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The potential of *Mythimna sequax* Franclemont eggs for the production of *Trichogramma* spp. after cryopreservation in liquid nitrogen[1]

Potencial de ovos de *Mythimna sequax* Franclemont criopreservados em nitrogênio líquido para produção de *Trichogramma* spp.

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ABSTRACT - The cryopreservation of noctuid eggs in liquid nitrogen has proved be a promising tool in the mass production of *Trichogramma*, however studies into this technique have only just begun. This study evaluated the response of different densities of the female of *Trichogramma pretiosum* Riley to the parasitism of *Mythimna sequax* eggs stored and not stored in liquid nitrogen, and the performance of females reared only in cryopreserved eggs. The study evaluated the influence of the number of *T. pretiosum* females (4, 8 and 12) released to parasitise 40 *M. sequax* eggs, stored and not stored for 15 days in liquid nitrogen, as well as the performance of *T. pretiosum* females reared in eggs stored for three generations and females reared in non-stored eggs. Parasitism by *T. pretiosum* in stored eggs was 84%, twice the value obtained in previous studies. The emergence of parasitoids was greater than 95% in both experiments. The performance of females raised in stored eggs did not differ from that of females raised in non-stored eggs. The data show that the technique of cryopreservation of *M. sequax* eggs may be a viable alternative in the mass production of *T. pretiosum*.

Key words: Egg storage. Ultra-low temperatures. Noctuids. Egg parasitoids.

RESUMO - A criopreservação de ovos de noctuídeos em nitrogênio líquido tem se mostrado uma ferramenta promissora para a produção massal de *Trichogramma*, entretanto, os estudos sobre esta técnica são incipientes. Neste trabalho, avaliou-se a resposta de diferentes densidades de fêmeas de *Trichogramma pretiosum* Riley ao parasitismo de ovos de *Mythimna sequax* estocados ou não em nitrogênio líquido e o desempenho de fêmeas criadas somente em ovos criopreservados. Para isso, avaliou-se a influência da quantidade de fêmeas de *T. pretiosum* (4; 8 e 12) liberadas para o parasitismo de 40 ovos de *M. sequax* estocados por 15 dias em nitrogênio líquido e não estocados, assim como, o desempenho de fêmeas de *T. pretiosum* criadas em ovos estocados por três gerações com fêmeas criadas em ovos não estocados. O parasitismo de *T. pretiosum* em ovos estocados foi de 84%, o dobro do número obtido em estudos anteriores. A emergência de parasitoides foi superior a 95% em ambos os experimentos. O desempenho das fêmeas criadas em ovos estocados não diferiu das fêmeas criadas em ovos não estocados. Os dados mostram que a técnica de criopreservação para os ovos de *M. sequax* pode ser uma alternativa viável para a produção massal de *T. pretiosum*.

Palavras-chave: Estocagem de ovos. Temperaturas ultrabaixas. Noctuídeos. Parasitoides de ovos.

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INTRODUCTION

Egg parasitoids of the genus *Trichogramma* Westwood are the most frequently used agents in programs of biological control of lepidopteran pests in several countries (PEREIRA *et al*., 2004). Their mass production is generally carried out using eggs of microlepidoptera, such as *Anagasta kuehniella* Zeller, *Sitotroga cerealella* Olivier and *Corcyra cephalonica* Staiton, as they have low production costs (PARRA, 1997). However, these hosts produce eggs of reduced size (CÔNSOLI; KITAJIMA; PARRA, 1999), which result in small and less vigorous parasitoids when compared to parasitoids from larger host eggs due to the greater availability of nutrients for the immature insects (BAI *et al*., 1992; SALT, 1941). Such factors as species, size and age of the host, as well as temperature and humidity, can affect the efficiency of *Trichogramma* parasitism under field conditions (DUROCHER-GRANGER *et al*., 2011; ZAMONER, 2005).

Studies carried out by Bai *et al*. (1992) showed that rearing *Trichogramma* from hosts that have eggs of greater volume resulted in a larger number of parasitoids per egg, and individuals that are longer-lived and more robust, favouring successful pest control. In this respect, the noctuid *Mythimna sequax* Franclemont, in addition to producing eggs of greater volume than the eggs of moths reared in flour, has high reproductive capacity, ease of rearing and reduced costs (MARCHIORO; FOERSTER, 2012).

Success in the mass production of parasitoids depends on synchronising with production in the host, especially in periods close to their being released in the field (TEZZE; BOTTO, 2004). One of the alternatives to meet the large-scale production demand of *Trichogramma* is the storage of host eggs at low temperatures for long periods (GRECO; STILINOVIC, 1998). This technique has been used to store eggs of pentatomid bugs in liquid nitrogen for the production of *Trissolcus basalis* Wollaston and *Telenomus podisi* Ashmead (Hymenoptera: Platygastridae) for release into soybean crops (CORRÊA-FERREIRA; OLIVEIRA, 1998; DOETZER; FOERSTER, 2013; FAVETTI; BUTNARIU; DOETZER, 2014).

With lepidoptera, the storage technique has mainly been carried out at low temperatures with parasitised eggs (JALALI; SINGH, 1992; ÖZDER, 2004; PITCHER *et al*., 2002). Cryopreservation of lepidopteran eggs in liquid nitrogen before parasitism remains poorly investigated. The main advantage of this technique is the storage of host eggs for prolonged periods (CORRÊA-FERREIRA; OLIVEIRA, 1998), and the inviability of the host, which avoids the risk of larvae hatching from eggs that may not have been parasitised (MILWARD-DE-AZEVEDO *et al*., 2004).

This technique has shown to be promising for the production of *Trichogramma*, as demonstrated by studies carried out with different hosts (GRECO; STILINOVIC, 1998; KRECHEMER; FOERSTER, 2016). Despite the promising results, the parasitism rate in these studies was below 70%, indicating a need to improve the technique.

The cryopreservation of noctuid eggs in liquid nitrogen may be a viable alternative for the mass production of *Trichogramma* spp., since these eggs have more volume than the eggs of *S. cerealella* (CÔNSOLI; KITAJIMA; PARRA, 1999; ZAMONER, 2005). However, such factors as female density and the quality of the host eggs may influence the performance of *Trichogramma* spp. (MOREIRA *et al*., 2004). The present study therefore evaluated the response of different densities of *T. pretiosum* females to the parasitism of eggs of *M. sequax* stored in liquid nitrogen, and the performance of females obtained from eggs stored for three generations, compared to females obtained from eggs that had not been stored.

MATERIAL AND METHODS

The experiments were carried out at the Integrated Insect Control Laboratory (LCII), and at the Laboratory of Cell Biology at the Biological Sciences Department of the Federal University of Paraná, Brazil.

The eggs of *M. sequax* used in the experiments were obtained from populations reared in the laboratory at a temperature of 20 ± 1 °C, 70 ± 10% relative humidity and a 12L:12D photoperiod (MARCHIORO; FOERSTER, 2012). The population of *T. pretiosum* was kept in 1.0 x 10 cm test tubes at the same temperature as the host. Every two days, eggs of *M. sequax* were exposed to parasitism by *T. pretiosum*. The adults were fed pure honey distributed in strands on the inside of the glass tube. *M. sequax* eggs, 24 hours old, were transferred to 2 mL capacity cryotubes (Corning®) at an approximate amount of 400 eggs per tube. The samples were placed directly in liquid nitrogen. After the storage period, the eggs were thawed in a water bath at 37 °C for 5 minutes and maintained at 20 ± 1 °C, a relative humidity of 70 ± 10% and a 12L:12D photoperiod for conducting the experiments.

The first experiment evaluated the influence of the number of *T. pretiosum* females on parasitism in eggs of *M. sequax*, both stored and not stored (control) in liquid nitrogen for 15 days. The treatments were D4 = 4♀:40 eggs, D8 = 8♀:40 eggs and D12 = 12♀:40 eggs. Clumps containing 40 eggs were transferred to glass tubes (1 x 10 cm) and exposed to parasitism for 24 hours by females up to 48 h old, who were fed on strands of

pure honey. The experimental design was completely randomised, with 6 treatments and 20 replications, each replication consisting of 40 eggs.

The second experiment evaluated parasitism by *T. pretiosum* females reared on eggs stored in liquid nitrogen for three generations (TPLN), and females reared in non-stored eggs (control) (TP). Eggs of *M. sequax*, cryopreserved in liquid nitrogen for 30 days, and eggs that had not been cryopreserved were exposed to parasitism by TPLN and TP females. Clumps of 20 eggs were transferred to glass tubes containing strands of honey on the inside for feeding the females. Two females (1♀:10 eggs) up to 48 h old were released into each tube for 24 hours to parasitise. The experimental design was completely randomised, with four treatments and 20 replications, each replication consisting of 20 eggs and two *T. pretiosum* females.

The parameters evaluated in the two experiments with the aid of a stereo microscope were the percentage of parasitised eggs, percentage of emerged adults (presence of emergence hole in the egg), length of cycle (egg-adult), number of emerged parasitoids and sex ratio.

Data that followed a normal distribution by the Shapiro-Wilk normality test and homogeneity by Levene's test, were submitted to ANOVA and the means compared by the Tukey test ($p \leq 0.05$). For data that did not follow these precepts, the Kruskal-Wallis test ($p \leq 0.05$) was used. The sex ratio was calculated with the formula SR = number of females / (number of males + number of females) and compared using the chi-square test (X^2) at an expected ratio of 1♂:1♀.

RESULTS AND DISCUSSION

In the two experiments, the highest rates of parasitism by *T. pretiosum* in the eggs of *M. sequax* were 84% for 30 days storage and 91% for 15 days storage. The mean percentage of eggs with emerged adults was 95%. These results were greater than for parasitism by *T. pretiosum* in eggs of *S. cerealella* cryopreserved in liquid nitrogen for 20, 30 and 130 days, which was 32, 41 and 43% respectively, with an emergence rate of less than 70% (GRECO; STILINOVIC, 1998), and for eggs of *A. kuehniella* stored in liquid nitrogen for up to nine months, which were not parasitised (LOHMANN *et al*., 2007). Both species are the most used in the mass production of *Trichogramma*. The low parasitism of *T. pretiosum* in the above-mentioned studies may be related to the size of the host egg, the thickness of the chorion (ZAMONER, 2005), and the period of storage. When the egg, that has a more fragile structure, is exposed to liquid nitrogen (-196 °C), it can undergo rupture of the cell membrane due to the formation of ice crystals inside the cell (CASTRO *et al*., 2011). This hypothesis is corroborated by studies carried out by Krechemer and Foerster (2016), who stored eggs of *M. sequax*, which have more volume than those of flour moths (ZAMONER, 2005). Those authors stored the eggs in liquid nitrogen for 30, 60 and 90 days, and obtained 61, 70 and 58% parasitism by *T. pretiosum* respectively. The results obtained in the present study, and confirmed by Krechemer and Foerster (2016), suggest that the eggs of *M. sequax* are tolerant to freezing in liquid nitrogen.

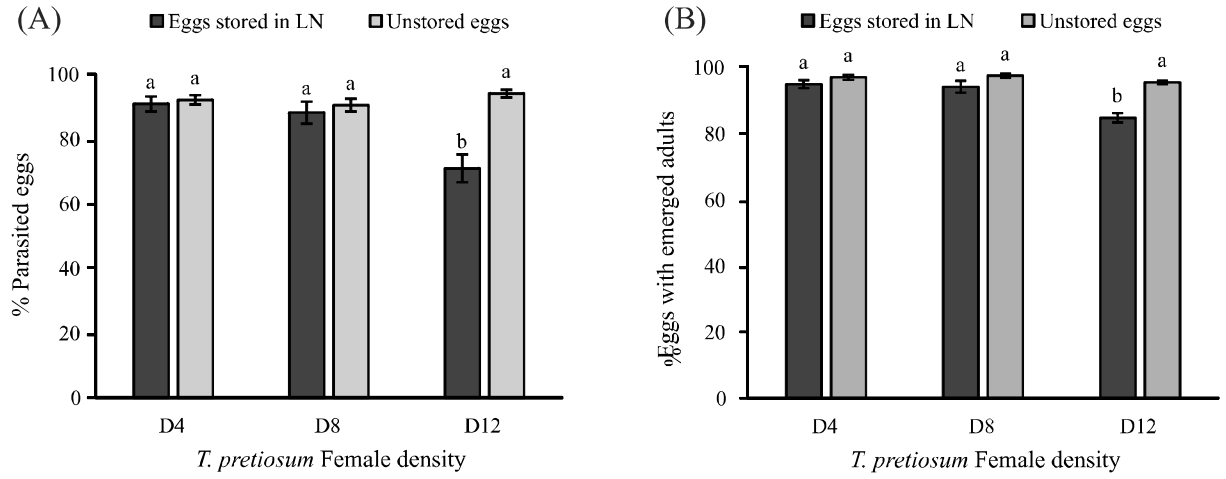
The density of *T. pretiosum* females did not affect parasitism in the non-stored eggs, with a mean value greater than 88% of parasitised eggs, but it did affect parasitism in the stored eggs, with only 71% of the eggs being parasitised in treatment D12 ($H = 21.0126$, $p<0.0008$) (Figure 1A).

An increase in the proportion of females (Treatment D4), the equivalent of one female to every 10 eggs, resulted in an increase in the parasitism of stored *M. sequax* eggs when compared to those obtained by Krechemer and Foerster (2016), who used a ratio of one female to 20 eggs. However, the proportion of 12 females caused a reduction in the rate of parasitism in stored eggs, produced by the high density of females in relation to the number of eggs offered, and resulting in superparasitism. Some eggs subjected to freezing may have undergone alterations in their structure, since they dehydrated after parasitism by several females.

Paron, Ciociola and Cruz (1998) found that an increase in the proportion of *T. atopovirilia* females affected parasitism and development of the parasitoids in eggs of *Helicoverpa zea* due to intraspecific competition for food by the parasitoid larvae inside the egg. Beserra *et al*. (2003) also saw a reduction in parasitism with an increase in the density of *T. pretiosum* females, since the females parasitise the same egg several times instead of parasitising various eggs. Parasitism of the same egg many times causes superparasitism, resulting in a drastic reduction in the rate of parasitism.

The results were similar in relation to the percentage of eggs with emerged adults, with the lowest value for emergence of 85% found in treatment D12 ($H = 44.2962$, $p<0.0000$) (Figure 1B). This result confirmed those obtained by Krechemer and Foerster (2016) for the emergence of *T. pretiosum* in the eggs of *M. sequax* stored in liquid nitrogen, and was superior to that of Greco and Stilinovic (1998), where the emergence of parasitoids in eggs of *S. Cerealella* stored in liquid nitrogen was 69%. Pratissoli *et al*. (2005) found values of greater than 85% emergence for *T. pretiosum* in the eggs of *Spodoptera frugiperda* (J.E. Smith) when a density of more than one female for 15 host eggs was used. According to Navarro

Figure 1 - Mean percentage (± SE) of parasitised eggs (A) and eggs with emerged *T. pretiosum* adults (B), for eggs of *M. sequax* stored in liquid nitrogen for 15 days and for non-stored eggs, parasitised at different female densities. Similar letters do not differ by the Kruskal-Wallis test (p≤0.05)



(1998), in quality control of *Trichogramma* production, emergence should be greater than 85%; the results obtained in this study are therefore above the standard for *T. pretiosum*.

The number of emerged parasitoids in the stored eggs differed from the non-stored eggs at all female densities (F: 42.319, p<0.0000) (Table 1). In stored eggs, the average number of parasitoids that emerged was lower than that seen for non-stored eggs (Table 1). The number of parasitoids not emerged from the eggs was proportional to the increase in female density. Treatment D12, for both the stored and non-stored eggs, showed a higher number of non-emerged parasitoids, differing from the other treatments (H = 56.5125, p<0.0000) (Table 1).

It was found that as the number of females increased, the percentage of parasitoid emergence decreased, both in stored eggs and in non-stored eggs. Although the number of parasitoids emerging from stored eggs was lower than from non-stored eggs, the number of emerged parasitoids, on average two parasitoids/egg, was still higher than the number of parasitoids emerged from eggs of *A. kuehniela* (0.98/egg), which are frequently used in the commercial mass production of *Trichogramma* (ZAMONER, 2005). The high number of females may have increased competition between them and caused them to parasitise the same egg several times (BESERRA *et al*., 2003), damaging the eggs or causing superparasitism, with less food available for development of the larvae (PARON; CIOCIOLA; CRUZ, 1998). Densities of four and eight females, which correspond to a ratio of 1♀:10 eggs and 1♀:5 eggs respectively, did not differ for the development parameters of *T. pretiosum* under evaluation. However, for mass production, it would be feasible to employ a density

Table 1 - Mean values (± SE) for the number of emerged and non-emerged parasitoids, sex ratio and length of cycle (egg-adult) for *T. pretiosum* in eggs of *M. sequax* stored in liquid nitrogen for 15 days and parasitised by different numbers of females (n =120)

Variable	Female density of *T. pretiosum*					
	D4		D8		D12	
	LN[1]	Control[2]	LN[1]	Control[2]	LN[1]	Control[2]
Emerged parasitoids	74.1 ± 4.10 c	111.2 ± 2.91 b	80.5 ± 6.32 c	125.6 ± 5.94 ab	51.3 ± 5.57 d	134.5 ± 4.33 a
Non-emerged parasitoids	10.1 ± 1.67 ab	5.1 ± 1.08 a	18.1 ± 2.56 b	10.2 ± 1.87 ab	32.4 ± 3.06 c	15.0 ± 1.91 b
Sex ratio*[ns]	0.76	0.71	0.75	0.66	0.72	0.66
Length of cycle (days)	14.0±0.00 b	13.6 ± 0.11 c	14.0±0.00 b	14.0 ± 0.05 b	14.5 ± 0.11 a	14.1 ± 0.08 b

Mean values followed by the same letter on a line did not differ by Tukey test (p≤0.05) for the number of emerged parasitoids, or by Kruskal-Wallis test (p≤0.05) for the other parameters. *[ns] = not significant for the chi-square test (p≤0.05). [1]LN = eggs stored in liquid nitrogen; [2]Control = non-stored eggs

of four females in the parasitism of stored eggs, since this density had the least effect on parasitoid development, resulting in a larger number of individuals.

The length of the biological cycle (egg-adult) of the parasitoids that developed in eggs stored at densities of four and eight females was 0.5 days less in relation to the density of 12 females (H = 26.481, p<0.001) (Table 1). The same difference was found in the parasitoids that developed in non-stored eggs at a density of four females when compared to 12 females (H = 17,605, p<0.001) (Table 1). The high density of females may have delayed development of the parasitoids due to the reduced availability of food inside the egg.

The sex ratio ranged from 0.60 to 0.80 and did not differ between treatments by the chi-square test (Tables 1 and 2). The results are within the standard for this species, and should be equal to or greater than 0.50 (NAVARRO, 1998). The stored and non-stored eggs parasitised by different densities of *T. pretiosum* females resulted in a greater number of females than males. The factor of nutrition may influence the sex ratio of the parasitoids, so that the eggs of *M. sequax* having greater nutritional reserves due to their volume, about three times greater than the eggs of *A. kuehniella* (ZAMONER, 2005), contributed to the increase in female density not affecting the sex ratio. These results confirm those obtained by Greco and Stilinovic (1998) and Krechemer and Foerster (2016), who found a predominance of *T. pretiosum* females in eggs stored in liquid nitrogen and in non-stored eggs.

Although parasitism of *T. pretiosum* in eggs stored in liquid nitrogen was lower in relation to non-stored eggs, the rate was higher than 84%, with no difference between females of *T. pretiosum* reared in stored eggs (TPLN) and those reared in non-stored eggs (TP) (Figure 2A). The percentage of stored eggs with emerging adults did not differ from the non-stored eggs, with an average of 97% for eggs parasitised by both TPLN and TP females (H = 4.9367, p<0.1765) (Figure 2B).

Cobert (1985) reports that parasitoids reared for several generations in a given host develop pre-imaginal conditioning, acquired during the immature phase, where the adults show a preference to parasitise eggs from the same host in which they are kept. However, this study found that females raised with non-stored *M. sequax* eggs and females raised with stored eggs both successfully parasitised eggs stored in liquid nitrogen, with no effect on progeny emergence. There is therefore no need to keep a strain of *Trichogramma* reared only with stored eggs, which contributes to a reduction in the labour and maintenance costs of rearing the hosts.

The average number of emerged parasitoids from stored eggs differed from the non-stored eggs parasitised by both TPLN females and TP females (F = 9.6953, p<0.0000) (Table 2). However, there was no difference between TPLN and TP females who received stored eggs, where the number of emerged parasitoids was between 30 and 33, and gave rise to approximately two parasitoids per egg.

Figure 2 - Mean percentage (± SE) of parasitised eggs of *M. sequax* (A) and eggs with emerged *T. pretiosum* adults (B), for eggs stored in liquid nitrogen for 30 days and for non-stored eggs, parasitised by females reared with eggs stored in LN for three generations (TPLN) and with non-stored eggs (TP). Similar letters do not differ by the Kruskal-Wallis test (p≤0.05)

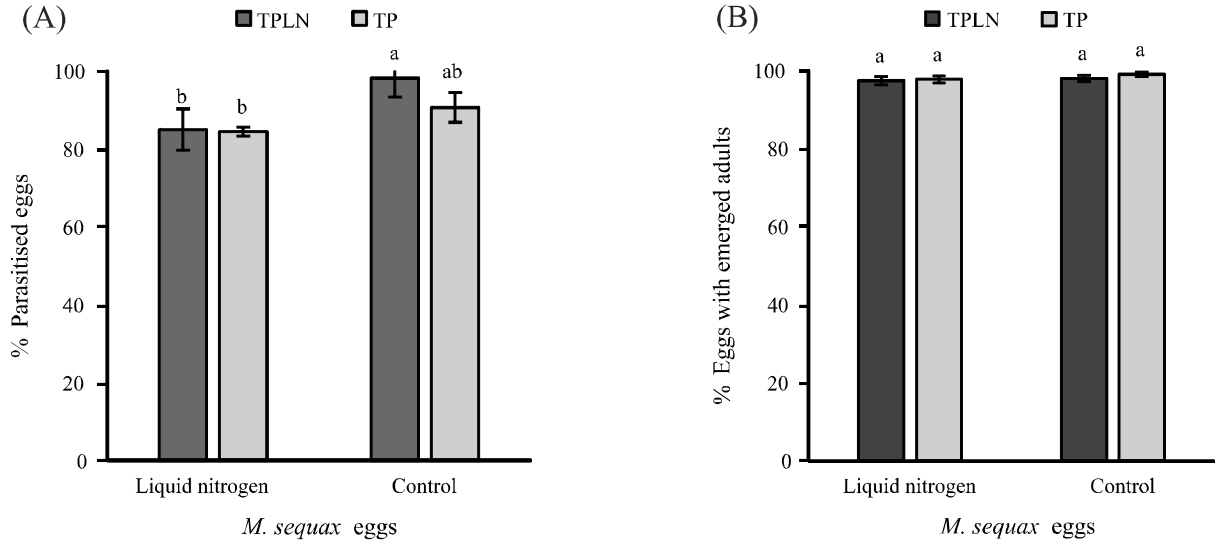


Table 2 - Mean values (± SE) for the number of emerged and non-emerged parasitoids, sex ratio and length of cycle (egg-adult) for *T. pretiosum* in eggs of *M. sequax* stored in liquid nitrogen for 30 days and parasitised by females reared from eggs stored in LN (TPLN) and non-stored eggs (TP) (N = 80)

Observed variable	Females of *T. pretiosum* reared in eggs stored and not stored in liquid nitrogen			
	TPLN		TP	
	LN[1]	Control[2]	LN[1]	Control[2]
Emerged parasitoids	32.8 ± 2.83 b	49.9 ± 2.83 a	30.3 ± 2.37 b	40.3 ± 3.33 ab
Non-emerged parasitoids	2.3 ± 0.48 b	1.0 ± 0.37 ab	2.1 ± 0.49 b	0.5 ± 0.16 a
Sex ratio*[ns]	0.80	0.60	0.80	0.80
Length of cycle (days)	16.3 ± 0.11 b	14.1 ± 0.07 a	16.6 ± 0.11 b	14.8 ± 0.09 a

Mean values followed by the same letter on a line did not differ by Tukey test ($p \leq 0.05$) for the number of emerged parasitoids, or by Kruskal-Wallis test ($p \leq 0.05$) for the other parameters. [*ns] = not significant for the chi-square test ($p \leq 0.05$). [1]LN = eggs stored in liquid nitrogen; [2]Control = non-stored eggs

In the treatments where TPLN and TP females received non-stored eggs (control), the number of emerged parasitoids ranged from 49.9 to 40.3 respectively, presenting around 2.2 to 2.6 parasitoids per egg. These values confirm those obtained by Zamoner (2005) when comparing *M. sequax* with eggs of *A. kuehniela*. On average, two parasitoids did not emerge from stored eggs parasitised by both TPLN and TP females, whereas in non-stored eggs the average was 1.0 and 0.5 non-emerged parasitoids ($H = 15.6085$, $p<0.0014$) (Table 2). These studies demonstrate that even when storage reduces adult emergence to two parasitoids per egg, the number of individuals will still be greater than obtained with eggs of *A. kuehniela*.

The length of the cycle (egg-adult) ranged from 14 to 16 days, with the parasitoids from non-stored eggs emerging earlier than those from stored eggs ($H = 67.0858$, $p<0.0000$) (Table 2). The development time of the parasitoids in stored eggs was around 16 days for females reared in stored eggs and for females reared in non-stored eggs, while for parasitoids developed in non-stored eggs, the time was 14 days (Table 2). Although there is no report in the literature, this observation may be explained by there being some physiological alteration in the nutritional components of the eggs during the freezing process. This longer cycle (egg-adult) was also reported by Krechemer and Foerster (2016) when storing eggs of *M. sequax* in liquid nitrogen for 30 days, and proves that as the period of egg storage increases, there is a tendency for the development time of the parasitoids to increase.

A comparison of the results of the TPLN and TP females shows that there was no difference for any of the biological parameters under observation. The results obtained in this study demonstrate that the technique of cryopreservation of *M. sequax* eggs for the mass production of *T. pretiosum* is viable. One of the hypotheses for the successful cryopreservation of eggs of *M. sequax* in liquid nitrogen may be related to the biochemical composition of the eggs, which include greater lipid reserves in their composition for being a winter host, and which may reflect in a higher tolerance of the eggs to low temperatures (AVANCI; FOERSTER, 2006).

This is therefore a pioneering study, as it achieves high rates of parasitism and viability in *Trichogramma* with noctuid eggs as the host. Studies by Krechemer and Foerster (2016) have demonstrated that eggs of *M. sequax* have a storage potential of up to 90 days with a parasitism of 58%. However, our hypothesis is that the high rates of parasitism and emergence may continue for longer periods than those evaluated in this study.

CONCLUSIONS

Based on the results, it can be concluded that:

1. As little as one female can be used for 10 eggs, without the need to increase the density to obtain a higher rate of parasitism;
2. The performance of *T. pretiosum* females reared in eggs stored in liquid nitrogen for three generations does not differ from that of females reared in non-stored eggs.

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