Multi-year survival of sugarbeet root maggot (*Tetanops myopaeformis*) larvae in cold storage

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Abstract

To test the hypothesis that long-term survival of sugarbeet root maggot in storage is facilitated by larvae undergoing prolonged diapause, respiration and gene expression patterns of field-collected diapausing larvae were compared with those of 1-, 2-, and 5-year laboratory-stored larvae. Additional assessments were made on post-storage survival, emergence, and reproductive fitness of stored larvae. Respirometry, carried out at 5 and 20 °C revealed no differences among respiration rates of initially diapausing and long-term stored larvae. A 15 °C increase in temperature elevated respiration in both diapausing and stored larvae, with levels of CO\textsubscript{2} release ranging between 8- and 14-fold higher at 20 °C than at 5 °C. Similarly, 6–10-fold increases in O\textsubscript{2} consumption levels were observed at the higher temperature. A transcript with sequence similarity to the fat body protein 2 (*Fbp2*) gene was highly expressed in diapausing larvae, and trace levels were expressed in some samples of 1-year stored larvae. However, no expression was detected in 2- and 5-year stored larvae. Survival and emergence studies of stored larvae revealed mixed populations of diapausing (i.e., the 5–17% of larvae that did not pupate) and post-diapausing (62–84% of larvae pupated) insects, with a high incidence of pupation (62%) and emergence (47%), even after 4 years in cold storage. Therefore, extended survival of *Tetanops myopaeformis* larvae in long-term cold storage is facilitated by two mechanisms, with a majority of larvae in post-diapause quiescence and a smaller fraction in a state of prolonged diapause.

Keywords: Diapause; Post-diapause quiescence; Respirometry; Sugarbeet root maggot; Fat body protein 2; Long-term storage survival

1. Introduction

Insects are able to overcome the challenges of changing seasons and associated cyclic fluctuations in environmental conditions by undergoing a genetically programmed, dynamic state of low metabolic activity known as diapause (Tauber et al., 1986). Insects also face unpredictable growing seasons that vary in length and suitability for growth and development (Hanski, 1988). Diapause can also serve as a potential adaptation to this type of seasonal variability in the form of extended diapause, which is synonymously referred to as superdiapause (Ushatinskaya, 1984), prolonged diapause (Tauber et al., 1986), and extra-long diapause (Hanski, 1988). The adaptive biological phenomenon underlying all of these terms is that a small percent of a population can remain in diapause more than 1 year to survive year-to-year variability; however, the mode of manifestation and conditions for its induction and termination are not fully understood. Studies by Soula and Menu (2005) revealed that the extended life cycle of the chestnut weevil, *Curculio elephas* Gyllenhal, was the result of prolonged diapause occurring secondarily to a developmental phase. This was in contrast to the usual hypothesis of extended, uninterrupted normal winter diapause previously described in the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Ushatinskaya, 1984). An additional form of dormancy, referred to as post-diapause quiescence, involves insects remaining in a dormant state beyond the typical duration of normal diapause due to the absence of favorable environmental conditions. Although no morphological differences are known to exist between diapause and post-diapause quiescent insects, there lies a fine line of difference in that the former are regulated...
endogenously while the latter are controlled exogenously (Tauber et al., 1986; Danks, 1987; Hodek, 1996, 2002; Kostal, 2006).

The sugarbeet root maggot, *Tetanops myopaeformis* (Röder) (Diptera: Ulidiidae), is a major economic insect pest of sugarbeet in the Red River Valley of Minnesota and North Dakota, and it is also a problem for growers in the western United States. Adult flies begin emerging during May and June, and females lay most eggs next to sugarbeet seedlings in June. Eggs hatch within 5–7 days, and larvae promptly begin feeding on roots of sugarbeet seedlings. Feeding continues as larvae develop through three instars. Larvae cease feeding after reaching full size (i.e., late July into September), and initiate diapause that lasts at least 6 months. Larvae overwinter in the soil at depths of 5–35 cm, and move to within 10 cm of the soil surface for pupation, in late March or early April (Callenbach et al., 1957; Harper, 1962; Whitfield, 1984; Anderson, 1986; Bechinski et al., 1989, 1990). Studies conducted by Whitfield (1984) and Whitfield and Grace (1985) indicated that sugarbeet root maggots are freeze tolerant and univoltine. Observations by previous investigators suggest that *T. myopaeformis* undergoes an obligate diapause that requires chilling up to 6 months for termination (Callenbach et al., 1957; Harper, 1962; Klostermeyer, 1973).

The ability of sugarbeet root maggots to survive prolonged (e.g., 51 months) periods in cold storage (5 °C) has been reported earlier by Kruger (1986), while the current data shows that the third-instar larvae collected in the months of August and September can be successfully maintained alive in cold storage (6 ± 1 °C) for up to 6 years (M.A.B., unpublished data). Based on the time of collection, it has been suggested by previous authors that those larvae are in diapause before entering the storage (Callenbach et al., 1957; Harper, 1962; Klostermeyer, 1973; Anderson, 1986); however, it was not known whether the larvae were maintaining the same physiological state throughout long-term storage. Besides, there were no previous reports on incidence of prolonged diapause in sugarbeet root maggot populations, either in the laboratory or in field conditions. Prolonged diapause, resulting from either a simple extension of normal winter diapause or larvae re-entering a second diapause following the winter diapause (Soula and Menu, 2005), could be allowing this phenomenon to occur. Alternatively, the larvae could be in a state of post-diapause quiescence (Tauber et al., 1986; Hodek, 1996) and waiting for the return of favorable temperatures.

The collective characteristics of diapausing individuals, often referred to as diapause syndrome, include several complex physiological and behavioral traits such as uniquely low metabolic activity associated with reduced respiratory rate, relative inability to respond to environmental cues, increased resistance to environmental extremes, and altered or reduced behavioral activity (Tauber et al., 1986; Danks, 1987). In addition, diapausing individuals exhibit specific gene expression patterns compared to those that are non-diapausing (Denlinger, 2002). Transition from diapause to post-diapause development, marked by a sudden shift in the expression pattern of diapause-regulated genes, was observed in the flesh fly, *Sarcophaga crassipalpis* Macquart when studied under laboratory conditions at 20 °C (Yocum et al., 1998; Rinehart and Denlinger, 2000; Rinehart et al., 2000, 2001). Recent findings demonstrate that the thermal history of an insect has a major influence on the gene expression pattern during its transition between diapause and post-diapause development (Hayward et al., 2005; Yocum et al., 2005, 2006).

A better understanding of the physiological status and mechanisms that enable prolonged survival of *T. myopaeformis* larvae in storage could provide insight regarding the phenology and field physiology of sugarbeet root maggot. In this study, we test the hypothesis that multi-year survival in *T. myopaeformis* larvae was facilitated by larvae maintaining a state of diapause throughout long-term (i.e., 1, 2, 3, 4, and 5 years) periods of cold storage.

2. Materials and methods

2.1. Insect sampling

Experiments were carried out on different life stages (i.e., second instars, diapausing third instars, post-diapause third instars, pupae, and adults) of *T. myopaeformis* collected from the field during the year of experimentation and on matured third instars that had been collected and maintained in laboratory storage, at 6 ± 1 °C for 1, 2, 3, 4, or 5 years. Larvae for emergence studies were collected from multiple fields in different years (1973–1985) near St. Thomas, North Dakota, during July through September. All insects used for respiration and gene expression studies were collected from fields located in Pembina County, North Dakota. Different developmental stages used for respiration studies were collected during the field season of 2004, whereas the 1-, 2-, and 5-year stored larvae used in respiration and gene expression studies were collected during August in 2003, 2002, and 1999, respectively. Larvae and pupae were dug from the soil, placed into plastic bags of loose field soil. The bags were transferred to a plastic cooler and transported to the laboratory where they were washed and transferred into clean plastic storage bags filled with moist silica sand. Insects in bags were then stored in an incubator at 6 ± 1 °C. Larvae that had been cold-stored were either in the pre-diapause phase (July collected) at the time of collection and had entered diapause soon after collection, or were already in diapause (August/September collected).

Adult flies for the experiments were collected from wooden utility poles during high periods of activity and placed in jars with a cotton plug soaked with honey and water. These experiments were carried out to develop a better understanding of various physiological phenomena
associated with diapause and long-term storage. Three independent experiments involving respirometry, differential gene expression evaluations, and assessments of post-storage survival, emergence, and reproductive performance were conducted on *T. myopaeformis* larvae. Metabolic rates of all post-egg stages were evaluated at 20 °C. Additional studies were done at 5 and 20 °C to compare the field-collected overwintering larvae with those that had been stored for extended periods in the laboratory. Larval responses to temperature increases were assessed to determine if prolonged diapause could explain the ability of larvae to survive long-term laboratory cold storage.

2.2. Respirometry

An automatic respirometer (Sable Systems, Henderson, NV, USA), programmed to operate at a flow rate of 100 ml/min, was used for measuring oxygen consumption and carbon dioxide production of test insects. Measurements were taken employing constant-volume respirometry (Lighton, 1991). Insects were acclimatized to the respective temperatures for 2–3 h before measurements, and both oxygen and carbon dioxide concentrations were measured on five to seven individual insects of each selected stage at each temperature. Measurement intervals ranged from 1 to 3 h for non-diapausing stages and 3 h to 3 days for diapausing and stored larvae. In a comparative study between field-collected diapausing and laboratory-stored larvae, respirometry was carried out at two different temperatures. First, the readings were taken at 5 °C, and later the same larvae were subsequently maintained at 20 °C to measure responses to an increase in temperature. Data were collected using the Sable Systems Data Acquisition Program (http://www.sablesys.com/datacan.html) in accordance with manufacturer’s protocol. Each insect was weighed to the nearest milligram after each respirometry session. Oxygen consumption and carbon dioxide production levels were calculated for each insect based on their weights and enclosure time and expressed as ml/g/h.

2.3. Survival rate, emergence, and potential fecundity of larvae subjected to long-term cold storage

Data from a study conducted between 1973 and 1988 is being introduced herein to provide a more complete characterization of the physiological status of these insects when subjected to long-term cold storage, and to explore the biological potentiality of *T. myopaeformis* larvae to survive and reproduce after being subjected to several years of cold (6 ± 1 °C) storage. Larvae used in this study were collected from sugarbeet fields during the months of July, August, and September, and were subjected to the same handling procedures as described above. The larvae intended for storage periods of 1, 2, 3, or 4 years were divided into 3–34 batches, based on locality of collection. The number of larvae in each batch ranged from 49 to 1215. During the months of May and June, the larvae were taken out of the incubator and maintained at 12L:12D, 25 °C, and 75–80% relative humidity (first sampling) to assess percent pupation, percent emergence, sex ratio, fecundity, and percent egg hatch. Observations were continued for 90 days to quantify percent pupation. Larvae that did not pupate (NP) but remained alive were returned to the incubator for storage and observations were repeated the following year (second sampling).

2.4. Differential gene expression

2.4.1. RNA extraction and differential display

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used according to manufacturer’s protocol to extract total RNA from field-collected diapausing (November 2004) larvae, and those stored in the laboratory for 1, 2, and 5 years. Differential display was carried out according to the manufacturer’s protocol by using GeneFishing™ DEG Premix kits (Seegene, Rockville, MD, USA). Fragments were cloned and sequenced, and primers were designed to synthesize probes using a DIG labeling kit (Roche Applied Science, Indianapolis, IN, USA). The BlastX program (Altschul et al., 1997) was used to search the GenBank sequence repository and establish sequence identity. The nucleotide sequence for clone D20A2b was deposited in GenBank and assigned accession number EV413920.

2.4.2. Northern blot analysis

Concentrations of RNA samples were determined using a spectrophotometer, and ethidium bromide was added to all samples to ensure equivalent loading. Total RNA (5 μg/sample) was separated on a 1% agarose denaturing gel (0.41 M formaldehyde, 1X MOPS-EDTA-sodium acetate). At the end of each gel run, the gel was photographed to compare rRNA intensity. RNA was subsequently transferred overnight to a positively charged nylon membrane (Roche, Indianapolis) with downward capillary action using a 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) transfer buffer (Schleicher and Schuell, Keene, NH, USA). The blots were air-dried, UV cross-linked at 12,000 μJ/cm², and stored at −20 °C. Blots were prehybridized and hybridized using the DIG Easy Hybridization Buffer™ (Roche, Indianapolis), and screened with probes at a concentration of 20 ng/ml of hybridization buffer. The digoxigenin-labeled probes were detected using the DIG High Prime DNA Labeling and Detection Start Kit II (Roche, Indianapolis).

2.5. Statistical analysis

Respiration and days-to-pupation data were analyzed by using one-way analysis of variance (ANOVA) (SAS Institute, 2006), and mean comparisons were carried out with Tukey’s multirange test. Results were expressed as
mean and mean + S.E.M. (standard error of mean), and considered significantly different at $P < 0.05$. Log transformation was performed on the respirometry data from different life stages of *T. myopaeformis* to normalize the distribution of data and to reduce the variation.

3. Results

3.1. Respiration during developmental transition

At 20 °C, a trend of declining respiration was observed as the larvae entered into diapause and a significant increase was seen during the adult stage (Fig. 1). Early second instars had the highest rate of respiration, making it the most metabolically active developmental stage, while the third-instar larvae collected in December had the lowest larval respiration rate, indicating that they were in a deep state of diapause. The phase of diapause initiation in August-collected third instars was marked by a significant decrease in the oxygen consumption rate from that of late second instars. The transition was signaled by a 45–50% decrease in the total respiration rate of August-collected third instars ($O_2 = 0.287 \text{ml/g/h}; \ CO_2 = 0.289 \text{ml/g/h}$) from that of late second instars ($O_2 = 0.578 \text{ml/g/h}; \ CO_2 = 0.52 \text{ml/g/h}$). Respiration rates of diapausing third-instar larvae remained fairly consistent with the progression of diapause from August to November and then decreased significantly in December. The difference between respiration rates of diapausing (December third instars) and post-diapausing larvae was insignificant ($O_2 = 0.133–0.183 \text{ml/g/h}; \ CO_2 = 0.126–0.186 \text{ml/g/h}$). Pupa had the lowest ($O_2 = 0.104 \text{ml/g/h}; \ CO_2 = 0.103 \text{ml/g/h}$) respiration rate among all stages, and respiration was significantly greater in adults.

3.2. Differences between laboratory-stored and field-collected diapausing larvae

3.2.1. Respiration at 5 °C

At 5 °C, a trend towards decreased respiration was observed between field-collected November and December diapausing larvae. A similar trend was also observed from 1 to 5 years in cold-stored larvae (Fig. 2). Oxygen use and carbon dioxide production levels indicated a significant reduction ($O_2$ from 0.04 to 0.016 ml/g/h; $CO_2$ from 0.032 to 0.013 ml/g/h) in respiration rate from 1 to 2 years of storage; however, no significant decrease ($O_2$ from 0.016 to 0.01 ml/g/h; $CO_2$ from 0.013 to 0.006 ml/g/h) in respiration was observed between larvae held for 2 and 5 years of cold storage. In comparison, the oxygen consumption levels of 1-year stored larvae were similar to those of diapausing (November and December) larvae, but carbon dioxide production levels of 1-year stored larvae were only similar to November diapausing larvae. Respiration rates of 2- and 5-year stored larvae resembled those of December diapausing larvae with respect to both oxygen and carbon dioxide levels. Oxygen consumption exceeded carbon dioxide production in both groups of insects.

3.2.2. Respiration at 20 °C

Increasing the ambient temperature by 15 °C resulted in a sharp increase in carbon dioxide production that ranged from eight-fold (i.e., 0.037–0.31 ml/g/h) in November-collected diapausing larvae to 14-fold (i.e., 0.01–0.15 ml/g/h) in December-collected diapausing larvae. A 6–10-fold rise in oxygen consumption was observed in both diapausing and stored larvae (Figs. 2 and 3). Respiration patterns at 20 °C clearly distinguished two groups: (1) 1-year stored and November diapausing larvae with high respiration; and (2) 2- and 5-year stored and December diapausing larvae.
larvae with low respiration. The higher temperature tended to increase carbon dioxide production rates of both diapausing and stored larvae to similar concentrations as those of oxygen consumption rates.

3.3. Survival rate, emergence, and potential fecundity of larvae following long-term storage

3.3.1. First sampling

Long-term storage had little effect on pupation. The percentage of larvae that pupated was surprisingly high (i.e., ranging from 74% to 84% in 1-, 2-, and 3-year stored larvae), and only dropped to 62% after 4 years of storage (Table 1). When larvae were taken out of storage and maintained at 25°C, 75% pupation occurred within 11–14 days in 1- and 2-year stored larvae while it took place within 8 days in 3-year stored larvae (Fig. 4). It took an additional week for 1- and 2-year stored larvae to achieve 90% pupation, and only two additional days for the 3-year stored larvae to reach 90%. In order to complete the remaining 10% of the total pupation, it took an average of 4–5 weeks for the 1- and 2-year stored larvae, while it occurred within 2 weeks for the 3-year stored larvae. An overall summation indicates that the average number of

![Fig. 3. Differences in oxygen consumption and carbon dioxide production (mean ± S.E.M.) at 20°C between diapausing *T. myopaeformis* larvae of November (D-1 Nov), diapausing larvae of December (D-2 Dec) and larvae stored in the laboratory for 1, 2, or 5 years. Bars sharing a letter are not significantly different (Tukey’s test). One-way ANOVA (*P* < 0.0001). N = 6 (D-1 Nov, D-2 Dec, 2 and 5 years); N = 7 (1 year).](image)

![Fig. 4. Number of days (mean ± S.E.M.) taken for pupation (75%, 90%, and 100%) in *T. myopaeformis* larvae stored in the laboratory for 1, 2, or 3 years. Bars with same letters are not significantly different (Tukey’s test). One-way ANOVA (*P* < 0.0001). Number of batches with size ranging from 49 to 1215 larvae per batch in parentheses.](image)

Table 1

Survival rate, emergence, and potential fecundity of *T. myopaeformis* larvae following prolonged long-term storage

<table>
<thead>
<tr>
<th>First sampling (mean ± s.e.m)</th>
<th>Years in storage</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Pupated (%)</td>
<td>84 ± 2 (31)</td>
</tr>
<tr>
<td>Emerged as adults (%)</td>
<td>81 ± 2 (31)</td>
</tr>
<tr>
<td>Males (%)</td>
<td>52 ± 1 (31)</td>
</tr>
<tr>
<td>Females (%)</td>
<td>48 ± 1 (31)</td>
</tr>
<tr>
<td>Eggs per female</td>
<td>53 ± 11 (4)</td>
</tr>
<tr>
<td>% Hatch</td>
<td>76 ± 1 (2)</td>
</tr>
<tr>
<td>NP (non-pupated) (%)</td>
<td>11 ± 1 (31)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Second sampling (mean ± s.e.m)</th>
<th>(n = 2)</th>
<th>(n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pupated (%)</td>
<td>67 ± 14</td>
<td>58 ± 4</td>
</tr>
<tr>
<td>Emerged as adults (%)</td>
<td>51 ± 4</td>
<td>75 ± 13</td>
</tr>
<tr>
<td>Males (%)</td>
<td>78 ± 22</td>
<td>56 ± 3</td>
</tr>
<tr>
<td>Females (%)</td>
<td>22°</td>
<td>44 ± 3</td>
</tr>
<tr>
<td>Eggs per female</td>
<td>12°</td>
<td>43 ± 10</td>
</tr>
<tr>
<td>NP (non-pupated) (%)</td>
<td>19 ± 0</td>
<td>24 ± 6</td>
</tr>
</tbody>
</table>

*n* (parenthesis in first sampling) — number of larval batches with size ranging from 49 to 1215 larvae/batch (first sampling) and 16 to 152 larvae/batch (second sampling).

*Females absent in one batch.*
days taken for the 3-year-old larvae to pupate was significantly lower (24 days) than 1- and 2-year (55 and 51 days) stored larvae (Fig. 4). The percentage of larvae that did not pupate (NP) and remained alive after 3 months of exposure to higher temperatures and moisture conditions ranged from 11% to 17% in 1-, 2-, and 3-year stored larvae, while only 5% of 4-year stored larvae failed to pupate (Table 1). The percentage of adult emergence declined gradually from 1 to 4 years of storage with male survival being greater than that of females. Although the rate of adult emergence declined with increasing time in storage, fecundity and percent hatch remained high for insects held in cold storage for up to 4 years (Table 1).

3.3.2. Second sampling

Data for the second sampling was available only for batches originating from 1- and 2-year stored larvae (Table 1), with fewer batches and very few larvae per batch. In the second sampling, pupation rates of the 1- and 2-year (NP) stored larvae were 67% and 58%, respectively, and of those pupated, 51% and 75% emerged as adults. A substantial drop in fecundity was observed in the second sampling of 1- and 2-year (NP) stored larvae compared to the first sampling. Interestingly, 19–24% of the larvae remained alive but did not pupate after the second sampling.

3.4. Differential gene expression

Differential display revealed approximately 30 gene fragments showing differential expression in diapausing and laboratory-stored larvae. However, northern blot analysis confirmed differential expression of only a single gene, D20A2b. A BlastX search of the GenBank sequence repository found Drosophila spp. fat body protein 2 gene (Fbp2) as the best match in sequence similarity to D20A2b. Expression of Fbp2 was detected in diapausing larvae, but not in 1-, 2-, or 5-year stored larvae (Fig. 5a).

3.4.1. Effect of temperature on regulation of Fbp2

To determine the effect of temperature on expression of Fbp2 in stored larvae, 1-year cold-stored (6 ± 1°C) larvae were exposed to 20°C for 0, 24, 48, or 72 h. Compared to diapausing control larvae, very low levels of Fbp2 expression were detected at 0 h and no signal was observed at 24, 48, or 72 h (Fig. 5b).

4. Discussion

Diapause is a dynamic event consisting of several successive phases rather than a single static state (Tauber et al., 1986; Danks, 1987; Hodek, 1996; Denlinger, 2002). Entry into diapause by insects in temperate climates is characterized by metabolic suppression, whereas termination of diapause involves an elevation in metabolic activity (Tauber et al., 1986; Danks, 1987; Kemp et al., 2004). In our study, diapause initiation was represented by a precipitous fall in the respiration rate of non-feeding third-instar larvae collected in August from that of late second instars. The trend of respiration levels being consistently lower from August to November indicates a progression into diapause. This supports the previous reports of sugarbeet root maggot beginning diapause in August and remaining in diapause for 6 months (Callenbach et al., 1957; Harper, 1962; Klostermeyer, 1973). Surprisingly, the phase of diapause termination was difficult to mark because there was no significant increase in respiration rate of post-diapause larvae from that of diapausing larvae in December. This could be due to larvae passing the actual phase of diapause termination and approaching the phase of metamorphosing into pupae, which is supported by the fact that most of the larvae in our collection pupated immediately after the recordings. Lowered metabolic activity during metamorphosis is common in endopterygote insects. Lowered oxygen consumption during larval-pupal ecdysis has been reported in wax mot Galleria mellonella (L.) (Sehnal and Slama, 1966). Oxygen consumption during the period of metamorphosis from larva to adult through a pupal stage was shown to follow a U-shaped curve in non-diapausing Sarcophaga argyrostoma (Robineau–Desvodi) at 25°C (Denlinger et al., 1972). Reduced metabolic activity could also account for the low levels of respiration observed in sugarbeet root maggot pupae in the present study. Significant respiration increases were only observed in adults (Fig. 1).

Respirometry, usually carried out measuring only oxygen consumption, has been used in the past as a reliable tool for distinguishing diapausing from non-diapausing insects because insect respiration rates are low during diapause and, with some exceptions, are relatively temperature-independent. At 5 and 20°C, the respiration rates of laboratory-stored larvae in this study resembled those of
field-collected diapausing larvae. Two groups were observed: (1) larvae stored for 1 year resembled the November-collected diapausing larvae; and (2) larvae stored for 2 and 5 years had similar respiration levels to those of diapausing larvae collected from the field in December (Figs. 2 and 3). This indicates that long-term cold-stored larvae are maintaining a low metabolic status that is comparable to that of diapausing larvae. A high level of respiration could be detrimental to the survival and development of stored larvae through extended periods at 5 °C, especially since energy sources are limited. Moreover, the metabolic similarity of stored larvae to diapausing larvae can be seen in their response to an increase in temperature. A 15° rise in temperature elevated the overall respiration rate in both groups (Figs. 2 and 3). Increases in respiration with increasing temperatures have been reported in diapausing pupae of *Pieris brassicae* (L.), with respiration increasing exponentially between 5 and 20 °C (Fourche, 1977). Similarly, a 1.5-fold increase in oxygen consumption was observed in diapausing larvae of the burnet moth, *Zygaena trifolii* Esper, following a temperature increase from 5 to 20 °C (Wipking et al., 1995). In the current study, increasing the temperature led to a 6–10-fold increase in oxygen consumption. Interestingly, carbon dioxide production rose by 8–14-fold (Figs. 2 and 3).

Recent advances in gene expression studies emphasize that diapause can indeed be characterized by upregulation of certain genes, in addition to a widespread gene shutdown (Denlinger, 2002). Differential display comparing diapausing *T. myopaeformis* larvae with those maintained in cold storage for 1, 2, and 5 years clearly demonstrated that stored larvae differ from diapausing larvae at the molecular level, with a transcript similar to *Fbp2* being highly upregulated in diapausing larvae and not being expressed at detectable levels in long-term stored larvae (Fig. 5a), except for traces of expression in some samples of 1-year stored larvae. In *Drosophila*, *Fbp2* is an ancient duplication of the alcohol dehydrogenase gene (*Adh*), which encodes a protein that differs substantially from ADH in its methionine content. FBP2 is functionally homologous to ADH, but differs in amino acid content by being high (20%) in methionine (Meghlouei and Veuille, 1997). Association of *Adh* with dormancy was established in nematodes where the *Adh* (dod-11) gene, was found upregulated in dauer larvae of *Caenorhabditis elegans* (Murphy et al., 2003). The fact that expression levels of a transcript for *Fbp2* (D20A2b) were high in diapausing larvae and low in only some stored larvae (1 year–0 h) suggests that a majority of the stored larvae are not in diapause. In addition, the absence of *Fbp2* expression upon exposure to a higher temperature in 1-year stored larvae (Fig. 5b) suggests that *Fbp2* is tightly diapause-regulated, but not affected by environmental factors such as temperature. This contrasts with the expression patterns observed with *purH* (Yocum, 2004), as well as *hsp23* and *hsp70* (Yocum et al., 2005, 2006; Hayward et al., 2005).

The post-storage performance data of stored larvae (Fig. 4) also offers reasonable support to the explanation that a majority of the long-term stored larvae are not in diapause. In this study, 90% of the larvae pupated within 20 days of exposure to higher temperature and relative humidity. The speed at which resumption of development occurred in stored larvae supports the contention that larvae have completed the refractory period of diapause and are maintaining a state of post-diapause quiescence in which development can be initiated with a sufficient rise in temperature (Lees, 1956; Tauber et al., 1986; Danks, 1987; Hodek, 1996, 2002). Respiration levels and gene expression pattern also indicate that the majority of long-term stored larvae are likely in a state of post-diapause quiescence. Post-diapausing insects in quiescence are known to have the advantage of retaining all the benefits of diapause (e.g., cold-hardiness, suppressed metabolism) without having to maintain diapause (Tauber et al., 1986).

Despite the fact that there was a high level of pupation (62–84%) in all groups of stored larvae following exposure to higher temperatures, a fraction (5–17%) of them exhibited a dissimilar response by remaining as dormant, non-pupated larvae (Table 1). The above results clearly show that two different groups of insects were represented within the stored larvae surveyed. The pupated individuals, which comprised the majority, represented post-diapause quiescent larvae, whereas the minor group of larvae that did not pupate remained in prolonged diapause. Although there are no previous reports on the incidence of prolonged diapause in *T. myopaeformis*, data from the second sampling in our study (Table 1) clearly support the occurrence of this phenomenon in this species. Among the non-pupated 1- and 2-year stored larvae subjected to second sampling, 67% and 58%, respectively, pupated in the following year (second sampling), while 19% and 24%, respectively, remained in a dormant state. Similar patterns of within-population variation in overwintering duration have been seen in Colorado potato beetles *L. decemlineata* (Tauber and Tauber, 2002), the yucca moth, *Prodoxus y-inversus* Riley (Powell, 1989, 2001), and *C. elephas* (Soula and Menu, 2005), where some individuals within a population maintained diapause for extended periods. The adaptive value of these long cycles has been suggested as contributing to synchronized mass emergences (Powell, 1989, 2001) and as a “bet-hedging” strategy to decrease the risk of population decimation by unpredictable catastrophic events (Menu et al., 2000).

In conclusion, the extended survival of *T. myopaeformis* in long-term cold storage is facilitated by two mechanisms, diapause and post-diapause quiescence. We arrive at this conclusion because all groups of stored *T. myopaeformis* included mixed populations of prolonged diapause and post-diapause quiescent larvae, with the majority being in the quiescent state. The fact that a fraction of the population remained in diapause reveals that the sugarbeet root maggot has an inherent ability to undergo prolonged diapause; however, quantifying the frequency or dynamics
of its occurrence in field populations will require further investigation.

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References


