Sugar Feeding Improves Survival of Nondiapausing Cold-Stored
*Culex pipiens*

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**ABSTRACT**

The continuous culture of mosquitoes is a costly endeavor for vector biology laboratories. In addition to the resources that must be committed to colony maintenance, biological costs, including genetic drift and accidental colony loss, also can occur. Although alternatives do exist, their application to mosquitoes is limited. Mosquito cryopreservation remains elusive, and many important species lack a well-defined diapause. Previously, we demonstrated that cold storing nondiapausing mated adult females of the northern house mosquito, *Culex pipiens* L. resulted in a nearly four-fold increase in longevity when measured at the LT50, allowing for cold storage for up to 10 wk. In the current study, we used sugar feeding during cold storage to significantly improve cold storage longevity. At 6°C, the LT50 of cold stored females was 23 wk, and 100% mortality was not realized until 43 wk. Cold-stored females did exhibit reduced fecundity, but egg production returned to normal levels within two generations. These results suggest that cold storage without diapause induction is a viable option for *Cx. pipiens*, and with the addition of sugar feeding, a colony could be maintained with less than two generations per year.

**KEY WORDS** *Culex pipiens*, northern house mosquito, cold storage, fecundity

The continuous culture of insects is an economically and biologically costly endeavor. The investment of material resources and personnel time always have been a significant issue for entomology labs, an issue that certainly will increase with the steady increase in characterized genomes and the concurrent increase in insect strains. The biological costs of continuous culture can include genetic drift (Mackauer 1972, Chambers 1977), which can lead to such deleterious effects as the loss of fecundity (Franz et al. 1996), and other significant characteristics (Mackauer 1976, Zheng et al. 1993, Goto et al. 2006).

Alternatives to continuous culture have been developed for several insect species. Cryopreservation protocols exist for a handful of dipteran species, including the screwworm, *Cochliomyia hominivorax* (Coquerel) (Leopold and Rinehart 2010); the house fly, *Musca domestica* L.; and several species of tephritid fruit flies (Leopold 2007), but the adaptation of this technique remains elusive for mosquitoes, because of issues with egg impermeability (Valencia et al. 1996a, b). Diapause can be implemented to reduce the incidence of generations in many insect species (Deningier 2008), although this seasonally reoccurring developmental arrest is either lacking or poorly understood in many important mosquito species, especially those of tropical origin. In addition, laboratory strains can lose the ability to diapause either through genetic drift (Goto et al. 2006) or through the development of nondiapausing transgenic lines. Another alternative to continuous culture is the development of cold storage protocols; subjecting insects to temperatures below their developmental threshold, but above freezing and in the absence of diapause. This technique has been proven successful for short-term to intermediate-term storage (days to weeks) for a variety of species (Özder 2008, Coudron et al. 2009, Yocum et al. 2010, Bourdais et al. 2011).

Regardless of the method used, insect cold storage can prove beneficial in several respects: operational costs can be reduced because cold stored insects generally require minimal maintenance, biological costs can be reduced as well, especially those that increase with increasing generations, and a certain degree of insurance is provided against the accidental loss of colonies. Previously we reported on a cold storage protocol for the northern house mosquito, *Culex pipiens* L., by which nondiapausing adults could be stored for up to 10 wk at 6°C, provided they had access to a water source (Rinehart et al. 2010). The current study describes the effect of access to a sugar source during storage, which substantially improves the longevity of nondiapausing adults.

**Materials and Methods**

**Insects.** These studies used the anautogenous Buckeye strain of *Cx. pipiens* (Robich et al. 2007). All life
stages of the colony were reared at 25°C with a photoperiod of 15:9 (L:D) h. Eggs, larvae, and pupae were maintained in shallow pans of water, with the larvae being fed powdered fish food flakes (TetraMin, Inc., Blacksburg, VA). Adults were housed in cubic cages 45 cm to a side, were provided honey and water soaked sponges, and were offered an anesthetized rat as a bloodmeal on a weekly basis. This rearing regime prevented diapause induction, as diapause in this species is triggered by rearing adults at 18°C and a photoperiod of 9:15 (L:D) h (Rinehart et al. 2006).

**Temperature Treatments.** Pupae destined for experiments were hand-picked from larval pans, transferred into cups of water, and placed into a clean adult cage. After a 3-d emergence period, the cups were removed from the cage and the resulting adults were kept for 1 wk at 25°C and a photoperiod of 15:9 (L:D) h with access to honey and water soaked sponges to allow for mating. The resultant 7–10-d-old adult mosquitoes were aspirated from the cage, anesthetized with carbon dioxide, and placed into 475-ml plastic “deli cup” cages cover with nylon window screen. Initial studies involved sets of 15 males or females per cage. To measure the effect of mosquito density on cold storage, subsequent studies involved either 30 or 45 females per cage. In all instances, a water soaked 4- by 6-cm sponge was placed on top of each cage, because this species is unable to absorb atmospheric water (Benoit and Denlinger 2007). In addition, a honey saturated 4- by 6-cm sponge also was placed on top of each cage. Three of these small cages were then placed inside a 25 cm diameter, 10-cm-high plastic container containing cups of saturated sodium chloride to maintain a relative humidity of 75% (Winston and Bates 1960), and were assigned randomly to either Percival Scientific model I30BL (Percival Scientific, Perry, IA) or Conviron model I25L (Conviron, Winnipeg, Manitoba) environmental chambers with a photoperiod of 9:15 (L:D) h and a temperature (hereafter referred to as lethal time 50 or LT50), after which each raft was moved to individual wells of a six-well culture plate. The larvae in each well were counted to determine hatching rates and were transferred to deli cup containers and fed pulverized fish food, with the eventual pupae produced in each cup documented to determine larval survival rates. Adults then were transferred to large cages and the process was repeated to assess the fecundity of the F1 and F2 generations. Fecundity values were compared with those determined for 12 egg rafts removed from a colony cage of females receiving their first bloodmeal.

**Assessment of Fecundity.** In addition to survival, the fecundity of stored females and subsequent generations also was assessed. Females were stored at each of the storage regimes until 50% mortality was reached (hereafter referred to as lethal time 50 or LT50), after which they were acclimated at 25°C for 1 wk with access to honey and water to ameliorate low blood feeding incidence immediately after storage. After acclimation, the mosquitoes were transferred to cubic cages 20 cm to a side for blood feeding and ovipositioning. Egg rafts were removed from the cages, photographed to count the number of eggs per raft by using a Moticam 2000 imaging system (Motic, Inc., Richmond, BC, Canada) attached to an Olympus SZH10 stereomicroscope (Olympus, Center Valley, PA), after which each raft was moved to individual wells of a six-well culture plate. The larva in each well were counted to determine hatching rates and were transferred to deli cup containers and fed pulverized fish food, with the eventual pupae produced in each cup documented to determine larval survival rates. Adults then were transferred to large cages and the process was repeated to assess the fecundity of the F1 and F2 generations. Fecundity values were compared with those determined for 12 egg rafts removed from a colony cage of females receiving their first bloodmeal.

**Statistical Analysis.** Data analysis was conducted using SigmaPlot version 12 (Systat Software, San Jose, CA). The analysis of the number of eggs, proportion hatch, and proportion pupation was carried out by Kruskal–Wallis one-way analysis of variance on ranks followed by Dunn’s multiple comparisons procedure. LT50 (time to 50% mortality) and LT75 (time to 75% mortality) were determined by Kaplan–Meier survival analysis, and significant differences between the curves were determined by the Gehan–Breslow test. Mean LT50 values were separated further by Holm–Sidak all pairwise multiple comparison procedure.

**Results**

**Cold Storage Survival.** Female adult non-diapausing Cx. pipiens demonstrated markedly increased longevity with lower incubation temperatures (Fig. 1b), whereas males exhibited little change in longevity when incubation temperatures were decreased (Fig. 1a). Although there were some differences in the time to 100% mortality in the male treatments (Fig. 1a), calculated LT50 values were identical for all four treatment groups (21 d regardless of temperature) (Fig. 2a). Furthermore, Gehan–Breslow analysis demonstrated that no significant differences were evident among the male survival curves (F3 = 5.648; P = 0.130).

Conversely, female mosquitoes stored at low temperatures survived substantially longer than their counterparts stored at 25°C. Although all individuals stored at 25°C died by 63 d of storage, 100% mortality was not realized until 126 d at 18°C, 203 d at 12°C, and 301 d at 6°C (Fig. 1b). The calculated LT50 values steadily increased with decreasing storage temperature as well, ranging from 28.0 ± 1.8 d at 25°C to 77.0 ± 2.4 d at 18°C, 126.0 ± 3.8 d at 12°C, and 161.0 ± 9.2 d at 6°C, with similar trends evident for calculated LT75 values as well, whereas LT25 values increased at 18°C but were nearly identical at 12 and 6°C (Fig. 2b). The steady increase in the difference between LT25 and LT75 values as storage temperatures decreased (Fig. 2b) can be attributed to the steady decrease in the steepness of the survival curves with decreasing storage temperature (Fig. 1b). Gehan–Breslow analysis confirmed significant variation among the female survival curves (F3 = 458.93; P < 0.001), with Holm–Sidak
post hoc analysis demonstrating significant differences among all treatment groups ($P < 0.05$).

**Effect of Population Density.** Mosquito density significantly affected female storage survival (Fig. 3). At 18°C, mosquitoes at the higher densities (henceforth referred to as 18° – n° for those stored at a density of 30 adults per cage and 18° – n°° for densities of 45 per cage) survived somewhat longer than those stored at 15 adults per cage (hereafter referred to as the 18° – n° treatment) as seen in Fig. 3a, with LT50 values of 77 ± 2.4 d for 18° – n°°, 84.0 ± 1.7 d for 18° – n°°°, and 84.0 ± 1.4 d for 18° – n°°° (Fig. 4a). The LT25 and LT75 values followed a similar trend (Fig. 4a). Gehan–Breslow analysis confirmed significant variation among the 18°C survival curves ($F_2 = 20.888; P < 0.001$), with Holm–Sidak post hoc analysis demonstrating significant differences among all treatment groups ($P < 0.05$). The effects of higher density storage were more pronounced at 6°C (Fig. 3c). At this temperature, the LT50 was 161 ± 9.2 d at 6° – n°°, 98 ± 6.5 d at 6° – n°°°, and 77 ± 2.8 d at 6° – n°°°° (Fig. 4c), showing a 9-wk decrease between the 6° – n°° and 6° – n°°° groups, and a 3-wk decrease between the 6° – n°°° and 6° – n°°°° groups. Gehan–Breslow analysis confirmed significant variation among the 6°C survival curves ($F_2 = 37.856; P < 0.001$), with Holm–Sidak post hoc analysis demonstrating significant differences among all treatment groups ($P < 0.05$).

![Figure 1](image_url)  
Fig. 1. Percent survival (mean ± SE) of sugar fed *Cx. pipiens* adults stored at different temperatures: circles (●) denote storage at 25°C, squares (■) storage at 18°C, triangles (▲) storage at 12°C, and diamonds (♦) storage at 6°C.
Effect on Fecundity. The size of egg rafts produced by cold stored females was affected significantly (one-way analysis of variance [ANOVA] $F_{9/11005} = 75.59, P < 0.001$) by cold storage when assessed at the calculated LT$_{50}$ of each treatment temperature and in subsequent generations (Fig. 5). Although egg raft sizes from females stored until the 18°C LT$_{50}$ (11 wk) were not significantly affected by cold storage, those sizes produced by females stored until the 12°C LT$_{50}$ (18 wk) and the 6°C LT$_{50}$ (23 wk) were significantly smaller than their counterparts reared at 25°C (Dunnett’s test $P < 0.05$). In both the 12 and the 6°C storage treatments, there was more than a 50% reduction in egg raft sizes as compared with those produced by nonstored females. Interestingly, egg raft size in these treatment groups was not fully restored in the F$_1$ generation. The progeny of females stored at 12°C exhibited a 20% decrease in egg production, whereas the progeny of females stored at 6°C showed a 47% decrease as compared with nonstored controls, although this decline was statistically significant for only the 6°C treatment group (Fig. 5). In all instances, egg production returned to normal levels in the F$_2$ generation.

The other measured parameters of fecundity were not affected by cold storage. One-way ANOVA demonstrated no significant differences in proportion of larva hatching ($F_{9/11005} = 12.38; P = 0.193$) nor proportion of successful pupation ($F_{9/11005} = 8.758; P < 0.05$) among any of the experimental groups tested (data not shown).

Discussion

Our results clearly demonstrate that cold storage of nondiapause adult Cx. pipiens can be greatly prolonged by sugar feeding. Storage success was sexually dimorphic, with males showing an LT$_{50}$ of 3 wk, regardless of temperature. This is a slight increase in longevity when compared with cold storage without sugar feeding, where LT$_{50}$ values ranged from less than 1 to a little over 2 wk (Rinehart et al. 2010). In addition, although the survival of sugar fed male mosquitoes did not vary by temperature, our previous
study did show a slight increase in survival with decreasing temperature (Rinehart et al. 2010). The inability of males to be cold stored has potential parallels to diapause induction, which is an exclusively female trait in this species (Spielman 1964). Although these observations have intriguing implications to the underlying cold physiology and diapause mechanisms in this species, they are largely irrelevant to the development of cold storage protocols, because mating occurred before cold storage.

Conversely, cold storage of adult females was much more successful. When comparing LT$_{50}$ values, mosquitoes incubated at 18°C survived 7 wk longer, those incubated at 12°C survived 14 wk longer, and those stored at 6°C survived 19 wk longer than mosquitoes reared at 25°C. Sugar feeding also substantially increased survival as compared with our previous study in which only water was available (Rinehart et al. 2010). Although the LT$_{50}$ of sugar fed mosquitoes maintained at 25°C was a little over a week longer than their nonfed counterparts, sugar feeding elicited a 7-wk increase at 18°C, an 11.5-wk increase at 12°C, and a 13-wk increase at 6°C.

What is the thermal optimum for cold storing Cx. pipiens? The steady increase in longevity with decreasing storage temperature leads us to believe that 6°C is very near the thermal optimum, especially when considering that storage temperatures below the critical thermal minimum (which based on circumstantial evidence is thought to be above 0°C for this species) would shorten longevity by preventing feeding and active water uptake. How do our results compare with the longevity of a diapausing individual? In field conditions, Cx. pipiens adult females usually seek over-wintering locations in late August through early October (Spielman and Wong 1973), and resume normal activity in March or April (Minar and Ryba 1971, Onyeka and Boreham 1987, Ciota et al. 2011). This would suggest a diapause duration of 6–8 mo, although the percent of individuals surviving overwin-
tering remains unclear. Our previous study using unfed mosquitoes resulted in a maximum LT\textsubscript{50} of 2.5 mo, well below what would be expected for a diapausing individual (Rinehart et al. 2010). In contrast, our current study using sugar fed mosquitoes resulted in an LT\textsubscript{50} of almost 6 mo and an LT\textsubscript{75} of 8 mo at 6°C, potentially approaching the survival rates one would expect for diapausing individuals.

Mosquito longevity varied inversely with insect density at the lower storage temperatures. Although the higher densities led to a LT\textsubscript{50} increase of 1 wk at 18°C, doubling the number of adults per cage resulted in a 17% decrease in the LT\textsubscript{50}, and tripling the number of adults per cage resulted in a 28% decrease in the LT\textsubscript{50} at 12°C. The effect was even more striking at 6°C, where doubling the number of individuals per cage resulted in a nearly 40% reduction in the LT\textsubscript{50}, and tripling the number of individuals resulted in more than a 50% reduction in the LT\textsubscript{50}. Hence, the optimal density for storage was 15 insects per cage at 6 and 12°C, with the higher densities proving equally successful at 18°C. The negative effects of adult insect crowding has been well documented (Graves and Mueller 1993, Carey et al. 1995) and is a concern in the development of mass-rearing protocols for mosquitoes (Benedict et al. 2009). Whether the limiting factor for insect density is the volume of the cage, the surface area available for resting sites, or the area of the feeding surface remains to be elucidated. However, even the optimal densities reported here should not be a

Fig. 4. Box and whisker plot of survival of sugar fed Cx. pipiens female adults stored at different temperatures and population densities. The midline represents the LT\textsubscript{50}, outside edges of the box denote the calculated LT\textsubscript{75} and LT\textsubscript{25} values, whereas the whiskers represent the 10th and 90th percentile. Circles signify outliers for each treatment group.
serious impediment to cold storage implementation, and cage engineering to optimize resting site area or feeding surface area may ameliorate the effects of high densities.

Similar to our previous observations, the increased longevity of cold-stored females was concurrent with a reduction in egg production. Although there were no statistically significant effects for those stored at 18°C, females stored at 12 and 6°C until their calculated LT50 showed an ≈50% drop in egg production as compared with their nonstored counterparts. This drop in egg production is similar to what was observed for females stored without access to sugar (Rinehart et al. 2010), although the effect of feeding on fecundity is difficult to determine because this data were acquired at the LT50 in both experiments rather than at a set storage length. How storage temperature affects the natural decay of fecundity with age in mosquitoes (Walter and Hacker 1974, McCann et al. 2009), or how the decline during storage compares to the decline during diapause that has been observed in other Dipteran species (Bourdais et al. 2011) remains to be elucidated.

Interestingly, the decrease in fecundity associated with cold storage persisted in the subsequent generation (although the decrease was significant only in the 6°C treatment) and did not return to normal levels until the F2 generation. This apparently epigenetic effect on insects after stress such as long-term exposure to suboptimal temperatures has been recorded in several other insect species (Leopold 1998, Pitcher et al. 2002, López and Botto 2005, Chen et al. 2008).

What are the practical implications of our results? When rearing Cx. pipiens by continuous culture, a discrete generation protocol produces ≈17 generations per year, or about one generation every 3 wk (Moll et al. 2008). Using the optimal conditions described in this study (6°C, and a population density of 15 mosquitoes per cage), and initiating a new generation at the LT50, a researcher could expect a generation of mosquitoes every 28 wk (1 wk of mating, 23 wks of storage, 1 wk of recovery, and 3 wk to produce the next generation). This would result in ≈1.85 generations per year. Hence, a repository could rear each strain of mosquito at a rate of just two generations per year, allowing for the maintenance of 8.5 times the number of colonies of a facility using a discrete generation protocol or twice the number of colonies of a facility using nonfed cold storage (Rinehart et al. 2010) without substantially increasing workload. An alternative interpretation is that sugar fed cold storage could result in a reduction of the maintenance cost per colony of almost 90% as compared with continuous culture maintenance, and a 45% reduction as compared with cold storage without feeding. The cold storage effects on fecundity could be potentially problematic to the development of cold storage protocols. For instance, if a facility implemented a continuous cold-storage protocol, storing the F1 generation from cold-stored females, and if the epigenetic effects were cumulative, the colony would perish quite quickly.

One possible solution would be discontinuous cold storage to regularly restore fecundity.

Although our results strengthen the case for cold storage of Cx. pipiens, and provide an improved model of mosquito cold storage in general, more research is warranted for high profile mosquito species such as Anopheles gambiae (Giles), for which diapause remains uncharacterized, and species for which mass-rearing protocols are being pursued (Alphey et al. 2010). However, cold storage on nondiapausing insects has been proven successful for a variety of species (Özder 2008, Coudron et al. 2009, Yocum et al. 2010, Bourdais et al. 2011), suggesting that other species of mosquitoes may be successfully cold stored as well.

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References Cited


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