Quality Testing of Three Species of Tephritid Fruit Flies After Embryo Cryopreservation

ROGER A. LEOPOLD,1 ARUN RAJAMOHAN,2 TODD E. SHELLY,3 AND ALFRED M. HANDLER4

ABSTRACT This study evaluates characteristics commonly used to define insect quality or fitness by using a complement of three species of tephritid fruit flies (Diptera: Tephritidae) obtained from cryopreserved embryos. Anastrepha ludens (Loew), Anastrepha suspensa (Loew), and Ceratitis capitata (Wiedemann) were used to assess embryo to adult emergence and adult longevity, flight ability, mating ability, fecundity, and genetic variability after cryopreservation. With the three species tested, embryo survival was reduced by 50–70% whereas adult eclosion seemed unaffected by cryogenic treatment. Laboratory cage survival of cryopreserved A. ludens paralleled that of controls when tested with or without food and water posttreatment. With C. capitata, field cage survival was also similar when the adult progeny of cryopreserved parents was compared with that of untreated flies of the same age. Assessment of flight ability of cryopreserved A. ludens over a 19-d period by using a flight mill showed no statistical difference when compared with the untreated groups over the same time period. Flight ability within field cages for newly emerged progeny of cryopreserved C. capitata also mirrored that of the controls. Observed matings occurring within laboratory cages containing equal numbers of A. ludens males and females did not differ from comparable control groups. Furthermore, male progeny obtained from cryopreserved C. capitata parents competed equally with untreated males for mates while housed in field cages. A laboratory analysis of fertility and fecundity of A. suspensa revealed that males mated with control females were unaffected by cryopreservation as embryos, whereas cryopreserved females exhibited a significantly reduced fecundity when mated with control males. The fecundity of C. capitata progeny of cryopreserved parents also did not differ from control levels while caged under laboratory conditions. A random amplified polymorphic DNA assay of the genetic diversity of A. ludens comparing cryopreserved males with control males showed that the coefficient of similarity was ≥85%. This study indicates that embryo cryopreservation had little or no effect on the reproduction, longevity and flight of the species tested and can be used to support maintenance of insect stocks and control programs supported by the mass-rearing process.

KEY WORDS Anastrepha ludens, Anastrepha suspensa, Ceratitis capitata, long-term cold storage, fitness testing
large *Drosophila* stock centers situated around the world. For example, the stock center at Bloomington, IN, reported in February 2009 that 25,109 strains of *Drosophila* were being maintained (http://flystocks.bio.indiana.edu/bloomington.csv). Although we do not know whether a lack of information on post cryopreservation quality is inhibiting use of the *Drosophila* techniques, we do know that the question of insect quality after liquid nitrogen storage has been posed by potential users of insect cryopreservation protocols. Maintaining insect quality can be problematic under laboratory or large factory-like rearing conditions. Preserving genetic integrity of insects used in research and competitiveness for released insects in area-wide control programs warrants continuous attention (Hooper 1987). Selection for undesirable traits may occur when rearing insects over many generations (Bush et al. 1976, Briscoe 1992, Mukhopadhyay et al. 1997). One of the main benefits of having the capacity to cryopreserve insects reared continuously under laboratory conditions is to prevent change from occurring. The one caveat of this statement is the process of cryopreservation cannot itself produce undesirable qualities. Otherwise, the benefits are lost.

Here, we present data on various quality control parameters obtained from three different species of economically important fruit fly species (Diptera: Tephritidae): *Ceratitis capitata* (Wiedemann), the Mediterranean fruit fly; *Anastrepha ludens* (Loew), the Mexican fruit fly; and *Anastrepha suspensa* (Loew), the Caribbean fruit fly. The tests performed provide assessment of survival, mating, fertility, fecundity, flight ability, and the maintenance of genetic diversity. These features are of considerable interest to those maintaining colonies for research purposes as well as those managing mass-rearing facilities involved with the sterile insect technique. It is important to realize that, owing to both logistical and regulatory (quarantine) restrictions, we were unable to perform all quality control tests on all three species. As such, the study did not focus on interspecific comparisons of the effects of cryopreservation on particular biological traits. Rather, it measured the effect of cryopreservation on a diverse array of fitness parameters to assess its utility as a tool in colony management. Although more complete, interspecific comparisons are warranted, the different tests described below nonetheless point to a general outcome of considerable importance, i.e., cryopreserved insects (or descendants of cryopreserved insects) suffered no apparent reduction in fitness.

### Materials and Methods

**Insects.** Eggs of *C. capitata* (Maui-Med strain) and *A. ludens* were obtained from USDA–APHIS mass-rearing facilities located in Waimanalo, HI, and Edinburg, TX, respectively. Eggs of the *A. suspensa* came from a colony maintained in our laboratory for \( \approx 4 \) yr and was descended from a laboratory colony maintained at the USDA–ARS Center for Medical, Agricultural and Veterinary Entomology, Gainesville, FL. For mating trials involving *C. capitata*, we used females from a recently established laboratory colony (six generations removed from the wild). This strain (referred to as “wild” type) was derived from coffee fruit, *Coffea arabica* L. (Rubiaceae) collected near Haleiwa, Oahu.

**Embryo Cryopreservation.** The embryos of *C. capitata* and *A. ludens* were cryopreserved as outlined in Rajamohan et al. (2003) and Rajamohan and Leopold (2007). The *A. suspensa* embryos were cryopreserved using the *A. ludens* protocol and by shortening the postoviposition incubation time to 21 h before starting the procedure. Storage time was generally <1 wk for all three species used in this study. This was done to facilitate accomplishing these studies within a reasonable time frame. The length of storage of biologics, whether long- or short-term, has been reported as of little consequence because potential harmful effects are largely eliminated at liquid nitrogen temperature (Mazur 1984).

**Postcryopreservation Rearing.** After recovery from liquid nitrogen storage, the embryos were placed into Schneider’s cell culture medium (Sigma-Aldrich, St. Louis, MO) and allowed to hatch. Depending on the species, the newly hatched larvae were counted and then transferred to petri dishes containing an artificial diet (*C. capitata*, Tanaka et al. 1969; *A. ludens* and *A. suspensa*, Busch-Petersen and Wood 1986). When the larvae reached the age of pupation, the plates were transferred to a box of vermiculite to permit the larvae to pop out of the diet and burrow into the vermiculite to pupate. The yield of pupae from the larvae and of the adults from the pupae was recorded for only *A. suspensa* in this study. For comparison purposes, similar data for *C. capitata* and *A. ludens* gained from previous studies are included in Table 1. Except for *C. capitata*, all tests were conducted on *A. ludens* and *A. suspensa* adults of the same generation that were cryopreserved as embryos. The adult diet for all three tephritid species was a sugar–yeast mixture (3:1, by weight) and water ad libitum.

Starting with \( \approx 200 * C. capitata* adults after cryopreservation, the flies were propagated as a colony for two generations to obtain an adequate number of insects for field testing. The larger numbers of flies were reared and held in plastic buckets covered with nylon screening (volume, 5 liters; 125–150 flies per bucket). Perforated plastic vials containing lemon

<table>
<thead>
<tr>
<th>Insect</th>
<th>Hatchinga</th>
<th>Pupationa</th>
<th>Emergencea</th>
<th>Adult yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. suspensa</em></td>
<td>37.4 ± 9.0</td>
<td>64.6 ± 9.5</td>
<td>94.5 ± 6.6</td>
<td>22.8</td>
</tr>
<tr>
<td><em>A. ludens</em></td>
<td>49.1 ± 12.2</td>
<td>66.5 ± 17.8</td>
<td>93.1 ± 7.6</td>
<td>29.2</td>
</tr>
<tr>
<td><em>C. capitata</em></td>
<td>47.3 ± 0.7</td>
<td>80.3 ± 7.9</td>
<td>92.9 ± 7.9</td>
<td>34.9</td>
</tr>
</tbody>
</table>

*Mean percentage ± SE.

Data from Rajamohan et al. (2003).

Data from Rajamohan and Leopold (2007).
juice-soaked sponges served as an ovipositional substrate, and eggs were placed on standard larval medium (Tanaka et al. 1969) in plastic containers over vermiculite for pupation. Adult C. capitata males were separated within 24 h of eclosion, before attaining sexual maturity (D. McInnis, personal communication). Flies were held at 23–27°C and received both artificial and natural light with a photoperiod of 12:12 (L:D) h. Females used in the trials were separated within 48 h of adult emergence (sexual maturity is attained at 9–11 d of age; T.E.S., unpublished data). The flies emerged in late August 2001, and all tests were conducted in September and October 2001.

**Adult Survival.** Data on adult survival were collected for A. ludens in the laboratory and for C. capitata in outdoor field cages where the mean temperature ranged from 27 to 28°C. Because our initial tests determined that A. ludens adults could survive upward to nearly 1 yr in the laboratory, we wanted to measure survival over a shorter, more manageable period of time. Thus, we evaluated survival in the absence of diet and/or water. Groups of 20 males, treated and untreated, were placed in cages with water only and examined at 24-h intervals. The dead insects were removed from the cages each day until all had succumbed.

Field survival for C. capitata males descended from cryopreserved parents was measured using two field cages (2.5 m in height, 3.0 m in diameter), each containing a single rooted guava, *Psidium guajava* L. (Myrtaceae) tree located at the University of Hawaii Agricultural Experiment Station, Waimanalo, Oahu. One tree (designated H) had few branches and a sparse canopy, whereas the other tree (designated I) had much denser foliage. For a given replicate, 100 control and 100 males descended from the cryopreserved line were released into field cages when they were 5 d old. Marking procedures followed those described below. No food or water was placed in the field cages (the guava trees bore no flowers or fruit during the study period), and survivors were collected 48 h later and counted. The mean temperature during the month when these experiments were conducted was 22 ± 5°C. This experiment was replicated six times.

**Fertility and Fecundity.** A laboratory evaluation of fertility and fecundity was performed for A. suspensa and a field test only on fecundity for C. capitata. Upon adult emergence, A. suspensa, cryopreserved as embryos, were separated by sex, and then 3–5 d later both sexes were allowed to mate with untreated flies that also had been separated by gender at emergence. Groups of 10–15 cryopreserved females that had mated with untreated males were allowed to oviposit for one hour per day for 5 d, and the total number of eggs collected per female was tabulated. Likewise, the number of eggs collected from groups of 10–15 untreated females that had mated with untreated males also was recorded. This experiment was replicated four times. Eggs were also collected from untreated females that had mated with either cryopreserved or untreated males and were allowed to hatch after being placed on moist filter paper within petri dishes (~100 eggs per sample). The hatching rates of both groups were compared after replicating the experiment three times.

A field evaluation of fecundity was conducted for C. capitata. Using 5-d-old flies, we obtained mating between control males and females and treated males and females within laboratory cages. Those pairs copulating for at least 60 min were selected for the experiment. The mating pairs were isolated, and after mating, the females were placed in field cages with water provided ad libitum. Groups of 30 mated control females and treated females mated with treated males were then placed in plastic buckets (diameter, 8 cm; height, 24 cm) and provided food and water ad libitum as described above. Two days after mating, we placed two perforated plastic vials in each bucket for oviposition and replaced these daily over the next 6 d. Daily egg counts were made by rinsing the vials with water, filtering the eggs on a piece of black cloth, and counting the eggs using a dissecting microscope. Female mortality was not monitored over the egg collection period, and dead individuals were not replaced.

**Mating Evaluation.** The mating index for A. ludens was determined by mating the adult male and female control flies with those that had been cryopreserved. The mating assay was conducted between 1400 and 1600 hours when the flies exhibited a higher propensity to mate under laboratory conditions. The experimental flies both from the cryopreserved stock and the control groups were separated by sex upon adult emergence and were then assayed on the seventh day after emergence. Thirty males from the cryopreserved groups and 30 females from the control groups were transferred to a cage 20 by 20 by 15 cm (length by width by height) equipped with three 9-cm-diameter circular windows covered with screen. A fourth window was covered with tube gauze to allow removal of the mating pairs. Mating pairs were removed from the cage every 30 min for 3 h and counted. An index was calculated using the formula \[ \frac{1}{n} \left( \sum_{i=1}^{n} x_i f_i \right) \], where \( n \) is equal to the number of mating pairs, and \( f_i \) is a time-dependent constant equaling 100, 50, 25, 20, and 15 at the consecutive 30-min observation periods. This index measures mating readiness over time and a greater index indicates a higher mating propensity.

Field mating trials for C. capitata were conducted at the University of Hawaii Agricultural Experiment Station. Groups of 100 treated (males from cryopreserved grandparents), 100 control males and 100 “wild” females were released between 0800 and 0830 hours in field cages that contained a single rooted guava tree. When tested, males were 5–7 d old and females were 10–14 d old. For each test, we marked only males from one group (i.e., treated or control) and alternated the identity of the marked group between successive trials. Males were marked 1 d before testing by cooling them for several minutes and placing a dot of enamel paint on the thorax. This procedure had no adverse effects, and males resumed normal activities within minutes of handling. The cages were monitored for 4 h, mating pairs were collected in vials, and the males identified. Individuals of both sexes were virgins when
tested, and new unmated flies were used for each of the six trials.

**Flight Ability.** The flight ability of the Mexican fruit fly males was assessed both in terms of speed and stamina. Individual flies from control and cryopreserved groups were tethered to a flight mill consisting of telescopic weight balanceable arms on a magnetic friction-reduced axis (Taylor et al. 1992). Each fly was attached to a 40-gauge copper wire on the dorsal side of the thorax using a small drop of tissue-tack adhesive (Ted Pella, Inc., Redding, CA) and then tethered to one of the arms of the mill. Each turn of the arm activated a photo sensor, which was in turn connected to a digital counter (Red Lion Controls, York, PA). The flies were permitted to fly for 6 h, and the revolutions were noted every hour.

To determine the proportion of *C. capitata* adults capable of flight, we placed 100 control or 100 treated pupae (sexes not distinguished) in PVC tubes (diameter, 9 cm; height, 10 cm) in a screened room (2.9 by 1.9 m, 1.8 m in height) at the USDA-APHIS Hawaii Fruit Fly Rearing Facility in Waimanalo. This room was maintained at 25–27°C and a photoperiod of 12:12 (L:D) h under fluorescent lights. The inside surfaces of the tubes were dusted with talcum powder to prevent escape by walking. Pupae were placed in the tubes 2 d before emergence, and the number of flies remaining in the tubes (either as unemerged or partially emerged pupae or as deformed adults) was counted 5 d later.

**Genetic Diversity Analysis.** The genomic DNA was isolated from the individual newly emerged adult male progeny of cryopreserved *A. ludens* and from untreated control males to determine whether the cryopreservation process had a diminishing effect on genetic diversity. The DNA samples were isolated (Cheung et al. 1993) and processed by the random amplified polymorphic DNA (RAPD)-polymerase chain reaction (PCR) technique of Roehrdanz (1996). The RAPD analysis was conducted using primers from QIAGEN Operon (Alameda, CA). We screened 20 of the 10-mer primers, examined ~30 loci for each primer, and used 30 insects for each primer. Primers producing reproducible polymorphic bands included OPH4, OPH5, and OPH20. The analysis was performed in 20-μl volumes containing PCR buffer (Applied Biotechnologies, Evansville, WI), 200 μM dNTPs, 0.5 μM primer, 4.0 mM MgCl₂, 1.25 U of Taq Polymerase (Applied Biotechnologies), and 10–20 ng of genomic DNA. The PCR reactions were carried out in a 480 Thermal Cycler (PerkinElmer Life and Analytical Sciences, Boston, MA) by using the following profile: 95°C for 1 min for one cycle, 95°C for 30 s, 46°C for 30 s for 40 cycles, and a final extension at 75°C for 30 s. The PCR products were size separated by electrophoresis in 1.2% agarose gel, stained with ethidium bromide, and photographed under UV illumination.

**Statistical Analysis.** Comparisons between control and treated flies were conducted either using the Student's *t*-test or analysis of variance (ANOVA) after the assumptions of normality and equal variances were tested using standard procedures in all cases (Stata 10.1, Stata Corporation, College Station, TX). Raw data were used in all cases except for Mediterranean fruit fly flight ability, where proportions of the flying *C. capitata* at emergence were arcsine transformed for analysis.

The relationship between *A. ludens* flight ability and age was found to be nonlinear; therefore, models were fit and compared using generalized linear models of the Stata 10.1 statistical package. The control and treated groups were then evaluated using a chi-square analysis.

The electrophoretic data were analyzed by using the Nei–Li coefficient of similarity as described by Nei and Li (1979). The polymorphic bands were scored on a binary basis either as present “1” or absent “0” and using this system a matrix was constructed for use in computing the coefficients of similarity. The equation $F = \frac{2ny}{(nx + ny)}$, where *x* is control fly band, *y* is cryopreserved fly band, and *n* is shared band. Thus, the genetic similarity between the control and cryopreserved groups was calculated as the ratio of the comparable to the total electrophoretic bands scored.

**Results**

**Embryo-to-Adult Survival.** Table 1 displays a comparison of the survival throughout the developmental stages to adulthood for all three species of tephritids after experiencing cryopreservation as embryos. The data from this study for *A. suspensa* and the previous studies as cited for *C. capitata* and *A. ludens* show that the cryogenic protocols developed for each species decrease survival during development to the adult stage after cryopreservation. The normalized hatching rates (=percentage of control hatch) ranged from 37 to 49%, development of larvae to pupae from 64 to 80%, and pupal to adult emergence from 87 to 94% for the three species. The yield of adults from the cryopreserved insects was ~23% for *A. suspensa*, 29% for *A. ludens*, and 35% for *C. capitata*, respectively.

**Laboratory cage survival of male *A. ludens* adults after cryopreservation during their embryonic stage is shown in Table 2. Longevity in the laboratory for these insects exceeds 7 mo when they are provided food and water. However, when deprived of food and water after adult emergence, the control and cryopreserved groups survived for only ~3.5 d. Supplying just water to both groups yielded 5.3-d survival for the control group, which was 1.3 d longer survival than the cryopreserved insects and significantly different ($F = 15.29; df = 1, 44; P = 0.0003$).

Field cage survival of the adult progeny derived from *C. capitata* parents cryopreserved as embryos is shown in Table 3. Although there was a large difference in the survival of control and treated flies between the two field cages containing the guava trees, the within cage variation for the control and cryopreserved groups was not significantly different for the 48-h observation period. The lower survival of the...
groups of flies caged with tree designated as H probably related to a sparse amount of foliage present on the trees during the time of that particular replicate test which provided less protection from the heat of the day and less opportunity to gain sustenance.

### Fertility and Fecundity.

The mean numbers of eggs hatching from the crosses consisting of adult *A. suspensa* males and females cryopreserved as embryos and mated with their untreated female and male counterparts were greater that of the control hatch but they were not statistically significant (Fig. 1). Larval hatching varied from a mean of $88\%$ from eggs in the control groups to nearly $97\%$ from the eggs obtained from cryopreserved females mated with control males. The numbers of eggs collected over the 5-d observation period from the cross involving cryopreserved males mated with untreated females was significantly greater than that collected from cryopreserved females mated with untreated males but was similar to that of the control group (Fig. 1). Furthermore, the control group and the cryopreserved females mated with untreated males did not differ statistically in the number of total eggs deposited.

Under field conditions, the total number of eggs oviposited over a 6-d period by *C. capitata* females descending from cryopreserved parents did not vary significantly from the control groups (Table 3).

### Mating Evaluation.

The mean number of matings by cryopreserved and control *A. ludens* males occurring over the 3-h observation period was $40\%$ and $31\%$, respectively (Table 2). The index for the cryopreserved males was also higher than the controls and it was a significant difference ($F = 37.53; df = 1, 4; P = 0.004$). The mean field cage mating of the male progeny derived from the treated and untreated *C. capitata* groups did not differ when tested in a competitive situation (Table 3). The males derived from cryopreserved parents mated at the same frequency as the control males in the head-to-head competition for females.

### Flight Ability.

Fig. 2 shows the results of testing the flight ability of cryopreserved and control *A. ludens* adult males over a 6-h period attached to a flight mill. There was a gradual decline in the distance in meters flown with increasing age. Within the span of eight to 27 d of age, the younger flies clustered $800–1200$ m and the oldest flies $200–400$ m. The greatest distance flown was nearly $1450$ m. The distribution of data points was analyzed using Stata 10.1 and found to be nonlinear for both the controls and cryopreserved males occurring over the 3-h observation period.

### Table 2. Survival and mating comparisons of control and cryopreserved (treated) *A. ludens* adult males

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (5)</th>
<th>Cryopreserved (5)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food and water</td>
<td>237.6 ± 32.2a</td>
<td>213.2 ± 85.9a</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>5.3 ± 1.3a</td>
<td>4.0 ± 0.9b</td>
<td></td>
</tr>
<tr>
<td>No food or water</td>
<td>3.6 ± 1.3a</td>
<td>3.5 ± 1.1a</td>
<td></td>
</tr>
<tr>
<td>Survival (d)</td>
<td>Total</td>
<td>Mating</td>
<td>propens index</td>
</tr>
<tr>
<td>Control (7)</td>
<td>31.4 ± 2.7</td>
<td>1.57a</td>
<td></td>
</tr>
<tr>
<td>Cryopreserved (5)</td>
<td>39.8 ± 1.0</td>
<td>2.09b</td>
<td></td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate replications. Numbers within columns with different lowercase letters are significantly different ($P < 0.05$; ANOVA).

### Table 3. Field evaluation of survival and mating plus laboratory testing of flight and fecundity of the F$_1$ progeny from cryopreserved (treated) and control groups of *C. capitata*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (± SE)</th>
<th>Treated (± SE)</th>
<th>t value</th>
</tr>
</thead>
<tbody>
<tr>
<td>% survival</td>
<td>21.7 ± 11.2</td>
<td>18.5 ± 10.7</td>
<td>0.45 NS$^b$</td>
</tr>
<tr>
<td>Tree H (6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tree I (6)</td>
<td>57.1 ± 10.2</td>
<td>61.2 ± 14.6</td>
<td>0.60 NS</td>
</tr>
<tr>
<td>No. eggs/group (8)</td>
<td>2,562.4 ± 645.5</td>
<td>2,265 ± 414.4</td>
<td>1.11 NS</td>
</tr>
<tr>
<td>% mating (10)</td>
<td>17.8 ± 9.3</td>
<td>16.4 ± 5.2</td>
<td>0.41 NS</td>
</tr>
<tr>
<td>% flying (10)</td>
<td>69.4 ± 7.2</td>
<td>78.2 ± 3.4</td>
<td>3.51**</td>
</tr>
</tbody>
</table>

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$^a$ Numbers in parentheses indicate replications.

$^b$ NS, not significant at $P > 0.05$.

$** P < 0.01$. 

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Fig. 1. Laboratory assessment of *A. suspensa* fertility and fecundity using control and cryopreserved groups. Diagonally striped bars having different letters are significantly different at $P < 0.01$ and hatching rates (black bars) among the groups did not differ at $P > 0.05$ according to Student-Newman-Keuls test.

Fig. 2. Flight mill testing of cryopreserved and control *A. ludens* males showing the relationship of flight distance with age. Each circle and dot represents an individual insect.
Furthermore, we found no significant difference between the control and cryopreserved flies when comparing distances flown to age ($\chi^2 = 0.3, df = 1, P = 0.59$).

The flight ability of *C. capitata* male and female progeny descended from cryopreserved parents was also unaffected when tested at adult emergence. In fact, the number of flies from cryopreserved parents flying from the confines of the emergence tubes was significantly greater than that of the control flies (Table 3). The reason for this difference is unknown.

**Genetic Diversity Analysis.** Analysis of the RAPD banding patterns elicited by the three OPH primers showed a high degree of similarity in comparisons between populations of control and adult *A. ludens* males cryopreserved as embryos (Fig. 3). The average percentage of shared polymorphic bands displayed by the primers among the insect samples for OPH-4, OPH-5, and OPH-20 was 71.0, 77.8, and 87.5%, respectively. The average range of percentages elicited by the primers was 69.4–71.1, 75.4–80.2, and 85.3–89.7%, and the coefficients of similarity were 0.85, 0.92, and 0.92 for the three groups. The average incidence of shared bands between the control and the combined treated groups was 0.72, further indicating that selection for insects tolerant to cryopreservation was not occurring and that genetic diversity using this system of analysis was not appreciably affected.

**Discussion**

The tests conducted in this study were mainly those that would be of interest to those scientists and insect production managers who maintain colonies of insects on a long-term basis. Most of the tests were the same as the routine assessments that mass-rearing facilities routinely perform on their production strain. Other tests are designed to examine traits that are not ordinarily assessed but are important to an evaluation of the overall fitness of a strain of insects that has been maintained in the laboratory for many generations.

The process of cryopreservation involves exposing the insect embryo to a number of potentially harmful physical and chemical stresses which may result in debilitated adults. Evidence of such stress is shown by the reduction of adult emergence of all three species of tephritids after cryopreservation (Table 1). Known chemical stresses include the solvents used to remove the lipid layer on the surface of the vitelline membrane and the cryoprotective agents (CPAs). For example, over exposure of Mediterranean fruit fly embryos to isopropanol when removing the lipid solvent was observed to cause necrosis along with a low survival rate (Rajamohan et al. 2003). Furthermore, CPAs have been shown to be solvents of certain subcellular components in cryopreserved cells and organisms (Pajot-Augy 1993).

Aside from chemical toxicity, the process of cryopreservation and recovery exposes the insect embryos to osmotic, thermal and possibly tissue distortion stresses (Armitage and Mazur 1984, Ashwood-Smith et al. 1988, Sathananthan et al. 1992). Rall (1987) emphasized the importance of maintaining osmotic equilibration with the CPA and recovery solutions and also by using appropriate cooling and warming rates to protect against cryoinjury when using the vitrification method for preserving mouse embryos. Furthermore, some of the potential for thermal stress has been eliminated by exposure of the Mexican fruit fly embryos to liquid nitrogen vapor before storage in liquid nitrogen or warming upon recovery (Rajamohan and Leopold 2007).
Although our previous investigations suggest that various physiochemical effects may reduce the overall yield of insects propagated from the embryos after recovery from storage (Leopold et al. 2001), the tests conducted in this study did not indicate that insects reaching adulthood suffered lasting debilitation. For example, the longevity of A. ludens in the laboratory and C. capitata under field conditions were basically unaffected by having a history of cryopreservation. Only A. ludens males were used in the assessment of laboratory longevity and, under these conditions, they proved to be especially long lived. Including females in the groups probably would have reduced their longevity because it has been observed that placing females with males decreased male longevity (Mangan 1997). C. capitata has been shown to be considerably shorter lived than the Mexican fruit fly (Carey et al. 2005), and their shorter life span was especially evident in our field cage studies.

Postcryopreservation fecundity was measured both in the laboratory and under field conditions. With C. capitata, we found no significant differences in the number of eggs laid by these females when compared with their respective control groups. Although cryopreserved A. suspensa males mated with untreated females produced a normal amount of eggs, the reciprocal cross produced significantly less eggs. However, oviposition by the cryopreserved females did not differ from the egg production by the controls. It should be noted that with each species the eggs were collected on a relatively short term basis ranging from 5 to 6 d and whether differences exist in total fecundity of the females after cryopreservation was not determined. Also, to obtain a sufficient number of insects to conduct all field and laboratory tests using C. capitata, it was necessary to use the F1 generation that descended from cryopreserved parents.

Flight ability relates not only to the capacity of the insect to move and forage in the wild but also is indicative of a functional flight musculature and its innervations. Wing movement is also involved in the defense, courtship, and mating functions of male tephritids (Sivinski et al. 1984, Burk 1991, Landolt and Sivinski 1992, Díaz-Fleischer and Aluja 2000). Flight ability was measured at emergence (Mediterranean fruit fly) using the standard method for testing mass-reared tephritids (FAO/IAEA/USDA 2003) as opposed to observations over a 19-d period with sexually mature male flies (A. ludens) by using a flight mill (Taylor et al. 1992). Our data showed that the ability to fly and flight endurance are unaffected by cryopreservation as the treated flies performed as well or better than their respective control groups regardless of the type of test that was used.

Further evidence for undamaged wing function after cryopreservation was gained by conducting the mating ability tests. The mating ability tests used in this study not only examined the quality of several aspects of tephritid mating ability, but they also indirectly yielded information on wing movement. For example, caged mating tests are often used to assess “mating speed” (Cayol 2000), whereas the culmination of successful mating indicates that male flies exhibited normal courtship and coupling activity through the use of their wing movement behavior. Thus, the mating test involving A. ludens specifically assessed the rate of male-female coupling over time in laboratory cages, whereas the C. capitata field cage experiments examined mating competitiveness by placing male flies from cryopreserved parents in competition with untreated flies for female mates. Because the latter test showed no differences in competitiveness between treated and untreated flies, it can be concluded that cryopreservation does not hinder the female calling by the males or other important elements of the male courtship behavior.

Recent studies suggest that certain cryoprotectants may harm cytoskeletal components of ovine embryos (Succu et al. 2007, Makarevich et al. 2008). This indicates that preparation for cryopreservation could ostensibly result in harm to the cellular integrity of embryos. Cryobionomics is a relatively new field of research and studies examining the genomic integrity of organisms are few in number (Harding 2004). To date, restriction fragment-length polymorphism, RAPD, and DNA fingerprinting by using specific probes have been used for studies of this nature (Angel et al. 1996). The RAPD method offers a simple yet efficient technique to evaluate genomic quality, and, under certain conditions, it has been reported capable of even assessing single base changes (William et al. 1990). Among 20 different primers that were assessed in this study on A. ludens, three were considered suitable and the estimated Nei–Li and Jaccard’s coefficients ranged from 0.85 to 0.92, which is indicative of ≈90% similarity (for assessment technique, see Lamboy 1994). Houle et al. (1997) examined D. melanogaster after embryo cryopreservation for the presence of mutations using a sex-linked recessive lethal assay. They found no quantitative increase in the lethal mutation rate after cryopreservation. New techniques to assess possible genomic alterations at the level of gene expression using cryopreserved tephritids have been initiated (Rajamohan et al. 2005). Nevertheless, the cumulative results obtained in this study on survival, flight and reproduction also suggest that overall genomic integrity was not harmed by cryopreservation.

It should be mentioned the above data are derived from samples that were in liquid nitrogen for <1 wk and thus did not experience long-term storage. Studies on the genetic and physiological quality after long-term storage of invertebrates are rare. Van Wyk et al. (2000) reported that five of eight species of gastrointestinal parasites remained infective after liquid nitrogen storage for 13.3–15.8 yr. Our prior studies on cryopreservation of A. ludens embryos showed survival was unaffected after nearly a year in storage (Rajamohan and Leopold 2007). Recent recovery of the stored A. ludens shows that the embryos are hatching after 5 yr of storage, albeit at a slightly lower level than previously observed (R.A.L., unpublished data). Over time, cryopreserved biologics presumably can be affected by free radical oxidation generated by cosmic
radiation, but the time frame for accumulating this type of damage is predicted to be extremely lengthy (Mazur 1984).

In summary, we have provided substantial evidence that indicates embryo cryopreservation by using the vitrification technique has little or no effect on various aspects of insect quality important to implementing control or research programs. It is hoped that this study will help ease any uncertainty that might arise for individuals considering the use of cryopreservation for increasing the economy and safety in the propagation of insects.

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