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Cold-Storage of *Piezodorus guildinii* (Hemiptera: Pentatomidae) Eggs for Rearing *Telenomus podisi* (Hymenoptera: Platygastridae)

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ABSTRACT

***Piezodorus guildinii* (Hemiptera: Pentatomidae) is an important soybean pest, and one of its main natural enemies is *Telenomus podisi* (Hymenoptera: Platygastridae). Rearing of the parasitoid is constrained by the hosts' egg quality, which deteriorates after few generations in laboratory, therefore, cold-stored host eggs utilization could be a useful tool for augmentative biological control. Thus, the objective was to evaluate the quality of *P. guildinii* cold-stored eggs, on the performance of parental and F_1 generation of *T. podisi*. Host eggs 24 hour old were stored at -18°C for one (N= 53), two (N= 28) or three months (N= 29), and approximately 40 host eggs were offered to a *T. podisi* female per treatment, for 48 hours. The control treatment consisted of 24-hour-old non-frozen host eggs, obtained and kept at 24°C (N= 55). Parental generation parasitism and progeny´s emergence on frozen eggs was significantly lower than on non-frozen eggs, even for the shorter storage period. Male proportion and preimaginal development time of the progeny increased with freezing period. However, parasitism proportion caused by adults of F_1, and emergence, male proportion, and preimaginal development time of F_2 were not affected. Although the performance of *T. podisi* on frozen *P. guildinii* eggs was significantly lower than on nonfrozen ones, host eggs storage for up to two months allowed obtaining a parasitism rate of 40% with a high emergence rate. This could be helpful enough to maintain mass rearings, mainly during the host hibernation period, and to enhance field parasitism when host is scarce.**

Keywords: Host storage, Mass-rearing, Parasitoids.

INTRODUCTION

Phytophagous hemipterans of the family Pentatomidae, commonly known as stink bugs, are important soybean pests. Among them, *Piezodorus guildinii* Westwood (Hemiptera: Pentatomidae) is the one that most affects the quality and viability of seeds and causes greater leaf retention when compared to other common bugs like *Nezara viridula* L. and *Euschistus heros* (F.) (Hemiptera: Pentatomidae) (Husch *et al.*, 2014). *Piezodorus guildinii* is a Neotropical multivoltine species, highly mobile and more difficult to control as it is less susceptible than other bugs to labeled insecticides such as pyrethroids and organophosphates (Temple *et al.*, 2013a). It is one of the most predominant stink bugs and a serious pest of soybean in the south Nearctic and whole Neotropical regions (Castiglioni *et al.*, 2010; Corrêa-Ferreira, 2008; Massoni and Frana, 2006; Temple *et al.*, 2013b). In the last two decades *P. guildinii* relative abundance has been increasing significantly and has currently become the most important species in Buenos Aires and other provinces of Argentina (Cingolani, 2012; Cingolani *et al.*, 2014).

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In the Neotropical region the most common egg parasitoid species of *P. guildinii* are *Telenomus podisi* (Ashmead), *Trissolcus urichi* (Crawford) and *Trissolcus basalis* (Wollaston) (Hymenoptera: Platygastridae) (Castiglioni *et al.*, 2010; Cingolani *et al.*, 2014; Corrêa-Ferreira, 2005; Molinari, 2005). Over 20 species of platygastrid wasps have been used for biological control of stink bugs in several countries (Luck, 1981; van Lenteren and Bueno, 2003). *Trissolcus basalis* releases mainly to control *N. viridula* have been made in Argentina (Crouzel and Saini, 1983), Brazil (Corrêa-Ferreira and Moscardi, 1995; Corrêa-Ferreira and Moscardi, 1996), Italy (Colazza and Bin, 1995) and the United States (Hoffmann *et al.*, 1991), and to control this stink bug and *Agonoscelis glitters* F. (Hemiptera: Pentatomidae) in Australia (Clarke, 1990). *Telenomus podisi* has been used to control *E. heros*, *P. guildinii* and *N. viridula* in soybean organic production fields in Brazil (Sujii *et al.*, 2002).

A frequent difficulty when implementing augmentative biological control is to obtain large numbers of control agents of appropriate quality, at the time they are required (Orr, 1988). Various techniques have been developed to optimize the maintenance of large parasitoids' rearing in the laboratory. Some of them are focused on parasitoids, such as cold storage of parasitized hosts (Bayram *et al*., 2005; Dass and Ram, 1983; Gautam, 1986; Noble, 1937), in vitro development of parasitoids (Shirazi, 2006), and cold storage of pupae or adults of the parasitoid (Bayram *et al*., 2005; Foerster *et al.*, 2004; Foerster and Doetzer, 2006; Gautam, 1986). Other techniques focus on ensuring a constant availability of enough host, such as the development of artificial diets (Fortes *et al.*, 2006; Panizzi *et al.*, 2000), irradiation of host eggs with gamma (γ) rays to increase the time during which they are likely to be parasitized (Nordlund *et al.*, 1983) and cold storage of healthy (not parasitized) hosts (Alim and Lim, 2010; Corrêa-Ferreira, 1998; Doetzer and Foerster, 2013; Favetti *et al.*, 2014; Kivan and Kilic, 2005; Mahmoud and Lim, 2007).

Several intrinsic platygastrids characteristics, such as simplicity of adults' diet, lack of hyperparasitoids and pathogens, good reproductive capacity, and small size (and therefore minimum space requirements) make the rearing of these wasps feasible. However, supporting large colonies of these parasitoids is strongly constrained by the quality of their hosts, which deteriorates after few generations with currently used laboratory techniques (Parra and Cônsoli, 2009). For the specific case of *P. guidinii*, the establishment of large colonies is quite difficult due to the fact that very few individuals, obtained from eggs collected from field, can reach the adult stage. In addition, reproduction begins just after the sixth week of life (15 days after the imaginal molt), only 60 or 70% of the females are fertile (Panizzi and Slansky, 1985), and each of them performs only three to five egg masses of on average of 14 eggs each, during its lifetime. Another important factor is the high nymphal mortality (60%) that is registered with the commonly used rearing techniques (Serra and La Porta, 2001) and the difficulty of maintaining the colony throughout the year, because even under optimal temperature and photoperiod conditions, reproduction is interrupted each year due to hibernation, at least in the geographic region between 30°-35° S latitude (Zerbino *et al.*, 2013).

There is evidence that cold storage of host eggs can have positive, negative or neutral effects on the performance of platygastrids parasitoids (Orr, 1988 and references therein), and no adverse effects were found in F_1 generation (Alim and Lim, 2010; Mahmoud and Lim, 2007). The objective of this study was to evaluate the quality of *P. guildinii* eggs preserved at low temperatures for different storage periods and to assess the parasitism rate, and progeny's emergence, sex ratio and preimaginal development time, of parental and F_1 generations.

MATERIALS AND METHODS

Insects and Rearing Procedures

Colonies of stink bugs and wasps were established from field collected individuals. Adults of *P. guildinii* were fed *Phaseolus vulgaris* (L.) pods replaced every two days, and maintained in cages (15x15x30cm) at 24±1°C, 70±10% RH and 14:10 L:D. Deposited eggs were collected daily, and *T. podisi* was reared on these eggs, and kept in test tubes under the same laboratory conditions with honey as food source. All parasitoids females used in experiments were inexperienced, two-days old and fed ad-libitum.

Cold Storage Treatments

Piezodorus guildinii eggs 24-hour-old were stored at -18°C for one, two, or three months (treatments) wrapped in aluminum foil, following the methodology proposed by Corrêa-Ferreira and Moscardi (1993). After the storage period, eggs were kept at 5°C for two hours and then at 24°C for another two hours for acclimation of stored eggs before being offered to the parasitoids (Albuquerque *et al.*, 2000). On each treatment, a group of approximately 40 host eggs were offered to *T. podisi* females. Fifty-three replicates for one month stored eggs, 28 replicates for two months stored eggs, and 29 replicates for three months stored eggs were performed. In order to avoid pseudoreplication, replicates of each treatment periods were performed starting at different times over the year. The control treatment consisted of 24-hour-old nonfrozen host eggs, obtained and kept at 24°C (N= 55). Eggs were exposed to one female parasitoid for 48 h and then were kept under controlled conditions (24±1°C, 70±5% RH, 14:10 L:D) until emergence of parasitoids. Eggs from which no parasitoid emerged after 12 days i.e. average preimaginal parasitoid development time at 24°C, (Corrêa-Ferreira, 1993) were dissected a week later to check presence of pupa or dead adult parasitoids inside the host.

We evaluated parasitism by parental generation, and emergence, male proportion, and preimaginal development time of F_1 on nonfrozen and frozen eggs. Additionally, we evaluated parasitism by F_1 and emergence, male proportion and preimaginal development time of F_2 (2^{nd} filial generation).

Statistical Analysis

Parasitism rate (number of eggs from which a pupa or an adult parasitoid was observed/total number of offered eggs) and emergence proportion (number of emerged wasps/number of eggs from which a pupa or an adult parasitoid was observed) were compared among treatments, using the Kruskal-Wallis test ($P< 0.05$). We performed the Kruskal-Wallis *H* test only when ANOVA assumptions were not met, even after applying transformations to our data. We counted the total number of females and males developed in each treatment. We used χ^2 of 2×4 contingency table to analyze if the number of adults of both sexes among treatments differed from what could be expected by chance, and subdivided contingency tables were analyzed to find significant differences between pairs of treatments (Zar, 1996). We also compared female progeny's preimaginal development time (from the beginning of the experiment to emergence) among treatments, using the Kruskal-Wallis *H* test.

In addition, the effect of one month host egg refrigeration on F_1 generation of the parasitoid was assessed. Forty *T. podisi* females randomly selected among the group of adult parasitoids emerged from one month stored host eggs, and 55 *T. podisi* females selected among group of adult

parasitoids emerged from nonfrozen eggs were provided with approximately 40 nonfrozen eggs of *P. guildinii* for 48 h in controlled conditions (24±1°C, 70±5% RH, 14 hours photophase). Proportion parasitism, emergence rate, and preimaginal development time were compared using ANOVA. Sex ratio was analyzed using χ^2 of a 2×2 contingency table.

RESULTS

No host nymphal development was recorded from frozen eggs. Parasitism rate by parental generation was significantly lower on frozen host eggs than on nonfrozen ones ($H_{(3,\ N=165)}$= 71.060; P< 0.001), and the lowest value was recorded for the storage period of three months (Figure 1). Similarly, the progeny of the parental generation had a significantly lower emergence proportion than those developed in nonfrozen eggs ($H_{(3,\ N=135)}$= 36.299, P< 0.001) (Figure 1). Also, the parental generation progeny´s sex ratio differed among treatments (χ^2= 28.925, df= 3, P= 0.05), and only in the three months frozen eggs, the sex ratio was different, as suggested by a subdivided contingency table considering three months frozen eggs versus all other treatments (χ^2= 23.865, df= 2, P= 0.05) (Table 1).

Preimaginal development time of the female progeny of the parental generation was significantly longer in wasps developed from one month frozen eggs ($H_{(3,\ N=107)}$= 26.796, P< 0.001) (Table 1). In every case, males emerged the day before females' emergence date.

For the F_1, parasitism rate caused by *T. podisi* females emerged from one month frozen eggs was similar to that of females emerging from nonfrozen eggs (F= 1.720, df= 1, P= 0.193) (Figure 2). Emergence rate (F= 0.74, df= 1, P= 0.393) (Figure 2), sex ratio (χ^2= 3.059, df= 1, P= 0.08) and preimaginal development time of the progeny of F_1 generation (i.e. F_2) (F= 0.24, df= 1, P= 0.625) (Table 2) were also similar between storage treatments.

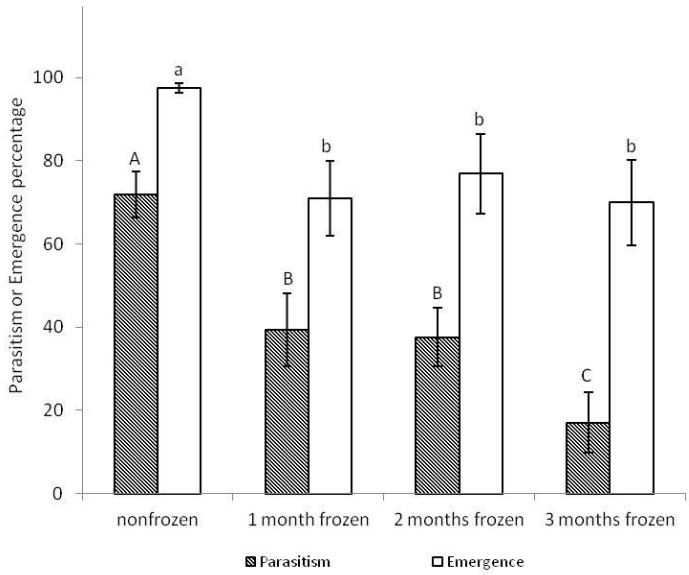

Figure 1. Parasitism and progeny's emergence proportion of the parental generation of *Telenomus podisi*, from nonfrozen or frozen *Piezodorus guildinii* eggs. Bars indicate the confidence interval at 95%. Different letters indicate significant differences (by Kruskal-Wallis) on parasitism (upper-case letters) and emergence (lower-case letters) among non-frozen and frozen eggs.

Table 1. Progeny's male proportion [Male/(Male+Female)] and females' preimaginal development time (days) (mean±SE) of the parental generation of *T. podisi* developed in nonfrozen and frozen eggs of *P. guildinii* stored during one, two or three months.[a]

Treatment	Progeny's male proportion	Females' preimaginal development time
Nonfrozen	0.126±0.008a (n= 55)	12.982±0.057a (n= 55)
1 month frozen	0.091±0.011a (n=32)	15.125±0.206b (n= 32)
2 months frozen	0.084±0.014a (n= 26)	13.125±0.189a (n= 16)
3 months frozen	0.250±0.015b (n= 16)	12.500±0.147a (n= 4)

[a] Numbers in each column followed by a different letter are significantly different (P< 0.05) for contingency table (progeny's male proportion) or Kruskal-Wallis.

Table 2. Progeny's male proportion [Male/(Male+Female)] and females' preimaginal development time (days) (mean±SE) of the parental generation and F_1 (1st filial generation) of *T. podisi* developed in non-frozen *P. guildinii* eggs. [a]

Parasitoid generation	Progeny's male proportion[ns]	Females' preimaginal development time[ns]
Parental	0.132±0.007 (df= 54)	12.982±;0.057 (df= 54)
F_1	0.106±0.007 (df= 39)	13.075±0.083 (df= 39)

[a] ns: Non significantly different (P< 0.05) for contingency table.

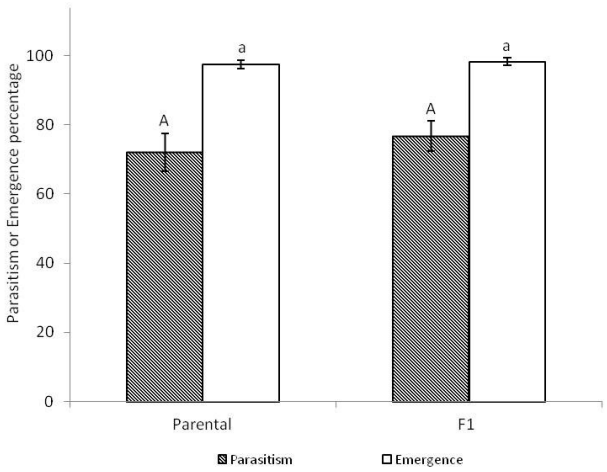

Figure 2. Parasitism and progeny's emergence proportion of the parental generation and F_1 (1st filial generation) of *Telenomus podisi* in nonfrozen *Piezodorus guildinii* eggs. Bars indicate the confidence interval at 95%.

DISCUSSION

Mass-rearings of platygastrid parasitoids demand large numbers of host eggs, as it has not been possible to maintain platygastrids species colonies without rearing the host up to the present (Orr, 1988 and references therein; Shirazi, 2006; Strand *et al.*, 1988; Volkoff *et al.*, 1992). Besides, stink bugs rearing are constrained, as they have a poorer development on artificial diets (Fortes *et al.*, 2006; Silva and Panizzi, 2008). In this sense, cold storage of host eggs seems to be interesting to obtain large numbers of control agents of appropriate quality, at the time they are required.

Our results showed that *P. guildinii* eggs storage for up to two months allowed obtaining an acceptable parasitism rate by *T. podisi* with high emergence. Parasitism on frozen host eggs was significantly lower than that observed on nonfrozen eggs, and had halved when eggs were preserved in freezer for one month. Other authors have shown that parasitism and progeny's emergence from cold stored hosts were variable with different temperatures and storage periods (Corrêa-Ferreira and Moscardi, 1993; Orr, 1988). *Nezara viridula* eggs preserved at temperatures between -2 and -10°C remained viable for parasitism by *T. basalis* for less than 60 days (Albuquerque *et al.*, 2000) while if the preservation of eggs occurred in freezer, as in this study, parasitoid development was not affected until a storage period of 150 days. In contrast, *Dolycoris baccarum* (L.) (Hemiptera: Pentatomidae) freezer stored eggs have remained viable for *Trissolcus nigripedius* Ashmead (Hymenoptera: Platygastridae) for only 8 days (Mahmoud and Lim, 2007).

Telenomus podisi progeny's emergence was lower from frozen eggs than from nonfrozen ones. We observed many parasitoid pupae and adults dead inside the host egg. This may indicate a decrease in egg quality as a resource, which could have diminished larval development and/or the capacity of developed adults to emerge. On the other hand, freezing and subsequent thawing could also have provoked changes in the hardness of the host egg chorion (Kivan and Kilic, 2005), making it more difficult for the wasps to cut it with their mouth parts for emergence.

The proportion of female wasps developed in frozen host eggs stored for three months was the lowest. As usually happens with hymenopteran parasitoids, *T. podisi* have a haplo-diploid sex-determination system, female being able to choose the sex of their progeny by controlling fertilization (Flanders, 1946). Distortions of sex ratio may come from a modification of the proportion of fertilized eggs oviposited i.e. primary sex ratio, or from a differential mortality between sexes i.e. secondary sex ratio. The sex ratio found in this study would indicate that an increase in storage time of frozen eggs made host quality to diminish, pushing females to oviposit more male progeny.

The longer preimaginal development time needed by wasps to complete their development on one month frozen eggs also denotes a decline in hosts' quality. In this study, only a low number of adults could emerge from eggs stored for longer period (three months), and 20% of the parasitoids died at pupal stage. This may suggest that the low quality of this host would have prevented wasps to complete their development.

Parasitism rate by the F_1 and emergence of F_2 from host eggs frozen for one month was not affected. Similarly, other authors did not find negative effects of pentatomids' eggs cold storage on platygastrids' second generation (Alim and Lim, 2010; Mahmoud and Lim, 2007).

Although the performance of *T. podisi* on frozen *P. guildinii* eggs was significantly lower than on non-frozen eggs, storage for up to two months allowed obtaining a parasitism rate of 40% with an emergence rate of almost 80%. This could be helpful enough to maintain mass rearing mainly during the host hibernation period, or to face adverse circumstances that can cause unsynchronization of host-parasitoids rearing.

An important technique for maintaining suitable host for longer periods is the storage of host eggs in liquid nitrogen. For example,

Graphosoma lineatum (L.) (Hemiptera: Pentatomidae) eggs remained suitable for *Trissolcus simoni* (Mayr) (Hymenoptera: Platygastridae) for as long as five years (Gennadiev and Khlistovskii, 1980). Even though, when a short storage period is sufficient, the use of frozen host eggs could be a cheaper and handier technique than maintaining hosts in liquid nitrogen.

Interestingly, cold-stored host eggs could also be useful to build up the population of natural enemies in the field by mass releasing non-viable host eggs when hosts is limiting in the field (Leopold, 1998). Lim and Mahmoud (2009), Alim and Lim (2011) and Mainali *et al*. (2012) have demonstrated that this tool could help to increase platygastrids field parasitism.

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نگهداری تخم *Piezodorus guildinii* (Hemiptera: Pentatomidae) در سردخانه برای پرورش *Telenomus podisi* (Hymenoptera: Platygastridae)

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چکیده

از آفت های مهم سویا حشره *Piezodorus guildinii* (Hemiptera: Pentatomidae) می باشد که یکی از دشمنان طبیعی آن *Telenomus podisi* (Hymenoptera: Platygastridae) است. پرورش این پارازیتویید محدود به کیفیت تخم میزبان است که بعد از چند نسل در آزمایشگاه تخریب می شود و بنا براین استفاده از تخم میزبان نگهداری شده در سرد خانه می تواند روش مفیدی برای رهاسازی انبوه در کنترل زیستی آن باشد. از این قرار، هدف این پژوهش ارزیابی کیفیت تخم های *P. guildini*i نگهداری شده در سردخانه و تاثیر روی عملکرد نسل والدی و F1 حشره *T. podisi* بود. به این منظور،تخم های میزبان که ۲۴ ساعت از تولیدشان می گذشت در شرایط ۱۸- درجه سانتی گراد به مدت های یکماه(تعداد *N=53*) ، ۲ ماه(*N=28*) و سه ماه (*N=29*) نگهداری شد و سپس در حدود ۴۰ تخم میزبان در طی ۴۸ ساعت به حشره ماده *T. podisi* در هر تیمار داده شد. در تیمار شاهد از تخم های میزبان که ۲۴ ساعت از تولیدشان می گذشت و در حرارت ۲۴ درجه سانتی گراد نگهداری شده بودند (*N=55*) استفاده شد. بر اساس نتایج، حتی در مورد تخم هایی که زمان کوتاهتری در سردخانه بودند رفتار انگلی (*parasitism*) نسل والدی و ظهور فرزندان (نتاج) در مورد تخم های یخ زده به طور معنی داری کمتر از تخم های یخ نزده بود. با افزایش دوره یخزدگی، نسبت نرها و دوره رشد قبل از بلوغ (*preimaginal*) نتاج زیاد می شد اما، نسبت رفتار انگلی ناشی از حشرات بالغ *F1* و نیز ظهور نتاج، نسبت حشرات نر، و طول دوره رشد قبل از بلوغ در *F2* تحت تاثیر دوره یخزدگی قرار نگرفت. هر چند عملکرد *T. podisi* روی تخم های یخ زده *P. guildinii* به طور معنی داری کمتر از تخم های یخ نزده بود، نگهداری تخم های میزبان در سردخانه تا دو ماه ، دستیابی به نرخ انگلی تا حد ۴۰٪ و نیز نرخ بالای ظهور نتاج را مقدور ساخت. از این قرار، این روش برای پرورش انبوه حشره به ویژه در طی دوره خواب زمستانی میزبان و تشدید رفتار انگلی آن در مزرعه در زمانی که شمار میزبان کم است می تواند به اندازه کافی مفید باشد.