol and then 40 s in hexane. After drying, all embryos were rinsed in tap water for at least 1 min and then placed in Schneider’s medium.

**Vitrification.** After permeabilization and before exposure to liquid nitrogen, the embryos were equilibrated in the vitrification solution by a two-step process. Embryos were placed as a monolayer inside a basket (consisting of a PVC ring and a 20 μm nylon mesh filter on the bottom) and incubated for 10 min in 10% 1,2-ethanediol [ED] with 0.5 M trehalose in Schneider’s medium) for 10 min. The samples were examined for depression of the freezing-point depression or the supercooling point, using cryomicroscopy. The embryo samples were first given the treatments as outlined in the shrinkage assessment but, instead of placing in the trehalose solution, they were placed in vitrification fluid (40% 1,2-ethanediol [ED] with 0.5 M trehalose in Schneider’s medium) for 10 min. The samples were examined for depression of the supercooling point using a cryomicroscope (Linkam Scientific Instruments Ltd., Tadworth, Surrey, United Kingdom). This procedure was carried out by transferring 5–10 cryoprotectant-treated embryos to a lint-free filter paper to remove excess surface fluids before loading on the cryostage. The temperature of each sample was lowered at 1°C per minute to −45°C, and the freezing point of each individual embryo in a sample recorded. If the embryos were permeable to the cryoprotectant, the freezing points would be lower than the controls.

**Permeabilization Estimation.** Two methods were used to assess whether the embryos were permeable to water and cryoprotectants after the various treatments. The first method involved a visual observation of shrinkage when embryos were exposed to 0.5 M trehalose solution in Schneider’s medium (trehalose dihydrate, Swanson Health Foods, Fargo, ND). The embryos that exhibited loss of water (i.e., wrinkling and flattening) after 1 min of treatment were recorded as permeable. The shrinkage of the following treatments was examined: dechorionation, permeabilization, reverse permeabilization, surfactant-treated, and surfactant-treated or dechorionation.

The second method involved determination of the freezing-point depression or the supercooling point, using cryomicroscopy. The embryo samples were first given the treatments as outlined in the shrinkage assessment but, instead of placing in the trehalose solution, they were placed in vitrification fluid (40% 1,2-ethanediol [ED] with 0.5 M trehalose in Schneider’s medium) for 10 min. The samples were examined for depression of the supercooling point using a cryomicroscope (Linkam Scientific Instruments Ltd., Tadworth, Surrey, United Kingdom). This procedure was carried out by transferring 5–10 cryoprotectant-treated embryos to a lint-free filter paper to remove excess surface fluids before loading on the cryostage. The temperature of each sample was lowered at 1°C per minute to −45°C, and the freezing point of each individual embryo in a sample recorded. If the embryos were permeable to the cryoprotectant, the freezing points would be lower than the controls.
trogen for 1 min in a zone previously determined to be between −120 to −135°C. The vapor-phase exposure permitted vitrification of the embryos without fracturing. Thereafter, the embryos were quenched in liquid nitrogen and the membrane placed in a plastic or stainless steel histological tissue-treatment cassette. The lid on the cassette was closed under liquid nitrogen and the cassettes containing the membranes with attached embryos stored in liquid nitrogen until thawing.

**Embryo Recovery.** The membranes with the attached embryos were removed from the cassette under liquid nitrogen and placed in the vapor, and held there for 1 min. Then, the membrane was submerged rapidly in 0.5 M trehalose, ensuring that the embryo-bearing side was facing the surface of the solution. The embryos were then gently agitated to ensure rapid thawing and equilibration to room temperature. After 2 min, the membrane was agitated rapidly to dislodge the embryos, and the trehalose removed and replaced with Schneider’s medium. The replacement of the medium was repeated three times; after this, the embryos were left in the medium until they hatched. The newly hatched larvae tended to float on the surface of the medium. They were collected and placed on a piece of tissue paper floating on the Pectinophora larval diet (Bioserve, USA). The number of larvae that underwent pupation and eclosion was recorded with respect to each step in the cryopreservation and recovery procedures.

**Transmission Electron Microscopy (TEM).** Untreated control embryos, embryos treated with 40% Clorox (≈2% sodium hypochlorite) for 1.5 min, embryos surface treated with hexane for 1 min, and embryos rinsed in 2% p-tert-octylphenoxymethyl alcohol for 1.3 min were examined for surface integrity by transmission electron microscopy (TEM). Embryos on filter paper were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.35 (Tousimis Research Corporation, Rockville, MD) for at least 2 h in the refrigerator. They were rinsed twice in sodium phosphate buffer and then placed in 2% osmium tetroxide in sodium phosphate buffer for 2 h at room temperature. After a buffer rinse, water rinse, and dehydration in a graded acetone series, samples were embedded in Epon-Araldite-DDSA with DMP-30 accelerator and sectioned at 50–70 nm thickness on a RMC MT XL ultramicrotome (Boeckeler Instruments, Tucson, AZ). Sections on copper grids were stained with lead citrate for 2.5 min and dried before being observed and photographed on a JEOL JEM-100CX II electron microscope (JEOL USA Inc., Peabody MA).

**Statistical Analysis.** The raw data were analyzed using Stata 10/IC (Stata, Inc., College Station, TX). Data comparisons were made using analysis of variance (ANOVA), and significant variation among the samples was defined as P value <0.05.

**Results**

**Dechorionation Treatment.** Light-microscopic observations made during and after the 1.5 min sodium hypochlorite treatment showed that the chorion of *P. gossypiella* embryos remained intact. Further, the hatch percentage was similar to that of the controls (Fig. 1). When the sodium hypochlorite treatment was extended to >1.5 min, mortality of the embryos rose to >95%.

Microscopic examination during the early periods of hypochlorite treatment revealed that the embryos remained hydrophobic in the bleach solution for a
period of at least 45–50 s. In addition, early stage embryos, between 24- to 29-h-old, were damaged more by the dechorionation procedure than were later stages of development (26 ± 8.2%; n = 5). When compared with untreated samples, embryos that were >48 h old exhibited no significant difference in hatchability because of the dechorionation treatment (96.9 ± 12.8%; P > 0.05; data not shown).

Permeabilization. The normal permeabilization process, which sequentially treats the embryos with 2-propanol and then hexane, resulted in 83.7 ± 2.06% hatch. The reverse-permeabilized samples had a hatch percentage of 79.5 ± 14.3%, while the surfactant treatment combined with hexane had a similar hatch rate (77.8 ± 7.1%). Surfactant treatment, followed by 1.5 min sodium hypochlorite rinsing, resulted in only 0.6 ± 0.3% hatching. In the absence of sodium hypochlorite treatment, 78.55 ± 16.3% of the embryos hatched after alkane treatment. Other than the sodium hypochlorite plus surfactant combination, the differences among all other treatments were statistically not significant (P > 0.05; Fig. 1).

Permeabilization (1.5 min in sodium hypochlorite, 20 s in 2-propanol, and 40 s in hexane) yielded negligible hatch when 24-h-old embryos were treated. However, >75% of the late-stage embryos (48 h and above) survived the same treatment and, as noted earlier, 83.7% of the 55-h-old embryos hatched after permeabilization treatment (data not shown).

Figure 1 also shows the effects of various permeabilization treatments on the subsequent supercooling points and eclosion rates for *P. gossypiella*. Permeabilized samples had a significantly lower puation rate (P < 0.01), although the rate of eclosion remained unaffected (P > 0.05). Pupation was significantly higher after the reverse-dechorionation treatments (P < 0.001) than in the controls. However, the rate of adult eclosion remained comparable to that of the controls.

Permeability Assays. Initial permeability assays were conducted by light microscopic observations of embryo shrinkage in 40% 1,2-ED after 1 min exposure. The embryos that were processed through a normal permeabilization procedure, with sodium hypochlorite, 2-propanol and hexane, showed significant shrinkage (Fig. 2), as did the embryos that were reverse-permeabilized. However, embryos treated only with sodium hypochlorite for 1.5 min did not show any sign of water loss.

While the detergent treatment by itself did not permeabilize the embryos to 1,2-ED, detergent treatment in combination with hexane caused shrinkage. Embryos that were rinsed in alkane alone also exhibited partial shrinkage. The rate and extent of shrinkage was not the same for the samples that were processed via the normal permeabilization procedure compared with the samples that were dewetted and rinsed in alkane or the embryos that were just rinsed in hexane.

Dechorionated and detergent-treated embryos (−18.5 ± 0.7°C) did not exhibit lower supercooling points compared with untreated embryos (−19.1 ± 4.8°C; P > 0.05). Embryos that were treated with 2-propanol and hexane, respectively, after sodium hypochlorite treatment exhibited a slightly lower but statistically insignificant supercooling point on treatment with 1,2-ED solution (−23.8 ± 3.6°C; P = 0.079; Fig. 2).

Significant depression in the supercooling point (−27.3 ± 2.5%; P < 0.001) was noted among embryos treated with sodium hypochlorite after 2-propanol and alkane treatments (reverse-permeabilization) compared with controls rinsed in 1,2-ED. Significantly lower supercooling points were also recorded from embryos rinsed only in 2-propanol and hexane.
Fig. 3. Transmission electron micrographs of sections of the chorion of Pectinophora gossypiella embryos. The effect of various treatments on the chorion was studied. Controls (A) had a layer of mucous on the surface that vanished after treatment with sodium hypochlorite (B), which did not disintegrate the chorion (c). The trebecular layer (t) of the chorion is visible near the bottom in all the images. Solvent (C) and detergent (D) treatments caused disruption to the subchorionic lipid plates, which accumulated as electron-dense layer (gray arrow). None of the treatments disrupted the chorion, which remained intact, including the electron-dense exo-chorion (white arrow). Bar in each figure represents 0.25 μm.

(-31.1 ± 1.7°C), compared with controls (P < 0.01) and compared with permeabilized (sodium hypochlorite-treated, followed by 2-propanol and hexane rinsed; P < 0.001) embryos. The embryos rinsed in alkane followed by detergent treatment supercooled to -32.9 ± 6.1 (significance vs. controls P < 0.001; significance vs. reverse permeabilized P > 0.1). However, detergent treatment by itself did not cause significant changes to the supercooling point (-18.5 ± 1.3°C; P > 0.1 vs. controls; Fig. 2).

Chorion Structure. The Untreated chorion of Pectinophora gossypiella embryos (55 h old at 24°C and 55% RH; Fig. 3A) was compared with those of embryos treated with Clorox (Fig. 3B), embryos permeabilized with hexane (Fig. 3C), and embryos treated with surfactant and permeabilized with alkane (Fig. 3D). The only layer on the egg shell that was removed by the treatments was the mucous layer seen in the untreated embryos (Fig. 3A). Figure 3C and D shows the embryos after treatments that involved use of hexane. In both cases, disruption to the electron-dense layer (see arrow) below the chorion is evident.

Vitrification. Before the vitrification assessments, the toxicity of the vitrification solution was assessed by withdrawing samples after 7, 10, 12, 15, 18, and 20 min in the ice-cooled vitrification solution. Embryos treated for 10 min in the vitrification solution appeared extremely shriveled, and ≤2% hatched (1.97 ± 0.33%; n = 5). Among the embryos treated for 7 min, 84.6 ± 9.5% (n = 5) hatched. Effects on pupation and eclosion after incubation in the vitrification solution were not assessed.

The ability of cryoprotected embryos to vitrify was tested for permeabilized, reverse-permeabilized, and surfactant/hexane-treated embryos. Embryos that were reverse-permeabilized, when vitrified and thawed, survived at a percentage nearly twice (15.0 ± 2.7%) that of samples that were just permeabilized and vitrified (9.5 ± 3.39%; Table 1). However, multiple assessments indicated that the difference in hatch percentage after vitrification between the reverse-permeabilized and detergent-permeabilized samples were statistically insignificant (P = 0.182).

Pupation and Eclosion. The reverse-permeabilized samples (that survived vitrification by exposure to liquid nitrogen) that yielded >10 larvae (eight samples) were reared on a bollworm diet. Of the larvae placed on diet, 76.4 ± 8.3% of them pupated, with adult moths emerging from 59.3 ± 1.6% of pupae.

Discussion

The current study aimed to develop successful cryopreservation of P. gossypiella embryos. For successful cryopreservation, it is essential to permeabilize, dehydrate, and ensure loading of the embryonic cells and extracellular spaces in the embryo with cryoprotectant. The permeabilization procedure assessed in this study was similar to the one used successfully on house fly embryos (Wang et al. 2000). Previously, we reported that P. gossypiella eggs could not be permeabilized using the standardized technique of dechorionation and dissolution of lipids over the vitelline membrane. We concluded that the chorion might be compositionally different in Pectinophora compared with dipterans (Rajamohan et al. 2011). For many cryopreservable insect species, the lipid layer(s) present over the vitelline membrane serves as the primary protective barrier to dehydration and cryoprotectant permeation (Papassideri et al. 1991).

Preliminary assessments in the current study indicate that chorion, in conjunction with the wax plates beneath the endochorion, might function as the primary barrier to permeabilization. Dechorionation

<table>
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<tr>
<th>Treatment</th>
<th>Permeabilized hatch % adjusted for permeabilized hatch</th>
<th>Hatch % after vitrification mean ± SD (n)</th>
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<tbody>
<tr>
<td>Permeabilization</td>
<td>83.7 ± 2.05 (6)</td>
<td>9.5 ± 9.39 (8)</td>
</tr>
<tr>
<td>Reverse permeabilization*</td>
<td>79.5 ± 14.3 (5)</td>
<td>15 ± 7.7 (8)</td>
</tr>
<tr>
<td>Detergent/permeabilization</td>
<td>79.6 ± 0.5 (5)</td>
<td>0.16 ± 0.41 (6)</td>
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Pupation and adult emergence rates are also presented for reverse-permeabilized samples. An asterisk marks samples for which the pupation and eclosion percentages are presented. *Pupation: 76.4 ± 8.33% (n = 8); adult emergence: 59.3 ± 1.6% (n = 8).
with hypochlorite and solvent rinses demonstrated the resilience of the chorion in maintaining its integrity. Before the treatments, the exposed surface of the exochorion displayed a fuzzy-appearing outer layer (Fig. 3A), which previous studies referred to mucous layer (Slifer and Sekhon 1963, Barbier and Chauvin 1974). However, the most intriguing layer is the undulating electron-dense surface of the exochorion that was not removed by sodium hypochlorite or hexane.

There are indications that both free hypochlorite ions and the solvents reached the embryo. The possible effect of hypochlorite was indicated by the high mortality of embryos exposed to 25% Clorox for 2 min. However, sodium hypochlorite-induced toxicity and mortality is not necessarily because of the permeation of ions. It could be because of the penetration of chlorine gas into the embryo. The effects of solvents and detergent manifested as disruptions in the electron-dense layer between the vitelline membrane and the chorion (see arrows in Fig. 3C and D). This electron-dense layer is often described as overlapping plates of lipids (Papassideri et al. 1991). Only if solvents penetrate the chorion can this layer be disrupted, which appears to be the case here. Beament (1946a and b) and Slifer (1945) proposed that this wax layer is the primary waterproofing mechanism of eggs and embryos. Although the current study indicates that the chorion is resistant to dissolution, the chorion does seem to be permeable to solvents, possibly aided by their very low viscosity. The disruption of the lipid layer permitted the movement of water and ethane 1,2-diol to and from the embryo, despite the presence of the chorion. This was evident from the embryo’s ability to shrink and the observed effects on the supercooling point of the embryo.

Neither the untreated control embryos nor the sodium hypochlorite-treated embryos shrank or creased in 40% ethylene glycol. Our earlier studies with diteran embryos showed that dechorionated embryos could, in fact, dehydrate in 0.5 M sugar (trehalose) or 8 M ethylene glycol solutions (Wang et al. 2000, Rajamohan et al. 2003). *Pectinophora* embryos that were treated with detergent and/or hexane showed significant creasing, indicating rapid loss of water from the embryos. Assays for uptake of cryoprotectants revealed that, while the control embryos froze at approximately −19°C, embryos that were treated with solvents and detergents and incubated in 40% ethylene glycol froze at significantly lower temperatures. Embryos treated with hexane froze at −31.1 ± 1.7°C.

Recent studies have evinced interest in using surfactants to effect permeabilization of insect embryos. Roversi et al. (2008) and Cosi et al. (2010) used Tween 80 to permeabilize the eggshell of *G. mellonella*, and Rand et al. (2010) assessed the efficacy of phytosurfactants on *Drosophila* embryos. In the current study, p-tert-octylphenoxyno polyethoxyethanol (Triton X-100) in water and propane 1,2-diol were used to permeabilize the *Pectinophora* embryos. While the surfactant, by itself, did not result in permeability changes in the embryo, when it was applied consecutively with a solvent a slightly lower freezing point depression was obtained than that when hexanes treatments alone was used. Rand et al. (2010) reported that surfactants are very efficient in the case of *Drosophila* embryos. However, their assays were performed on dechorionated embryos, in which the lipid layers are fully exposed to surfactant action. In the case of *Pectinophora*, the nonremovable chorion protects the wax layer from exposure to the high molecular weight surfactant (Triton X-100, MW. 646.8), while relatively low molecular weight hexanes might be able to penetrate through smaller openings in the chorion, such as the aeropyle, to disrupt the wax layer. However, Cosi et al. (2010) reported that, in the case of *G. mellonella*, detergents were as effective as hexane and resulted in a much higher survival because of the elimination of solvent toxicity. However, Roversi et al. (2008) had used Tween 80 and reported a post-cryopreservation hatch percentage of 1.6 ± 0.5%. Although sufficient to rejuvenate a healthy colony of waxmoths, the hatch percentage is significantly lower than previously reported studies on insects. This could be attributed to insufficient permeation of the cryoprotective agents into the embryo.

An assumption borne out of our previous study (Rajamohan et al. 2011) was that the chorion might be reinforced with wax, preventing sodium hypochlorite from degrading the chorion. This assumption led us to design a reverse-permeabilization procedure to augment the permeability of the embryos beyond that observed with solvent or solvent plus surfactant treatments. When embryos were treated with solvents, followed by sodium hypochlorite, the chorion did not show any degradation (Fig. 3D), ruling out the possibility that lipids over the chorion might be protecting the embryos. This also highlights the role played by the electron-dense layer on the surface of the chorion, previously termed by Barbier and Chauvin (1974) as “external chorion” in the case of the moth, *G. mellonella*. Reverse-permeabilized embryos exhibited much lower supercooling points on incubation in ethane 1,2-diol, but it was not significantly lower than that observed after use of the normal permeabilization procedure.

Embryos from permeabilization treatments that resulted in higher percentages of hatch, pupation, and eclosion (see Table 1) were used in testing vitrification in liquid nitrogen. Embryos were dehydrated and loaded with 40% ethane 1,2-diol + 0.5 M trehalose solution in Schneider’s insect cell culture medium. Trehalose is a nonpermeating sugar that increases the rate of dehydration and improves the vitrification characteristics of 1,2-ED, which is a poor glass-forming compound (Kuleshova et al. 1999). Embryos that were reverse permeabilized often yielded higher percentages of hatch; however, these were not significantly different compared with embryos that were permeabilized by the normal procedure. The embryos permeabilized using surfactants did not survive the vitrification process. One of the reasons for this could be that surfactants that are trapped in the aeropyles in the embryo can constitute what is termed “a surfactant monomer.” Surfactant monomers attract water mole-
cules, forming clathrates, which have structural similarities to ice and, although not very efficient as ice nucleators, they have the potential to behave as such (Zhang et al. 2004).

We conclude that the chorionic barrier is a significant impediment to cryopreservation of *P. gossypiella* embryos. However, we also notice that the chorionic barrier does not completely impede the disruption of the sub-chorionic lipid plates by solvents. This barely allows for sufficient dehydration of the embryos, making cryopreservation feasible. Even though this study reports a significantly higher hatch percentage (9–15% viable embryos, postvitrification) than previous reports among other lepidopterans (Luo et al. 2006; Roversi et al. 2008), the results are accompanied by large standard deviations. The current procedure seems to be affected by numerous factors, including very high dispersion among the embryonic stages. Studies are in progress to obtain lepidopteran embryos in highly synchronous developmental stage. Multi-generational quality control assessments after vitrification are also in progress. This study details significant progress over previous reports on species of Lepidoptera, with a nearly sevenfold increase in postvitrification hatch percentage. Even relatively small increases in cryopreservation success could represent important advances for conservation of lepidopteran species and use of cryopreserved embryos in pest-management strategies.

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