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Assessment of frozen larvae of *Callosobruchus maculatus* as hosts for rearing *Pteromalus cerealellae* (Ashmead) (Hymenoptera: Pteromalidae)

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ABSTRACT

The suitability of frozen host larvae for rearing *Pteromalus cerealellae* (Ashmead) (Hymenoptera: Pteromalidae), an ectoparasitoid of *Callosobruchus maculatus* (F.) (Coleoptera: Chrysomelidae) and other stored-product insects was investigated. The reproductive potential (number and sex ratio of progeny) of female *P. cerealellae* was compared on live (fresh) *C. maculatus* larvae (concealed within cowpea seeds) versus frozen larvae (obtained by freezing infested cowpea seeds at −20 °C for 48 h) which were subsequently thawed and held at ambient conditions (~25 ± 1 °C, 50 ± 5% RH) for 4, 24, 48, 72, 96, and 120 h before exposure to female parasitoids. No significant differences were recorded in the numbers and sex ratios of the progeny produced by female *P. cerealellae* on live larvae compared to frozen host larvae that were thawed and held at ambient conditions for up to 96 h, suggesting that live and frozen larvae of *C. maculatus* are equally suitable for rearing *P. cerealellae*. However, the data showed that progeny production on frozen hosts gradually declined with thawing duration and was significantly reduced at the thawing duration of 120 h. When live and frozen host larvae were simultaneously presented together to female *P. cerealellae* at different exposure periods, relatively greater progeny production was recorded on live hosts than on frozen hosts at 12, 24, and 48 h of exposure. This may suggest preference of female *P. cerealellae* for live versus frozen host larvae. These results are discussed in relation to the life history strategy and host location behavior of *P. cerealellae*, and may have practical implications in the development of efficient mass rearing systems for the parasitoid.

1. Introduction

Parasitoids are potentially important regulators of host insect populations and some are commercially produced as biological control agents of various pests (Mills, 1994; Cranshaw et al., 1996; Donnelly and Phillips, 2001; Floate and Spooner, 2002). Mass rearing and release of parasitoids and other natural enemies are critical components of any biological control program to suppress pest populations (Rueda and Axtell, 1987; Petersen and Cawthra, 1995; Kaufman et al., 2001; Geden and Hogsette, 2006; Geden and Kaufman, 2007). Typically, parasitoids are reared on living immature hosts and this strategy has many limitations. Use of live (fresh) larvae or pupae as rearing hosts for parasitoids may reduce the window of opportunity for parasitism due to rapid development of the hosts from potentially suitable stages for parasitism to unsuitable stages. Furthermore, rearing of parasitoids on live hosts has the inherent risk of accidental releases of non-parasitized pests in the field (Geden and Kaufman, 2007).

The use of killed (frozen or irradiated) hosts to rear parasitoids could potentially mitigate these limitations and may even have some advantages over the use of live hosts in certain applications, such as in foreign exploration efforts to establish colonies of exotic parasitoids in locales where live hosts are not available (Pickens and Miller, 1978; Geden et al., 2006). Use of frozen (freeze-killed) hosts for maintenance of parasitoid colonies can increase the efficiency of rearing programs. For instance, when hosts are reared in excess of needs, they can be frozen and used when normal supplies are low or can be stockpiled and used later in mass release programs (Klunker and Fabritius, 1992; Geden and Kaufman, 2007). In addition, rearing parasitoids on frozen hosts may reduce the risk of contamination of one population with another because any potential contamination which may occur during host maturation can be eliminated by freeze-killing (Geden and Kaufman, 2007).

Several studies have reported on the ability of some pupal parasitoids to successfully develop on frozen pupal hosts (Richerson and Borden, 1972; Petersen and Matthews, 1984; Rueda and Axtell, 1987; Rivers and Delinger, 1995; Floate and Spooner, 2002). For instance, Pickens and Miller (1978) successfully reared a fly pupal parasitoid, *Pachycrepoideus vindemiae* (Rondani) Hymenoptera: Pteromalidae) on frozen pupae of the housefly, *Musca domestica* Linnaeus (Diptera: Muscidae) and concluded that continued, peri-

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odic additions of frozen house fly pupae could increase the effectiveness of the parasitoid in chicken houses. Rueda and Axtell (1987) also reported that frozen pupae of *M. domestica* were as suitable as fresh pupae for mass rearing of three pteromalid parasitoids (Hymenoptera: Pteromalidae): *Muscidifurax raptor* Girault and Sanders, *P. vindemiae*, and *Spalangia cameroni* Perkins. The majority of the available literature on the ability of parasitoids to develop on frozen hosts has however, focused on pupal parasitoids of flies; not much is known about the development of larval parasitoids on frozen hosts (Kaschef, 1959).

Pteromalus cerealellae (Ashmead) (Hymeoptera: Pteromalidae) is a solitary larval ectoparasitoid of several stored-product pests including *Callosobruchus maculatus* (Fabricius) (Coleoptera: Chrysomelidae), *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae), *Lasioderma serricorne* (Fab.) (Coleoptera: Anobiidae), *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae), and *Sitophilus* spp. (Coleoptera: Curculionidae) (Ashmead, 1902; Brower, 1991; Howard, 2001; Onagbola et al., 2007). Females of *P. cerealellae* lay eggs in the larvae of these insects, which typically are concealed within grain seeds. This parasitoid is considered as a promising candidate for utilization in the biological control of stored grain pests (Brower, 1991; Mbata et al., 2005; Onagbola et al., 2007). The ability of *P. cerealellae* or other parasitoids of stored-product insects to successfully develop on frozen host larvae has not been previously investigated. This study was therefore conducted to determine if *P. cerealellae* could be reared successfully on frozen larvae of *C. maculatus*, one of its principal hosts. First, the reproductive potential (number and sex ratio of progeny) of female *P. cerealellae* when presented frozen fourth-instar larvae of *C. maculatus* versus live (fresh) larvae of the same instar stage was compared. To determine the effect of thawing duration on the viability of frozen larvae as hosts, reproductive potential of female *P. cerealellae* was compared on frozen larval hosts which were subsequently thawed and held at ambient conditions for various lengths of time (4, 24, 48, 72, 96, and 120 h) prior to exposure to female *P. cerealellae*. Finally, the relative suitability of frozen versus live larvae was compared by presenting both host types simultaneously to female *P. cerealellae* in the same arena.

2. Materials and methods

2.1. Insects: cowpea bruchids

Callosobruchus maculatus was utilized as the host for *P. cerealellae* in this study. The starting culture of *C. maculatus* was obtained from Fort Valley State University, Fort Valley, GA, USA (contact: Dr. George Mbata), where it had been reared continuously on cowpea seeds (*Vigna unguiculata* Walp.) for several years. *Callosobruchus maculatus* was reared in our laboratory on cowpea seeds (California Black Eyed variety) in 1-L wide-mouthed Mason glass jars. A fresh culture was started every 5 days by placing ~25 pairs of 3-day-old mated *C. maculatus* in a glass jar containing ~100 g of cowpea seeds held at 30 ± 1 °C, 70 ± 5% RH, and L12:D12 h (Mbata et al., 2005; Onagbola et al., 2007). The beetles were allowed to lay eggs on the seeds for 24 h after which they were removed with an aspirator. The infested seeds were incubated at the conditions specified above until the larvae had reached the fourth-instar stage, which were then provided to *P. cerealellae* for parasitization.

2.2. The parasitoid

The original culture of *P. cerealellae* was obtained from Fort Valley State University, Fort Valley, GA, USA where the parasitoid had been reared continuously for several years. The *Pteromalus cerealellae* culture was maintained in our laboratory by transferring about 30 adult pairs into a glass jar containing *C. maculatus*-infested cowpea seeds at a stage when most of the bruchid larvae were at the fourth larval instar. This was determined to occur, in a preliminary experiment approximately 15 days after infestation of cowpea seeds under our rearing conditions. The jars were held at the environmental conditions stated above for *C. maculatus*. Adult *P. cerealellae* were removed from the jars after 5 days of oviposition and the attacked *C. maculatus* larvae were incubated in a growth chamber at the above environmental conditions until the emergence of adult parasitoids.

2.3. Reproductive potential of P. cerealellae on live (fresh) and frozen C. maculatus larvae

The development of *P. cerealellae* was compared on live (<1-day-old) and frozen (<1-day-old) fourth-instar larvae of *C. maculatus* (concealed within cowpea seeds). Frozen larvae were obtained by freezing infested cowpea seeds containing a fourth-instar larva of *C. maculatus* at −20 °C for 48 h (Johnson and Valero, 2003) and thereafter exposing (thawing) it to ambient laboratory conditions (~25 ± 1 °C, 60 ± 5% RH) for ~4 h prior to exposure to a female parasitoid (frozen larvae were confirmed dead by dissection). Eighty infested cowpea seeds containing live or frozen larvae (1 larva per seed) were placed in a 10-cm diameter plastic Petri dish. A mated 2-day-old female *P. cerealellae* was then placed in each Petri dish and allowed to parasitize the larval hosts for 120 h (5 d). At the end of the 120 h exposure period, the female parasitoid was removed and the Petri dish containing host larvae was incubated at 30 ± 1 °C, 70 ± 5% RH, and L12:D12 h until the completion of emergence of parasitoid F_1 progeny (~10 days after the start of parasitoid emergence). Each treatment was replicated 20 times (i.e. 20 female parasitoids each exposed to 80 host larvae). The number and sex of offspring produced by each female parasitoid was recorded daily and summed at the end of the incubation period. Data were analyzed by using the Student's *t*-test (JMPIN Version 5.1, SAS Institute, 1998) to determine significant differences in reproductive potential of female *P. cerealellae* on live versus frozen host larvae.

2.4. Effects of duration of thawing of frozen host larvae on parasitoid reproductive potential

Having demonstrated the ability of *P. cerealellae* to develop on frozen host larvae in the preceding experiment, a second experiment was conducted to test for possible effects of duration of thawing of frozen host larvae on reproductive potential of female *P. cerealellae*. Infested cowpea seeds containing fourth-instar (<1-day-old) *C. maculatus* larvae were placed in a 10-cm diameter plastic Petri dish (80 seeds per dish each containing 1 larva) and frozen at −20 °C for 48 h as described in the preceding experiment. The Petri dishes were then removed from the freezer (thawed) and subsequently held at ambient laboratory conditions for various lengths of time: 4, 24, 48, 72, 96, and 120 h before exposure to female parasitoids. The control treatment consisted of infested cowpea seeds containing live fourth-instar (<1-day-old) *C. maculatus* larvae (80 seeds per dish each containing 1 larva). A mated (2-day-old) female *P. cerealellae* was then placed in each Petri dish and allowed to parasitize the larval hosts for 24 h as described in the preceding experiment. At the end of the 5 day exposure period, the female parasitoid was removed and the Petri dish containing host larvae was incubated as described in the preceding experiment. Each treatment was replicated 20 times (i.e. 20 female parasitoids each exposed to 80 host larvae). The number and sex of off-

spring produced by each female parasitoid was recorded daily and summed at the end of the incubation period. Progeny production data obtained for each treatment were square-root transformed ($\sqrt{(x+0.5)}$) and then analyzed with one-way ANOVA followed by Tukey's HSD test ($P < 0.05$). Data on offspring sex ratio were subjected to Student's *t*-test (JMPIN Version 5.1, SAS Institute, 1998) to test for significant deviation ($P < 0.05$) from an expected 1:1 sex ratio of emerged progeny per treatment.

2.5. Relative suitability of live and frozen C. maculatus larvae when presented simultaneously

The relative suitability of live versus frozen *C. maculatus* larvae as hosts for *P. cerealellae* was tested when presented simultaneously in a Petri dish. Infested cowpea seeds containing (<1-day-old) live or frozen fourth-instar *C. maculatus* larvae (frozen larvae obtained as previously described and held at ambient conditions for ~4 h prior to exposure to female parasitoid) were marked with Sharpie® permanent marker for later identification. Forty marked seeds containing live larvae and 40 marked seeds containing frozen larvae were placed together in a 10-cm diameter plastic Petri dish for a total of 80 seeds (mixed live and frozen larvae). A mated (2-day-old) female *P. cerealellae* was then placed in the Petri dish and allowed to parasitize the larval hosts for a period of 12, 24, 48, 72, 96, or 120 h (parasitism exposure period), as described in the preceding experiments. At the end of each exposure period, the female parasitoid was removed and the Petri dish containing the marked host larvae was incubated (separately for each host type) as described in the preceding experiments. This experiment was replicated 20 times. The number and sex of offspring produced by each female parasitoid was recorded daily and summed at the end of the incubation period. Data obtained on the total number of progeny produced per female *P. cerealellae* at each exposure period were analyzed by using the Student's *t*-test (JMPIN Version 5.1, SAS Institute, 1998) to determine any significant differences in reproductive potential of female parasitoid on live versus frozen larvae. The number of progeny produced on each host type at the different exposure periods were square-root transformed ($\sqrt{(x+0.5)}$) and then analyzed with one-way ANOVA followed by Tukey's test to determine significant effects ($P < 0.05$) of exposure period on progeny production.

3. Results

3.1. Reproductive potential of P. cerealellae on live (fresh) and frozen C. maculatus larvae

No significant differences were recorded in progeny production by female *P. cerealellae* on live versus frozen *C. maculatus* larvae (Students' "*t*"-test: $t = 0.230$, $df = 1$, $P = 0.819$): approximately 42.9 ± 5.0 and 40.8 ± 5.1 (means ± SE) progeny were produced per female on live and frozen larvae, respectively (Fig. 1). Also, no significant differences were recorded on the sex ratios of progeny produced on live larvae ($t = 0.019$, $df = 1$, $P = 0.985$) compared to frozen larvae ($t = -0.093$, $df = 1$, $P = 0.927$).

3.2. Effects of duration of thawing of frozen host larvae on parasitoid reproductive potential

One-way ANOVA revealed significant effects of host type on the mean number of progeny produced by female *P. cerealellae* ($F = 3.692$, $df = 6$, $P = 0.002$). Significantly fewer progeny were produced from frozen larvae which had been thawed and held at ambient conditions for 120 h (5 d) compared to those that were held for 4 or 24 h, or to live larvae. Progeny production was however, not significantly different between live larvae and frozen larvae which were held at ambient conditions for 4 or 24 h, prior to exposure to female *P. cerealellae* (Fig. 2). In general, a gradual decline in progeny production on frozen larvae was recorded with increasing thawing duration (Fig. 2). However, Students' *t*-test analysis showed no significant difference in the sex ratios of progeny produced on live larvae ($t = 0.884$, $df = 1$, $P = 0.382$) or frozen larvae thawed and held for 4 h ($t = 0.932$, $df = 1$, $P = 0.357$), 24 h ($t = -0.609$, $df = 1$, $P = 0.546$), 48 h ($t = -1.712$, $df = 1$, $P = 0.095$), 72 h ($t = 0.733$, $df = 1$, $P = 0.468$), 96 h ($t = 0.525$, $df = 1$, $P = 0.603$), or 120 h ($t = -0.489$, $df = 1$, $P = 0.628$).

3.3. Relative suitability of live and frozen host larvae when presented together

When live and frozen hosts were presented simultaneously in a Petri dish to female *P. cerealellae*, significant differences were recorded in the mean numbers of progeny produced on each host type at the shorter exposure periods (Fig. 3). Significantly more progeny was recorded on live hosts than on frozen hosts at expo-

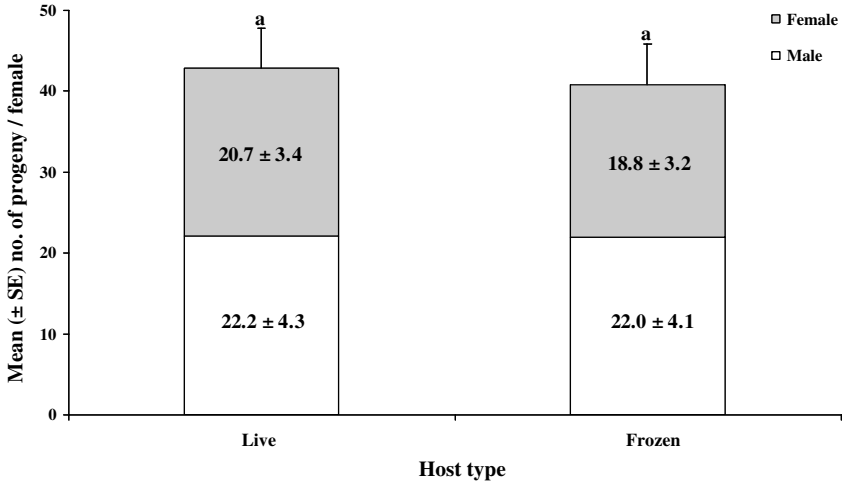

Fig. 1. Reproductive potential of female *P. cerealellae* on live and frozen *C. maculatus* larvae. Figure shows mean (±SE) number of progeny produced per mated female *P. cerealellae* ($n = 20$) on live and frozen *C. maculatus* larvae. A female parasitoid was exposed to 80 larvae of each type for 120 h (5 d). White and gray bars indicate female and male progeny, respectively. Bars showing the mean total number of progeny produced from live or frozen host followed by different letters are significantly different (Students' *t*-test, $P < 0.05$).

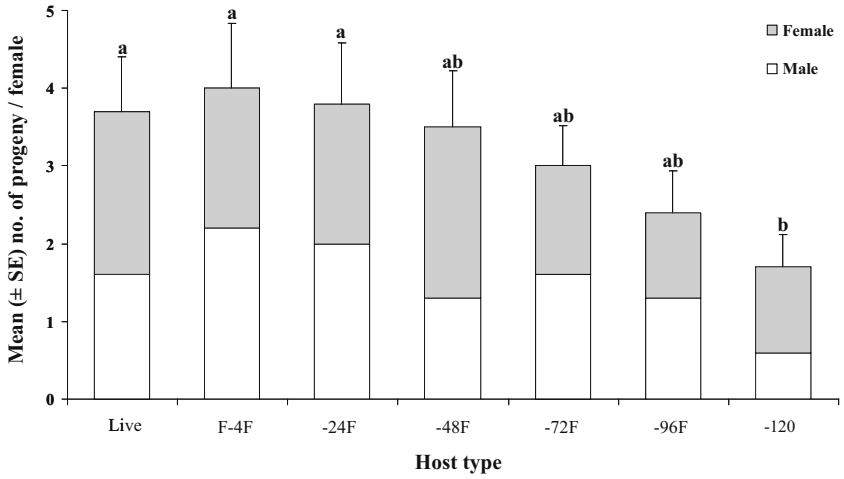

Fig. 2. Reproductive potential of female *P. cerealellae* on previously frozen *C. maculatus* larvae which were subsequently thawed and held at ambient laboratory conditions (~25 ± 1 °C, 60 ± 5% RH) for various periods of time. Figure shows mean (±SE) number of progeny produced per mated female *P. cerealellae* on live compared to frozen *C. maculatus* fourth-instar larvae thawed and held for 4 h (F-4), 24 h (F-24), 48 h (F-48), 72 h (F-72), 96 h (F-96), or 120 h (F-120) prior to exposure to female *P. cerealellae*. A female parasitoid was exposed to 80 larvae of each type for 5 d. White and gray bars indicate numbers of male and female progeny, respectively. Bars showing the mean total number of progeny per female for the different exposure periods having different letters are significantly different (Tukey's HSD test, $P < 0.05$).

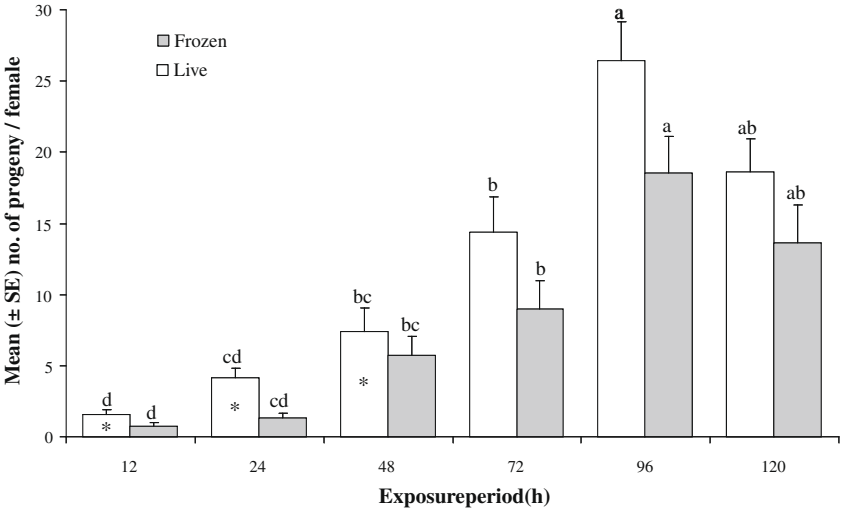

Fig. 3. Relative suitability of live and frozen *C. maculatus* larvae when presented simultaneously to female *P. cerealellae*. Figure shows mean (±SE) number of progeny per female at various exposure periods: 12, 24, 48, 72, 96, and 120 h. At each exposure period, means followed by asterisks (*) are significantly different between live and frozen larvae (Students' *t*-test, $P < 0.05$). For each host type (live or frozen larvae), means for the different exposure periods having different letters are significantly different (Tukey's HSD test, $P < 0.05$).

sure periods of 12 h ($F = 5.61$, $df = 1$, $P = 0.023$), 24 h ($F = 16.85$, $df = 1$, $P = 0.0002$), and 48 h ($F = 5.84$, $df = 1$, $P = 0.021$) (Fig. 3). However, no significant differences were recorded in progeny production on the two host types at the 72 h ($F = 2.21$, $df = 1$, $P = 0.145$), 96 h ($F = 2.83$, $df = 1$, $P = 0.101$) and 120 h ($F = 2.35$, $df = 1$, $P = 0.134$) exposure periods. Analysis of variance (ANOVA) showed significant effects of exposure period on progeny production on both live ($F = 20.04$, $df = 5$, $P < 0.0001$) and frozen hosts ($F = 16.46$, $df = 5$, $P < 0.0001$). In general, progeny production increased on each host type with exposure period (Fig. 3).

4. Discussion

There were no significant differences in the reproductive potential of female *P. cerealellae* on live versus frozen larvae of *C. maculatus* suggesting that both host types are equally suitable for rearing *P. cerealellae*. Similar results showing parasitoids ability to develop on alive and dead (killed) hosts have been reported for other hymenopteran parasitoids, including other Pteromalid species (Kaschef, 1959; Petersen and Matthews, 1984; Morgan et al., 1986; Rueda and Axtell, 1987; Roth et al., 1991; Rivers and Delinger, 1995; Floate and Spooner, 2002). For example, Kaschef (1959) reported that female *Lariophagus distinguendus* Först (Hymenoptera: Pteromalidae) successfully parasitized living and CO_2-killed prepupae of *Stegobium paniceum* L. (Coleoptera: Anobiidae), various species of pupal parasitoids of flies (Diptera) including *P. vindemiae*, *S. cameroni*, and several *Muscidifurax* spp. (Pickens and Miller, 1978; Petersen and Matthews, 1984; Rueda and Axtell, 1987; Petersen et al., 1992; Petersen and Currey, 1996; Floate and Spooner, 2002; Geden and Kaufman, 2007) have been successfully reared on frozen housefly, *M. domestica* pupae, Morgan et al. (1986) found Housefly pupae killed by exposure to gamma radiations to be suitable for production of *Spalangia endius* Walker and Roth et al. (1991) also showed frozen hornfly pupae to be suitable as hosts for *S. cameroni*.

The ability of *P. cerealellae* to develop on frozen host larvae may be related to its idiobiontic (host killing prior to oviposition) life history strategy, which has been observed in several other pteromalid parasitoids including *Cyrtogaster vulgaris* Walker (Askew, 1965), *Trichomalopsis apanteloctena* (Crawford) (Nakamatsu and Tanaka (2004) and *Nasonia vitripennis* (Walker) (Pexton and Mayhew, 2005). During oviposition, female *P. cerealellae* injects venom into its host through the ovipositor resulting in the paralysis and subsequent death of the host very soon thereafter (personal observation). In essence, most of the developmental period of *P. cerealellae* is spent on a dead host since the parasitized (live) host is likely to be dead by the time of larval hatch. Based on these results, we predict that other idiobiont larval parasitoids may also have the ability to develop successfully on killed hosts, as demonstrated for *P. cerealellae*.

Given that frozen *C. maculatus* larvae held at ambient conditions for up to 96 h (4 days), produced statistically similar numbers of progeny as live larvae with no significant differences in sex ratios, further supports the case for use of frozen larvae for rearing *P. cerealellae*. However, finding out that progeny production on frozen hosts gradually declined with thawing duration and was significantly reduced at longer thawing durations (5 days) is interesting and should be considered in future applications of this strategy. Similar results showing reduced progeny production on frozen hosts which had been exposed to ambient conditions for 5 days have been reported for other pteromalid wasps including *M. raptor*, *P. vindemia*, *S. cameroni*, and *Spalangia endius* Walker (Rueda and Axtell, 1987). The authors attributed the reduced suitability of frozen larvae with thawing duration to faster deterioration of frozen pupae during exposure to thawing temperatures and the disintegration of insect cells (cytolysis) which normally occurs after slow thawing (Losina-Losinsky, 1967; Rueda and Axtell, 1987). Deterioration and cell disintegration may also explain the results of this study showing a gradual reduction in progeny production by female *P. cerealellae* on frozen host larvae which were subsequently exposed to ambient conditions for 3–5 days.

The results of the third experiment in which both live and frozen host larvae were presented simultaneously to female *P. cerealellae* at different exposure periods showed relatively greater progeny production on live hosts than on frozen hosts at 12, 24, and 48 h of exposure. This may be indicative of the preference of female *P. cerealellae* for live host larvae over frozen host larvae, or the relatively greater suitability of live host larvae for parasitoid development. Although, this experiment was not designed to evaluate host preference or host location cues in *P. cerealellae*, it is likely that these results may be due to preference of female *P. cerealellae* for live larvae, given that live and frozen larvae were equally suitable for development of this parasitoid.

Little is known about the cues used by female *P. cerealellae* to locate larval hosts, which typically are concealed within seeds. However, parasitoids of endophytic hosts (that specialize on hosts covered by hard substrate) have been reported to locate their hosts by using various cues in hierarchical order (e.g. Godfray, 1994; Hailemichael et al., 1994; Meyhöfer and Casas, 1999; Fischer et al., 2004). In particular, many parasitoids of endophytic/concealed hosts are known to use vibrational cues to locate their hosts (Kaschef, 1964; Sokolowski and Turlings, 1987; Meyhöfer et al., 1994, 1997; Meyhöfer and Casas, 1999; van Dijken and van Alphen, 1998; Fischer et al., 2004). For example, the leaf miner parasitoid, *Dapsilarthra rufiventris* (Nees) (Hymenoptera: Braconidae) (Sugimoto et al., 1988a,b) and *Biosteres longicaudatus* Ashmead (Hymenoptera: Braconidae) (Lawrence, 1981) were reported to show preference for live hosts than dead hosts. Also, two parasitoids of *Drosophila* spp., *Asobara tabida* Nees (Hymenoptera: Braconidae) and *Leptopilina longipes* Spier (Hymenoptera: Eucoilidae) failed to locate dead larvae in the substrate, suggesting the use of host vibrational signals for host location (Sokolowski and Turlings, 1987). The larvae of *C. maculatus* are concealed within cowpea seeds and produce vibrational signals during feeding (Kaschef, 1964; Shade et al., 1990). Thus, our results may suggest the involvement of host vibrational cues in host location by female *P. cerealellae*. However, the ability of female *P. cerealellae* to parasitize live and freeze-killed hosts suggest that female parasitoids do not rely exclusively on larval vibrational signals for host location. Ongoing studies on the host location behavior of this parasitoid may provide insights into the relative importance of vibrational cues for host location.

To our knowledge, this study represents the first report on successful development of *P. cerealellae* on frozen host larvae. Our results showing that frozen host larvae are as suitable as live larvae for rearing *P. cerealellae* may have practical implications in the development of efficient mass rearing systems for *P. cerealellae*, which is a prerequisite to its future utilization for biological control of stored-product insects. It remains to be determined; however, if prolonged freezing or storage of frozen host larvae will have an effect on their suitability for rearing *P. cerealellae*.

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