

The effect of diapause, cold acclimation and ice-nucleating bacteria on the cold-hardiness of *Plodia interpunctella*

Fields, P.G.*^{#1}, Timlick, B.²

¹ Agriculture and Agri-Food Canada, Cereal Research Centre, 195 Dafoe Rd., Winnipeg Manitoba R3T 2M9, Canada. Email: paul.fields@agr.gc.ca

² Canadian Grain Commission, 844 – 303 Main St., Winnipeg, Manitoba R3C 3G8, Canada.

* Corresponding author

Presenting author

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Abstract

Laboratory tests showed that over 50 d at 0°C, over 10 d at -5°C or 1 d at -10°C were required to control non cold-acclimated 5th instar larvae of *Plodia interpunctella* (Lepidoptera: Prylidae) (Indian meal moth). To control the most cold-hardy stage, diapausing cold-acclimated 5th instar larvae over 14 d at -10°C or 1 d at -15°C were required. A freeze-out of a single floor of a seed warehouse was carried out from 23 December 1993 until 2 January 1994 by shutting off the heat and opening the windows. The lowest temperatures achieved varied with the size and location of the seed bulk. Seed packets, that had a few grams of seed, reached -17°C, whereas, the middle of a bag stack with forty 50-kg bags of maize only reached -9°C. By the end of the 10-d freeze-out all non cold-acclimated *P. interpunctella* larvae were killed in the packets, 90% of the diapausing cold-acclimated larvae were killed in a single bag and 65% were killed middle of the stack. Mortality in the freeze-out was higher than we would have predicted from the laboratory data. The supercooling points (temperature at which freezing begins) of the *P. interpunctella* larvae range from -7°C for the non-diapausing non-acclimated to -13°C for the diapausing acclimated.

Keywords: Indian meal moth, Warehouse, Larvae, Supercooling point

1. Introduction

To survive the winter, insects in temperate areas often enter into diapause and increase their cold-hardiness before the onset of cold temperatures. The relationship between diapause, endocrine-mediated dormancy, and cold hardiness (an increase in the capacity to survive low temperatures) is unclear for most species, and it is only recently that there have detailed studies in this area (Denlinger, 1991). Denlinger (1991) describes four possible cases of the interaction between diapause and cold-hardiness: cold-hardiness without diapause, cold-hardiness coincidental but independent of diapause, cold-hardiness dependant on diapause and diapause independent of cold-hardiness (eg. summer diapause).

We believe that *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) the Indian meal moth offers a way to study this relationship, because it has an extensively studied facultative diapause (Tzanakakis, 1959; Bell, 1976; Bell et al., 1979; Kikukawa and Masaki, 1984). *Plodia interpunctella* is a cosmopolitan pest of stored grain, commodities and processed food (Rees, 2004). In comparative tests, it is one of the most cold-hardy stored-product insects (Mansbridge, 1936; Solomon and Adamson, 1955). There have been a few studies examining the cold-hardiness of *P. interpunctella* (Cline, 1971; Le Torch, 1977; Fields, 1992; Carrillo et al., 2006). However, there is only one study examining the relationship between diapause and cold-hardiness (Tzanakakis, 1959), but this study only used insects that had a short cold acclimation, (1 d at 5°C and 1 d at 0°C) and only used one exposure temperature (-5°C).

The use of extreme temperatures, in particular extreme low temperatures has been used to control stored product pests for many years (Fields, 1992; Mason and Strait, 1998). In western Canada, flour mills regularly use "freeze-out" techniques in their facilities during the winter to control insect pests (Worden, 1987). The effect of the insect's developmental stage and degree of acclimation, as well as the temperature, relative humidity and the duration of exposure are all variables, which will impact the success of a freeze-out (Fields, 1992).

The purpose of this study was to examine the cold-hardiness of *P. interpunctella* with and without diapause and with and without cold acclimation under a range of low temperatures, and to determine the effectiveness of freeze-outs in a seed warehouse.

2. Materials and methods

2.1. Low temperature survival in laboratory

Three times weekly, approximately 50 mg of *P. interpunctella* eggs were placed in 4-L jars with 1.5 kg of hard spring wheat and 250 g of wheat : wheat germ : honey : glycerol (ratio by weight, 40:5:1:1). The culture originated from individuals captured at a seed packaging plant in southern Manitoba, Canada. New insects captured at the seed plant were added to the culture regularly throughout these experiments.

To induce diapause, insects were reared at 25°C, 60% r.h. and 16 h light and 8 h dark for 2 wk, then placed at 20°C, 12 h light and 12 h dark for 4 wk. These conditions were shown to induce diapause in *P. interpunctella* (Kikukawa and Masaki, 1984). Corrugated cardboard discs were introduced into the jars when the wandering stage appeared. Larvae crawled into the discs and spun cocoons and entered into the prepupal stage. Discs were left for approximately 2 wk after the cessation of wandering stage to ensure that all the individuals were in diapause. Non-diapausing insects continued development and emerged as adults within two wk. To obtain non-diapausing pre-pupae, insects were held at 25°C, 60% r.h. and 16 h light and 8 h dark. Discs from the non-diapausing colony were removed for testing after the cessation of wandering. Any adults emerging from these discs were immediately counted and removed. These rearings were simultaneous, so that there was a regular supply of diapausing and non-diapausing larvae.

Discs with diapausing and non-diapausing larvae were divided into two groups; one group was subjected directly to low temperatures (non-acclimated) while the other was placed at 10°C for 4 wk (acclimated) before being placed at the low temperatures. Groups of discs from each of the four categories (acclimated diapausing, acclimated non-diapausing, non-acclimated diapausing, non-acclimated non-diapausing) were subjected to one of four temperatures (0, -5, -10, or -15°C) for a number of predetermined time periods (1 to 63 d) to ascertain the mortality due to cold injury. Sets of controls from each group were established on three occasions over the course of the experiment to determine if acclimation caused mortality before the insects were subjected to low temperatures. There were 75 to 200 insects per time-temperature-stage combination.

After the discs were removed from the low temperatures, they were placed at 25°C and 16:8 L:D photoperiod. Adults were counted and removed regularly. After adults ceased to emerge, discs were dismantled and counts were made of the dead prepupae, pupae intact (dead) and empty pupal cases.

2.2. Supercooling points

The supercooling point of *P. interpunctella* was determined for pupae and the 5th instar larvae of the four previously described stages (non-diapausing non-acclimated, non-diapausing acclimated, diapausing non-acclimated and diapausing acclimated). To determine the supercooling point for insects influenced by ice nucleators, eggs were placed on wheat treated with 0, 100 or 500 ppm of SnomaxTM (Johnson Controls Inc, USA). Snomax (Fields et al., 1995) is an ice nucleating bacteria product used in artificial snowmakers. To obtain this potent source of ice nucleators, *Pseudomonas syringae* (strain 31a), a common foliar bacterium isolated from maize leaves, is grown under conditions that maximize its ice-nucleating activity. Approximately 1.5 kg of wheat was placed in 4 -L jars with Snomax. Jars were rolled for 2 min to thoroughly mix the Snomax into the wheat. Approximately 500 one-d old eggs were placed on each treatment and were incubated as described previously to induce diapause. The insects were not cold-acclimated.

To determine supercooling points insects were placed on thermocouples within a Styrofoam container. When placed into a freezer at -40°C the inner part of the container cooled at approximately 1°C /min. The container was made 2 pieces of circular Styrofoam, each 5 cm deep with a radius of 20 cm. Forty-two wells, each 2.5 cm deep with 0.75 cm radius were constructed in the Styrofoam, with thermocouples emerging through the base of each. Insects were placed into small plastic funnels placed over each thermocouple to ensure the insect came to rest against the thermocouple. After placing the container in the freezer, temperatures were recorded every second until the heat of crystallization, or supercooling point occurred.

2.3. Freeze-out in commercial seed packing plant

The seed packing plant received seeds from across North America in bags with 2 to 50 kg of seed per bag. Seed was packaged into small retail packets with a few grams of seed and sold to home owners for small gardens. The bulk-seed floor at a seed packaging plant was used as a freeze-out area for controlling *P. interpunctella* that may be present in bulk-seed bags or retail packets. This area was conducive for to a freeze-out situation for a number of reasons:

- 1) no water pipes to drain and risk freezing
- 2) windows on all walls
- 3) plant operations cease for the Christmas holidays. Therefore, when the plant closes and staff goes on vacation, windows in this area can be opened and the temperature can be allowed to drop.

There were of two types of *P. interpunctella* larvae tested. One set of insects were non-diapausing and non-acclimated larvae placed into seed packets and placed on the bulk-seed floor 2 d before the freeze-out. These insects represent individuals found in the heated areas of the plant. The temperatures in this part of the plant were between 15-25°C. Nineteen packets were removed on 17, 26, 31 December 1993 and 6 January 1994 or at 0, 9, 14 and 20 d after being set up on the bulk-seed floor. There were 10 insects/seed packet.

The second type of insect was composed of individuals allowed to develop on the bulk-seed floor. These insects were considered to be diapausing and acclimated prepupae at the onset of the freeze-out. The temperatures on this floor were between 8-17°C during the 4 mo preceding the freeze-out. These insects were placed into tubes and put into various spots in the bulk-seed floor. Fifty insects were placed per 25 mL tubes containing approximately 15 g of wheat. Six tubes were placed at each of 4 locations: 1) Single bag: tubes inserted into the centre of a 50-kg bag of maize that was alone on a pallet, 2-4) Stack: tubes inserted into the middle of the top, middle and bottom bags of a stack of forty 50-kg bags of maize located on a pallet. Two tubes from each location were removed on the same dates as the seed packets.

The windows were opened on 23 December 1993 and closed on 2 January 1994. Temperatures are measured hourly by placing thermocouples at various locations and within bulk and packaged material to determine the effect of thermal mass. After removal from the freeze-out, packets and tubes were placed at 25°C and 70% r.h., and the number of emerging adults noted.

3. Results

3.1. Low temperature survival in laboratory

Regardless of the insects state, the lower the exposure temperature, the more rapidly the insects died (Fig. 1). At -15°C, all insects died within 1 d, at -10°C, all insects had over 89% mortality, after 14 d and at 0°C, it took 49 d before the non-diapausing groups had over 90% mortality. Diapausing acclimated larvae were the most cold-tolerant of all groups. Diapausing non-acclimated larvae were the next most cold-hardy. For the non-diapausing insects, there was not a great difference in acclimated and unacclimated insects at 0°C, but at -5°C and -10°C the acclimated insects were more cold hardy. However all non-diapausing insects were dead after 14 d at -5°C or -10°C.

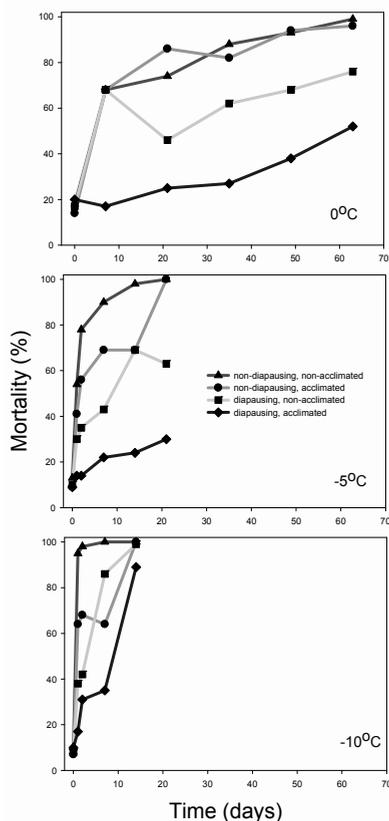


Figure 1 Mortality of 5th instar *Plodia interpunctella* larvae reared under various conditions.

3.2 Supercooling points

Pupae had the highest supercooling point at -5.0°C . The non-diapausing non-acclimated insects had a supercooling point of -7.4°C (Table 1). This explains why only 5% survived 1 d at -10°C (Fig. 1). *Plodia interpunctella* in other stages have supercooling points around -10°C or lower and had much greater survival at -10°C (Table 1, Fig. 1). Acclimation lowers the supercooling point a few degrees (-9.8°C), as does diapause (-10.7°C). The most cold hardy stage, diapausing acclimated, also had the lowest supercooling point (-13.4°C). Ice nucleating bacteria at 500 ppm significantly reduced the supercooling points, but had no effect at 100 ppm (Table 2).

Table 1 Supercooling points of pupae and 5th instar larvae of *Plodia interpunctella*, with different physiological states.

Stage	Diapause ¹	Acclimation ²	Snomax (ppm)	Supercooling points (+ SEM, $^{\circ}\text{C}$) ³	N
Pupae	ND	NA	0	-5.0 ± 0.2 a	11
Larvae	ND	NA	0	-7.4 ± 0.6 b	16
Larvae	ND	A	0	-9.8 ± 0.6 c	13
Larvae	D	NA	0	-10.7 ± 0.4 c	18
Larvae	D	A	0	-13.4 ± 0.5 d	11

1. ND = non-diapausing (25°C , 16L:8D), d = diapausing (20°C , 12L:12D); 2. NA = non-acclimated (25°C), A = cold-acclimated (10°C for 4 weeks); 3. Means with the different letter are significantly different, Tukey's multiple range test, $P < 0.05$.

Table 2 Supercooling points of 5th instar larvae *Plodia interpunctella* in different forms, (see Table 1 for footnotes).

Stage	Diapause ¹	Acclimation ²	Snomax (ppm)	Supercooling points (+ SEM, °C) ³	N
Larvae	D	NA	0	-10.7 ± 0.4 a	16
Larvae	D	NA	100	-9.8 ± 0.4 a	20
Larvae	D	NA	500	-7.2 ± 0.5 b	18

3.2. Freeze-Out

The non-acclimated non-diapausing insects had 100% mortality at the end of the freeze-out. Insects held in a single bag on a pallet had 90% mortality at the end of the freeze-out. *Plodia interpunctella* larvae placed in the top bag of a stack had 80% mortality, while those placed in the middle and bottom of the stacks had the least mortality at 65 and 69% at the end of the freeze-out (Fig. 2A). These mortalities were related to the temperatures that the insects were exposed (Fig. 2B). The seed packets on the table reached -17.1°C, the single bag reached -15.2°C, the top bag of a stack reached -12.5°C, the middle bag of a stack reached -9.3°C and the bottom bag of a stack reached -10.6°C. Mortality of insects in the seed plant was greater than what we predict from the laboratory tests with diapausing acclimated insects. For example, at the end of the freeze-out the insects in the middle bag of the bag stack had 65% mortality, and were exposed to about 6 d between -5 and -10°C. In the laboratory diapausing acclimated insects held for 7 d at -5 or -10°C had only 22 to 33% mortality (Table 1).

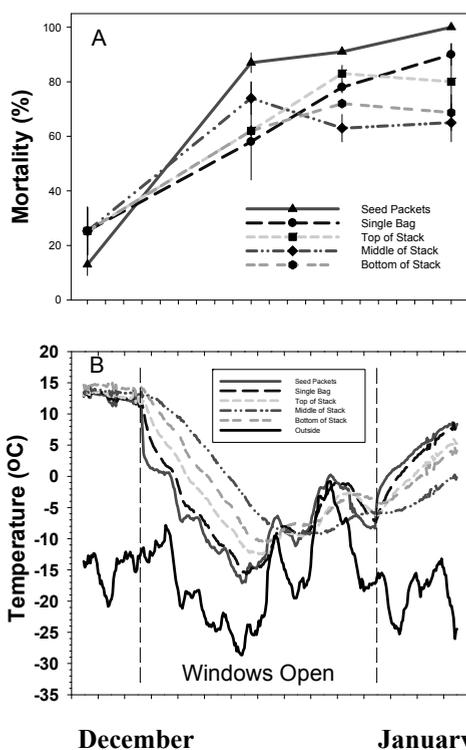


Figure 2 **A:** Mortality of 5th instar *Plodia interpunctella* larvae placed in small seed packets, at the centre of a single 50-kg bag of maize placed on a wooden pallet, or in the centre of a 50-kg bag of maize in the top, middle or bottom bag of a forty-bag stack placed on a pallet. **B:** Temperatures outside a seed warehouse in Manitoba Canada and inside the warehouse (see above). Windows were opened on 23 December 1993 and closed on 6 January 1994.

4. Discussion

Plodia interpunctella larvae increased cold-hardiness both with and without diapause. Although, as is noted by Denlinger (1991), the cold-hardiness that is associated with diapause is increased by the onset of cool temperatures. Tzanakakis (1959) showed that diapause increases the cold-hardiness of *P. interpunctella* compared to non-diapausing insects. Non-diapausing insects increased their cold-hardiness after a brief period of cold acclimation (1 d at 5°C and 1 d at 0°C), however diapausing insects did not. Our study showed that, acclimation increased the cold-hardiness of both diapausing and non-diapausing larvae. This is probably due the longer cold acclimation period in our studies compared to Tzanakakis (1959).

We obtained similar cold-induced mortality with the non-acclimated non-diapausing larvae as previous studies (Tzanakakis, 1959; Le Torc'h, 1977, Carrillo et al., 2006). There was 100% mortality at -5°C after 4 d (Tzanakakis, 1959), whereas there was 78 and 90% mortality after 2 and 7 d at -5°C respectively in this study. There was 94% mortality at 2°C after 40 d (Le Torc'h, 1977), similar to our study, where we exposed insects to 0°C for 35 or 49 d and obtained 88 and 93% mortality respectively. There was 100% mortality of non-diapausing non-acclimated larvae after 16 h at -10°C (Carrillo et al., 2006) compared to 95% mortality after 24 h in our study. For field cold-acclimated larvae, Carrillo et al. (2006) found it required 13 d at -10°C for 100% mortality, compared to 89% mortality after 14 d in this study. It is more difficult to make direct comparisons for the other treatments, as our regime for diapause induction and cold acclimation was different than Tzanakakis (1959).

Carrillo and Cannon (2005) did an extensive study of the supercooling points (SCP) of *P. interpunctella*. They found that the SCP of unacclimated 5th instar larvae was -14.1°C, (-7.4°C, this study) which dropped to -22°C (-13.4°C, diapausing acclimated this study) when larvae were reared outside to enter diapause and cold acclimate. They measured pupal SCP at -22.2°C, much lower than the -5.0°C SCP we observed for pupae. In general, they found much lower SCP than we observed. They did observe some differences between strains, but it is unlikely that the large differences in the SCP between studies could be just because of different strains. Calibration errors could account for some differences. Field acclimation they subjected the insects to was likely to yield more cold-hardy individuals with lower SCP than the laboratory acclimation in our study. These insects in Carrillo and Cannon (2005) may have been subjected to a different expenditure of energy reserves and/or stresses on their metabolic pathways that regulate cryoprotection that impacts the populations success through diapause (Hahn and Denlinger, 2007)

Freeze-outs provided this company with a simple, non-chemical, inexpensive method to control insects in their seed warehouse. Not all insects were controlled in the large bag stacks with pallets of 40 bags. There are a number of options to lower the temperatures of the seed to completely control infestations; break up the bag stacks into smaller stacks that would have a smaller thermal mass, prolong the freeze out, use fans to move cold air into the warehouse, or have an area constantly exposed to outside temperatures to do freeze-outs on high-risk product throughout the winter. This particular company captured only 9 mo in pheromone traps the 5 mo after the freeze-out compared to 478 mo over the same period in the pervious year. We can not attribute the decline conclusively to the freeze-out as they implemented other control measures in parallel to the freeze-outs.

Although the freeze-out has been demonstrated in a geographical location that is blessed with particularly cold winters, this method could be used in other locations by using large freezers. In some regards, freeze-out are like fumigations in that once the building and grain warms above 20°C it can become reinfested. So monitoring for insect activity would be an important part of using freeze-out to control *P. interpunctella* populations.

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