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ANIMAL SCIENCE

Biological behavior of *Chrysomya putoria* (Wiedemann, 1819) (Diptera: Calliphoridae) after refrigeration: Logistics for use in Biotherapy

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Abstract: The influence of refrigeration on the post-embryonic development of *Chrysomya putoria* larvae was evaluated, regarding its resistance in the logistics of storage and distribution in biotherapy. Previously sterilized larvae were submitted to four periods of storage under refrigeration (T1=12 h, T2=24 h, T3=48 h and T4=72 h) and control (without sterilization and refrigeration). Newly hatched larvae (0.200 g) were stored between 3 and 9ºC. After refrigeration, 40 neo-larvae (in triplicate) were transferred to 50 g of protein diet and incubated in an acclimatized chamber. There was a significant difference in the larval body mass (T1 and T2) and in the duration of larval, pupal and total development (T3 and T4). The sex ratios found in the four treatments did not differ from what was expected. Normality rates were 100% for all treatments. There was no significant difference between the Control, T1 and T2 treatments for larval, pupal and total viability. There was a significant difference between control (C) and T4 (larval viability), between C, T3 and T4 (pupa) and between C and T4 (total). *C. putoria* has resistance under refrigeration and storage of up to 56 h, presenting viability above 70% for use in biotherapy.

Key words: biotherapy, entomotherapy, storage, logistics.

INTRODUCTION

Nature's pharmacy is vast and its products have been used throughout human history as raw material for use in traditional medicine. Plants were the first to be used by early humans in the search for cures of diseases, and after positive experiences with medicinal formulations obtained from vegetables, early humans sought to use products of animal origin. Today, we have several medicines that are derived from plants, microorganisms, algae or animals, such as aspirin, penicillin, cytarabine, heparin, and captopril, among others (Ratcliffe et al. 2011). Although insects are seen as pests in many crops, recent developments in the identification and bioengineering of natural products with potential therapeutic use have underpinned the emergence of a series of companies created to explore insect-derived bioactives, the so-called "Drugs from bugs" (Zainzinger 2019).

The best known among the natural therapies that use insects is biotherapy, also known as larval therapy or larval debridement therapy. This consists in applying sterile live larvae of necrophagous flies (Diptera: Calliphoridae) in wounds with devitalized tissues in order to remove the necrotic material and promote local healing. Many health professionals have recognized the importance of this biotherapy, especially in cases of wounds that are difficult to heal or infected with antimicrobial-resistant bacteria. Clinical-laboratory studies point to

several beneficial actions of biotherapy on these lesions: debridement of necrotic tissue (Mumcuoglu 2001), microbial decontamination (Sherman 2014, Nigam et al. 2010), preventing the development of bacterial resistance (Chernysh et al. 2015), immunomodulation of the local inflammatory response (Elkington et al. 2009, Chernysh et al. 2012), anti-inflammatory activity (Van Der Plas et al. 2009), pro-angiogenic activity (Wang et al. 2020, Morgan & Nigam 2013), proliferation and migration of fibroblasts for extracellular matrix remodelling and stimulation of granulation tissue (Prete 1997), and disaggregation action of bacterial biofilms (Van der Plas et al. 2008).

The choice of a dipteran species that has ideal biological characteristics for biotherapy is fundamental. Thus, the *Chrysomya putoria* (Weidmann 1819) species was selected for this study, which is a necrophagous dipteran found in abundance in Brazil, mainly in urban centers (Marinho et al. 2006).

The mastery of breeding techniques for this species enables obtaining females with a high capacity for oviposition, generating a large amount of egg masses to be submitted to the sterilization process, which is an important prerequisite for its use in biotherapy. In addition, the possibility of storing the larvae obtained under refrigeration improves their distribution logistics for use (Dallavecchia et al. 2015, Ferraz et al. 2014).

Thus, the objective of this work was to evaluate the larval, pupal and total viability, as well as the body mass of the larvae, the development of the insects after storage at low temperatures for different time periods (12, 24, 48 and 72 h) and the sex ratio of adult insects of the *C. putoria* species.

MATERIALS AND METHODS

The study was conducted at the Laboratório de Estudos de Dípteros (LED) (Diptera Study Laboratory) and at the Laboratório de Microbiologia do Departamento de Microbiologia e Parasitologia do Instituto Biomédico da Universidade Federal do Estado do Rio de Janeiro (UNIRIO). The sterilization of the egg masses (0.200g) was performed with 2% glutaraldehyde, a liquid sterilant commonly used in hospital materials and which has a fast and effective action on gram-positive and gram-negative bacteria, mycobacteria, fungi and viruses (Gomes et al. 2007).

Sterile newly hatched larvae from eggs sterilized according to a previously developed protocol (Dallavecchia et al. 2014) were placed in Petri dishes containing gauze and a filter paper disc moistened with sterile saline in triplicate (Fig. 1a, b). Then, the plates were transferred to a refrigerator with temperature and humidity recorded by a thermohygrograph (Sigma II) and kept for the following time periods: 12, 24, 48 and 72 h (Fig. 1c) which corresponded to T1, T2, T3 and T4 treatments, respectively. The control consisted in the use of larvae without sterilization and without refrigeration, in triplicate, kept in a climatized chamber under controlled conditions an acclimatized chamber (Quimis) regulated at 30 ºC/day and 28 ºC/night, with 60±10% relative humidity and 12 h of photophase (beginning at 6:00 am).

The plates were removed from the refrigerator after 12, 24, 48 and 72h, then 40 first-instar larvae were transferred to 50 g of chicken gizzards placed in 100 mL beakers. These were inserted into beakers larger than 500mL with sterilized wood shavings as a substrate for pupariation, and then sealed with scaline fabric secured with an elastic band and labelled (Fig. 1d, e). The rearing containers were transferred to

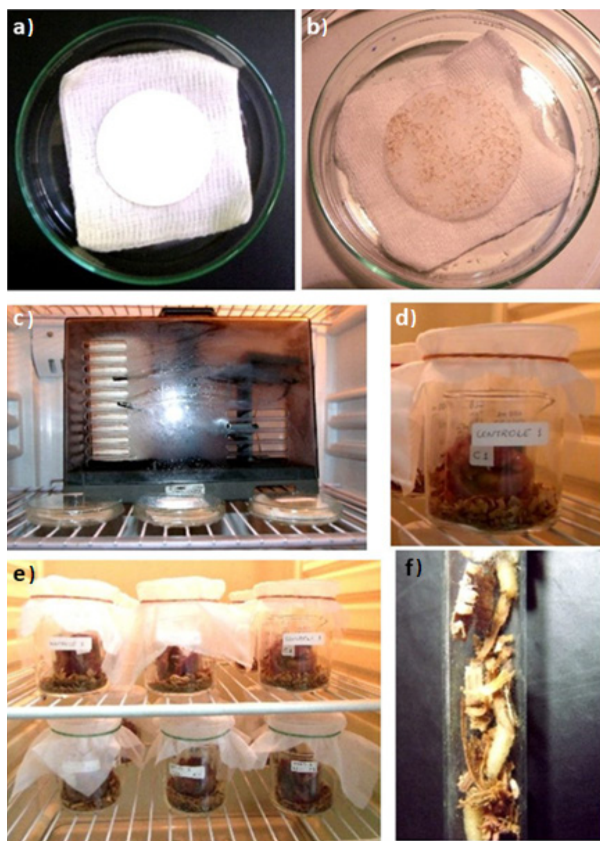

Figure 1. a) Petri dish with gauze and filter paper; **b)** Petri dish containing sterilized eggs of *Chrysomya putoria* in the hatching phase on filter paper and gauze moistened with sterile saline; **c)** Thermohygrograph; **d)** and **e)** 40 first instar larvae incubated in beakers containing chicken gizzard for all treatments; and **f)** Test tube with *C. putoria* larvae and wood shavings after diet abandonment.

an acclimatized chamber regulated at 30 ºC/day and 28 ºC/night, with 60±10% relative humidity and 12 h of photophase (beginning at 6:00 am) to observe the viability of the immature stages and larval, pupal and total development.

After abandoning the diet, samples with five larvae (3rd instar) had their body mass determined using then weighed on semi-analytical scales, then were transferred to test tubes with sterilized wood shavings until the insects emerged (Fig. 1f). All five treatments (Control, T1, T2, T3 and T4) were performed in triplicate.

The Microsoft Excel program was used to analyze the raw data and the other analyses were performed with the R program, version 3.4.4. Variations in mean larval body mass and duration of larval, pupal and total development stages (from first instar to adult) were analyzed by Kruskal-Wallis test followed by Pairwise comparisons using Wilcoxon rank sum test with adjust *p*-value by BH method (Benjamini & Hochberg 1995). The viability was analyzed by proportion test. In all tests a significance level of 0.05 was considered. The sex ratio (SR) was determined as follows: SR = F/M+F, where F is the number of females and M is the number of males. The expected frequency (%) is a 1:1 ratio (= 0.50). The normality of the insects, that is, the absence of morphological changes, were also observed in terms of their frequency.

RESULTS

The maximum temperature recorded in the refrigerator during the experiment was 9 ºC and the minimum was 3 ºC, while the maximum air humidity was 100% and the minimum was 19% (Fig. 2). The body mass of third instar larvae that abandoned the diet (treated and control) differed significantly between treatments ($p < 0.05$) (Table I), the same we can see duration of larval stages. The development time of T4 larvae was significantly longer than in the other treatments and in the control ($p < 0.01$). In pupal stage the treatments T3 and T4 was lower than T1, T2 and control treatments, and the difference was significantly. The complete developmental stage was significantly higher for T4 ($p < 0.01$).

The average time of development from neo-larvae to adult was: 8.113 days for control; 8.104 days for T1; 8.067 days for T2; 8.063 days for T3; and 9.138 days for T4 (Table I). The diet abandonment rate of *C. putoria* larvae reached its peak on the 4th day after the beginning of

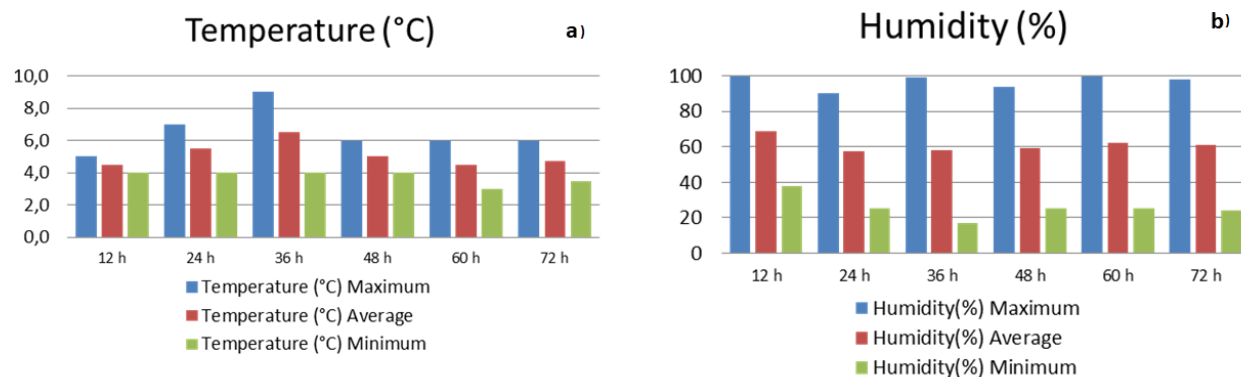

Figure 2. a) Record of the variation of temperature and b) Record of the variation of relative humidity of the air during the days of experimentation.

the experiment in all treatments, indicating homogeneity in the development of these insects. Only 8 (n=120) larvae from the control and 17 (n=120) from the T1 abandoned on the 3rd day. Pupariation peaked on the 5th day after the start of the experiment in all treatments, except for T4, which peaked on the 6th day. The emergence peak in all treatments was on the 8th day; however, emergence at T4 started on the 9th day (Fig. 3).

The larval viability differed significantly between the treatments tested ($p < 0.05$), being significantly lower for T4 (70%) when compared to the other treatments ($p < 0.05$). Could be observed in pupal viability significantly difference between control, T1 and T2 against T3 and T4 ($p < 0.05$). In T4 (72h of refrigeration) only 38% of the insects reached adulthood ($p < 0.05$), there was significantly difference between other treatments. The sex ratio of adult insects was below the expected value (SR= 0.5) for the control - SR= 0.38 and T3 - SR= 0.33. Normality was 100% for all insects in all treatments (Table II).

DISCUSSION

Chronic wound debridement therapy with larvae is simple, and highly effective. It can even be used in patients undergoing antibiotic therapy without the danger of these interfering with the therapeutic action of the larvae (Ferraz et al. 2014). It is indicated for different types of infected wounds and its effectiveness has already been widely proven (Sherman et al. 2013, Siribumrungwong et al. 2018, Borkataki et al. 2018).

Another increasingly important use of larval therapy is in the context of wars and disasters where production of medicinal maggots needs to be located close to the point of care (Stadler et al. 2016). In these extreme situations, it is important to know the entire process of rearing, maintaining, sterilizing and storing the larvae (Stadler 2020).

Mumcuoglu et al. (2001) reported the survival of larvae (1st intar) of *Lucilia sericata* for up to 5 days at a temperature between 5 and 8 ºC without interfering with their viability. The same could be observed in the present study after the storage of larvae from sterilized eggs under a minimum temperature that varied between 3 and 9 ºC for a period of up to 72 h. The storage of newly hatched larvae from sterilized eggs enables these sterile larvae to be sent to places far from their original place of production. The low temperature slows the metabolism of the larvae, delaying their development and allowing them to withstand starvation longer,

Table I. **Mean (standard deviation) body mass (mg) and mean duration of post-embryonic development stages* of *Chrysomya putoria* from control and refrigeration treatments** reared in chicken gizzards.**

Body mass and duration of stages in days				
Treatment	**Mass (g)**	**Larvae (days)**	**Pupae (days)**	**Adult (days)**
	$\bar{X} \pm p$	$\bar{X} \pm p$	$\bar{X} \pm p$	$\bar{X} \pm p$
Control	0.053**a** ± 0.008	5.025**a** ± 0.406	3.138**a** ± 0.351	8.113**a** ± 0.166
T1 (12 h)	0.056**b** ± 0.004	4.904**a** ± 0.318	3.313**a** ± 0.469	8.104**a** ± 0.148
T2 (24h)	0.052**a** ± 0.001	5.000**a** ± 0	3.067**a** ± 0.105	8.067**a** ± 0.105
T3 (48h)	0.057**b** ± 0.003	5.000**a** ± 0	3.027**b** ± 0.128	8.063**a** ± 0.179
T4 (72h)	0.053**a** ± 0.002	6.160**b** ± 0.339	3.000**b** ± 0	9.138**b** ± 0.341

***Means followed by the same letter in the same column do not differ significantly by Kruskal-Wallis followed by Pairwise comparisons. ** Control - without treatment, T1- sterile larvae submitted to 12 h of refrigeration, T2- sterile larvae to 24 h of refrigeration, T3- sterile larvae submitted to 48 h of refrigeration, T4- sterile larvae submitted to 72 h of refrigeration (experiments with 40 first instar larvae in triplicate).**

thereby reaching their final destination in viable condition.

Ferraz et al. (2016) obtained a similar mean body mass (control - 0.560 mg, T1 - 0.560 mg and T2 - 0.1520 mg) in testing different diets for *C. putoria* larvae to that found in the present study. This fact shows that, although there was a significant difference in the body mass of T1 and T3 compared to the control, the larvae remained healthy, even after the egg sterilization, starvation and refrigeration process, reaching equivalent body masses to those obtained under non-adverse conditions, reflecting an adequate post-embryonic growth.

Regarding development, the larvae almost entirely abandoned the diet on the 4th day in all treatments. The same could be observed with pupariation, which mostly peaked on the 5th day, with the exception of T4, which peaked on the 6th day. The birth of adults occurred on the 8th day after beginning the experiment for the control and T1, T2 and T3 treatments. The exception was T4, which had its birth peak on the 9th day. Although T4 had pupated and emerged within a day of the other treatments, the development generally showed homogeneity in all evaluated conditions. This fact shows that the egg sterilization process followed by refrigeration did not affect the duration of the post-embryonic development stages, nor the larval abandonment rate, which is the most important phase for biotherapy. Larvae stay in patient wounds for up to 72 h (Sherman 2014), which is the period of time they need to consume enough food for their full development. After this period, in nature or in the laboratory, the larvae abandon the diet to pupate. With this, it is necessary that the dressings of the patients are changed after 72 h, for the larvae to be removed from the wound bed. This periodicity is also convenient for the maintenance of colonies in the laboratory.

In one study to evaluate the post-embryonic behavior of *C. putoria* larvae in different diets under an average temperature of 20.6 ºC and an average Relative Humidity of 67.7%, Ferraz et al. (2012) observed that the larvae completed their biological cycle (larva to adult) in 8.88 days for insects raised on beef; 8.68 days in chicken gizzards; and 9.07 days in gizzard agar. The larvae in the present study completed their total cycle more quickly, completing it in 8.113

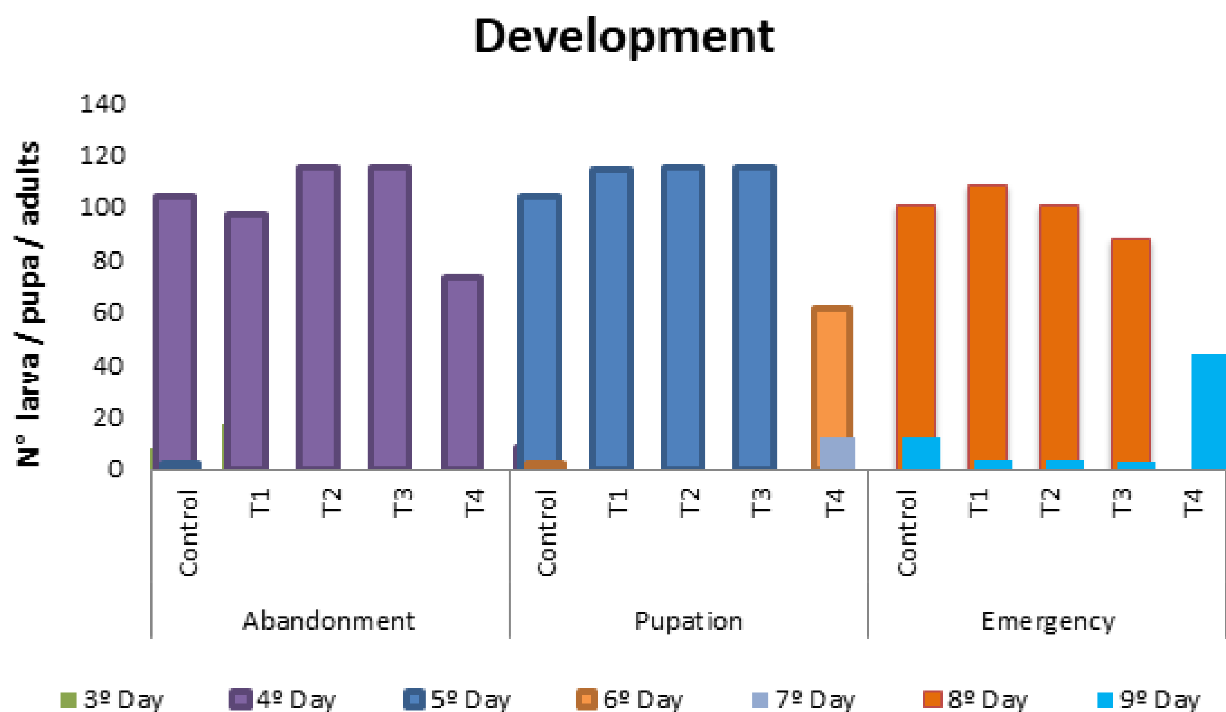

Figure 3. Abandonment rate of mature larvae from the diet, pupariation and emergence of *C. putoria* after sterilization of eggs with glutaraldehyde and submitted to refrigeration (treatments: T1 - 12 h, T2 - 24 h, T3 - 48 h and T4 - 48 h). In the control, eggs without sterilization and submitted to refrigeration.

days for the control, 8.104 days for T1, 8.067 days for T2, and 8.063 days for T3; however, the insects in T4 needed a longer time (9.138 days) to complete their development. This acceleration in development time is probably due to the higher temperature of the climatized chamber (30 ºC day/28 ºC night) where the insects were kept after the refrigeration process. However, the insects required a longer time period in T4, which can be explained by a longer refrigeration period (72 h), significantly interfering in this biological parameter. These results generally showed that *C. putoria* larvae have great resistance to the sterilization, starvation and refrigeration process without affecting their biological cycle, except when submitted to 72 h of refrigeration.

The significant difference observed in T4 larval viability (70%) was probably due to the fact that the larvae remained without any food for up to 72 h after hatching and were kept at low temperatures (between 3 and 9 ºC). Even so, 70% of the larvae were able to adapt to the adverse conditions (previous sterilization process, starvation, low temperature) to which they were subjected.

The high viability of *C. putoria* larvae observed in the T1 to T3 treatments is of paramount importance for implementing larval therapy. This is because the larvae are subjected to processing before being applied to the wounds which could compromise their viability, such as egg separation (mechanical disturbance), chemical sterilization, rinsing and filtration at the end of sterilization, and storage at low temperature (physical stress) (Dallavecchia et al. 2019).

Laboratories which commercially produce and distribute larvae for medicinal use generally need to maintain larval viability for a considerable period of time, since the larvae are highly perishable and go through a long

Table II. Viability of larval, pupal and total stages of *Chrysomya putoria* reared in chicken gizzards in treatments (T1-12 h, T2-24 h, T3-48 h, T4-72 h after sterilization and refrigeration, Control - larvae from eggs without treatment).

% Viability*							
Treatment	**Cooling time(h)**	**Larvae**	**Pupae**	**Adults**	**$\overline{X}$ Female**	**$\overline{X}$ Male**	**Sex Ratio****
C	Control	95,00a	97,36a	92,50a	14	23	0,38
T1	12	95,00a	94,73a	90,00a	17	19	0,47
T2	24	95,00a	89,47a	87,50a	16	19	0,46
T3	48	95,00a	78,94b	75,00a	10	20	0,33
T4	72	70,00b	53,57b	37,50b	8,7	6	0,59

*** Larval, pupal and adult viability were analyzed by the proportion test and a significance level of 0.05 was considered. **The Sex Ratio (SR) was obtained as follows: SR = F / M + F, where F is the number of females and M the number of males.**

logistical process until they reach their final destination, which can be in another city or even country, including dispatch by air. The recommended time period for the larvae to be distributed and applied to the wound is up to 48 h (Stadler 2020).

The pupal viability ranged from 97,36% to 78,94% in all treatments, with the exception of T4, where only 53,57% of the insects pupated. This same behaviour could be observed in the work by Ferraz et al. (2011) when testing three diets for raising Diptera, where pupal viability ranged from 98% to 71%.

Furthermore, approximately 90% of flies successfully completed development to adult stage in the control, T1, and T2 groups, but only 75% in the T3 and 37,50% in the T4 group. Total viability was lower for T4, which can be explained by the starvation time (72 h) to which the larvae were submitted. Feeding after hatching larvae is extremely important for the normal life cycle of dipterans. Although T4 showed a total viability below 50%, this fact is not important for larval therapy, since adult insects are not used in biotherapy, only larvae.

The pupa and total viability are important for maintaining colonies in the laboratory; however, the larvae used for the colonies do not undergo chemical or refrigeration processes, which were the objectives of this study.

The sex ratio in T1, T2 and T3 treatments was as expected (0.5), based on the formula presented in the methodology, indicating stability in the population. Fisher (1930) advocates that there will only be stability in the population when the sex ratio is 1:1; a deviated sex ratio is not evolutionarily stable, because there will be a disproportion in the sex of individuals in the population in future generations. The control and T3 had a lower sex ratio than expected. Although this fact is relevant for maintaining dipteran colonies in the laboratory, it does not affect the development of larvae for biotherapy which feed voraciously when inserted into the bed of infected wounds.

We can conclude in this study that *C. putoria* larvae are very resistant to the various stressful logistical processes for their use in biotherapy. They remained healthy after feeding and development even in a state of starvation. The observed viability of almost 70% after 72 h at low temperatures allows enough time to transport the larvae to distant locations until their final destination: the patient.

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