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Evaluation of cold storage techniques to improve mass rearing of *Cleruchoides noackae* from *Thaumastocoris peregrinus* eggs

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Research Paper

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Abstract

The egg parasitoid *Cleruchoides noackae* Lin & Huber, 2007 (Hymenoptera: Mymaridae) is originated from Australia and the main biological control agent of *Thaumastocoris peregrinus* Carpenter & Dellapé, 2006 (Hemiptera: Thaumastocoridae) on *Eucalyptus* L'Hér (Myrtaceae). Companies that grow *Eucalyptus* are in need of a mass rearing protocol to increase the number of individuals produced and improve the quality of this parasitoid. The aim of this study was to define a protocol for mass rearing *C. noackae* in *T. peregrinus* eggs, based in the evaluations of the key biological attributes of this parasitoid in the parental and F1 generations, after the cold storage of the parasitised host eggs. Two methods were tested as *C. noackae* rearing protocols. In the first, parasitised eggs of *T. peregrinus* by *C. noackae* were cold stored for 7 days after being left in a climatic chamber at 24 ± 2°C, 60 ± 10% RH and a photoperiod of 12:12 (light:dark) h (standard environmental conditions) for 3, 6, 9 or 12 days. In the second, *T. peregrinus* eggs parasitised by *C. noackae* were maintained in a climatic chamber under standard environmental conditions for 6 days, after which these eggs were cold-stored for 0 (control), 7, 14 or 21 days. Parasitism (%), and the development period (parasitism to adult) and female proportion (%) of *C. noackae* were evaluated. Based on the results (parental generation: parasitism, around 45%; F1 generation: parasitism, around 55%; development period, around 16 days; female proportion, around 60%), eggs should be stored at 5°C on the sixth day after parasitism by *C. noackae* and maintained at this temperature for 7 days. The cold storage of *T. peregrinus* eggs, after parasitism, can be included in the mass rearing protocols of the parasitoid *C. noackae*.

Introduction

The bronze bug, *Thaumastocoris peregrinus* Carpinteiro & Dellapé, 2006 (Hemiptera: Thaumastocoridae) is native to Australia and specific to *Eucalyptus* L'Hér (Myrtaceae) plants (do Nascimento-Machado *et al.*, 2019). This insect is a major pest of *Eucalyptus scoparia* Maiden and *Eucalyptus nicholii* Maiden & Blakely in urban areas and of *Eucalyptus* spp. in commercial plantations in Sydney, Australia (Noack *et al.*, 2009; Lo *et al.*, 2019). The first report of *T. peregrinus* as a *Eucalyptus* pest outside Australia was in South Africa in 2003 and, later, in about 30 other countries (Jacobs and Neser, 2005; Montagu *et al.*, 2020; Mutitu *et al.*, 2020). The first outbreak of *T. peregrinus* in Brazil was reported in 2008 with about 245,000 hectares of commercial *Eucalyptus* plantations infested (Wilcken *et al.*, 2010) and caused by the haplotype A of this species, introduced into South America from Australia (Machado *et al.*, 2020). This infestation led to an estimated wood production loss of 10–15% at a value of 330 million USD between 2010 and 2015 (Wilcken *et al.*, 2019). Damage to *Eucalyptus*, by nymphs and adults of *T. peregrinus*, is due to the suction of sap, mainly from the palisade parenchyma cells (Santadino *et al.*, 2017). The injuries by this insect to plants include changes in the leaf colour, starting with yellowing (chlorosis), silvering and browning of the infested leaves, with losses of the photosynthetic area. This can cause the death of young trees in cases of single severe infestations or successive ones at low or intermediate levels (Jacobs and Neser, 2005; Nadel *et al.*, 2010; Nadel and Noack, 2012).

Cleruchoides noackae Lin & Huber, 2007 (Hymenoptera: Mymaridae) is a solitary egg parasitoid originated from Australia that is reared in the laboratory by forest companies around the world and released under different environmental conditions to parasitise *T. peregrinus* in *Eucalyptus* spp. plantations (Lin *et al.*, 2007; Nadel *et al.*, 2012; Barbosa *et al.*, 2018).

Cleruchoides moackae was introduced to Brazil after a quarantine procedure in 2012 from individuals imported from Australia (Barbosa *et al.*, 2018). The establishment of mass rearing is a key obstacle to the use of *C. noackae* as a biological control agent, but it can be achieved after biological studies (Barbosa *et al.*, 2019; Becchi *et al.*, 2023). The longevity of *C. noackae* females and males increased by 2.4 and 1.7 days, respectively, when fed honey solutions. The highest offspring production of 7.7 wasps per female was achieved with this food, but the proportion of males was slightly higher than females. The average parasitism was 32.2%. The development period from parasitism to adult emergence was 15.7 days in host eggs with a maximum age of 5 days (Mutitu *et al.*, 2013).

Cleruchoides noackae became established in *Eucalyptus* spp. plantations in Brazil shortly after its first release in 2012 with parasitism reaching 60% from recovered *T. peregrinus* eggs from the field (Barbosa *et al.*, 2017). The *C. noackae* rearing in the laboratory in Brazil, for biological control programmes with augmentative field releases, demands a large number of *T. peregrinus* eggs due to the monophagous nature of *C. noackae* – the lack of alternative hosts – and a cold storage protocol to maintain the viability of the parasitised eggs of this host (de Souza *et al.*, 2016). In addition, *T. peregrinus* feeds, exclusively, on *Eucalyptus* shoots (Soliman *et al.*, 2012; Barbosa *et al.*, 2019) increasing the importance of cold storage protocols for parasitised eggs of this insect to produce *C. noackae* with quality and quantity (Colinet and Boivin, 2011; Spínola-Filho *et al.*, 2014). The release of *C. noackae* to manage *T. peregrinus* is compatible with the application of entomopathogens, such as *Beauveria bassiana* (Bals.-Criv.) Vuill. (1912) and *Metarhizium anisopliae* (Metchnikoff) Sorokin (1883) (Hypocreales: Cordycipitaceae), but this parasitoid dies in areas applied with the insecticide bifenthrin (Domingues *et al.*, 2020).

The storage of immature parasitoids in host eggs at low temperatures can increase the production of biological control agents with low rearing costs (Colinet and Boivin, 2011; Spínola-Filho *et al.*, 2014). The parasitised host storage can reduce production costs by different ways, including (a) the parasitised eggs produced in numbers above weekly releasing targets can be cold stored for further releases; (b) impossibility to carry out releasing activities because of operational problems such as damage on drones and vehicles, poor road accessibility, lack of labours, rain, etc. – parasitised eggs can be cold stored for further releases; (c) workers can focus on parasitoid production during certain days and in other activities in the remaining ones (Sinulingga *et al.*, 2021). Lower temperatures reduce metabolic activities and induce dormancy in insects, which later resume their development when removed from these conditions (Rodrigues and Sampaio, 2011). However, extreme storage temperatures can reduce survival, body size, number of offspring and cause physiological and morphological changes in parasitoids (Pitcher *et al.*, 2002; Levie *et al.*, 2005). The establishment of parasitoid mass rearing depends on the quality of the progeny, per generation, after the storage of their immatures in host eggs (Barbosa *et al.*, 2018).

Cold storage of *T. peregrinus* eggs, parasitised by *C. noackae*, could increase the survival period and production of this biological control agent, which should be studied for rearing protocols for these insects (Barbosa *et al.*, 2019). Our hypothesis is that the cold storage of *T. peregrinus* eggs, after parasitism by *C. noackae*, could improve the mass rearing of this parasitoid. The objectives of this work were to evaluate the potential of two cold storage techniques to improve mass rearing of *C. noackae* in eggs of *T. peregrinus*. The potential of the rearing techniques was identified by assessing the biological parameters of parasitism, development and female proportion of individuals from the parental and F1 generations of *C. noackae* in eggs of *T. peregrinus* cold stored after parasitism.

Materials and methods

Rearing of T. peregrinus

Thaumastocoris peregrinus eggs were obtained from a colony at the Entomology Laboratory at Embrapa Florestas in Colombo, state of Paraná, Brazil and maintained in a climatic chamber at 23 ± 2°C, 50 ± 10% RH and a photoperiod of 12:12 (light:dark) h. Nymphs obtained and adults were kept together in bouquets of *Eucalyptus benthamii* Maidan & Cambage shoots fixed by a 15 cm wide foam strip in the same chamber used to maintain its eggs. The petiole of these shoots was kept in a 500 mL glass Erlenmeyer® flask filled with distilled water. A bouquet of fresh shoots was placed near the dried ones after approximately 2 days to stimulate the migration of *T. peregrinus* to the new shoots to feed (Barbosa *et al.*, 2016). Paper towel strips (Scott®; state of São Paulo, Brazil) were attached to some shoots as a substrate for oviposition and collected daily. These strips were, once removed, kept in the same chamber used for its rearing until establishing the experiments. The eggs of *T. peregrinus*, used in the experiments, were younger than 24 h.

Rearing of C. noackae

Cleruchoides noackae was obtained from the colony of the Entomology Laboratory at Embrapa Florestas in Colombo, which started in 2012 with the introduction of individuals, imported from Australia and quarantined in Brazil (Barbosa *et al.*, 2018). This parasitoid was reared by inserting paper towel strips laid with *T. peregrinus* eggs in the polystyrene flasks (7 cm long × 3 cm diameter) and its adults fed with a 50% aqueous honey solution in drops placed on filter paper strips (Whatman®, n° 2; state of Minas Gerais, Brazil) in a climatic chamber at 24 ± 2°C, 60 ± 10% RH and photoperiod of 12:12 (light:dark) h. This temperature is adequate for the reproduction and population increase of *C. noackae* parasitising eggs of *T. peregrinus* (Becchi *et al.*, 2023). Ten eggs, which were less than 24 h, were exposed per *C. noackae* couple for 24 h and placed in new flasks kept in the same chamber used for rearing adults. The adults of *C. noackae*, used in the experiments, were younger than 24 h and fed with 50% aqueous honey solution supplied in filter paper strips (0.5 cm wide × 5 cm long).

Experiment 1: optimal age of C. noackae *immatures for cold storage in* T. peregrinus *eggs*

Paper towel strips each with 10 eggs of *T. peregrinus* per replication, with 15 replications per treatment, were exposed to parasitism per couple of *C. noackae* (parental generation) in a polystyrene flask (7 cm long × 3 cm diameter) for 24 h in a climatic chamber at 24 ± 2°C, 60 ± 10% RH and a photoperiod of 12:12 (light:dark) h (standard environmental condition). After the parasitism period, the exposed eggs were removed from the flasks and divided into groups each with 15 strips. These eggs were left under the standard environmental conditions for 3

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(treatment 2), 6 (treatment 3), 9 (treatment 4) or 12 days (treatment 5), resulting in eggs with immature parasitoids of different ages inside them. These periods were selected based on the development period of this parasitoid in the standard rearing conditions (Barbosa *et al.*, 2019). After these periods, the eggs were cold stored in a climatic chamber at 5 ± 2°C, 60% RH and darkness (standard cold temperature used in insect rearing; Barbosa *et al.*, 2018). Seven days after storage, the eggs were returned to the climatic chamber where they were kept under the same standard environmental conditions until the emergence of their adults or hosts. The cold storage period of 7 days was utilised because it is the median embryonic period in the standard environmental conditions (Barbosa *et al.*, 2019). Eggs in the control were, permanently, kept under the standard environmental conditions (no cold storage; treatment 1).

Fifteen couples, from the F1 progeny of *C. noackae*, obtained from each treatment were individualised in polystyrene flasks (7 cm long × 3 cm diameter) each with 10 eggs of *T. peregrinus*. Parasitism was allowed for 24 h and, after this period, the parasitoids were removed and the eggs of *T. peregrinus* kept in a climatic chamber under the standard environmental conditions.

The percentage of parasitism [(number of parasitoids emerged + number of parasitoids retained in the egg) ÷ total number of eggs × 100] was evaluated in the parental generation and in the F1 progeny of *C. noackae*, except for those that emerged from immature cold stored after 12 days, with a reduced number of adults. The period of development (parasitism to emergence, in days, excluding the storage period) and the female proportion [(number of ♀ ÷ number of ♂ + ♀) × 100] of the F1 generation of *C. noackae* were evaluated for the insects emerged from immature cold stored at different ages. The eggs with the parasitoid retained inside were distinguished by observing them under a stereomicroscope (Nikon® P-DSL32; Tokyo, Japan).

Experiment 2: cold storage of C. noackae *immatures in* T. peregrinus *eggs*

Twenty *T. peregrinus* eggs per polystyrene flask (60 flasks in the experiment; 7 cm long × 3 cm diameter) were exposed to a couple of *C. noackae* (parental generation) for 24 h in a climatic chamber under the standard environmental conditions. After parasitoid exposition, the eggs were remained under the same environmental conditions for 6 days (defined in the first experiment). After this period, the *T. peregrinus* eggs were divided into four groups each with 300 eggs and cold stored in a climatic chamber at 5 ± 2°C, 60% RH and darkness for 0 (control – treatment 1), 7 (treatment 2), 14 (treatment 3) or 21 days (treatment 4) and, after storage, returned to the climatic chamber, where they remained under the standard environmental conditions with the eggs of the control. Fifteen replications were performed per treatment.

A couple of *C. noackae* from the F1 progeny was placed per polystyrene flask (7 cm long × 3 cm diameter) with 10 eggs of *T. peregrinus* and 15 replications. After exposition, the parasitoids were removed and the eggs kept in a climatic chamber under the standard environmental conditions.

The percentage of parasitism in the parental generation and the F1 progeny of *C. noackae* was evaluated, except for 14 and 21 days (in the F1 progeny) where a low number of parasitoids emerged. The development period (parasitism to emergence, in days, excluding storage time) and the female proportion of the F1 progeny of *C. noackae* were evaluated for insects that emerged after different periods of cold storage.

Statistical analysis

The parasitism and female proportion data of the parasitoid *C. noackae* were analysed using the generalised linear models function of binomial error distribution (*logit* link function) and considering overdispersion (Hinde and Demétrio, 1998). The development period of the parasitoid was analysed with the Gaussian distribution. The quality of the fitted model was based on the half probability plot with a simulation envelope, using the *hnp* function of the *hnp* package (Moral *et al.*, 2017). The data of treatments were submitted to the Tukey's range test ($P < 0.05$) (Tukey, 1949) with the *glht* function of the *multcomp* package (Hothorn *et al.*, 2008). Statistical analysis was performed using R language software, version 3.3.2 (R Core Team, 2016).

Results

Experiment 1

Optimal age of C. noackae *immatures for cold storage in* T. peregrinus *eggs*

The percentages of parasitism, of the parental generation of *C. noackae*, in *T. peregrinus* eggs cold stored for 7 days and after being left under standard environmental conditions for 3 (28.66 ± 7.29%), 6 (39.33 ± 7.46%) or 9 (30.00 ± 7.74%) days were not significantly different to that in eggs not cold stored (49.33 ± 3.71%) and it was higher than in those kept under standard environmental conditions for 12 days before refrigeration (12.00 ± 4.70%) ($F_{4,70} = 4.5299$, $P = 0.0026$) (fig. 1a).

The parasitism of *T. peregrinus* eggs by adults of the F1 progeny of *C. noackae* emerged from eggs without storage (control) (60.00 ± 5.57%) or cold stored for 7 days and left under standard environmental conditions for 3 (43.00 ± 3.95%) and 6 (54.00 ± 7.63%) days was greater than with 9 days (7.00 ± 4.95%) ($F_{3,36} = 13.498$, $P < 0.0001$) (fig. 1b).

The development period (parasitism to adult) ($F_{4,45} = 1.1464$, $P = 0.3471$) (fig. 2a) and the female proportion ($F_{4,45} = 1.9664$, $P = 0.1159$) (fig. 2b) of the F1 progeny of *C. noackae*, originated from *T. peregrinus* eggs cold stored for 7 days after being left under standard environmental conditions for different periods, was not significantly different to the control (without refrigeration).

Experiment 2

Cold storage of C. noackae *immatures in* T. peregrinus *eggs*

The parasitism, by individuals of the parental generation of *C. noackae*, in eggs of *T. peregrinus* in the control (without cold storage) (55.50 ± 4.00%) or cold stored for 7 days (50.50 ± 5.10%) after being left under standard environmental conditions for 6 days was higher than in those cold stored for 14 (8.50 ± 2.21%) and 21 days (7.50 ± 2.89%) ($F_{3,76} = 39.57$, $P < 0.0001$) (fig. 3).

The parasitism of *T. peregrinus* eggs, by the F1 progeny of *C. noackae*, emerged from eggs cold stored for 7 days (54.50 ± 4.78%) and after being kept under standard environmental conditions for 6 days or not cold stored (57.50 ± 5.01%) was not significantly different ($F_{1,38} = 0.187$, $P = 0.6679$).

The development period (parasitism to adult) ($F_{1,36} = 0.0662$, $P = 0.7984$) and the female proportion ($F_{1,36} = 0.3907$, $P = 0.5359$) of the F1 progeny of *C. noackae*, originated from eggs cold stored

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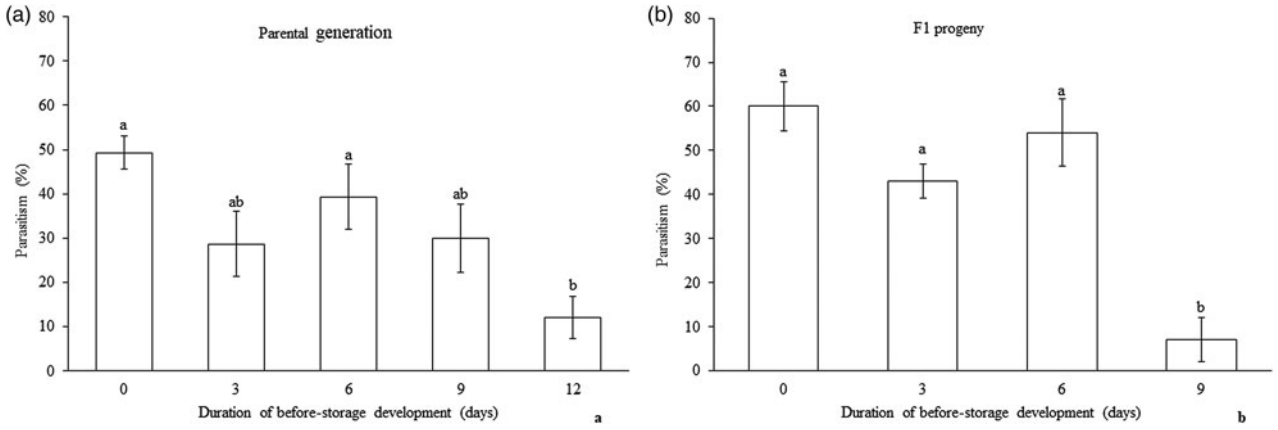

Figure 1. Parasitism (mean ± standard error) of *Cleruchoides noackae* (Hymenoptera: Mymaridae) on eggs of *Thaumastocoris peregrinus* (Hemiptera: Thaumastocoridae) developed in standard conditions for 3, 6, 9 or 12 days before the standard cold storage, (a) parental generation, (b) F1 progeny. Bars with different letters, per figure, are statistically different by the Tukey's range test ($P < 0.05$).

for 7 days or without storage after being left under standard environmental conditions for 7 days, was not significantly different.

Discussion

The first experiment assessed the percentages of parasitism, from the parental generation of *C. noackae*, in *T. peregrinus* eggs cold stored for 7 days after being left under standard environmental conditions for 3, 6 or 9 days. These periods were not significantly different to the unstored control and greater than that in eggs kept under standard environmental conditions before refrigeration for 12 days. These results were better with the cold storage of immatures in less advanced development of *C. noackae*. Immature parasitoids at initial development – smaller body size – are better protected in eggs from hosts with a greater fluid volume. In addition to protection, this liquid regulates temperature, is a source of nutrients and facilitates the maturation of parasitoids (Colinet and Boivin, 2011). Storage of parasitoids at more advanced development in host eggs for prolonged periods can induce damage due to nutritional changes (reduction in lipid body mass) and sterilise males or reduce reproductive potential (Colinet *et al.*, 2006; Colinet and Hance, 2009).

The decrease in the parasitism rate of the parental generation of *C. noackae* with the cold storage of its immatures at 12 days old agrees with the lower survival and number of offspring and the greater impact on the sterility of males and females of *Aphidius rhopalosiphi* Stefani-Peres, 1902 (Hymenoptera: Braconidae) mummies at 3 and 1 day old when stored at −5°C for 10 days. Three- and 1-day-old *A. rhopalosiphi* parasitoids are in post-metamorphosis and pre-pupal stages, respectively. The post-metamorphosis stage demands a higher nutritional quality of the host egg for transformation into an adult (Levie *et al.*, 2005), which did not happen with the parasitised eggs of *T. peregrinus* by *C. noackae* after storage.

The higher parasitism of *T. peregrinus* eggs by adults of the F1 progeny of *C. noackae* obtained from eggs without storage

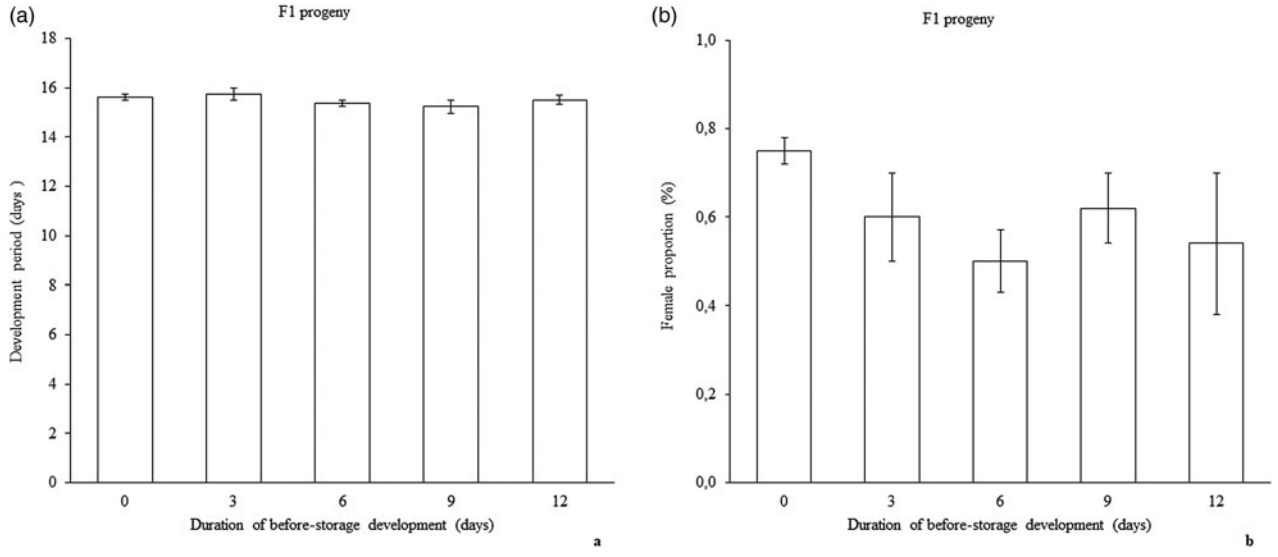

Figure 2. (a) Development (days) and (b) female proportion (%) (mean ± standard error) of the F1 progeny of *Cleruchoides noackae* (Hymenoptera: Mymaridae) in eggs of *Thaumastocoris peregrinus* (Hemiptera: Thaumastocoridae), originated from those not stored (control) or cold stored for 7 days after developed in standard conditions for 3, 6, 9 or 12 days. Means between treatments, per figure, are not statistically different by the Tukey's range test ($P > 0.05$).

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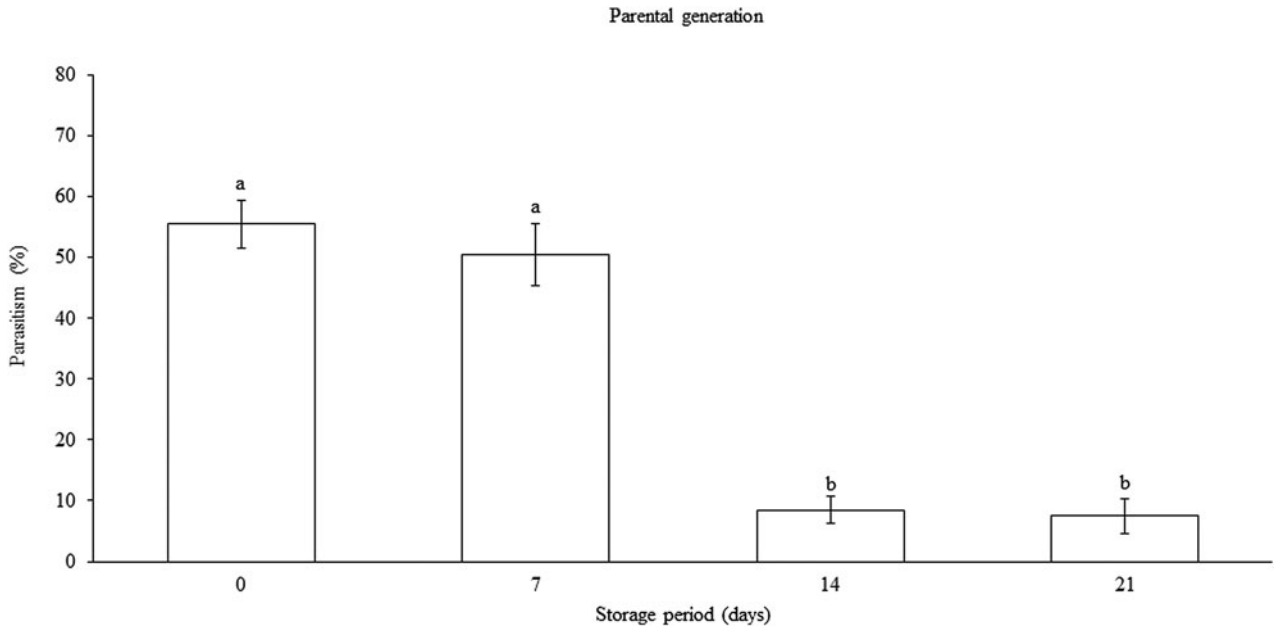

Figure 3. Parasitism (mean ± standard error) by the parental generation of *Cleruchoides noackae* (Hymenoptera: Mymaridae) on eggs of *Thaumastocoris peregrinus* (Hemiptera: Thaumastocoridae) cold stored for different periods (including 0 days in the control) after developed in standard conditions. Bars with different letters are statistically different by the Tukey's range test ($P < 0.05$).

(control) or cold stored for 7 days and left under standard environmental conditions for 3 and 6 days than with 9 days determines the optimal age of immatures of this parasitoid to start cold storage.

The lower parasitism of the F1 progeny of *C. noackae* in *T. peregrinus* eggs kept under standard environmental conditions for 9 days before being cold stored for 7 days may be due to the production of weaker adults. A similar result was reported for parasitism after cold storage for periods of up to 6 days of *Trichogrammatoidea bactrae* Nagaraja, 1979 (Hymenoptera: Trichogrammatidae) for parental and F1 generations. A more drastic reduction was seen when more advanced larval stages of *T. bactrae* were stored at 4°C in *Sitotroga cerealella* (Olivier, 1789) (Lepidoptera: Gelechiidae) eggs (Mohamed and El-Heneidy, 2020). The not significantly different development period and female proportion of the F1 progeny of *C. noackae* in *T. peregrinus* eggs, from those cold stored for 7 days after being left under standard environmental conditions for different periods and in the control, shows that the cold storage reduced parasitism of F1 adults, but not the development and female proportion of this biological control agent. Female-biased sex ratio shows that cold storage is harmless in causing defects in haplodiploid sex determination. An increase of males in the progeny could indicate genetic defects or alterations by endosymbiotic microorganisms (Kageyama *et al.*, 2021).

The higher parasitism, by individuals of the parental generation of *C. noackae*, in *T. peregrinus* eggs in the control (without cold storage) or cold stored for 7 days after being left under standard environmental conditions for 6 days, than those cold stored for 14 and 21 days, confirms the report that cold storage is more suitable for immature *C. noackae* at initial development (Barbosa *et al.*, 2018, 2019). The period of cold storage of host eggs can determine the survival of the parasitoid and its developmental and reproductive attributes, as reported for the reduction of parasitism and survival, the number of females and the quality of adults of subsequent generations of *Psix saccharicola* (Mani, 1941) (Hymenoptera: Platygastridae) parasitising eggs of *Acrosternum arabicum* Wagner, 1959 (Hemiptera: Pentatomidae) after being stored at 4°C for periods longer than 120 days, due to lower nutritional quality of the host eggs (Forouzan *et al.*, 2018).

The parasitism by individuals of the F1 progeny of *C. noackae*, not significantly different in eggs of *T. peregrinus* offered to the parental generation of this parasitoid in the control (without cold storage) or cold stored for 7 days is important, because the storage of these eggs for a week facilitates release programmes of this parasitoid (Barbosa *et al.*, 2017, 2018). This indicates the innocuity of cold storage for a maximum period of 7 days to parasitoids, including their organs associated with reproduction.

The not significantly difference in developmental period and female proportion of the F1 progeny of *C. noackae*, emerged from eggs cold stored for 7 days or without such storage, shows female-biased (Becchi *et al.*, 2020) and the limit period for storage of these eggs without impact on *T. peregrinus* (Mutitu *et al.*, 2013; Barbosa *et al.*, 2017).

The need of large numbers of hosts at appropriate times is a problem for mass rearing parasitoids. Refrigeration of parasitised eggs of *T. peregrinus* while maintaining their viability for the mass rearing of *C. noackae* can improve the production of this parasitoid for biological control programmes in *Eucalyptus* plantations. The protocol for cold storage of *T. peregrinus* eggs after parasitism by *C. noackae* should be followed in biological control programmes with augmentative releases of this parasitoid, especially, in forest companies with certification or that wishing to obtain certification from the Forest Stewardship Council (FSC). *Thaumastocoris peregrinus* eggs, parasitised by *C. noackae*, can be kept for a period lower than 9 days under standard environmental conditions before being stored for 7 days at 5°C, facilitating plans for releasing this parasitoid in the field. This period did not affect the main attributes of the parental generation of this parasitoid, allowing the transport of parasitised eggs to areas of release far from production laboratories. The storage of 9-day

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eggs, however, decreased successful parasitism in the F1 generation. This time should not be utilised to rear F1 generations.

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Competing interests. Wagner de Souza Tavares is employed at PT. Itci Hutani Manunggal (IHM).

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