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Cold storage of *Trichogramma nerudai* using an acclimation period

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Abstract The availability of suitable storage methods for parasitoids is a valuable tool in biological control programs. Studies were conducted to investigate the effects of cold storage with acclimation period on the quality of *Trichogramma nerudai* Pintureau and Gerding. Prepupae were stored 50, 75 and 100 days at 5 °C with a previous acclimation period of 10 or 20 days at 12 °C. It was possible to arrest the development of *T. nerudai*. All the treatments with acclimation period of ten days had emergence values under 10% that were not useful to establish a cold storage protocol. Twenty days of acclimation had a positive impact on cold storage tolerance at 50 and 75 days. The adult emergence, the emergence time, the sex ratio, the parasitism and the progeny quality have not been affected by the storage of *T. nerudai* using an acclimation period of 20 days and until 50 days under cold temperature.

Keywords Acclimation · Biological control · Cold storage · Hymenoptera · Parasitoids · Trichogrammatidae

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Introduction

The life cycle of many insects can be interrupted by a period of dormancy that results in protecting a vulnerable stage from adverse environmental conditions. In most of parasitoids, dormancy could be developed by either quiescence or diapause (Rossi 1993; Boivin 1994). Quiescence is halted or slowed development as a direct response to no-suitable environmental conditions. The development resuming occurs when favourable conditions return (Saunders 1982; Chapman 1998). On the other hand, diapause is a period of developmental arrest combined with adaptive physiological changes and, in this case, the return of favorable conditions does not mean that development is always resumed (Chapman 1998). In addition, diapause is often induced by cues predicting the unfavourable season. This is not a direct response to unfavourable conditions. These cues serve as a trigger to synchronize the life cycle along with environmental conditions (Saunders 1982; Chapman 1998).

One of the major problems to the successful implementation of biological control programs is the difficulty and cost of rearing beneficial insects, since most of them have a relatively short shelf-life, and they must be produced shortly before they are used (Glenister and Hoffmann 1998). The availability of suitable storage methods of biological control agents is a valuable tool in order to maintain a high production level, to provide flexibility and efficiency

in mass production, to allow synchronized field releases of natural enemies during the critical stages of pest outbreaks, to facilitate insect transport for long distances and to maintain insect colonies under laboratory conditions for research purpose (van Lenteren and Tommasini 2002; Colinet and Boivin 2011).

Parasitoids can be stored at low temperatures within a range of 0–15 °C, although under these moderately low temperatures most species had some level of mortality (van Lenteren and Tommasini 2002). Temperature is one of the main abiotic factors affecting survival during cold storage. The design of cold storage protocols often involves the use of specific temperatures and the determination of the lower threshold temperature for insect development (Leopold 2007). In general, for most of the species, there is a temperature at or above which permanent detrimental effects will not occur (Colinet and Boivin 2011). The optimal temperature for cold storage should be based upon the relative balance between the need to reduce metabolic rate and/or development and the risk of chilling injuries accumulation.

Egg parasitoids of the family Trichogrammatidae have been successfully employed worldwide to control many Lepidoptera pests via inundative biological control (Smith 1996; Desneux et al. 2010; Tabone et al. 2010). Two main cold storage methods, with and without previous diapause induction, have been used in mass rearing of *Trichogramma* spp. (Tezze and Botto 2004; Colinet and Boivin 2011; Reznik 2011; Lessard and Boivin 2013). Storage techniques with diapause induction require a gradual or rapid acclimation, with a long-term (days or weeks) or a short term (minutes to hours) pre-exposure to sublethal low temperature, respectively (Chown and Nicolson 2004; Chown and Terblanche 2006; Anguilletta 2009). In parasitoids, acclimation has a positive impact on cold storage tolerance (Marwan and Tawfiq 2006; Luczynski et al. 2007). However, some cases with detrimental effects of acclimation have also been recorded (Bernardo et al. 2008).

Both the host and the parasitoid could be affected by prolonged exposure to cold resulting in lethal or sublethal consequences (Hance et al. 2007). The consumption of energy reserves, particularly lipids, during cold exposure is thus expected to affect the fitness cost on either survival or reproduction (Colinet et al. 2006). The reduction of fitness-related traits in surviving individuals can be observed immediately after storage, later in development or even in the next generations (Colinet and Boivin 2011). Although there are many studies concerning storage of Trichogrammatidae species (Jalali and Singh 1992; Krishnamoorthy and Mani 1999), it is important to focus on the amenability to cold store of certain particular species since not all of them are able to be cold stored.

Trichogramma nerudai Pintureau and Gerding (Hymenoptera: Trichogrammatidae) is a neotropical haplodiploid species. Because of the wide range of hosts that *T. nerudai* is able to parasitize (various agricultural and forest lepidopteran pests like *Rhyacionia buoliana* Denis and Schiffermüller, *Tuta absoluta* Meyrick, *Plutella xylostella* Linnaeus and *Cydia pomonella* Linnaeus), this species is considered an important natural enemy (Estay and Bruna 2002; Botto et al. 2004; Tezze and Botto 2004; Desneux et al. 2010). For this reason, it is important to develop a storage method for this species to determine a period of storage that allows us to synchronize the availability of the parasitoid with the presence of each target pest species in the field. The aim of the present study was to investigate the effects of cold storage with acclimation period on the quality of *T. nerudai*.

Materials and methods

Laboratory colony

The research was carried out at the Insectario de Investigaciones para Lucha Biológica (IILB), IMYZA-INTA, Castelar, Buenos Aires, Argentina. *T. nerudai* used in these studies was obtained from a colony maintained in this laboratory. This parasitoid was produced since 1997 on UV-irradiated eggs of the Angoumois grain moth *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae), in accordance with standardized production procedures (modified from Hassan 1997). It was reared in a controlled environment at 25 ± 5 °C, 40–65% RH and L:D 12:12. Since the beginning of the rearing, we have been performing quality controls in accordance with standardized procedures of the IOBC for other *Trichogramma* spp. (van Lenteren 2003). The parasitoids have demonstrated an appropriate performance in field trials against economic important pests of apple and tomato, *C. pomonella* and *T. absoluta*, respectively

(Botto et al. 2005; Cáceres et al. 2007; López and Argerich 2007; Hernández et al. 2015).

Cold storage treatments

Cold storage experiments were carried out on the prepupal stage of *T. nerudai*. To obtain parasitoid prepupae, host eggs of *S. cerealella* stuck in a sheet of cardboard (aprox. 12 cm × 17 cm) were placed within the parasitoid rearing glass jar (height 20 cm, diameter 10 cm) to be parasitized by newly emerged females, during 24 h. These parasitized eggs were kept under standard rearing conditions. Once the parasitoids reached the prepupal stage (checked by dissections), a piece of the cardboard holding ca. 600 *S. cerealella* parasitized eggs was placed in a glass tube (height 10 cm, diameter 1.5 cm) and stoppered with a piece of film. Ten tubes (replicates) were randomly allocated to each cold storage treatments.

There were nine treatments: a control treatment with no acclimation/cold storage periods (maintained at standard rearing conditions) and eight treatments corresponding to the combination of two acclimation periods (10 and 20 days at 12 ± 0.5 °C, 75 ± 5% RH and complete darkness) and four cold storage periods (0, 50, 75 and 100 days at 5.4 ± 0.1 °C, 75 ± 5% RH and complete darkness). Acclimation was provided by a Instrumentalia MGC-350HP growth chamber. The cool conditions were given by a CONVIRON E7 bioclimatic chamber. Once the acclimation/storage period was over, each treated group was transferred to standard conditions.

The effect of acclimation/cold storage on the quality of the parasitoid was evaluated by measuring the following variables in each replicate: adult emergence (emerged adults per parasitized pupae), adult emergence time (period in days between the end of the acclimation/cold storage and the adult parasitoid emergence), sex ratio (percent females) and parasitism (number of parasitized eggs by the females along their lives/number of total eggs). To evaluate this last variable, eggs of *S. cerealella* stuck in a sheet of cardboard (ca. 600) were exposed to all the females emerged, in each acclimation/cold storage replicate until the death of parental adults. Then, the piece of cardboard containing the exposed eggs was transferred to a new glass tube until the emergence of adult parasitoids (F1). For offspring generation, adult emergence, adult emergence time and sex ratio were recorded. The adult emergence (both in parental and offspring generation) and parasitism (in parental generation) were registered in two circular subsamples (diameter 0.5 cm) in each of the replicates for each treatment combination.

Statistical analysis

Adult emergence (F0 and F1), sex ratio (F0 and F1) and parasitism (F0) were analyzed as binary responses using logistic regression (logit link function) with the cold storage treatment (combination of the levels of the two factors (acclimation period and cold storage time) and the control) as categorical factor. Model fit was checked by deviance and means were separated using LSD Fisher multiple comparison test (Di Rienzo et al. 2016). Data in results and figures are presented as mean + SE. The level of significance of the parameters considered was 0. 05.

Results

The adult emergence of stored parasitoids decreased significantly with an increase in the duration of storage but for a longer acclimation period the values of emergence were higher than for a shorter period (χ^2 = 13,727.93, d.f. = 8, $P < 0.0001$). More than 60% of parasitoids emerged after being exposed to 12 °C for 20 days and subsequently stored at 5.4 °C for 50 and 75 days while emergence was less than 20% when the pupae were acclimated for 20 days and cold stored during 100 days or acclimated during ten days, independently of the subsequent cold storage time (Fig. 1a).

All the individuals in each treatment emerged at the same time. A descriptive analysis showed a general trend in which the parasitoids subjected to an acclimation period of ten days took longer to emerge compared to the control. Meanwhile, the time of emergence of those with an acclimation of 20 days was similar to the control (Fig. 1b).

The acclimation and storage periods studied affected significantly the sex ratio F0 (χ^2 = 52.09, d.f. = 8, $P < 0.0001$). Regardless of the time of cold storage, there were more females when the acclimation period was 20 days compared to ten days (Fig. 1c). Besides, for all the treatments, the sex ratio

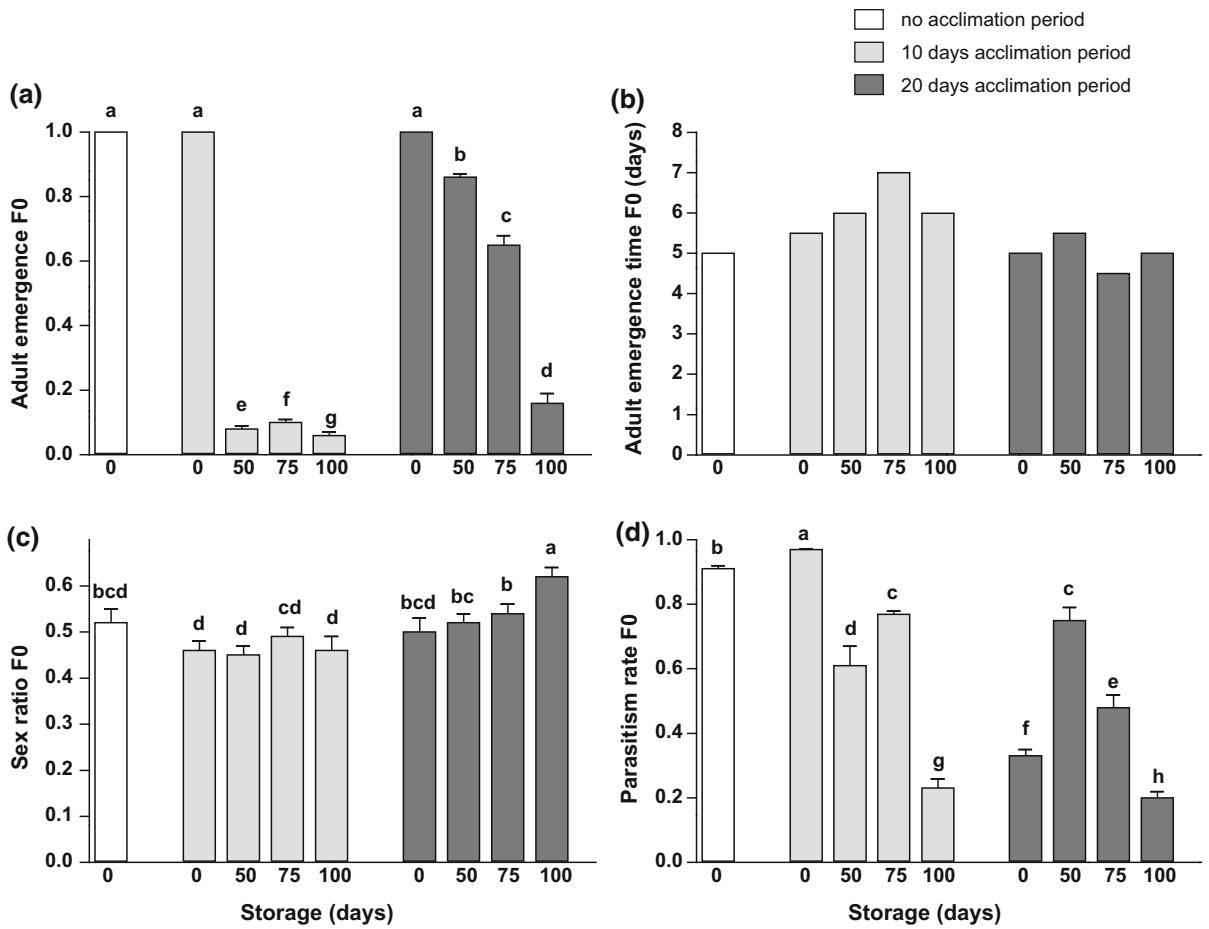

Fig. 1 **a** Percentage of emerged adults F0, **b** adult emergence time F0, **c** sex ratio (percent females) F0 and **d** parasitism rate F0 of *T. nerudai* after nine treatments: a control with no acclimation/cold storage periods and eight treatments corresponding to the combination of two acclimation periods (10 and 20 days at 12 °C) and four cold storage periods (0, 50, 75 and 100 days at 5.4 °C). Different letters show significant differences between treatments ($P < 0.05$). Data are presented as means + SE

was similar to that of the control, except for an acclimation of 20 days with 100 days of storage.

The parasitism rate of the parental generation (F0) was significantly affected by the treatment applied ($\chi^2 = 4768.70$, d.f. = 8, $P < 0.0001$). In general, it was observed that for both acclimation periods the higher the time of cold storage the lower the parasitism rate. With the exception of the treatment in which the prepupae were exposed to an acclimation period of ten days without cold storage, the parasitism was lower than in the control treatment. However, it was higher than 0.60 when the parasitoids were stored during 50 days independently of the acclimation period or during 75 days after ten days of acclimation (Fig. 1d).

The proportion of adults emerged in the progeny (F1) was affected by the acclimation period and the cold storage ($\chi^2 = 3047.96$, d.f. = 8, $P < 0.0001$). This proportion was higher than 90% for all treatments except for 100 days of storage after both acclimation periods (15–18%; Fig. 2a).

As in the case of the F0, all the individuals F1 in each treatment emerged at the same time. When the acclimation of their parents lasted ten days, the emergence time of adult offspring was similar (0 and 50 days of cold storage) or lesser (75 and 100 days) than in the control. When the acclimation lasted 20 days this time was similar (0 and 75 days of cold storage) or higher (50 and 100) than in the control (Fig. 2b).

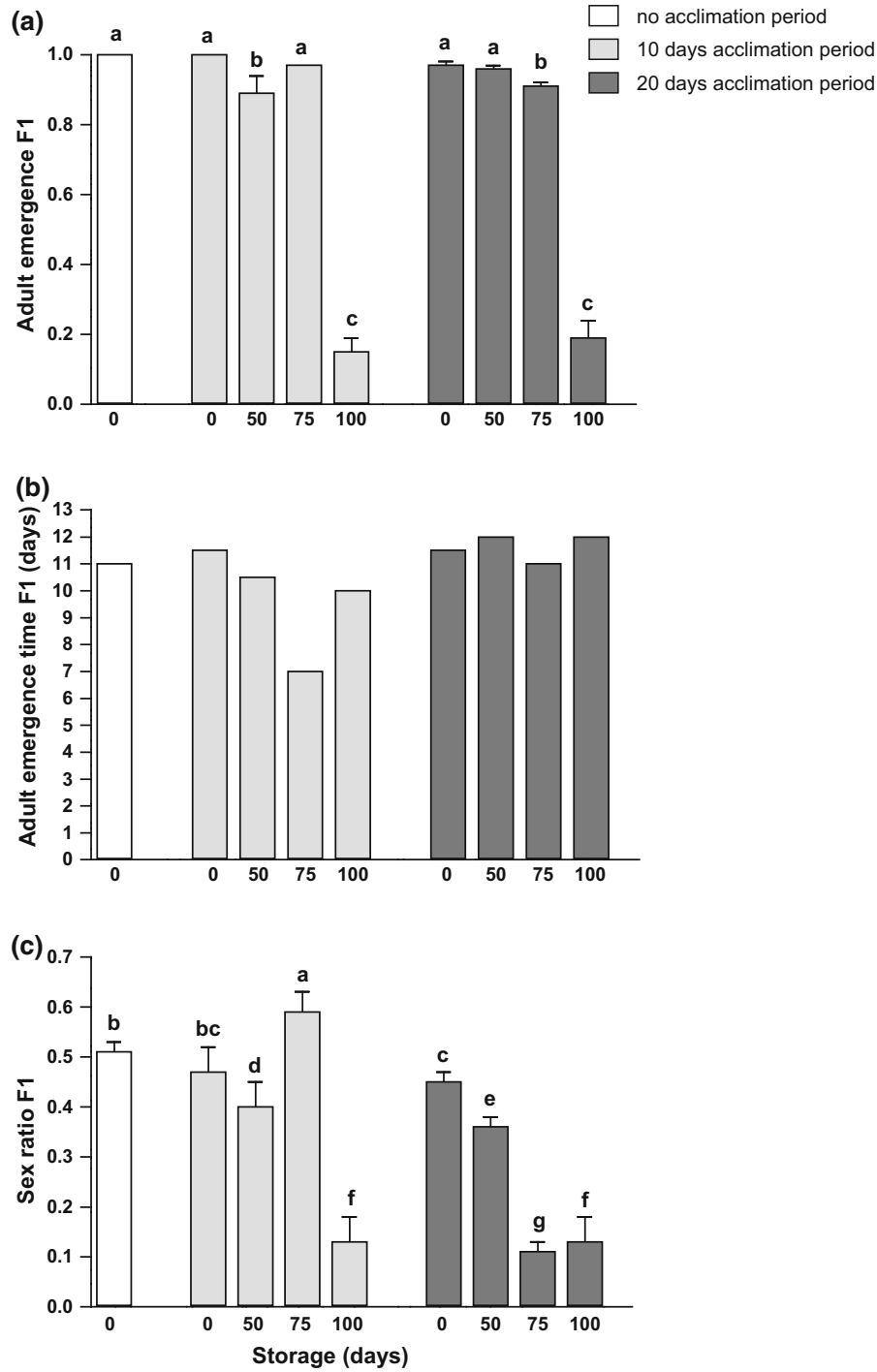

Fig. 2 **a** Percentage of emerged adults F1, **b** adult emergence time F1 and **c** sex ratio (percent females) F1 of *T. nerudai* after nine treatments: a control with no acclimation/cold storage periods and eight treatments corresponding to the combination of two acclimation periods (10 and 20 days at 12 °C) and four cold storage periods (0, 50, 75 and 100 days at 5.4 °C). Different letters show significant differences between treatments ($P < 0.05$). Data are presented as means + SE

Sex ratio F1 was significantly affected by the acclimation and storage treatments applied to the parental generation ($\chi^2 = 928.28$, d.f. = 8, $P < 0.0001$). In general, we observed that for both acclimation periods the higher the time of cold storage the lesser the sex ratio (Fig. 2c).

Discussion

Parasitoids that develop at suboptimal temperature usually suffer major fitness costs (Hance et al. 2007; Colinet and Boivin 2011). In addition to chilling injuries, parasitoids stored as prepupae may not have sufficient energy resources to complete their development and/or to emerge (Colinet and Boivin 2011). However, when *T. nerudai* prepupae were stored at the acclimation temperature after either 10 or 20 days they showed emergences comparable to the control (100%), that indicated high survival at that temperature. The lower developmental temperature threshold for several *Trichogramma* spp. closely related to *T. nerudai*, is between 9 and 11 °C (Foerster and Foerster 2009; Lessard and Boivin 2013). Thus *T. nerudai* probably has a similar lower development threshold value and this is approximately the acclimation temperature used at the present study.

Prolonged cold exposure to 5.4 °C was detrimental to *T. nerudai* survival. This effect was also observed in other *Trichogramma* species like *Trichogramma carverae* Oatman and Pinto, *Trichogramma brassicae* Bezdenko and *Trichogramma funiculatum* Carver (Rundle and Hoffmann 2003; Rundle et al. 2004), *Trichogramma cacoeciae* Marchal and *Trichogramma evanescens* West. (Rossi 1993) and *Trichogramma cordubensis* Vargas and Cabello (Ventura García et al. 2002).

In our study, the negative effect of the cold storage duration on the *T. nerudai* emergence was pronounced when individuals were preconditioned with ten days of acclimation (5–9% emergence). In these cases, the emergence of parasitoids did not reach 10% in any period of cold storage. However, when the parasitoids were preconditioned at 12 °C for 20 days, there was an increased parasitoid survival in comparison to the acclimation for ten days. This indicated that the acclimation for 20 days had a positive impact on cold storage tolerance. This impact is lost when the cold storage is very prolonged (100 days). Similar results were also observed in *T. cordubensis* that showed a higher percentage of adult emergence after a cold storage temperature of 3 °C with a previous acclimation at 10 °C during for longer exposure periods (30 and 40 days) compared to shorter periods (10 and 20 days) (Ventura García et al. 2002). Moreover, *T. nerudai* exposed to cold storage at 4 °C without an acclimation period (Tezze and Botto 2004) showed emergence values higher than those obtained for *T. nerudai* exposed to an acclimation of ten days but lower than those observed for an acclimation of 20 days in our study. However, it is important to remark that Tezze and Botto (2004) stored *T. nerudai* parasitoids in the stage of pupa (probably more resistant to cold temperatures) (Jalali and Singh 1992; Nakama and Foerster 2001).

We found that parasitoids subjected to an acclimation of ten days delayed the development in all the studied periods of cold storage and, compared to the control, more time was necessary to complete their development. The additional time required to emerge post-storage period compared to the control could be due to some effect of the cool temperature on insect physiology (Pandey and Johnson 2005). It could produce a retarded growth rate or selective survival of individuals that have slower growth rate (Colinet and Boivin 2011). In the present study, since only a few individuals (< 10%) emerged with the acclimation for ten days and cold storage for 50, 75 and 100 days, it might be possible that only a few parasitoids that had a slower growth rate could have been selected. Parasitoids treated with an acclimation of 20 days showed a time to emerged post-storage period similar to the control. Comparable results were found in other genus of parasitoids (Colinet and Boivin 2011; Pandey and Johnson 2005; Chen and Leopold 2007; Chen et al. 2008; Bernardo et al. 2008; Lessard and Boivin 2013) and in other *Trichogramma* species (Jalali and Singh 1992; Ventura García et al. 2002).

According to our results, quiescence was observed with acclimation (both 10 and 20 days) because the response occurred immediately after the temperature changed and the time to emerge after the storage was similar to the control (acclimation of 20 days) or higher for no more than two days (acclimation of ten days). Quiescence was observed in prepupal stage in other *Trichogramma* species like *T. carverae*, *T. brassicae* and *T. funiculatum* (Rundle and Hoffmann 2003; Rundle et al. 2004), *T. cacoeciae* Marchal and *T. evanescens* (Rossi 1993) and *T. cordubensis* Vargas and Cabello (Ventura García et al. 2002).

On the other hand, a characteristic feature of the facultative winter diapause is that the period of sensitivity to environmental cues and the diapause per se usually take place at different life cycle stages (Saulich and Volkovich 2004; Reznik 2011).

Concerning *Trichogramma* thermal response, the highest thermosensitivity is characteristic of embryos and young larvae. For these periods, even occasional long periods of decrease in temperature to 10–12 °C could increase significantly the portion of diapausing prepupae (Reznik et al. 2008; Reznik 2011). It could be the reason why we observed quiescence and not diapause in our assays with *T. nerudai*, since they were maintained at 25 °C as embryo and larva and exposed to cold storage as prepupa.

There were not significant differences in the sex ratio F0 comparing the control with the treatments with ten and 20 days of acclimatation and 20 days of acclimation with 50 and 75 days of cold storage. This result shows that under these conditions there was not differential pupal mortality related to sex due to effect of cold storage. This is important in biological control programs where the number of released females is critical (Hassan et al. 1990). As in our study Tezze and Botto (2004) found not differences in the sex ratio of *T. nerudai*. However, we observed that the acclimation for 20 days and cold storage for 100 days produced a higher sex ratio. This was probably due to a prolonged exposure to cold that affected the emergence (16%) and probably generated a differential mortality between sexes. However, this appraisal was done on a very low number of individuals. On the other hand, a higher sex ratio would have no impact on the storage of parasitoids for biological control purposes (Leopold 1998).

Respect to the parasitism rate F0 in *T. nerudai*, it decreased significantly with the increase of the duration of cold storage for both acclimation periods. Similar results were also observed in *T. carverae* (Rundle et al. 2004) and in *Trichogramma achaeae* Nagaraja, *Trichogramma chilonis* Ishii and *Trichogramma japonicum* Asmead (Jalali and Singh 1992). On the other hand, Tezze and Botto (2004) did not study the effect of cold storage on the parasitism in their experiments with *T. nerudai.* Thus, the incorporation of this biological trait is of great relevance in studies of cold storage since this is a valuable tool in biocontrol programs. Moreover, the proportion of parasitized hosts in relation to the total number of offered hosts contrary to fecundity (which refers to an absolute number of eggs produced) indicates the quality of the parasitoids treated with cold storage. Thus, in the parasitism rate is considered host recognition, acceptation, and discrimination (Colinet and Boivin 2011).

It is important to mention that, for unclear reasons, in our study parasitism was unexpectedly higher in treated parasitoids with 20 days of acclimation and 50 days of cold storage (76%) than for those with 20 days of acclimation and without cold storage (33%). One possible explanation for this result is that this particular combination of treatments (long acclimation and intermediate cold storage) could produce a reduction of host defenses that can improve the performance of the parasitoid development. On the other hand, this acclimation period followed by more than 50 days of storage could affect survival of the parasitized eggs leading to a low parasitism (Bueno et al. 2009; Lessard and Boivin 2013). A result similar to this was observed by Rundle et al. (2004) in *T. carverae* where the number of parasitized eggs was higher in the parasitoids stored at 4 °C for one week than in parasitoids maintained at 25 °C.

From a practical point of view, only the treatment with 20 days of acclimation and 50 days of cold storage is useful to establish a cold storage protocol for *T. nerudai*. At these conditions the parasitoids had high emergence (aprox. 90%) and parasitism (aprox. 75%), and the adult time emergence and sex ratio were similar to the controls. According to our results, Jalali and Singh (1992), Zhu et al. (1992) and Ventura García et al. (2002) concluded that other *Trichogramma* sp. were tolerant to cold storage up to 50–60 days, because after this period their biological traits were affected. Despite Tezze and Botto (2004) did no studied parasitism, they found *T. nerudai* tolerant to cold storage within the same range. In our work this tolerance could be improved through a prior acclimation leaving the total storage time to 70 days.

Cold storage of *T. nerudai* for 50 days and acclimation for 20 days at the studied temperatures could be useful for applying in biological control programmes since we found the proportion of adult emerged F1 was similar to the control and the adults F1 delayed their development one day. Ultimately, the success of cold storage protocols depends on their impact on field performance. We are planning to study the influence of cold storage conditions on the field success of *T. nerudai.*

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