
Short-term storage conditions for transport and farm delivery of the stink bug *Perillus bioculatus* for the biological control of the Colorado potato beetle

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de Ladurantaye, Y., Khelifi, M., Cloutier, C. and Coudron, T. A. 2010. **Short-term storage conditions for transport and farm delivery of the stink bug *Perillus bioculatus* for the biological control of the Colorado potato beetle.** Canadian Biosystems Engineering/Le génie des biosystèmes au Canada. 4.1–4.7. The most common method of controlling the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), is the application of chemical insecticides. Several alternatives to chemical insecticides have been proposed, but are in general not yet applicable for current commercial scale production. One of them is augmentative biological control using a natural insect predator. Considering the need of more sustainable agriculture, the objective of this study was to develop adequate short-term storage conditions for the second instar nymphs of the predaceous pentatomid, *Perillus bioculatus*. This instar has previously been found to be suitable for release to control eggs and early instars of the CPB in the spring on potatoes. Many laboratory tests on *P. bioculatus* second instar nymphs have been carried out to investigate the following storage conditions: air temperature, photoperiod, and period of storage. Results show that storage temperatures within the range of 9 to 15°C and photoperiod (16:8 h L:D and 0:24 h L:D) did not significantly affect the survival and post-storage development of *P. bioculatus* nymphs stored for periods of 2 to 8 d. Compared with shorter periods (i.e., less than 8 days), storage for 10 days significantly reduced the survival rate by about 20%. Development time to the next instar at 20°C following storage was not significantly affected by experimental conditions, although development was slightly faster at 15°C for 10 days of storage. Future studies should investigate the dispersal and predaceous performance of *P. bioculatus* following various combinations of storage conditions. **Keywords:** Storage, temperature, photoperiod, Colorado potato beetle, chemical insecticides, alternative, biological control, *Perillus bioculatus*.

Les insecticides chimiques sont couramment utilisés pour contrôler le doryphore de la pomme de terre, *Leptinotarsa decemlineata* (Say). Plusieurs alternatives à l'utilisation des insecticides chimiques ont été explorées, mais demeurent inapplicables à grande échelle. Parmi ces alternatives, on distingue le contrôle biologique augmentatif utilisant des prédateurs naturels. Dans l'optique d'une agriculture durable, l'objectif de ce travail de recherche était de déterminer les conditions adéquates d'entreposage des nymphes de deuxième stade larvaire du prédateur *Perillus bioculatus*. Il a déjà été démontré que ce stade larvaire en particulier convient bien à la distribution au champ pour contrôler les œufs et les petites larves de doryphore

sur les plants de pomme de terre au printemps. Plusieurs expériences sur des larves de deuxième stade de *Perillus bioculatus* ont été réalisées en laboratoire pour examiner les conditions d'entreposage suivantes: température de l'air, photopériode et durée d'entreposage. Les résultats ont démontré que les températures d'entreposage entre 9°C et 15°C et une photopériode de (16: 8 h L: N et 0:24 h L: N) n'ont pas affecté de façon significative la survie et le développement après l'entreposage des nymphes de *Perillus bioculatus* et ce pour des périodes d'entreposage de deux à huit jours. Comparé à des périodes plus courtes, l'entreposage pendant 10 jours a réduit la survie d'environ 20%. Le temps de développement à 20°C pour atteindre le troisième stade suite à l'entreposage n'a pas été significativement affecté par les conditions expérimentales, bien que le développement ait été légèrement plus rapide à 15°C pendant 10 jours d'entreposage. Comme travaux futurs, il sera intéressant d'examiner la dispersion et la performance de prédation de *Perillus bioculatus* suite à un tel entreposage sous diverses conditions. **Mots clés:** Entreposage, température, photopériode, doryphore de la pomme de terre, insecticides chimiques, alternative, contrôle biologique, *Perillus bioculatus*.

INTRODUCTION

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) is the most important defoliator of potato, *Solanum tuberosum* (L.), in Canada (Hare 1980; Boiteau et al. 1992; Howard et al. 1994), the United-States (Radcliffe et al. 1993), and many countries in Europe and Asia (Jolivet 1991). In North America, attention has been focused for about 25 yr on both the increasing threat of the CPB resistance to chemical insecticides and the development of other alternatives (Boiteau et al. 1987; Duchesne and Boiteau 1995; Cloutier et al. 2002). Chemical insecticides have often been efficient in controlling the CPB on a short-term basis. Since the CPB evolved resistance to DDT in the 1950s, it also acquired resistance to many other chemical insecticides (Forgash 1981; Martel 1987; Boiteau et al. 1987; Whalon et al. 1993). Currently, resistance to imidacloprid, a recently introduced insecticide that is widely used to control this insect pest, has been reported

(Mota-Sanchez et al. 2006; Alyokhin et al. 2007). The over use of chemical insecticides can lead to environmental and health problems for people living in regions where potato is intensively cultivated. For decades, biological control using arthropod predators and parasitoids has been investigated as an alternative to chemical insecticides to control CPB populations. To date, most of the developed alternatives have not been efficient enough or difficult to apply on a commercial scale.

Relatively recent invasive pests, such as the CPB in Canada, often have few coevolved natural enemies and need specific control management. More specific natural predators of CPB eggs and larvae have received particular attention. The two-spotted stink bug, *Perillus bioculatus* (Fabricius) (Hemiptera: Pentatomidae), native to North America, is among the most specialized insect predators of the CPB (Knight 1923, 1952; Saint-Cyr and Cloutier 1996; Hough-Goldstein et al. 1993; Cloutier et al. 2002) with potential to attack eggs, all larval instars, and even adults of the CPB. *Perillus bioculatus* is predaceous on other chrysomelid larvae, caterpillars, and other insect herbivores, but it has most frequently been recorded as a predator of the CPB. The biological control of the CPB using *P. bioculatus* has been efficient in small-scale release trials and in cage studies in Europe (Jermy 1980), Canada (Cloutier et al. 2002), and the United States (Hough-Goldstein and Keil 1991; Biever and Chauvin 1992a, 1992b; Hough-Goldstein and Whalen 1993; Poprawski et al. 1997). In Quebec, Cloutier and Bauduin (1995) and Cloutier and Jean (1998) experimentally used *P. bioculatus* nymphs to control the CPB and observed its predation activity under a range of temperatures. In augmentative releases, a ratio of three *P. bioculatus* second instar nymphs (Fig. 1) to one CPB egg cluster during spring egg laying provided adequate foliage protection. The marked preference of *P. bioculatus* for the CPB may imply a minimal risk of non-target predation (Saint-Cyr and Cloutier 1996). The field studies of Saint-Cyr and Cloutier (1996) confirmed the pre-emptive strategy that



Fig. 1. The two spotted second instar stink bug, *Perillus bioculatus* (Fab.), feeding on a CPB egg.

would consist of releasing *P. bioculatus* nymphs as soon as spring CPB egg mass density can be monitored and used to estimate the potential for damage to potato crops by first generation resident beetles. In this case, it would be possible to save on the treatment cost by a more precise management of the predators.

Compared with eggs and first instars, second instar *P. bioculatus* would be more suitable for mass release because of their immediate predation potential on small CPB instars, especially the egg clusters and newly hatched larvae, and predator dispersal capabilities in the field, which interacts with CPB density through predator hunger and satiation (Lachance and Cloutier 1997; Cloutier 1997). On the other hand, eggs and first instar nymphs are more fragile, but they do not need to feed, and hence are potentially easier to keep alive during storage and pre-release management. However, their release synchronization with early CPB instars would be more difficult because of the offset time before developing their predation ability, which is manifest only after molting to the second instar (Hough-Goldstein et al. 1996). Also, they are possibly more vulnerable to predation and many other potential risks. During our laboratory experimentation, we observed that the hatching rate of eggs from mass reared females is variable, even under laboratory conditions. Thus, more eggs would need to be released to compensate potential loss. Hough-Goldstein et al. (1996) found that eggs released directly on plants survived poorly, whereas those placed in a refuge gave as good control of CPB as second instar nymphs that were also released in a refuge.

For efficient use of predators for biological control in large fields, proper storage and transport techniques are vital from the production/rearing facility until their field distribution. Transportation over long distances and variable temperatures make the storage of suitable predator instars essential until they are released in the field. At cold temperatures, insect metabolism and activity slow down, which is desirable, but, over time, cold stress is potentially harmful, as shown for other insect biological control agents (e.g., Colinet et al. 2006). Cold storage is helpful in directly delaying growth and development of the insect, and also importantly in this particular case in preventing potential cannibalism. De Clercq and Degheele (1993) found that nymphs of two related *Podisus* stinkbugs were less resistant to cold storage than eggs and adults. Nymphs of first to fourth instars hardly survived 5–7 days at temperatures below 10°C although fifth instar nymphs were more tolerant. Their work suggested that *P. bioculatus* second instar nymphs might not survive cold storage or would do so at substantial fitness costs. Knowing the storage limits of second instar nymphs of *P. bioculatus* should help to establish suitable conditions for their transport, storage, and release in commercial potato fields to control the CPB and subsequently develop an appropriate delivery system. The main focus of this research was therefore to examine conditions for *P. bioculatus* second instar nymphs' tolerance to short-term (1–2 weeks) cold storage.

MATERIALS and METHODS

Perillus bioculatus

Perillus bioculatus eggs were collected directly from a colony maintained on an artificial diet for about 20 generations at the Biological Control of Insects Research Laboratory, Columbia, MO, USA. These eggs were mixed with vermiculite in small containers and then shipped by air in a box with newspaper and cold pack to keep them cool. On their arrival at our laboratory (Département de biologie, Université Laval, QC, Canada), the eggs were placed in ventilated plastic Petri dishes (1×5 cm) over layers of damp paper towelling. Each plastic Petri dish was placed in a walk-in growth chamber (Model PGW-36, Conviron, Winnipeg, MB, Canada) at $20 \pm 1^\circ\text{C}$, $65 \pm 10\%$ RH, and a photoperiod of 16:8 h (light:dark) and to prevent desiccation, they were gently sprayed daily with distilled water.

Colorado potato beetle

A CPB colony was also maintained in a greenhouse to serve as food for the predators. Colorado potato beetle eggs laid on potato leaves were picked daily and kept in a refrigerator at 4°C until used and for a maximum of 9 days. The stored eggs served as food for the second instar *P. bioculatus* nymphs under storage conditions, especially during subsequent development to instar three at 20°C (see below).

Experimental design and procedure

A split-split-plot design was used to test three factors: (1) air temperature as the main plot (T) with three levels (9, 12, and $15 \pm 1^\circ\text{C}$), (2) photoperiod in the subplot (Ph) with two levels (16:8 h L:D vs. 0:24 h L:D), and (3) period of storage in the sub-subplot (P) with five levels (2, 4, 6, 8, and 10 days), for a total of 30 factor combinations ($3 \text{ T} \times 2 \text{ Ph} \times 5 \text{ P}$). The basic design consisted of three growth chambers, each held at one of the three experimental temperatures. Photoperiod within temperature was

implemented as two subdivisions in each chamber, where the no light (0 L – 24 D h) treatment was realized by fully shading the insects. The observation units consisted of small cages made of ventilated plastic Petri dishes (1×5 cm), each one containing 5 or 10 nymphs, each unit being randomly assigned among treatment combinations. Since we had a limited number of nymphs, we used two nymph densities during the experimentation: 10 nymphs for the trials at the critical storage temperature of 9°C and 5 nymphs elsewhere.

As found by De Clercq and Degheele (1993), survival of young *Podisus* nymphs at temperatures below 10°C was reduced, and therefore 9°C was chosen as the minimum experimental temperature for *P. bioculatus*. Cloutier and Bauduin (1995), estimated from field observations that 12°C is near the minimum for predation activity, and finally 15°C was also included for modelling purposes. The technique used for shading (keep insects in the dark for the whole storage period in the 0–24 h photoperiod treatments) was to place caged insects underneath two upside down plastic flower pots (Fig. 2a), so that their drip holes were not aligned. In this way, nymphs were not exposed to light, but ventilation was possible to keep the same air conditions inside and outside the pots. An electronic thermistor and a photometer were used to check air temperature and darkness conditions, respectively, inside cages and pots.

Different arrivals of *P. bioculatus* eggs were considered as different blocks for experimental design purposes ($N =$ three blocks). Newly arrived eggs were held in a growth chamber at 20°C and regularly monitored until hatching. Thereafter, nymphs were observed once daily until they molted to the second instar. Newly molted second instars (0–24 h old) were randomly collected and combined in groups of 5 or 10 individuals and placed in the Petri dishes (Fig. 2b). The nymphs were continuously supplied with CPB eggs and water throughout the storage periods. At the end of storage treatment, the number of survivors was recorded and they were transferred to a new growth chamber set at 20°C and monitored daily for survival and

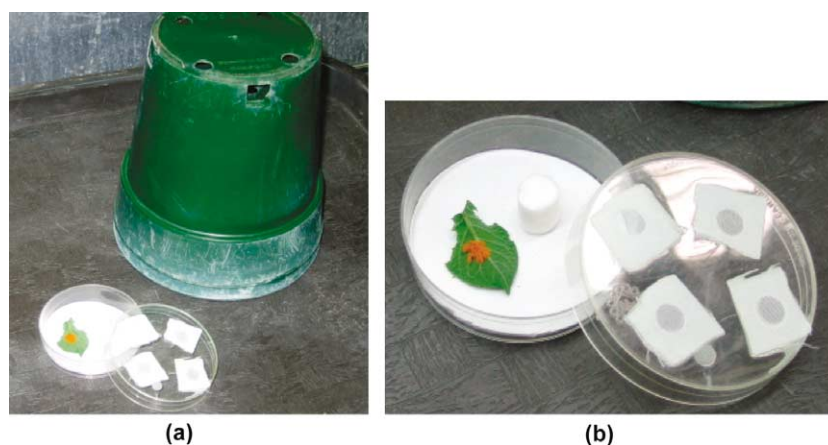


Fig. 2. Experimental setup: (a) technique used to keep caged stinkbug nymphs in continuous darkness in the no light (0–24 h) treatments, (b) newly molted *Perillus bioculatus* second instar nymphs placed in disk-vented plastic Petri dishes with water-soaked dental cotton and CPB eggs on a piece of potato leaf.

development to instar 3, as determined by the presence of exuviae shed at moulting. For post-storage conditions, 20°C was selected as close to optimal for *P. bioculatus* development and predation on CPB (Lachance and Cloutier 1997). The same food/water combination was provided as during the storage period at a rate of one egg cluster per unit.

Statistical analysis

An ANOVA was performed on the data using the Proc Mixed and Proc Glimmix procedures of SAS software (SAS Institute, Inc. 2003). When ANOVA results were significant, *a posteriori* pairwise comparisons were done using protected LSD, with the Bonferroni correction to adjust experimentwise significance values.

The angular transformation [$\arcsin \sqrt{(P/100)}$] was applied to the survival data to improve homogeneity of variances (Steel and Torrie 1980), with number of observed nymphs per Petri dish (5 or 10) being included as a covariate in the ANOVA.

RESULTS and DISCUSSION

Effects of photoperiod on the nymphs survival during storage

Photoperiod had no significant effect on the survival during storage ($F_{1, 6} = 0.28$, $P = 0.6185$) (Table 1) or the survival up to instar 3 ($F_{1, 6} = 0.05$, $P = 0.8367$), and neither was photoperiod involved in any significant interactive effect with temperature or storage period (Table 1). The results suggest that the young nymphs could be safely kept in complete darkness with no photoperiod for a storage period of 10 days or less and at any of the three experimental temperatures, which simplifies the storage process.

Effects of storage temperature and duration on survival

There was no significant effect of temperature ($F_{2, 4} = 1.42$, $P = 0.3423$) on percent survival to the end of storage, survival being significantly affected only by the storage period ($F_{4, 46} = 10.68$, $P < 0.0001$) (Table 1). For the 10 days storage period, a significant number of nymphs had died (i.e., survival rate of $79.03 \pm 0.40\%$) compared with those kept for 8 days or less (Fig. 3). Similarly, there was no significant effect of rearing temperature on survival to instar 3 ($F_{2, 4} = 0.47$, $P = 0.6534$), with storage period being also significant ($F_{4, 48} = 6.85$, $P = 0.0002$). Overall, this suggests that young nymphs could be successfully stored for approximately 1 week at any of the tested temperatures before release.

Effects of storage conditions on post-storage development

After storage, the additional time required to complete the second instar at 20°C was recorded to determine any storage effects on fitness, as measured by the developmental rate of *P. bioculatus* nymphs. ANOVA results show that overall second instar developmental time to the third instar was not significantly affected by photoperiod ($F_{1, 6} = 0.67$, $P = 0.4439$) (Table 2), nor were there any significant interactions among experimental factors

Table 1. ANOVA results for the survival data after storage.

| Source of variation | D.F. | F Values | Pr > F |
|--|------|----------|-------------|
| Block | 2 | 0.99 | 0.4476 NS |
| Temperature | 2 | 1.42 | 0.3423 NS |
| Block × temperature (Error1) | 4 | | |
| Photoperiod | 1 | 0.28 | 0.6185 NS |
| Temperature × photoperiod | 2 | 0.17 | 0.8475 NS |
| Error2 | 6 | | |
| Storage period | 4 | 10.68 | < 0.0001*** |
| Storage period × temperature | 8 | 0.82 | 0.5921 NS |
| Storage period × photoperiod | 4 | 1.09 | 0.3721 NS |
| Storage period × temperature × photoperiod | 8 | 0.86 | 0.5582 NS |
| Error3 | 46 | | |
| Corrected total | 87 | | |

***Significant at $P < 0.05$; NS, not significant.

($P \geq 0.3863$). The highly significant effect of storage period ($F_{2, 4} = 54.64$, $P < 0.0001$) (Table 2), simply reflects the fact that development time, as measured here, includes the period of storage which was experimentally manipulated. The slight marginal effect of temperature ($F_{2, 4} = 5.53$, $P = 0.0705$) (Table 2) suggests that some development did occur under storage at the higher temperatures. Fig. 4 shows that at 15°C average development time was slightly shorter (10.7 ± 0.3 days), compared with 9°C and 12°C (average development times of 12.1 ± 0.3 and $11.9 \pm$

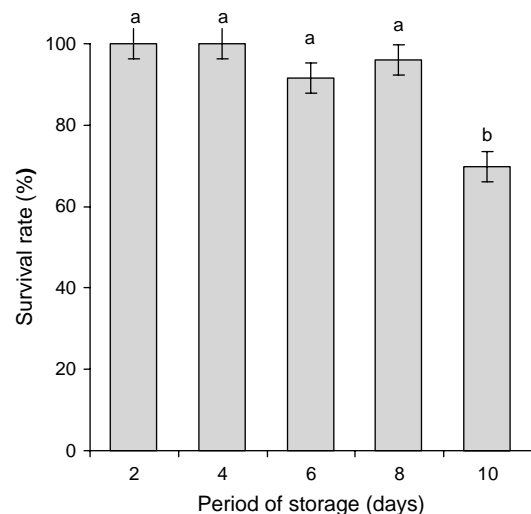


Fig. 3. Survival of second instar *Perillus bioculatus* nymphs for different storage periods. For each period, data were pooled across rearing temperatures. Bars with the same letter are not significantly different in multiple comparisons with the Bonferroni correction.

Table 2. ANOVA results for the development time data.

| Source of variation | D.F. | F Values | Pr > F |
|--|------|----------|-----------|
| Block | 2 | 4.17 | 0.1050 NS |
| Temperature | 2 | 5.53 | 0.0705 NS |
| Block × temperature (Error1) | 4 | | |
| Photoperiod | 1 | 0.67 | 0.4439 NS |
| Temperature × photoperiod | 2 | 0.02 | 0.9791 NS |
| Error2 | 6 | | |
| Storage period | 4 | 54.64 | <.0001*** |
| Storage period × temperature | 8 | 0.72 | 0.6697 NS |
| Storage period × photoperiod | 4 | 1.06 | 0.3863 NS |
| Storage period × temperature × photoperiod | 8 | 0.26 | 0.9740 NS |
| Error3 | 45 | | |
| Corrected total | 403 | | |

***Significant at $P < 0.05$; NS, not significant.

0.3 days, respectively). Overall, the results indicate that some development may occur under 15°C over 10 days of storage, with no obvious detrimental effect of storage conditions on capacity or time to reach the next instar.

The storage limits of the second instar nymphs of *P. bioculatus* were successfully investigated to optimize their storage and transport conditions for an eventual release in commercial potato fields, without affecting their survival. The storage conditions suggested in this study will slow the growth of the nymphs until their release in the field. Cold temperatures, in the range of 9 to 15°C, will slow the predators' metabolism and activity with no apparent harmful stress. The results obtained also show that it is possible to keep the nymphs in darkness making their storage and transport easier by using common delivery boxes and the existing refrigerated chambers once they have been delivered to the farm. More important, organic farmers will be able to order the required number of nymph predators in advance from any insect rearing/distributing facility and keep them for up to at least 1 week to coincide with the targeted CPB instars or until adequate weather conditions allow their release in the field.

CONCLUSIONS

Based on the results of our experiments, short-term storage conditions of *P. bioculatus* second instar nymphs can be optimised by considering these facts:

- Storage temperatures in the range of 9 to 15°C did not significantly affect the survival of second instar nymphs kept for a maximum of 8 days under storage conditions. For 10 days of storage, which was the longest period tested here, nymphal survival was reduced by about

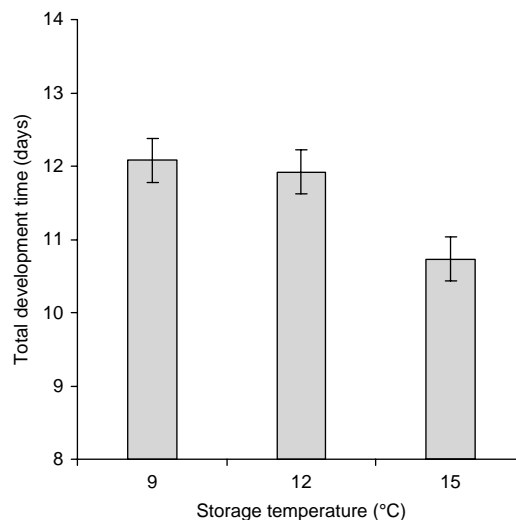


Fig. 4. Effects of the three temperatures tested (9, 12, and 15°C) on the development time of the second instar nymphs to instar 3 (development time data include the storage period).

20% and thus the performance of survivors might be expected to decline.

- Storage temperatures in the range of 9 to 15°C did not significantly affect total time to the next instar, which included post-storage at 20°C. But for storage at 15°C, time to the next instar was on average 1–1.5 days shorter than at 9 or 12°C, suggesting some development during storage. This must be considered for biocontrol purposes, especially when predator-prey synchronization is important.

- For storage periods of 10 days or less in complete darkness, the lack of a photoperiod did not significantly affect survival or development time to the next instar.

Future studies investigating the predaceous performance of *P. bioculatus* (i.e., dispersal and predation rate) following storage should be conducted to provide additional insight into potential effects of storage and aid in designing parameters that will assist in maximizing the use of this beneficial predator in controlling CPB.

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