A PROTOCOL FOR STORAGE AND LONG-DISTANCE SHIPMENT OF MEDITERRANEAN FRUIT FLY (DIPTERA: TEPHRITIDAE) EGGS. I. EFFECT OF TEMPERATURE, EMBRYO AGE, AND STORAGE TIME ON SURVIVAL AND QUALITY

CARLOS CÁCERES[1], EDWIN RAMÍREZ[2], VIWAT WORNOAYPORN[1], SHEIKH MOHAMMAD ISLAM[1] AND SOHEL AHMAD[1]
[1]Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture
International Atomic Energy Agency, Agency's Laboratories, A-2444 Seibersdorf, Austria

[2]Programa Moscamed-Guatemala, Planta de Producción de Mosca del Mediterráneo km 48
Carretera a El Salvador, El Cerinal, Barberena, Santa Rosa, Guatemala

ABSTRACT

The operational use of Mediterranean fruit fly (medfly) *Ceratitis capitata* (Wiedemann), genetic sexing strains in Sterile Insect Technique applications can be maximized by developing methods for effective shipment of eggs. This would enable a central production facility to maintain the relevant mother stocks and large colonies to supply eggs to satellite centers that would mass produce only males for irradiation and release. In order to achieve this, the survival of medfly embryos of different ages was assessed after storage at 5, 10, 15, 20, and 25°C in water for different periods of time. Survival was affected by all 3 variables, i.e., embryo age, water temperature, and length of storage. Storage of embryos at any temperature for 120 h resulted in almost no survival. Controlling the age of the embryo at the time of the temperature treatment is crucial for the success of this procedure. Embryos collected between 0 to 12 h after oviposition and pre-incubated at 25°C for 12 h provide a suitable 72 h window for shipment when maintained between 10 to 15°C. Under these conditions, no significant reductions in survival during all the developmental stages were observed.

Key Words: Mediterranean fruit fly, *Ceratitis capitata*, egg shipment, SIT, genetic sexing

RESUMEN

El uso operacional de cepas de la mosca del mediterráneo *Ceratitis capitata* (Wiedemann) en las cuales es posible separar los sexos a través de mecanismos genéticos para su utilización en la Técnica del Insecto Estéril (TIE), puede ser maximizado con el desarrollo de métodos efectivos para el envió y transporte de huevos. Esto permite que un laboratorio de producción centralizada mantenga las respectivas colonias responsables por la producción de huevos para este abastecer laboratorios satélites responsables por la producción masiva de solamente machos para subsiguiente irradiación y liberación. Para ser posible esta alternativa fue evaluada la supervivencia de embriones de diferentes edades después de su almacenamiento en agua a 5, 10, 15, 20 y 25°C por diferentes periodos de tiempo. La supervivencia fue afectada por las 3 variables evaluadas, la edad del embrión, la temperatura del agua y el periodo de almacenamiento. El almacenamiento de los embriones a cualquier temperatura por 120 horas dio como resultado la casi no supervivencia. Una edad controlada de los embriones a tratar es crucial para el éxito de este protocolo. Embriones colectados entre 0 a 12 horas después de la oviposición y su previa incubación a 25°C por 12 horas brinda un margen de hasta de 72 horas de duración del almacenamiento y transporte, siempre y cuando estos se mantengan en una temperatura de entre 10 a 15°C. En estas condiciones, fue registrada una reducción no significante de la supervivencia de los diferentes estados de desarrollo.

Currently, a majority of area-wide programs that integrate the Sterile Insect Technique (SIT) against the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann), are using genetic sexing strains based on male-linked translocations that generate temperature sensitive lethality in female embryos (Robinson et al. 1999). In order to supply field programs with sterile flies for release, sterilized male pupae are shipped to emergence and release centers (FAO/IAEA/USDA. 2003; Enkerlin et al. 2003) with the shipment being carried out 1 or 2 d prior to adult emergence and immediately after irradiation (Schwarz et al. 1981; Vargas 1989; Cáceres 2002). This approach can reduce insect quality through prolonged exposure of pupae to reduced-oxygen atmospheres during transport.

Males of the genetic sexing strains carry a translocation that reduces fertility and the females have reduced viability due to being homozygous for the *temperature sensitive lethal* (*tsl*) mutation. These 2 factors impact design and

implementation of production systems in mass rearing facilities (Cáceres et al. 2004). Consequently it is obligatory to have 2 specific systems, one for the production of only male pupae for sterilization and release and the second for maintenance of the production colony (Cáceres et al. 2000). In addition, a Filter Rearing System (FRS) is needed in order to maintain the genetic integrity of the genetic sexing mother stock (Fisher and Cáceres 2000). In rearing systems for colony production, where females are required, the larval rearing conditions have to be very carefully controlled so as to keep temperatures below those that would impact viability of females. For male only production no such precautions are needed and larval rearing is straightforward. The relatively complex, but manageable, rearing systems associated with the use of *tsl* based genetic sexing strain has led to the idea of separating the colony production process, including the FRS, from that of the production of male pupae for irradiation and release. In this concept, a large central facility would be responsible for the more complex task of maintaining the mother stock and large production colony, and this central facility would supply eggs to satellite facilities where male only production would be carried out.

In order to determine the feasibility of this concept, a first step is to assess if protocols can be developed which enable routine long-distance shipments of eggs to be carried out. The objective of this study was to analyze the effects of embryo age, storage periods, and storage temperatures on egg viability and egg to adult survival.

Materials and Methods

Egg Storage Experiment

Eggs of the medfly genetic sexing strain VIENNA-8/D53 (Franz 2005) were collected 1 h after oviposition and 150 aliquots were transferred to moist black filter paper for incubation at 25°C and 90% RH for different incubation periods. To obtain embryos of different ages, 25 aliquots were incubated for each the following periods: 1, 6, 12, 18, 24, and 36 h. After incubation the aliquots were transferred to flasks with water and for each incubation period 5 flasks were placed at each of the following storage temperatures: 5, 10, 15, 20, and 25°C, with 1 of the 5 flasks being held for the following storage periods: 12, 24, 48, 72, or 120 h. Immediately after this treatment 5 samples of 200 eggs were collected from each flask and placed on moist black filter paper located over standard carrot powder larval diet (Heather & Corcoran 1985) in a Petri dish held at 25°C. Each Petri dish was kept individually in a small plastic box with sand as pupation medium and covered with a mesh lid that allowed ventilation. The percent egg hatch for each sample of 200 eggs was determined. The larvae completed development in the Petri dish, left the diet, and pupated in the sand. 2 days before adult eclosion, pupae were collected and placed in a separate Petri dish to determine adult emergence. The effect of storage time on egg hatch and egg to adult survival was analyzed by 1-way analyses of variance (ANOVA) for each temperature and embryo incubation period and means compared by the Tukey multiple range test.

Male Mating Competitiveness

In a separate experiment, following incubation for 24 h at 25°C, eggs of strain VIENNA 8/D53 were stored in water for 120 h at 5, 10, 15, and 20°C. Mating competitiveness of the males emerging from these different treatments was measured in field cage tests by comparing 50 individually marked, protein-fed, virgin 5-d-old males from each temperature treatment with 50 untreated VIENNA 8/D53 control males, introduced into a field cage that was 2 m high and 3 m diameter (Calkins & Webb 1983) and containing orange trees (FAO/IAEA/USDA 2003). The field cage was set up in an environmentally controlled greenhouse at 25°C and 60% RH. Males were released early in the morning of the day of the test and 50 protein fed laboratory wild type females of strain Egypt-II) were released 1 h after the males. Propensity of Mating (PM) (Cayol 2000) and the proportion of males that participated in mating were calculated based on the number and type of mating recorded for the control and treatment flies. The experiment lasted for 3 h and was replicated 12 times for each temperature treatment. The effect of the storage time and temperature treatment on the percent of participation of mating was analyzed by one-way analyses of variance (ANOVA) and means compared by the Tukey multiple range test. Results from all experiments were analyzed with statistical software MINITAB for windows.

Results

Egg Hatchability

The data for egg hatchability following the different treatments are shown in Fig. 1a-e. Fig. 1a shows the data for storage at 5°C and it is clear that the shorter the incubation period (i.e., the younger the embryos), the more susceptible they are to long-term storage. In general longer storage times for any age of embryos result in lower egg hatch ($F = 2, P < 0.01$). Egg hatch for embryos incubated for 1 h and stored for 12 h was 48.9 ± 0.5%, however, this value declined to 0.1% following storage for 120 h (Fig. 1a). In general, egg hatch improved at 10°C, with only the 2 shortest incubation periods showing a decline following the longer storage periods (Fig. 1b). Egg hatch for embryos stored at 15°C showed a similar pattern

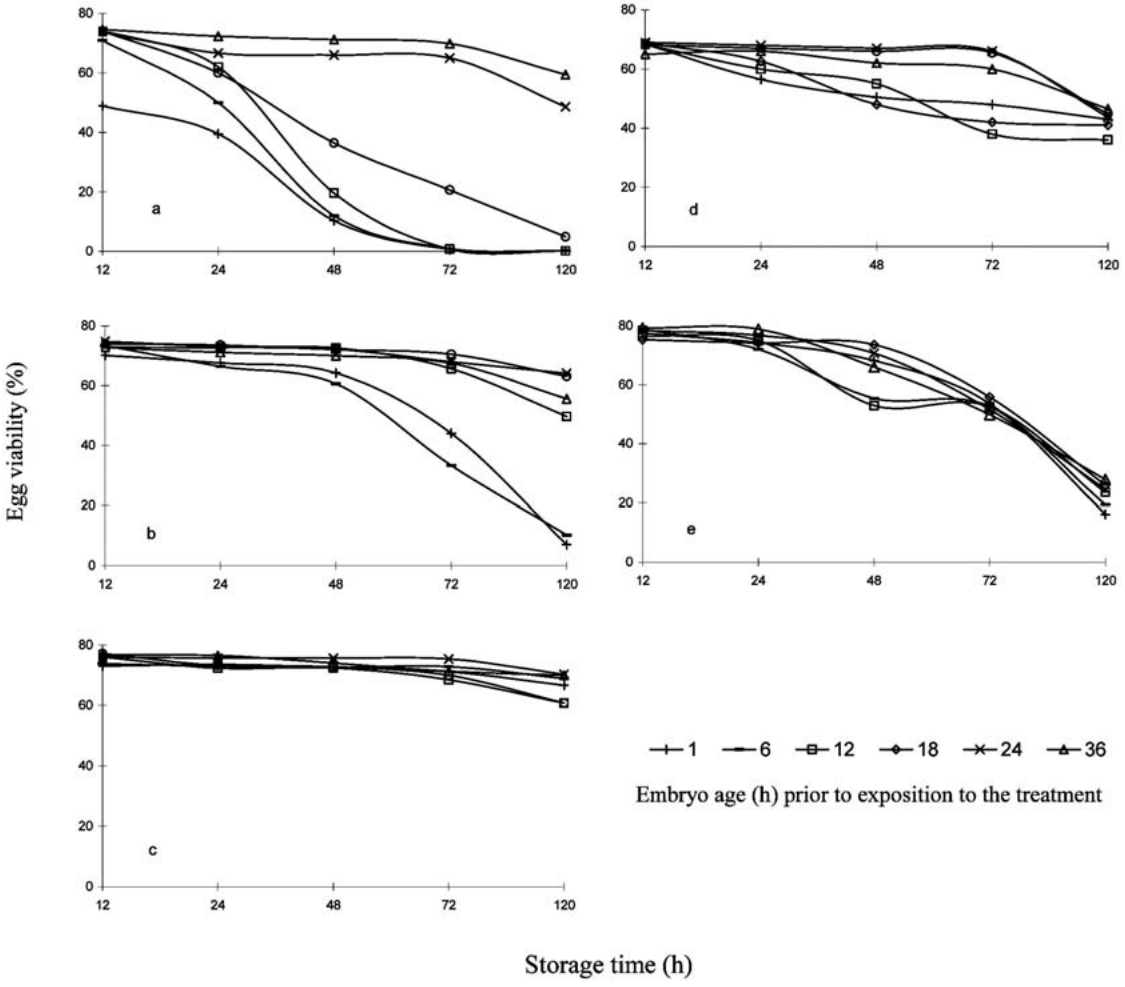

Fig. 1. Survival of Medfly embryos of different ages held at different treatment temperatures (a, 5; b, 10; c, 15; d, 20 and e, 25°C) for periods lasting between 12 and120 h.

for all incubation periods and storage times, with only long storage times significantly reducing survival ($F = 10, P < 0.01$) (Fig. 1c). Egg hatchability values at 15°C ranged from 77.2 ± 9.4% to 60.8 ± 3.1%. There was a general decline in egg hatch when storage temperature was increased to 20°C with again shorter incubation periods leading to higher lethality (Fig. 1d). Egg hatch for embryos incubated for 1, 6, and 12 h was reduced significantly after 24 h of storage ($F = 25$, $P < 0.05$), while embryos incubated for 18, 24 and 36 h showed significant reductions in egg hatch only after 72 h of storage ($F = 34$, $P < 0.05$). Egg hatchability values ranged from 69.0 ± 1.0% to 36.0 ± 0.1%. Egg hatch of embryos stored at 25°C showed significant reductions in survival after 24 h of storage ($F = 16, P < 0.05$) and egg hatchability ranged from 79.2 ± 0.4% to 16.2 ± 0.9% (Fig. 1e).

Egg to Adult Survival

The effect of storage for different periods of time at different temperatures following egg incubation for different times on the overall egg to adult survival is shown in Fig. 2a-e and Table 1. The egg to adult survival is calculated based on the number of adults produced from a certain number of eggs; it therefore includes the egg hatch data shown in Fig. 1. Egg to adult survival declined with time of storage for all embryo ages and temperature treatments. Egg to adult survival was lowest for embryos stored at 5°C reaching nearly zero after 72 h of storage ($R^2 = 0.95$, $P < 0.05$). The same tendency was observed for embryos incubated for 6, 12, and 18 h, where the values ranged from 45 to 0%, 48 to 0%, and 49 to 4%, respectively, ($R^2 = 0.95$, 0.94, and 0.97; $P <$

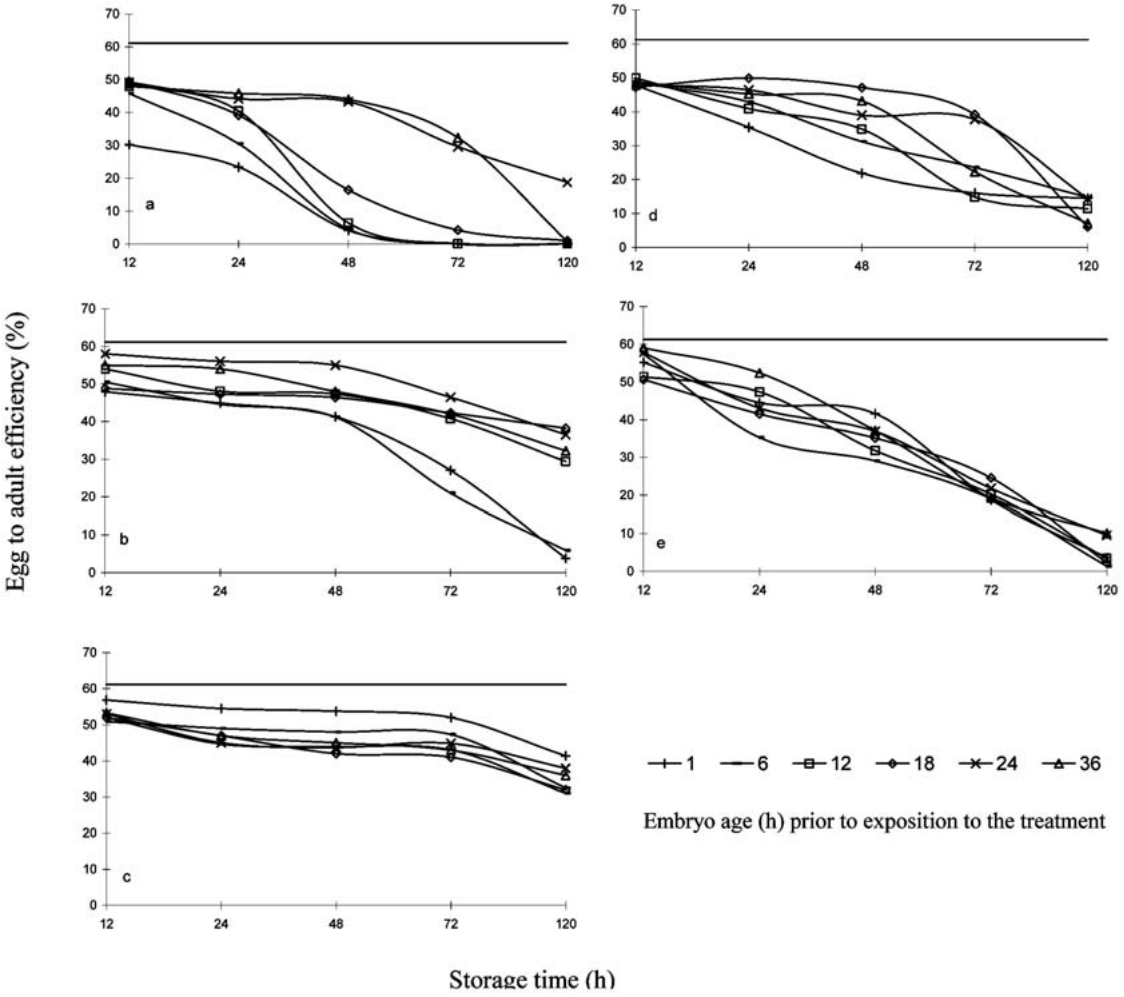

Fig. 2. Rate of adult emergence (%) from medfly embryos of different ages held at different treatment temperatures (a, 5; b, 10; c, 15; d, 20 and e, 25°C) for periods lasting between 12 and 120 h.

0.05). Egg to adult survival from embryos incubated for 24 and 36 h fell significantly after 48 h of storage ($F = 189$ and 170 respectively, $P < 0.05$), and values ranged from 49 to 19% and 48 to 1%, respectively.

For storage at 10°C the trend of egg to adult survival was similar to that for 5°C, but values were in general higher. Embryos incubated for shorter periods had the lowest values. Egg to adult survival for embryos incubated for 18, 24, and 36 h was significantly reduced following 48 h of storage, but the effect was not as great as for the 5°C treatment ($P < 0.05$).

Following storage at 15°C, egg to adult survival decreased significantly only after 72 h of storage for embryos incubated for 1 and 6 h ($P < 0.05$). Values for these incubation periods ranged from 57 to 41% and 51 to 32%, respectively. For embryos incubated for 12 h maximum values were observed when they were stored for 12 h. When embryos of the same incubation period were stored for 24, 48, and 72 h, values were significantly lower ($F = 12$ $P < 0.05$). As expected, the trend for these incubation periods was a progressive reduction in egg to adult survival. Egg to adult survival values for 12 h incubation ranged from 52 ± 1% to 31 ± 1.8%. For embryos incubated for 18 and 24 h, a significant reduction was only observed after 24 h of storage ($F = 2.72$ and 0.10 respectively, $P < 0.05$). Values ranged from 52 ± 5.2% to 32 ± 1.6% and 53 ± 0.8% to 38 ± 3.3%, respectively. For embryos incubated for 36 h significant differences were only observed after 48 h of storage ($F = 44$, $P < 0.05$).

For embryos stored at 20°C the adult emergence pattern for all incubation periods was similar. Egg to adult survival of embryos incubated for 1 h was the lowest for most of the storage

TABLE 1. ADULT EMERGENCE (%) FROM 1, 6, 12, 18, 24, AND 36 H OLD EMBRYOS HELD AT DIFFERENT TEMPERATURE (5, 10, 15, 20, AND 25°C) FOR PERIODS BETWEEN 12 AND 120 H. MEANS FOLLOWED BY THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT ($P < 0.05$). *CONTROL, EGGS WERE COLLECTED 1 H AFTER OVIPOSITION THEN INCUBATED AT 25°C FOR 48 H THEN TRANSFERRED TO THE LARVAL MEDIUM.

Temperature treatment	Storage time (h)	Embryo age (h)					
		1	6	12	18	24	36
5°C	12	30.3 ± 0.3 a	45.8 ± 2.8 a	48.8 ± 0.2 a	49.5 ± 0.6 a	49.2 ± 0.6 a	48.0 ± 1.6 a
	24	23.4 ± 3.5 b	30.5 ± 2.3 b	40.5 ± 2.9 a	39.2 ± 2.7 b	44.2 ± 2.0 b	45.8 ± 0.3 ab
	48	4.1 ± 0.2 c	4.5 ± 0.1 c	6.4 ± 0.5 b	16.4 ± 0.7 c	43.3 ± 0.1 b	44.0 ± 1.1 b
	72	0.1 ± 0.0 d	0.0 ± 0.0 d	0.0 ± 0.0 c	4.2 ± 0.4 d	29.6 ± 0.9 c	32.3 ± 0.6 c
	120	0.0 ± 0.0 e	0.0 ± 0.0 d	0.0 ± 0.0 c	1.0 ± 0.1 e	18.7± 0.9 c	0.7 ± 0.0 a
10°C	12	47.9 ± 6.2 a	50.5 ± 0.6 a	54.0 ± 0.3 a	48.8 ± 4.7 a	58.0 ± 1.5 a	55.0 ± 2.0 a
	24	44.9 ± 3.4 a	44.6 ± 0.8 ab	48.1 ± 0.5 ab	47.3 ± 6.0 a	56.0 ± 3.2 a	54.0 ± 0.2 a
	48	41.3 ± 2.4 a	41.2 ± 1.7 b	47.3 ± 0.8 bc	46.4 ± 0.7 a	55.0 ± 0.0 a	48.0 ± 0.4 ab
	72	27.0 ± 2.3 b	21.0 ± 1.1 c	40.8 ± 0.1 c	42.3 ± 2.9 ab	46.4 ± 1.3 b	42.0 ± 5.9 b
	120	3.8 ± 0.1 c	5.9 ± 0.2 d	29.4 ± 1.8 d	38.2 ± 1.6 b	36.5 ± 3.8 c	32.2 ± 2.8 c
15°C	12	56.9 ± 3.4 a	50.9 ± 1.1 a	52.0 ± 1.1 a	52.0 ± 5.2 a	53.0 ± 0.8 a	53.3 ± 1.0 a
	24	54.5 ± 0.3 a	49.0 ± 2.9 a	44.7 ± 2.7 b	47.0 ± 5.2 a	45.0 ± 0.4 b	47.0 ± 1.5 ab
	48	53.8 ± 10.7 a	48.0 ± 1.2 a	44.0 ± 1.1 b	42.0 ± 1.5 b	43.7 ± 0.6 b	45.0 ±1.7 ab
	72	52.0 ± 3.3 a	47.3 ± 0.9 a	43.0 ± 0.8 b	41.0 ± 6.3 b	44.8 ± 0.5 b	43.0 ± 4.8 ab
	120	41.4 ± 7.12 b	32.5 ± 1.3 b	31.0 ± 1.8 c	32.0 ± 1.6 c	38.0 ± 3.3 c	36.0 ± 3.1 c
20°C	12	47.8 ± 4.1 a	48.7 ± 0.3 a	49.9 ± 2.9 a	47.2 ± 6.3 a	47.8 ± 0.7 a	48.8 ± 0.4 a
	24	35.4 ± 1.0 b	42.9 ± 0.0 ab	40.9 ± 2.5 b	49.9 ± 6.7 a	46.4 ± 1.0 a	45.2 ± 1.4 ab
	48	21.9 ± 2.7 c	31.2 ± 1.6 b	34.9 ± 2.1 c	47.1 ± 2.5 a	39.0 ± 2.9 b	43.2 ± 1.9 b
	72	16.0 ± 3.3 cd	23.6 ± 1.3 c	14.9 ± 0.5 d	39.2 ± 0.2 b	37.7 ± 1.6 b	22.3 ± 0.4 c
	120	14.5 ± 2.7 d	14.8 ± 0.8 d	11.4 ± 0.0 e	6.1 ± 0.1 c	14.4 ± 1.2 c	7.1 ± 0.9 d
25°C	12	55.2 ± 4.0 a	57.4 ± 6.4 a	51.4 ± 2.1 a	50.7 ± 0.2 a	57.8 ± 0.7 a	59.0 ± 0.3 a
	24	44.5 ± 0.0 b	35.3 ± 2.4 b	47.4 ± 4.5 a	41.5 ± 2.7 b	43.1 ± 1.1 b	52.4 ± 1.3 b
	48	41.6 ± 0.8 b	29.1 ± 0.3 bc	31.8 ± 1.0 b	35.1 ± 0.5 c	36.9 ± 2.7 b	37.1 ± 0.0 c
	72	18.89 ± 0.6 c	19.1 ± 0.5 c	20.3 ± 3.0 c	24.6 ± 0.9 d	21.9 ± 1.4 c	19.4 ± 0.9 d
	120	3.7 ± 0.2 d	1.4 ± 0.9 d	3.4 ± 0.2 d	2.3 ± 0.0 e	9.6 ± 2.6 d	10.0 ± 1.1 e
*Control		61.2 ± 2.2					

periods with decreasing values as storage times increased. A similar trend was shown for embryos incubated for 1, 6, 12, and 36 h (R^2 = 0.92, 0.97, 0.95 and 0.89, respectively). The egg to adult survival of embryos incubated for 18 and 24 h was not correlated with storage time (R^2 = 0.65 and 0.79, respectively).

The egg to adult survival for embryos stored at 25°C showed a reduction as a function of storage time (R^2 = 0.94, 0.96, 0.97, 0.93, 0.99, and 0.99; $P < 0.05$, respectively) for 1, 6, 12, 18, 24, and 36 h of incubation periods. Values ranged from 55 to 4%, 57 to 1% 51 to 3%, 51 to 2%, 58 to 10%, and 59 to 10%, respectively, for these incubation periods.

Male Mating Competitiveness

In all field cage tests the Proportion in Mating (PM) index was high (91.3 ± 5%) indicating that there was a high degree of sexual activity during the test period. The proportion of copulations by control males was 19.3 ± 3.8% while that for males that emerged from the different temperature treatments oscillated between 16.5 ± 5.3% to 19.3 ± 4.4%. Results on the basis of the proportion of male participation in mating have shown no significant reduction in the sexual performance of males emerging from embryos stored for 120 h at any of the temperatures tested ($F = 0.71, P < 0.5$) (Fig. 3).

DISCUSSION

The most appropriate procedure for egg storage or for egg shipment allowing the subsequent male only production in satellite facilities is to select eggs 0 to 12 h post oviposition and incubate them for at least 12 h at 25°C. The embryos can then be stored or shipped for up to 72 h when maintained at 10 to 15°C without any significant reduction in egg to adult survival or adult male quality.

Overall, the optimal conditions for storage or shipping were to maintain the embryos in water up to 72 h at a temperature of 15°C. Eggs can be placed into storage at any development stage 1 h

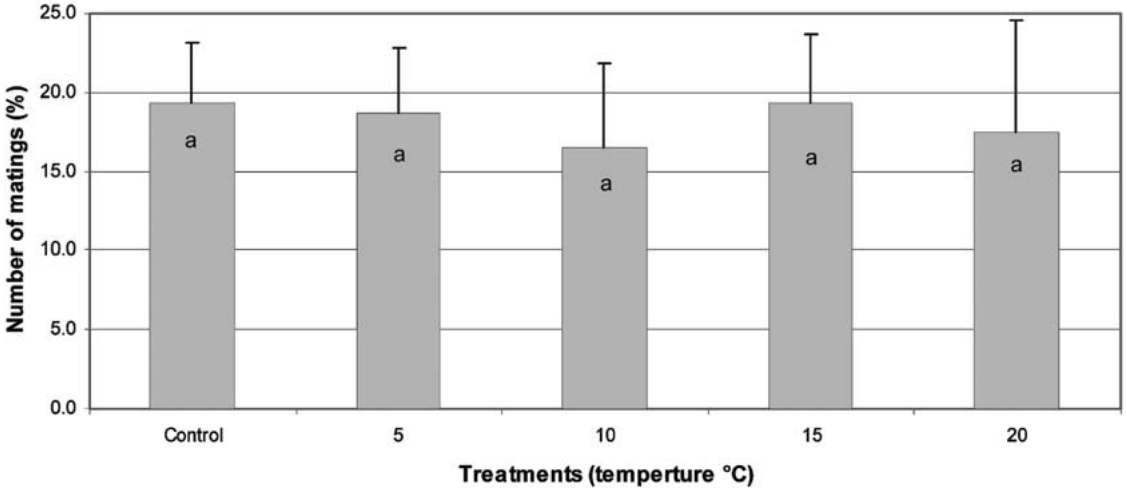

Fig. 3. Average number of matings of control VIENNA-8/D53 genetic sexing strain males (c) and treatment VIENNA-8/D53 genetic sexing strain males with virgin Eg II females. Treatment males were derived from embryos stored for 120 h at 5, 10, 15, or 20°C. Different letters represent significant difference ($F = 0.71$; $P > 0.05$) after Generalized Linear Model ANOVA followed by Tukey multiple comparisons.

after oviposition. Hatching and egg to adult survival was relatively unaffected after storage at this temperature for up to 72 h. After 72 h storage, hatching of the 1 h embryos was still 94% of the control, but egg to adult survival fell to 85% of the control. Hatching of older embryos (18, 24, and 36 h old) was unaffected; however adult emergence was significantly reduced after 72 h of exposure (70; 71 and 70% of the control, respectively).

Storage at 10°C affected the rate of adult survival when the embryos were younger than 12 h and a significant reduction in egg to adult survival was observed when embryos of 1 or 6 h of age were stored for more that 48 h. Consequently, for storing eggs in water at 10°C, the embryos should be at least 12 h old. Following 120 h storage, hatching was still 66% of the control, but the rate of egg to adult survival fell to 50% of the control. A similar pattern was observed for any of the storage times tested for embryos 18, 24, and 36 h old.

Storage at temperatures higher than 15°C had a negative effect on embryo survival probably due to the fact that at these temperatures normal embryo development continues. For embryos stored at 20°C and incubated for 1, 6, and 12 h, this effect was more obvious after 24 h of storage. Results showed that short storage of embryos at 20°C is possible but the embryos should be at least 18 h old. After 72 or 120 h storage, hatching of the embryos incubated for 18 h was still 86 and 59% of the control, respectively, but rate of egg to adult emergence fell to 63 and 10% of the control, respectively.

In general, the most sensitive development stages are the 1s during early embryogenesis, i.e., 1-6 h old eggs. Similar effects were observed by Leopold (2000) in housefly *Musca domestica* (L.), but Arakaki et al. (1984) reported no effect in melon fly *Bactrocera cucurbitae* Coquillett on egg hatch, larval growth, pupal and adult development when 20-h-old embryos were stored in water at 5 and 10°C after 168 h of storage. There is good evidence that chilling intolerance of the very young embryos is related to the formation of the blastoderm (Strong-Gunderson & Leopold 1989; Callaini & Marchini 1989). In recent studies, Stefani et al. (2004) determined that in medfly, 1-2 h after oviposition, the eggs are at stage embryonic stage 2 with 2 nuclei. At 2.5 h the number of nuclei had multiplied to 64 nuclei, and at about 3.5 h embryos reach stage ten and the beginning of the formation of the syncytial blastoderm. At about 8.5 h, more than 256 nuclei can be observed, and cellularization of the blastoderm occurs.

In conclusion, the optimal protocol for storage or long distance shipment of medfly eggs is to collect eggs between 0 to 12 h post oviposition and incubate them for 12 h at 25°C, when these embryos reach the optimal storage age of 12 to 24 h old and can then be transported during a 72 h window when maintained at 10 to 15°C. N1 of the treatments affected male mating competitiveness. Similar results were obtained by Rajamohan et al. (2003) during quality assurance tests carried out to compare cryopreserved medfly eggs. Thus, these results support the viability of routine long distance egg shipment from central egg production centers to male-only satellite production facilities.

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