Larval summer diapause of *Chrysocharis pubicornis* (Zetterstedt) (Hymenoptera: Eulophidae), a pupal parasitoid of agromyzid leafminers: Sensitive stage for diapause induction and effects of cool exposure on diapause termination

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**Abstract**

We examined the sensitive stage to summer diapause induction of *Chrysocharis pubicornis*. Individuals of different ages were transferred from diapause-preventing (15°C–12L : 12D) to diapause-inducing conditions (25°C–16L : 8D) throughout the 22-day egg-larval period. Transferring 15-day-old (third instar larvae about to complete feeding) or younger individuals resulted in more than 60% diapause; however, less than 25% of larvae entered diapause when transferred at 18 days (full-grown, resting third instar) or older. This suggests that sensitivity is high in younger instars, but decreases in the last instar. Moreover, individuals transferred at 18 days emerged 15 days earlier than control individuals continuously reared at 15°C–12L : 12D. We also examined the effects of cooling treatment duration on diapause termination by exposing diapausing larvae to 15°C–12L : 12D for increasing periods, followed by transfer to 25°C–16L : 8D. The percentage of diapause termination and the time to adult emergence increased with the cooling period, and it took between 12 and 16 days for 50% of individuals to complete diapause. The diapause termination rate after 20-day cooling was similar to that under continuous cooling (≈90%), but the time to emergence was significantly shortened by 15 days. The implications of our findings in an eventual biocontrol program are discussed.

**Key words:** *Chrysocharis pubicornis*; biological control; sensitive stage; storage; mass rearing

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**INTRODUCTION**

Diapause, a state of arrested development, is one of the major adaptations that parasitoids have evolved to overcome stressful periods and to keep in synchrony with the seasonal occurrence of their biotic requirements (Tauber et al., 1986). In biological control programs, however, diapause can be a major obstacle preventing the continuous rearing of hosts and their parasitoids or predators, and also by lowering the efficacy of the natural enemies released (Parrish and Davis, 1978; Onyango and Ochieng’-Odero, 1994; van den Meiracker, 1994; Salom et al., 2001; Velarde et al., 2002; Musolin et al., 2004). On the other hand, diapause has been recognized as an asset because it can allow or enhance the storage of biocontrol agents (Laing and Corrigan, 1995; Leopold, 1998; Ventura García et al., 2002; Rundle et al., 2004; Mehrnejad and Copland, 2005; Broufas et al., 2006). Thus, knowledge of diapause characteristics in the species involved is crucial for developing efficient mass-rearing techniques and fulfilling the potential of natural enemies to suppress pests in the field (Tauber et al., 1986).

*Chrysocharis pubicornis* (Zetterstedt) (Hymenoptera: Eulophidae) is a solitary pupal endoparasitoid of agromyzid leafminers (Hansson, 1987) that in Japan usually occurs as one of the dominant species attacking the indigenous garden pea leafminer *Chromatomyia horticola* (Goureau) (Diptera: Agromyzidae) (Mitsuda and Yamasaki, 2003). Therefore, this parasitoid could eventually contribute to suppress *C. horticola* populations, dramatic outbreaks of which have been recently reported in crops throughout Japan (Saito, 2004).

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Moreover, *C. pubicornis* may have a role in controlling exotic *Liriomyza* leafminers (Diptera: Agromyzidae); laboratory experiments showed that it can attack and develop on *Liriomyza trifolii* (Burgess) and *Liriomyza sativae* Blanchard (unpublished data); thus, further research on the biology and seasonal adaptations of this little-studied parasitic wasp is considered essential.

We have found that under long daylengths and warm temperatures most *C. pubicornis* individuals enter summer diapause at the larval stage inside the host puparia, whereas the rest develop to adults in a relatively short time. On the other hand, short daylengths and low temperatures stimulate continuous development (diapause induction virtually 0%) but also significantly prolong the egg-adult development time (Baeza Larios et al., 2007); therefore, from the standpoint of a mass-rearing program, there is a trade-off between diapause induction and development time.

Diapause in *C. pubicornis* terminates at low temperature or a combination of moderate temperature and short daylength, and adults emerge after a relatively long period (more than a month) of continuous exposure to diapause-terminating conditions (Baeza Larios et al., 2007); however, the sensitive stage to diapause-inducing stimuli and the minimum period of exposure to diapause-terminating conditions required to complete diapause have not been determined. These diapause characteristics would not only help to better understand the ecology of this species, but also to manipulate environmental conditions to increase the efficiency of an eventual mass-rearing and release program.

In this study, we examined the sensitive stage of *C. pubicornis* to diapause-inducing stimuli and the minimum period of exposure to diapause-terminating conditions required for diapause completion.

**MATERIALS AND METHODS**

**Host culture.** A colony of *Chromatomyia horticola* was established with flies obtained from infested garden pea (*Pisum sativum* L.) leaves collected at several locations in Miyazaki City (31°56.3’N, 131°24.8’E), from March to May 2002. Flies were then reared at 22°C, 16L : 8D photoperiod and 40–60% RH using potted kidney bean (*Phaseolus vulgaris* L.) as the host plant. To maintain genetic diversity, field-collected adult flies were periodically added to the colony during the springs of 2003 and 2004 (approximately 100 individuals per week in February and March). The flies had been reared for approximately twenty generations by the time the experiments started. Host pupae for the experiments were obtained as described in an earlier paper (Baeza Larios et al., 2007).

**Parasitoid culture.** The initial *C. pubicornis* colony was established in the spring of 2002. Wasps were obtained from the *C. horticola* infested garden pea leaves collected in the field as described above, by keeping the leaves in an emergence device (Ohno et al., 1999). Rearing was conducted at 25°C and 16L : 8D, using laboratory-reared *C. horticola* pupae as the host. Under those conditions, less than half of the parasitoid larvae pupate and emerge as adult wasps, and the rest enter diapause (Baeza Larios et al., 2007); therefore, to maintain genetic diversity, diapausing individuals were exposed to 15°C and 12L : 12D to terminate diapause (Baeza Larios et al., 2007), and the emerging adults from both diapausing and nondiapausing groups were pooled in the culture. Field-collected wasps were also periodically added to the colony during the springs of 2003 and 2004 (approximately 25 individuals per week in March and April). The wasps had been reared for approximately twenty generations by the time the experiments started. Parasitized *C. horticola* pupae for the experiments were prepared as described in Baeza Larios et al. (2007). The wasps used for parasitization were only those that emerged as adults when reared at 25°C and 16L : 8D (nondiapausing individuals).

**Sensitive stage to diapause induction.** *C. pubicornis* has three larval instars (Cameron, 1939), and diapause is clearly expressed in the third instar. Examining the sensitivity of each particular instar, however, was not possible because their duration is unknown, and their morphological characteristics are difficult to observe due to the endoparasitic nature of this species. Therefore, since the egg-larval period at 15°C and 12L : 12D is approximately 22 days (unpublished data), we examined the sensitivity to diapause-inducing stimuli by transferring parasitized host pupae from diapause-preventing (15°C–12L : 12D) to diapause-inducing (25°C–16L : 8D) conditions (Baeza Larios et al., 2007) when the parasitoids were either 3, 6, 9, 12, 15, 18, 21,
or 24 days old. As control treatments, parasitized pupae were held continuously at either 25°C–16L : 8D (control 1), or at 15°C–12L : 12D (control 2) from egg to adult emergence. The treatments were replicated 4 to 6 times with an average of 80 individuals per replicate.

Starting from the day when the first adult wasp emerged, the sex and date of emerging individuals were recorded daily for 3 weeks. The host puparia from which no parasitoid emerged were then dissected and all living larvae were considered to be in diapause. Parasitoid pupae and unemerged adults found during dissection were considered as non-diapausing individuals. The diapause induction rate was calculated as \[ \frac{\text{No. of diapausing larvae}}{\text{No. of diapausing individuals} + \text{No. of non-diapausing individuals}} \times 100. \]

Effect of duration of cool exposure on diapause termination. Diapause in *C. pubicornis* terminates in similar proportions (>90%) by simply cooling to 15°C (under either short 12L : 12D or long 16L : 8D photoperiod) or by exposure to 20°C and a short photoperiod (Baeza Larios et al., 2007). In this experiment we evaluated the effect of different periods of exposure to 15°C and 12L : 12D as diapause-terminating conditions, considering the favorable effects of diapause development under low temperatures (Hodek and Hodková, 1988) and that, in nature, diapause is likely to terminate under a low temperature and short photoperiod combination (Baeza Larios et al., 2007). Kidney bean leaves bearing parasitized *C. horticola* pupae were kept at 25°C–16L : 8D to obtain diapausing *C. pubicornis* larvae. After the emergence of the last non-diapausing adult wasp, the leaves were stored for 3 months under the same conditions and then transferred to diapause-terminating conditions (15°C–12L : 12D) for 4, 8, 12, 16, and 20 days. Thereafter, the leaves were returned to the original storing conditions until emergence of adult wasps. As controls, diapausing larvae were either kept under the original diapause-inducing conditions (control 1) or transferred to diapause-terminating conditions until the emergence of adult wasps (control 2). Six replicates of approximately 35 larvae were used for each treatment. After adult emergence started, the number and sex of emerged wasps were recorded daily for 30 days, and then all puparia in the leaves were dissected. Since pupation was the criterion used for diapause termination, all parasitoid larvae found alive during dissection were considered to be in diapause. The diapause termination rate was calculated as \[ \frac{\text{No. of larvae that pupated}}{\text{No. of diapausing larvae} + \text{No. of non-diapausing larvae}} \times 100. \]

RESULTS

Sensitive stage to diapause induction

The age of *C. pubicornis* individuals when transferred from diapause-preventing to diapause-inducing conditions significantly affected the diapause induction rate (ANOVA, \( F_{3,41} = 58.81, \ p < 0.001 \)) (Fig. 1). Diapause was induced in more than 60% of individuals when transferred from 3–15 days of age, and the induction rates were not significantly different from when rearing for the full cycle under diapause-inducing conditions (control 1); however, when 18-day-old or older individuals were transferred, the diapause rate significantly decreased below 25%. Transferring at 18 or 24 days resulted in a diapause induction rate not significantly different from that when rearing the whole cycle under diapause-preventing conditions (control 2). The total development time (egg-adult) of non-diapausing individuals significantly increased with the age of transfer to diapause-inducing conditions [ANOVA for treatments that resulted in less than 50% diapause (18 days and more); males: \( F_{3,246} = 406.65, \ p < 0.001 \); females: \( F_{3,409} = 549.08, \ p < 0.001 \)].

![Fig. 1. Diapause induction rate (mean±SE) after rearing *C. pubicornis* individuals under diapause-preventing conditions (15°C–12L : 12D) and transferring to diapause-inducing conditions (25°C–16L : 8D) at different ages. Means with the same letter are not significantly different at 0.01 level (Tukey HSD). C1, control 1: individuals reared from egg-adult at 25°C–16L : 8D; C2, control 2: individuals reared from egg-adult at 15°C–12L : 12D. Percent diapause data were arcsin square-root transformed prior to analysis.](image-url)
Table 1. Egg-adult development time of non-diapausing individuals and diapause induction rate (percentage for both sexes combined) when transferring *C. pubicornis* individuals of different ages from diapause-preventing (15°C–12L:12D) to diapause-inducing conditions (25°C–16L:8D)

<table>
<thead>
<tr>
<th>Age when transferred (days)</th>
<th>Egg-adult development time in days (mean±SE)</th>
<th>Diapause induction rate (%)(mean±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male (n)</td>
<td>Female (n)</td>
</tr>
<tr>
<td>18</td>
<td>27.9±0.27 (52)</td>
<td>28.6±0.21 (66)</td>
</tr>
<tr>
<td>21</td>
<td>29.4±0.24 (62)</td>
<td>29.8±0.24 (72)</td>
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<tr>
<td>24</td>
<td>31.0±0.23 (63)</td>
<td>31.4±0.18 (74)</td>
</tr>
<tr>
<td>C2a</td>
<td>42.0±0.71 (73)</td>
<td>44.5±0.34 (197)</td>
</tr>
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</table>

*C2, control 2: individuals reared from egg-adult at 15°C–12L:12D.*

Individuals transferred at 18 days emerged after 28 days (cooling period included), whereas those kept under continuous cooling (control 2) emerged after 42–44 days. Upon dissection of puparia, some dead larvae, dead pupae, and adults that failed to emerge were found sporadically, but the overall rate was considered negligible (1.3%, <0.1% and <1% respectively).

**Effects of duration of cool exposure on diapause termination**

The percentage of diapause termination significantly increased with the exposure period to 15°C–12L:12D (ANOVA, *F*<sub>6,35</sub>=85.81, *p*<0.001) (Fig. 2A). At 0 (control 1) and 4 days of exposure there was absolutely no diapause termination, but after 8 days, an average of 22.3% of the larvae broke diapause, pupated and emerged as adults. It took between 12 and 16 days of exposure for 50% of individuals to terminate diapause, and the highest diapause termination rates (>90%) were obtained with either 20 days of exposure to 15°C–12L:12D and transfer to 25°C–16L:8D or by rearing continuously at 15°C–12L:12D (control 2). The time to adult emergence also significantly increased with the length of exposure period to 15°C–12L:12D (ANOVA; males: *F*<sub>4,291</sub>=507.68, *p*<0.001; females: *F*<sub>4,325</sub>=831.76, *p*<0.001), ranging from 23 days at 8 days of exposure to 46 days under constant cooling at 15°C–12L:12D (control 2). Some dead larvae, dead pupae, and adults that failed to emerge were found sporadically upon dissection, but the overall rate was considered negligible (<2%, <0.5% and <1.5% respectively).

**DISCUSSION**

**Sensitive stage to diapause induction**

In insects, diapause-inducing stimuli are perceived only during specific, genetically determined stages, and these sensitive stages have a vital function in the life cycle because they determine if development is to proceed on a reproduction-destined or diapause-destined pathway (Tauber et al., 1986). Cameron (1939) described 3 larval instars for *C. pubicornis* (*Chrysocharis syma*, C. Hansson, personal communication) parasitizing the pupa of *Phytomyza ilicis* (Diptera: Agromyzidae). The third larval instar completes feeding and then enters a period of rest before changing into prepupa (Cameron, 1939). Since diapausing larvae found during dissection in the present study appeared to have completed feeding, it can be inferred that the diapausing stage of *C. pubicornis* is the full-grown, resting third larval instar.

By extrapolating Cameron’s data on the duration of *C. pubicornis* immature stages, the 22-day duration of the egg-larval period when *C. pubicornis* develops on *C. horticola* at 15°C–12L:12D (unpublished data) can be broken down as follows: Egg, 3–5 days; first, second and third larval instar until maturity and completion of feeding, 8–10 days (6–15 days after oviposition); third instar in larval rest, 4–5 days (16–20 days after oviposition) and prepupae, 1 to 2 days (21–22 days after oviposition). Based on the previous estimation, it can be...
inferred that when transferred from diapause-preventing to diapause-inducing conditions, most 15-day-old individuals were third instar larvae about to complete feeding. This stage seems critical because although diapause sensitivity was still high (60%, Fig. 1), it dropped significantly in the following 3 days, so that individuals transferred at 18 days (most likely third instar larvae that had completed feeding and were in the middle of the rest period) or older had little or no sensitivity to diapause. Therefore, sensitivity appears to be high in young larval instars, but it decreases in the last instar, and most sensitivity is lost during the period of larval rest. Similar findings have been reported for hibernal diapause in a review (Mousseau and Dingle, 1991) and several studies (Tauber and Tauber, 1972; Christiansen-Weniger and Hardie, 1999; Kurota and Shimada, 2001; Sato, 2003; Huang et al., 2005), indicating that the sensitive stage for larval diapause occurs in the one or two instars immediately prior to the diapause stage; however, there are few summer diapause studies of parasitic Hymenoptera (Noyes, 2003), and ours is apparently the first report of summer diapause in a eulophid parasitoid. Our findings suggest that, in the field, *C. pubicornis* larvae that develop from eggs laid from early to mid-April onwards (when diapause-inducing stimuli are likely to begin) are those that will eventually enter diapause in a gradually increasing proportion as daylength and temperature increase (Baeza Larios et al., 2007).

**Effect of duration of cool exposure on diapause termination**

Summer diapause generally requires specific stimuli for termination (Masaki, 1980; Tauber et al., 1986), and in some species diapause development occurs rather rapidly when exposed to diapause-terminating conditions. In the onion maggot *Delia antiqua* (Diptera: Anthomyiidae) only five days at 16°C are enough to complete diapause development (Ishikawa et al., 2000), and aestivating pupae of its congener, the cabbage root fly (*Delia radicum*) start to develop into flies as soon as the temperature falls below 20°C (Finch and Collier, 1985). Our findings suggest, however, that diapausing *C. pubicornis* larvae need to spend a certain period under diapause-terminating conditions to acquire reliable environmental information before diapause development can proceed. Such a period varied significantly among individuals, because 8 days at 15°C–16L:12D were sufficient for only 23% of diapausing larvae to terminate diapause and pupate, whereas 90% of completion was achieved only after 20 days under the same conditions (Fig. 2A). In nature, this may help to prevent accidental pupation when there is unusually cool weather for a few days. Moreover, the variation in the response of diapausing individuals to diapause terminating conditions may help to prevent the synchronous emergence of wasps in early fall after
diapause completion. At the beginning of fall, host availability in the field would be just starting to increase, and thus emerging at a slow rate and over a wide period of time appears to be advantageous rather than the synchronous emergence of most individuals (Baeza Larios et al., 2007).

Our data also suggest that the postdiapause development rate is related directly to temperature, and thus, is subject to manipulation in the laboratory. Figure 2B shows that individuals who spent a greater proportion of the time to adult emergence at 25°C–16L : 8D emerged earlier. In particular, exposing diapausing larvae for 20 days to 15°C–12L : 12D and subsequently transferring them to 25°C–16L : 8D not only resulted in a diapause termination rate similar to that of continuous exposure at 15°C–12L : 12D (control 2) (Fig. 2A), but also the time to adult emergence (females) was shortened by more than 15 days (Fig. 2B) most likely due to the effect of high temperature.

The mortality of immature stages and unemerged wasps cannot be attributed only to the nearly 5-month storage period that elapsed between the induction of diapause and dissection of host puparia. The exact time of death is unknown and similar values were observed in the previous experiment in which there was no storage period; thus, it is unlikely that storage affected the survival rate of diapausing individuals.

**Implications for biological control**

Knowledge of the mechanisms of diapause induction and termination also has practical applications such as improving the rearing methods of economically important insects (Kato et al., 1979) or the species (hosts or natural enemies) involved in IPM programs (Parrish and Davis, 1978; Onyango and Ochieng’-Odero, 1994; Salom et al., 2001; Velarde et al., 2002; Bloem et al., 2004). *C. pubicornis* is an indigenous parasitoid under consideration as a potential candidate to control agromyzid leafminers in greenhouses (Ohno et al., 2004); however, summer diapause prevents the continuous rearing process in the laboratory under the commonly used 25°C and 16L : 8D conditions. Currently, low temperatures must be used to minimize diapause induction or to induce diapause termination, but this implies an increase in energy costs and in the time to adult emergence (diapause induction vs. development time trade-off). The results of this study showed that by manipulating the environmental conditions, continuous rearing is possible without the protracted egg-adult development time when rearing at low temperatures. This could be accomplished by first rearing individuals under diapause-preventing conditions (15°C–12L : 12D) for 18 days until the sensitive stage to diapause conditions has been completed in most developing larvae. Thereafter, individuals must be transferred to 25°C–16L : 8D, which will shorten the time to adult emergence by 15 days in comparison to continuous rearing at 15°C–12L : 12D (Table 1), but without a significant increase in diapause rate (Fig. 1).

Storage is another practical application of diapause in IPM programs. Cold storage of diapausing hosts or natural enemies is being applied in commercial mass-rearing systems to provide flexibility in production and release (Leopold, 1998 and references therein). Moreover, the reduction of natural enemy mass-rearing costs through improved storage techniques is considered essential to promote the practical use of natural enemies in protected culture in Japan (Yano, 2003). This makes *C. pubicornis* an attractive candidate to control agromyzid leafminers in greenhouse crops in Japan, particularly because summer diapause in this species is maintained at warm temperatures, thus there would be no additional energy costs of cold storage. In this study we were able to shorten the normally long time to emergence of postdiapause *C. pubicornis* adults by exposing diapausing larvae to diapause terminating conditions (15°C–12L : 12D) for only 20 days and then transferring them to 25°C–16L : 8D. This could be done without a significant reduction in the diapause termination rate (Fig. 2A). Thus, a high number of individuals can be expected to complete diapause and emerge as adults in the shortest time possible (Fig. 2B), which would be advantageous, for instance, for timing the release of parasitoids. On the other hand, although long-term storage did not cause significant mortality in this study, some sublethal effects on offspring fitness components have been reported in some species (Leopold, 1998; Ellers and van Alphen, 2002; Tezze and Botto, 2004). The magnitude of these effects, however, is generally proportional to the time spent in storage; therefore, it could be possible to determine an optimum storage period (Leopold, 1998). Further research on this area
would be needed, should C. pubicornis prove to be a good candidate for use in a biocontrol program against agromyzid leafminers.

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REFERENCES


