

Diapause induction in *Trichogramma embryophagum* Htg. (Hym., Trichogrammatidae): the dynamics of thermosensitivity

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Abstract

It is known that the low temperature is the most important factor inducing the pre-pupal diapause in *Trichogramma* species. The position of the thermosensitive period over the life cycle and temporal variation of the degree of responsiveness were investigated in *T. embryophagum* Htg. by transferring pre-imaginal stages between 'neutral' temperature of 15°C and 'diapause-inducing' temperature of 10°C. Our experiments showed that 6 days long exposure at 10°C significantly increased the percentage of diapausing pre-pupae when started during rather large part of development: from embryo up to early pre-pupa. The highest thermosensitivity was recorded during the embryo and the larval stages, with some decrease during the hatching period. Treatments with shorter cold exposures (2–3 days) gave similar results. Even 24 h long exposure at 10°C increased the percentage of diapausing pre-pupae when applied during egg or early larval stage. Being started at the same stage of development, longer cold exposures caused stronger increase in the percentage of diapausing individuals. The experiments did not reveal any significant daily changes in thermosensitivity: at 12 : 12 h light : dark, larvae subjected to the low temperature during six photophases showed practically the same percentage of diapausing individuals as those subjected to the low temperature during six scotophases, and as those subjected to the 3 days long uninterrupted cold exposure. Hence, in natural conditions even occasional short-term cold periods could be accumulated.

Introduction

It is known that facultative diapause is an anticipatory reaction induced by environmental tokens long before the onset of the adverse seasonal conditions. Thus, the sensitive stage and the diapausing stage of the insect's life cycle could be separated. The period of sensitivity to a key factor can last from several days to several months. However, a shorter duration is usually sufficient to induce the diapause. In addition, even within the sensitive stage, the relative sensitivity may vary with time (Tauber et al. 1986; Zaslavski 1988; Danks 2002, 2003; Denlinger 2002; Saunders 2002).

The diapause in *Trichogramma* species has already attracted much attention because these minute egg parasitoids are widely used as biocontrol agents (Boivin 1994; Smith 1996). It was repeatedly shown that the low temperature is the most important factor acting on the larvae and inducing the pre-pupal diapause almost independently of the day length (Boivin 1994; Garcia et al. 2002; Ma and Chen 2006). Later, it was demonstrated that the progeny diapause may depend on the photoperiodic conditions of the parental generation development, although this maternal influence could be revealed only under near-threshold temperatures (Zaslavski and Umarova 1990; Boivin 1994; Laing and Corrigan 1995; Reznik et al. 2002; Reznik and Kats 2004).

As for the temperature-sensitive phase and the sensitivity dynamics, relatively little is known about *Trichogramma* in this regard. Particularly, judging from the published data, the minimum time required for the diapause induction is surprisingly long. In *T. cordubensis* Vargas and Cabello, it was possible to induce diapause by exposing larvae to 10°C for at least 30 days (Garcia et al. 2002). In *T. dendrolimi* Mats., the minimum effective duration of the cold exposure was ca 15 days (Ma and Chen 2006). For comparison, the minimum time required for the diapause induction by photoperiod can be as short as 2–3 light : dark (L : D) cycles (days).

On the assumption that photoperiodic and temperature diapause-regulating reactions are nothing more than two extremes of a photo-thermal reaction based on the single mechanism (Zaslavski 1988, 1996), two important hypotheses could be proposed.

First, in spite of relatively long-term period of thermosensitivity, the minimum duration of the cold exposure causing detectable changes in *Trichogramma* may be as short as few days (by analogy with few L : D cycles in the photoperiodic reaction), although the proportion of diapaused individuals could increase with the time spent under the low temperature conditions.

Second, the role of the night (scotophase) temperature in *Trichogramma* diapause induction could be much more important than that of the day (photophase) temperature, which was shown for many insect species (Tauber et al. 1986; Zaslavski 1988; Saunders 2002; see 'Discussion' for more references). The present study was aimed at experimental verification of these hypotheses.

Materials and Methods

Insects and general methods

A laboratory parthenogenetic strain of *T. embryophagum* Htg. originating from Moscow province of Russia was cultivated on the eggs of the grain moth, *Sitotroga cerealella* Oliv., under constant laboratory conditions (20°C, 18 : 6 h L : D) for more than 100 generations.

For each replicate of each experiment, paper cards with 500–600 grain moth eggs glued by non-toxic water-soluble glue were subjected for 2 h to parasitization by 100–200 *T. embryophagum* females. Then these cards with parasitized host eggs (the maternal generation) were individually placed in large test tubes and incubated at the same temperature conditions (20°C) and under 18 : 6 or 16 : 8 h L : D, depending on the experiment. At the day of mass

emergence of the maternal generation, a block of 36 paper cards with fresh grain moth eggs (ca 100 eggs per card) was placed in a large test tube with emerged wasps and also subjected to parasitization for 2 h. To ensure uniformity, in all replicates of all experiments parasitization was conducted at the same time of the day: between 13:00 and 15:00, (i.e. 4–6 h after the light-on) and then all wasps were removed from the host eggs.

Rate of development

The aim of this experiment was to create a reference time scale for the further tests. To avoid the diapause induction, the maternal generation was reared at 20°C and 18 : 6 h L : D. Samples of parasitized eggs of the progeny generation were periodically dissected and percentages of individuals at different stages of development (eggs, larvae, pre-pupae, pupae and adults) were separately recorded for each card (Volkoff et al. 1995; Dahlan and Gordh 1996; Jarjees and Merritt 2002 for morphological description of *Trichogramma* development). In addition, darkening of parasitized host eggs was recorded. As *Trichogramma* females usually lay only one egg in each egg of the grain moth, the number of emerged adults was estimated by the number of parasitized eggs with emergence holes. The larval eclosion (the eggs to larvae ratio) was recorded every day. Percentages of pre-pupal, pupal and adult stages were recorded every second day. Twelve replicates of this experiment were conducted.

Diapause induction

In these tests, the maternal generation was reared at 20°C, 16 : 8 h L : D, which was shown to be 'moderately' diapause-inducing conditions (Reznik et al. 2002). Immediately after the parasitoid oviposition, the cards with the newly parasitized host eggs (the progeny generation) were separated, randomly placed in small test tubes and then treated according to the particular experimental design. In all treatments, the progeny generation developed under 12 : 12 h L : D. Two temperature regimens were used: a 'neutral' or 'threshold' temperature of 15°C, usually inducing diapause in a relatively small fraction of *T. embryophagum* pre-pupae (Zaslavski and Umarova 1990; Reznik et al. 2002) and a 'diapause-inducing' temperature of 10°C, which was shown to induce maximum diapause rate in *T. embryophagum* and in most of the other studied *Trichogramma* species (Zaslavski and Umarova 1990; Boivin 1994; Garcia et al. 2002; Rundle et al. 2004;

Ma and Chen 2006). The developing wasps were transferred between these temperature regimens, and the experimental treatments differed in the onset time and duration of the 'cold exposure'. In addition, two controls were run. In the 'high-temperature control', the insects developed at the constant temperature of 15°C until the adult emergence. In the 'low-temperature control', the parasitized eggs were stored at 10°C during 30 days after the moment of parasitization, which was shown to be strongly diapause-inducing conditions (e.g. Ma and Chen 2006). Then the 'low-temperature control' cards were transferred to 15°C, to speed up the development and to facilitate the emergence of active individuals.

After the mass emergence of the non-diapausing fraction of adults (i.e. in 40–45 days after the parasitoid oviposition in the 'high-temperature control' and in 60 days after the parasitoid oviposition in the 'low-temperature control') all parasitized host eggs were dissected, the non-diapausing individuals (emerged adults, few dead adults inside the host chorion, sporadic dead or alive pupae) and diapausing pre-pupae were recorded. Each alive pre-pupa was assumed to be diapausing. The few insects that died during the larval or pre-pupal stages were excluded from the consideration. The percentage of diapausing individuals was separately calculated for each card, i.e. for each replicate in each treatment in each experiment. As the proportion of diapausing progeny may vary even in successive generations reared under constant laboratory conditions (see 'Dynamics of thermosensitivity: absence of daily cycles' for references) all replicates in all treatments in each experiment were conducted with simultaneously emerged females of the same generation.

The first experiment was aimed at the estimation of the temporal dynamics of thermosensitivity. In this experiment, the parasitized eggs were transferred to the low temperature conditions every second day from the day of parasitoid oviposition to the 18th day of development at 15°C, when mass pupation occurred (fig. 1e). The duration of the cold exposure in different treatments of this experiment varied from 1 to 6 days.

The second experiment concerned the possible role of photoperiod in the dynamics of thermosensitivity and was particularly aimed at the detection of daily cycles. In all treatments of this experiment, insects were kept at 12 : 12 h L : D and were subjected to cold exposure (or exposures) during the 4–9th days of their development. In the first treatment, the cold exposure lasted 6 days (the 4th–9th days from the

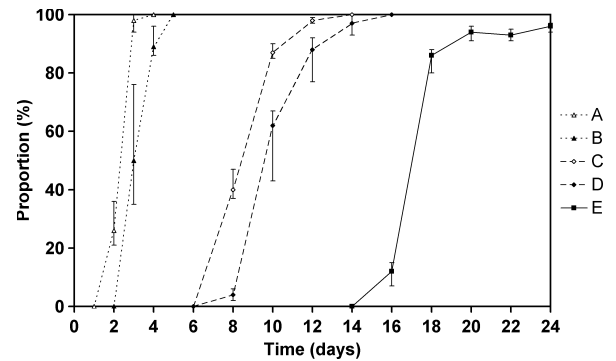


Fig. 1 Developmental milestones of *T. embryophagum* at 15°C (cumulative per cent at the day of dissection). A and B – per cent of egg hatch; C – per cent of darkening of parasitized host eggs, D – per cent of pre-pupa; E – per cent of pupa, F – per cent of adult. A – the X-axis indicates the period of time (days) between parasitization and transfer from 15° to 10°, dissection was made 3 days later, B–F – the X-axis indicates the period of time (days) between parasitization and dissection. Medians and quartiles (n = 12) were shown.

moment of parasitoid oviposition). In the second and the third treatments, the cold exposure lasted 3 days: the 4–6th or the 7–9th days from the day of parasitoid oviposition respectively. In the fourth treatment, the wasps were subjected to the low temperature conditions during only photophases of the 4–9th days of development, and in the fifth treatment, during only scotophases of the same period of time. In addition, as in the first experiment, the high- and low-temperature controls were conducted.

Statistical treatment

Each card with the progeny generation was considered as an experimental unit. As in most of treatments the data were non-randomly distributed, medians and quartiles were used as descriptive statistic. Then percentages were square root–arcsine transformed and treated with ANOVA and the Tukey honestly significant difference (HSD) test (Lloyd and Ledermann 1984). To pool the data of the first experiment, percentages were replaced by their ranks, separately estimated for each duration of the cold exposure (see 'Dynamics of thermosensitivity: developmental changes' for more details). All calculations were performed using SYSTAT (Chicago, IL, USA).

Results

Rate of development

From fig. 1b it is seen that at 15°C, the egg hatching began between the second and the third days after

the oviposition. At the third day, about a half of larvae eclosed, and at the fourth day, the hatching was almost completed. As expected, additional 3 days long exposure at 10°C caused an increase in the percentage of hatched eggs (fig. 1a). In host eggs transferred to the low temperature conditions at the second and the third days after parasitoid oviposition, correspondingly, ca 25% and ca 100% of *Trichogramma* larvae eclosed during this cold exposure. However, when the parasitized eggs were transferred from 15° to 10° in <2 days after the oviposition, larvae were not recorded even at the end of the cold exposure (fig. 1a).

As for the further pre-imaginal development at 15°C, the darkening of the parasitized host egg and the pre-pupal molt in most of individuals occurred during the 8–10th and during the 10–12th days respectively (fig. 1c and d). First pupae were recorded at the 16th day and at the 20th day practically all of active individuals have pupated (fig. 1e), although 5–10% of (supposedly, diapausing) pre-pupae were recorded up to the 28th day. Adult emergence commenced at the 30th day after parasitoid oviposition (fig. 1f).

Dynamics of thermosensitivity: developmental changes

First, our experiments showed that a 6-day-long cold exposure significantly increased the percentage of diapausing pre-pupae when started during a rather large part of development, although the highest thermosensitivity was recorded during the first 8 days (fig. 2a). Treatments with shorter cold exposures (3 or 2 days long) gave similar results, but the period of a statistically significant thermosensitivity lasted during, respectively, 12 and 10 days (fig. 2b and c). Moreover, even 24 h long cold exposure caused a significant increase in the percentage of diapausing pre-pupae when applied during the first 4 days after the moment of parasitization (fig. 2d). On the other hand, none of these experimental results reached the level of the low-temperature control, where 98% of pre-pupae diapaused.

Second, some decrease in thermosensitivity was recorded when the cold exposure started at the second day after the oviposition, although this effect was not always significant (fig. 2). To clarify the situation, the data for all durations of the cold exposure were pooled together, percentages being replaced by their ranks, separately estimated for each duration. These pooled data (fig. 3) clearly showed that the cold exposures started at the second day

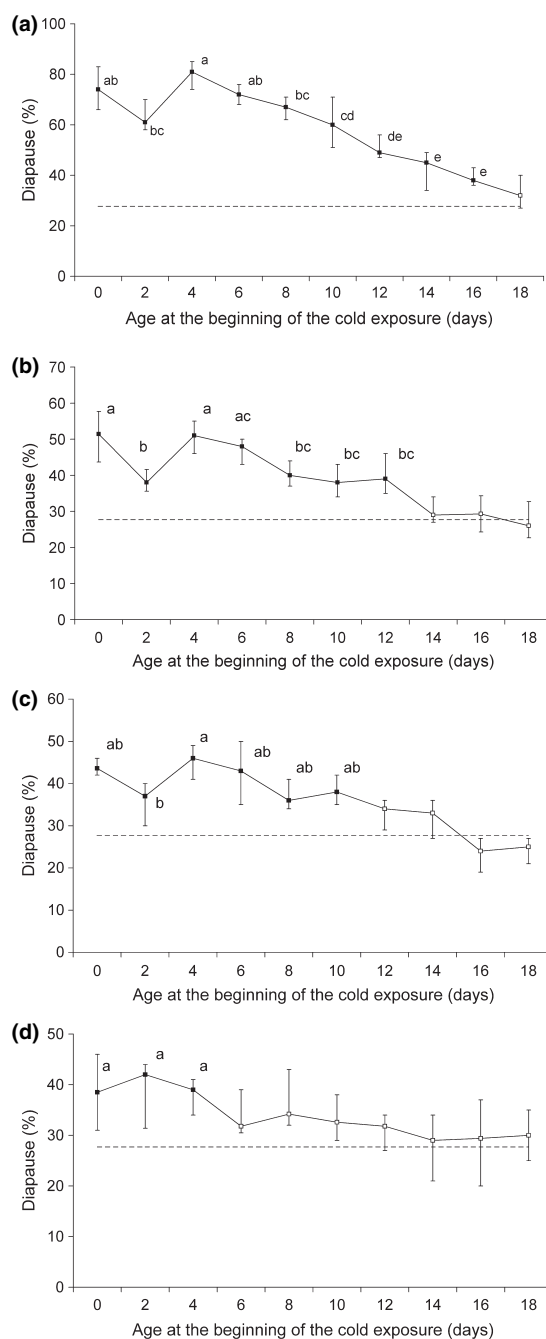


Fig. 2 Developmental changes in thermosensitivity of *T. embryophagum* measured by the percentage of diapausing pre-pupae. Duration of the cold exposure: a – 6 days, b – 3 days, c – 2 days, d – 1 day. For experimental treatments, medians and quartiles ($n = 12$) were shown. Horizontal dashed line indicates the median of the high-temperature control ($n = 48$). Symbols accompanied with different letters in the same graph are significantly different at the $P < 0.05$ level (hereafter, the Tukey HSD test of square root–arcsine transformed data was used). Filled symbols indicate significant difference from the high-temperature control.

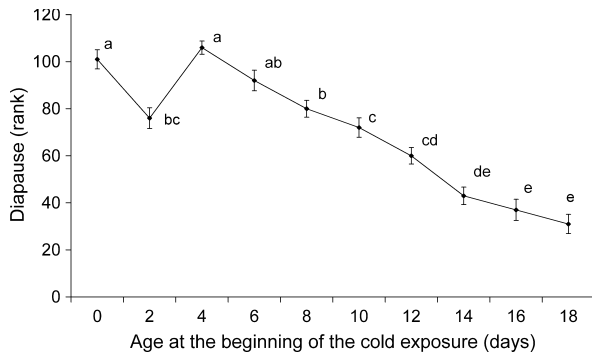


Fig. 3 Developmental changes in thermosensitivity of *T. embryophagum* measured by the ranked percentages of diapausing pre-pupae (pooled data of treatments with different durations of the cold exposure). Mean \pm SE ($n = 48$) are shown. Symbols accompanied with different letters are significantly different at the $P < 0.05$ level (the Tukey HSD test).

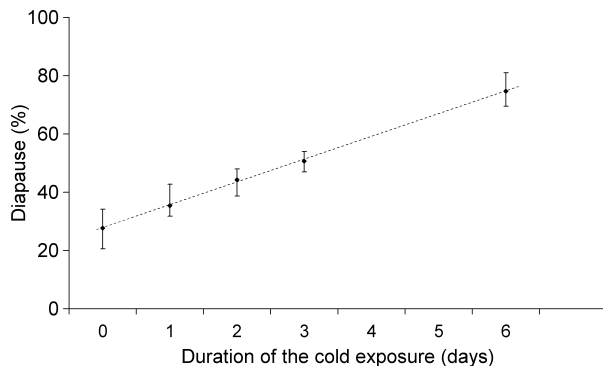


Fig. 4 Influence of the duration of the cold exposure on the percentage of diapausing pre-pupae of *T. embryophagum*. Zero duration means the high-temperature control. Medians and quartiles ($n = 36-48$) were shown. Dashed line indicates the linear regression $Y = 7.74X + 28.2$ ($r = 0.89$, $n = 192$ and $P < 0.001$).

after the oviposition caused significantly lower increase in the percentage of diapausing individuals, when compared with the two neighbouring experimental variants (immediately or 4 days after parasitoid oviposition).

Third, a comparison of fig. 2a–d suggests that longer cold exposure started at the same stage of development caused stronger increase in the percentage of diapausing individuals. This tendency is clearly seen from the data for cold exposures of different durations started during the period of maximum thermosensitivity (i.e. immediately after, 4 days, or 6 days after the moment of parasitoid oviposition). Within the range tested, the percentage of diapausing pre-pupae linearly increased with the duration of the cold exposure (fig. 4).

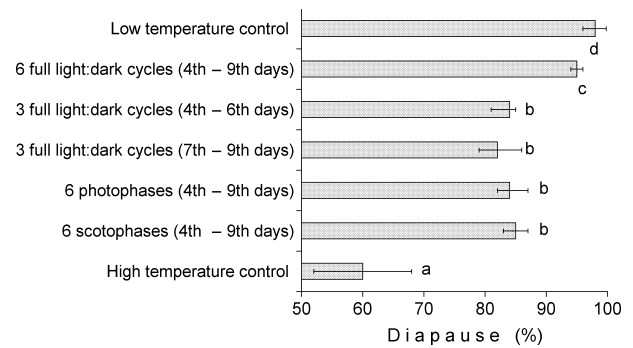


Fig. 5 Percentages of diapausing pre-pupae in two controls and five treatments of the second experiment. On the left, duration and timing of the cold exposure is indicated. Horizontal bars show the percentage of diapausing individuals (medians and quartiles, $n = 18$). Treatments marked with different letters are significantly different at the $P < 0.05$ level (the Tukey HSD test of square root–arcsine transformed data).

Dynamics of thermosensitivity: daily cycles

This experiment did not reveal any significant daily changes in thermosensitivity. *T. embryophagum* larvae subjected to the low temperature during six successive photophases showed practically the same percentage of diapausing individuals as those subjected to the low temperature during six successive scotophases. Moreover, uninterrupted cold exposures of the same total duration (3 days) produced the same effect, while 6-day-long cold exposure caused a further increase in the tendency to diapause, and all abovementioned treatments were significantly different from the ‘low-temperature control’ (fig. 5).

Discussion

Rate of development

Our data on the duration of *T. embryophagum* egg development (ca 3 days at 15°C, i.e. ca 10% of the total time of development) agree well with the results of other authors. In most of *Trichogramma* species studied, the period of time from the parasitoid oviposition to the larval eclosion constituted 10–12%, the larval and the pre-pupal stages constituted, respectively, 15–20% and 20–25%, while the pupal stage occupied about a half of the total duration of the pre-imaginal development (Hutchison et al. 1990; Corrigan et al. 1995; Dahlan and Gordh 1996, 1998; Consoli et al. 1999; Takada et al. 2000; Jarjees and Merritt 2002), which is rather close to our data (fig. 1).

Dynamics of thermosensitivity: developmental changes

First, we conclude that the period of thermosensitivity in *T. embryophagum* is very long: from the early embryo up to the early pre-pupal stage (compare figs 1 and 2). When the cold exposure started immediately after the parasitoid oviposition and lasted up to 3 days, *Trichogramma* eggs had still not hatched even at the end of the cold exposure (fig. 1a), but showed relatively high thermosensitivity (fig. 2b–d). On the other hand, at the 16th day of development, when all larvae had molted to the pre-pupal stage and even first pupae were already recorded (fig. 1d and e), a 6-day-long cold exposure still significantly increased the percentage of the diapausing individuals (fig. 2a). The most detailed of the previous studies conducted with the other *Trichogramma* species (Ma and Chen 2006) gave similar results, although these authors did not test the embryo stage.

Note that the end of the high sensitivity period (ca the eighth day of development, see fig. 3) closely coincided with the mass darkening of the host eggs and the beginning of molting to the pre-pupal stage (fig. 1c and d) suggesting that the egg and larva were the most thermosensitive stages. As for the sharp decrease in thermosensitivity recorded at the second day of development (figs 2 and 3), it was never observed before. Just at this period, the egg hatching occurred (fig. 1b) suggesting that this decrease may be explained by the gap between the embryonic and larval sensitivity periods.

The sensitivity period markedly increased with the duration of the cold exposure (compare fig. 2a–c). This suggests that the loss of thermosensitivity is not a qualitative, but a quantitative process that may be considered as an increase of the threshold level. The higher the threshold, the stronger stimulus is needed to cause the reaction.

Second, we conclude that our first working hypothesis was experimentally confirmed: the minimum duration of the cold exposure causing detectable increase in the proportion of diapausing individuals in *T. embryophagum* was as short as 1 day, similarly to the photoperiodic reaction, where in some species even a single 'long' or 'short' photoperiod is sufficient to produce a measurable effect (Tauber et al. 1986; Zaslavski 1988; Saunders 2002).

Note that the minimum significantly effective duration of the cold exposure estimated by other researchers was much longer: 20 days (Garcia et al. 2002) and 15 days (Ma and Chen 2006). The striking disagreement between our data and the results

of the earlier studies could be possibly explained by the difference in the methods. In the cited works, insects were subjected to diapause-inducing temperature conditions (ca 10°C) against the background of very low (3°C) or very high (26°C) temperature which both were shown to be strongly diapause averting (Ma and Chen 2006). In our study, we used another method (recommended by e.g. Tauber et al. 1986): insects were reared under the 'neutral' temperature of 15°C and transferred for a particular time to the diapause-inducing conditions of 10°C. This method was shown to be more sensitive and, in addition, it seems to be much closer to the natural temperature fluctuations. The latter is particularly important considering that *Trichogramma* species are used not only for inundative releases, but also for inoculative biological control (Smith 1996; Hoffmann et al. 2002; Wright et al. 2002; Yong and Hoffmann 2006) and constitute an important component of 'natural biological control' (Gururaj et al. 2001).

Third, our experiments supported the hypothesis that, similarly to the L : D cycles, the cold exposures could be accumulated. In the first experiment, the proportion of diapaused individuals linearly increased with the number of days spent under the low temperature conditions (fig. 4). A similar effect was described e.g. for *Locustana pardalina* Walker (Acrididae), but in this case the percentage of egg diapause depended linearly on the duration of the high temperature exposure (Matthée 1978).

Moreover, even 12 h long exposures were rather accurately summated, so that six 12 h long exposures and three 24 h long exposures caused practically the same effect (fig. 5). Hence, in natural conditions even occasional short-term cold periods could be accumulated by pre-imaginal stages of *Trichogramma* species and could finally induce a diapause. Similarly, e.g. in the aphid *Megoura viciae* Buckton, the cumulative effect of several long photoperiods was proportional to the total number given, regardless of whether they were placed consecutively or separated by a varying number of short days (Lees 1973).

Thus, by analogy with the photoperiodic reaction, the 'critical' or 'required' day number, i.e. the period of time needed to produce a 50% response (Saunders 2002) could be estimated. In our case, considering that in the 'high-temperature control' (15°C) ca 30% of pre-pupae diapaused (fig. 2), while the 'low-temperature control' (10°C) resulted in almost 100% diapause, it would be more correctly to use 60–70% as the threshold level. This yielded the 'required day number' of 4–5 days (fig. 4), which is

rather close to the results obtained in certain photoperiodic experiments (Lees 1973; Zaslavski 1988; Vaz Nunes and Hardie 1999; Saunders 2002).

Dynamics of thermosensitivity: absence of daily cycles

First, note the difference in percentage of diapause between 'high temperature controls' in two experiments conducted with different *Trichogramma* generations (compare figs 2 and 5), similar variations were often recorded in laboratory lines (Zaslavski and Umarova 1990; Reznik et al. 2002). As for experimental treatments, a series of six 12 h long cold exposures induced the pre-pupal diapause regardless of whether the low temperature coincided with the scotophase or with the photophase. Moreover, uninterrupted 72 h long cold exposure caused practically the same effect (fig. 5). Thus, our second working hypothesis was refuted by the experimental data: the low temperature promoted the diapause induction independently of the L : D cycle.

It is generally accepted that the role of the night (scotophase) temperature in the diapause induction is much more important than that of the day (photophase) temperature (Tauber et al. 1986; Zaslavski 1988; Saunders 2002). This tendency was demonstrated in many insect species including parasitoids (e.g. Brown and Phillips 1991), although there are a few exceptions to this rule (Eizaguirre et al. 1994). Evidently, *T. embryophagum* (and, possibly, other *Trichogramma* species) represent another exception.

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