doi:10.12741/ebrasilis.v12i2.823

e-ISSN 1983-0572
Publication of the project Entomologistas do Brasil
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EntomoBrasilis
e-ISSN 1983-0572

Forensic Entomology/Entomologia Forense

Cooling and freezing effects on development of flies of forensic importance

Cesar Carriço[1]✉, Rebecca Leal Caetano[2], Júlio Vianna Barbosa[3] & Zeneida Teixeira Pinto[3]

1. Instituto Brasileiro de Medicina de Reabilitação-IBMR/Laureate International Universities. 2. Universidade Estácio de Sá (UNESA), Petrópolis. 3. Instituto Oswaldo Cruz /Fundação Oswaldo Cruz.

EntomoBrasilis 12 (2): 57-62 (2019)

Abstract. Cooling and freezing experiments were carried out at Oswaldo Cruz Foundation (Fiocruz / RJ). Flies colonies were established from specimens collected at the campus using a baited trap, as described for the species: *Chrysomya putoria* (Wiedemann), *Chrysomya megacephala* (Fabricius), *Lucilia cuprina* (Wiedemann), *Musca domestica* Linnaeus, *Peckia ruficornis* (Fabricius) and *Peckia chrysostoma* (Wiedemann). Prolonged exposures at lethal low temperatures can produce deleterious effects (including death) even if the insect does not freeze; during long-term exposure to low temperatures, the organisms may finally die from the exhaustion of energy reserves.

Keywords: Brazil; Diptera; Forensic Entomology; Temperature.

Efeitos do resfriamento e congelamento no desenvolvimento de moscas de importância forense

Resumo. Experimentos de resfriamento e congelamento foram realizados na Fundação Oswaldo Cruz (Fiocruz / RJ). Colônias de moscas foram estabelecidas a partir de espécimes coletados no campus usando uma armadilha com isca, conforme descrito para as espécies: *Chrysomya putoria* (Wiedemann), *Chrysomya megacephala* (Fabricius), *Lucilia cuprina* (Wiedemann), *Musca domestica* Linnaeus, *Peckia ruficornis* (Fabricius) e *Peckia chrysostoma* (Wiedemann). Exposições prolongadas em baixas temperaturas podem produzir efeitos deletérios (incluindo morte) mesmo se o inseto não congelar; durante a exposição prolongada a baixas temperaturas, os organismos podem morrer pelo esgotamento das reservas de energia.

Palavras-chave: Brasil; Diptera; Entomologia Forense; Temperatura.

Insects of the Diptera order, especially the muscoids, are the first to be attracted by decaying bodies (Carvalho *et al.* 2000; Smith 1986). These are sometimes, the only evidences that can determine the time and exact location of individual's death (Benecke 1998). Dipterans from Calliphoridae, Muscidae, and Sarcophagidae families are the most common used in forensic entomological studies.

Most species of flies from Neotropical Region have medical, health and/or forensic importance (Oliveira *et al.* 2007; Oliveira Costa 2011). Calliphoridae flies, also known as blowflies, are among the first colonizers of cadavers, being used as biological clock in measuring the time of death (Amendt *et al.* 2004). *Chrysomya putoria* (Wiedemann) was introduced in Brazil in the 1970s and lives in close association with humans (Ebeling 1978). *Lucilia cuprina* (Wiedemann) are found mainly in garbage in the cities, and can also be found in decaying meat, fruit and human faeces, being incriminated as a carrier of enteropathogens for man (Linares 1981; Furlanetto *et al.* 1984; Almeida & Mello 1996; Mariconi *et al.* 1999). *Chrysomya megacephala* (Fabricius) is responsible for secondary myiasis in humans and animals, and is capable of transmitting pathogens. This species is found throughout the national territory, but reproduces more easily in urban than in rural or forest areas (D'Almeida *et al.* 1998).

The housefly, *Musca domestica* (Linnaeus), is considering a forensically-important fly in many parts of the world (Smith 1986; Byrd & Castner 2001). Previous work has revealed the importance of fly puparia as entomological evidence in investigation not only to estimate the postmortem interval (Lord 1990; Benecke 1998; Erzincliogluo 2000; Greenberg & Kunich 2002), but also to demonstrated the presence of drug or poisonous substances (Miller *et al.* 1994; Bourel *et al.* 2001).

Sarcophagidae specimens, also known as fleshflies, are found in Neotropical Regions being associated to carcasses of vertebrate animals (Barros *et al.* 2008, Carvalho & Linhares 2001) and human cadavers (Oliveira-Costa *et al.* 2001; Barros *et al.* 2008). According to Dias *et al.* (1984) the species *Parasarcophaga ruficornis* (Fabricius) presents a high degree of synanthropy in Belo Horizonte, the same was observed by Linhares (1979) in

Edited by:
William Costa Rodrigues

Article History:
Received: 04.xi.2018
Accepted: 27.v.2019

✉ **Corresponding author:**
Júlio Vianna Barbosa
julio.jub@gmail.com
No ORCID record

Funding agencies:
Without funding declared

the city of Campinas. Several studies evaluate the effect of drugs in decomposing tissues on the development of *Parasarcophaga ruficornis* (Fabricius) and implications of this effect on the estimations of postmortem intervals (Goff *et al.* 1992; Goff *et al.* 1993; Goff *et al.* 1994; Goff *et al.* 1997). *Peckia* (*Peckia*) *chrysostoma* (Wiedemann) is reported intimately associated to carcass in Brazil (Salviano 1996; Carvalho & Linhares 2001; Oliveira-Costa *et al.* 2001; Oliveira-Costa 2011) and Vasconcelos *et al.* (2014) published the first evidence of *P.* (*P.*) *chrysostoma* as colonizers of human bodies.

According to Rodrigues *et al.* (2004), temperature is one of the environmental factors that directly interfere with the development of the insect population, since it does not have a thermostatic regulation system. The temperature ranged between 15 and 38 ºC is considered the best range for the development of most species. When the temperature drops to 15 °C, most insect species hibernate temporarily. At 0 °C or below occurs cooling or freezing of fluids, at - 4.5 °C, complete freezing may occur, causing the insect death.

Many authors report the influence of temperature on the development, abundance, richness and seasonality of muscoid dipterans, evidencing their importance of bionomy for forensic studies (Sousa & Linhares 1997; Paes *et al.* 2001; Vianna *et al.* 2004; Batista-da-Silva 2010; Neto *et al.* 2011). In many researches in the area of medical and forensic entomology, freezing techniques were used to evaluate the bionomy of muscoid dipterans (Oliveira *et al.* 2002; Cerigatto 2009; Mastrangelo *et al.* 2014).

The aim of this work was to evaluate the cooling and freezing effects on third instar larvae and pupae of the species *C. putoria, C. megacephala, L. cuprina, M. domestica, P. ruficornis* and, *P.* (*P.*) *chrysostoma*.

MATERIAL AND METHODS

Insects. Experiments were carried out at the Oswaldo Cruz Foundation (Fiocruz / RJ). Flies colonies were established from specimens collected at the campus using a baited trap, as described by Ferreira (1978) for *C. putoria*, *C. megacephala, L. cuprina*, *M. domestica*, *P. ruficornis* and *P.* (*P.*) *chrysostoma*.

The insects were kept in cages in a climatized chamber at a temperature of 27±1 ºC, relative humidity of the air 70±10% and photophase of 12:12 h and supplied with water and sugar *ad libitum*. Ground rotten meat was given to stimulate oviposition, and the females readily oviposited on this medium. The new generation was reared as described earlier, and immature stages from the second laboratory hatched generation were used for this study

Cooling assays. All experimental groups were performed in quintuplicate using ten immature stages for each replicate. In addition, a control group was performed for each experiment.

For each species, 50 third instar larvae (L3) and 50 newly formed pupae were random selected and individually transferred to glass tubes previously sterilized containing cat's sand to one-fourth of their volume sealed with cotton.

Both third instar larvae and newly formed pupae from test group of each species were submitted to refrigeration temperature of 2±1 ºC, 51±10% relative air humidity (U.R.A.) and, and photophase of 12:12h, while control groups were maintained in a climatized chamber at a temperature of 27±1 ºC, 70±10% U.R.A. and photophase of 12:12 h.

After a twenty-four hour period, samples were taken from the refrigerator and placed in cages on ventilated shelves at a temperature of 27±1 ° C and 60% U.R.A. Experiments were observed daily.

Viability of pupal period, *i.e.*, percentage of third instar larvae turned pupa and newly-hatched larvae to adult period, i.e., percentage of adults emerged from each experiment, since these adults came from a viable egg. In addition, general aspects of morphology, reproductive capacity and generation of fertile offspring of the individuals tested was observed.

Freezing assays. All experimental groups were performed in quintuplicate using ten immature stages for each replicate. In addition, a control group was performed for each experiment.

For each species, 50 third instar larvae (L3) and 50 newly formed pupae were random selected and individually transferred to glass tubes previously sterilized containing cat's sand to one-fourth of their volume sealed with cotton.

Both third instar larvae and newly formed pupae from test group of each species were submitted to freezing temperature of -26±1 ºC, 39±10% U.R.A. and photophase of 12:12h, while control groups were maintained in a climatized chamber at a temperature of 27±1 ºC, 70±10% relative air humidity and photophase of 12:12h.

After a twenty-four hour period, samples were taken from the freezer and placed in cages on ventilated shelves at a temperature of 27±1 °C and 60% U.R.A. Experiments were observed daily.

Viability of pupal period, *i.e.*, percentage of third instar larvae turned pupa and newly-hatched larvae to adult period, *i.e.*, percentage of adults emerged from each experiment, since these adults came from a viable egg. In addition, general aspects of morphology, reproductive capacity and generation of fertile offspring of the individuals tested was observed.

RESULTS AND DISCUSSION

The range between 15 - 38 ºC is considered the optimum range of development of most insect species, allowing a rapid development and consequently a greater number of offspring (Rodrigues 2004).

Cooling assays

Chrysomya putoria. All the 50 cooled third instar larvae (L3) of *C. putoria* turned pupa, but only seven adults emerged (Figure 1) and were not capable of reproduce and generate fertile offspring. The surviving larvae presented a different appearance from the control group: reduced size and black coloration. Already from pupae replicates, only eight adults emerged (Figure 2) and were unable to reproduce and generate fertile offspring. The pupae presented no morphological differences when purchased with the control.

Batista-da-Silva *et al.* (2010) observed that *C. putoria* has a preference for temperature range between 31 and 38 ºC with relative humidity between 47 and 87%. Souza & Linhares (1997) reported the presence of *C. putoria* in pig carcasses in the months with the highest temperatures of the year in Campinas, São Paulo. However, in the work of Vianna *et al.* (2004) carried out in Pelotas in Rio Grande do Sul, *C. putoria* was collected at temperature range of 20.0 and 22.5 °C, already between the ranges of 10.0 and 12.5 ºC the same specie was not collected. According to Vianna *et al.* (2004) *C. putoria* has its development compromised at low temperatures, demonstrating that for this species, temperature is an important factor in the analysis of population fluctuation.

Chrysomya megacephala. All the 50 cooled third instar larvae (L3) of *C. megacephala* turned pupa, but only eight adults emerged (Figure 1) and were not capable of reproduce and generate fertile offspring. The surviving larvae presented a different appearance from the control group: reduced size and black coloration. Already from pupae replicates, only eight

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adults emerged (Figure 2) and were unable to reproduce and generate fertile offspring. The pupae presented no morphological differences when purchased with the control.

The development time of neolarva to adult of *C. megacephala* observed by Milward-de-Azevedo *et al.* (1996), created with equine meat diet, was 10,8 days under a temperature of 24ºC and 7,92 days under a temperature of 30 ºC. Gabre *et al.* (2005) observed that at 26ºC the larval development time is 5.4 days, the pupal development 5.3 days and the total development time 11.7 days.

Lucilia cuprina. All the 50 cooled third instar larvae (L3) of *L. cuprina* turned pupa, but only seven adults emerged (Figure 1) and were not capable of reproduce and generate fertile offspring. The surviving larvae presented a different appearance from the control group: reduced size and black coloration. Already from pupae replicates, only nine adults emerged (Figure 2) and were unable to reproduce and generate fertile offspring. The pupae presented no morphological differences when purchased with the control.

These results demonstrate low resistance to low temperatures. Paes *et al.* (2001) conducted a study on the thermal determination for post-embryonic development of *L. cuprina* under laboratory conditions, using the following temperatures: 25, 30 and 35 °C. The authors observed that the temperature of 25 °C was the one that best allowed the development of this species under laboratory conditions.

Musca domestica. All the 50 cooled third instar larvae (L3) of *L. cuprina* turned pupa, and 46 adults emerged (Figure 1) and were able to reproduce and generate fertile offspring. The surviving larvae presented a different appearance from the control group: reduced size and black coloration. Already from pupae replicates, 43 adults emerged (Figure 2) and were able to reproduce and generate fertile offspring. The pupae presented no morphological differences when purchased with the control.

Weigert *et al.* (2002), when evaluating the influence of temperature on the development of larvae of *M. domestica*, observed that there was a higher production under temperatures of 20, 23 and 26 ºC, than at temperatures of 29 and 32 ºC. Rosenthal *et al.* (2013) observed that temperatures in the range of 5 and 10 °C negatively influenced the viability of *M. domestica* eggs when compared to temperatures in the range of 25±2 °C. In addition, low temperature can influence lifecycle longevity.

Peckia ruficornis. All the 50 cooled third instar larvae (L3) of *P. ruficornis* turned pupa, and 46 adults emerged (Figure 1) and were able to reproduce and generate fertile offspring. The surviving larvae presented a different appearance from the control group: reduced size and black coloration. This experiment showed that the temperature of 2 °C and 51% U.R.A. was not able to prevent the development of the treated larvae of this species. In contrast, from pupae replicates, none adults emerged (Figure 2). The pupae presented no morphological differences when purchased with the control.

In a study by Amoudi *et al.* (1994), *P. ruficornis* flies reached maximum development at pupae and adult stages at 25 and 28 ° C, while minimum weights were recorded at 16 and 37 °C. The ideal temperature in terms of rapid development, low mortality and increased weight varied between 22 and 28 °C.

Peckia* (*Peckia*) *chrysostoma. All the 50 cooled third instar larvae (L3) of *P.* (*P.*) *chrysostoma* turned pupa, and 45 adults emerged (Figure 1) and were able to reproduce and generate fertile offspring. The surviving larvae presented a different appearance from the control group: reduced size and black coloration. Already from pupae replicates, 45 adults emerged (Figure 2) and were not able to reproduce and generate fertile offspring. The pupae presented no morphological differences when purchased with the control. Salviano *et al.* (1996) observed that the temperature of 25.9 ºC and 79% of U.R.A. allows a high viability of larvae and pupae of *P.* (*P.*) *chrysostoma* in laboratory conditions.

Freezing assays. In relation to the freezing experiments, none of the third instar larvae nor newly formed pupae remained viable after the twenty-four-hour period (Figures 3 and 4).

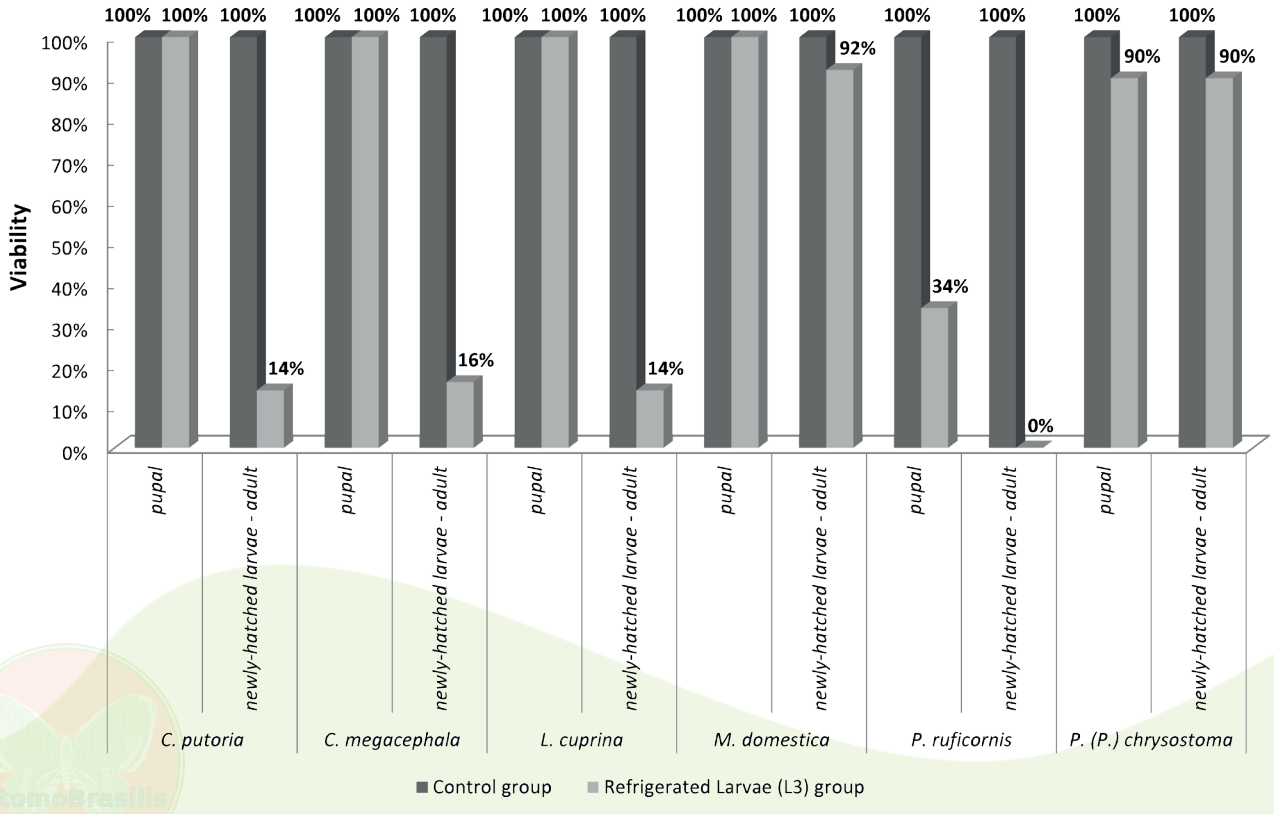

Figure 1. Pupal and newly-hatched larvae viabilities of refrigerated third instar larvae (L3) of *Chrysomya putoria*, *Chrysomya megacephala*, *Lucilia cuprina*, *Musca domestica*, *Parasarcophaga ruficornis* and *Peckia* (*Peckia*) *chrysostoma*.

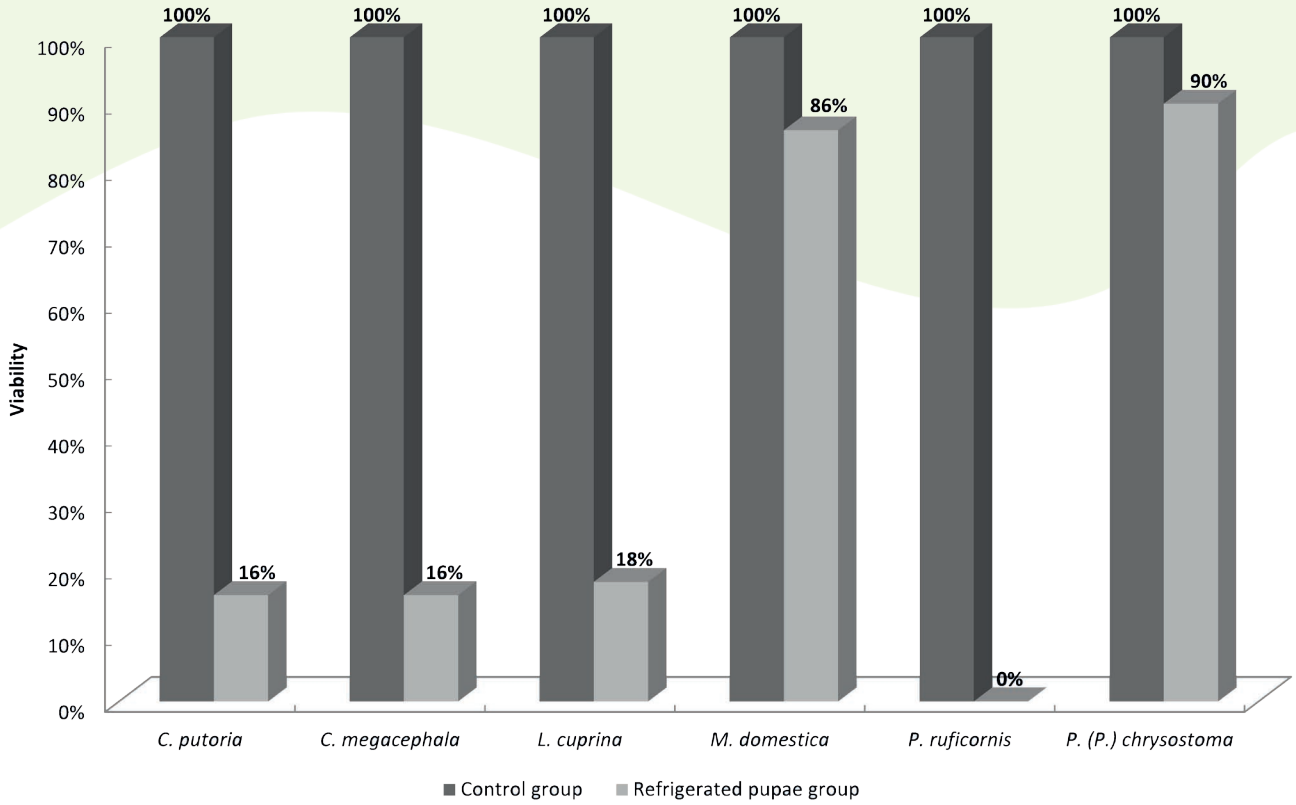

Figure 2. Newly-hatched larvae viability of refrigerated third instar pupae of *Chrysomya putoria*, *Chrysomya megacephala*, *Lucilia cuprina*, *Musca domestica*, *Parasarcophaga ruficornis* and *Peckia* (*Peckia*) *chrysostoma*.

The control group of each experiment presented 100% viability both for third instar larvae (L3) and pupae for all species (Figures 3 and 4).

Temperature and humidity are extremely important factors in the life cycle of muscoid dipterans, since certain species and families react differently to these factors. To understand the behavior of colonization in bodies or carcasses of some necrophagous species against temperature oscillation under laboratory conditions is very important since multiple freeze–thaw cycles are common in alpine, polar and temperate habitats. Research on the effects of freezing on decomposition is minimal in the literature and the effect that it has on insect biology.

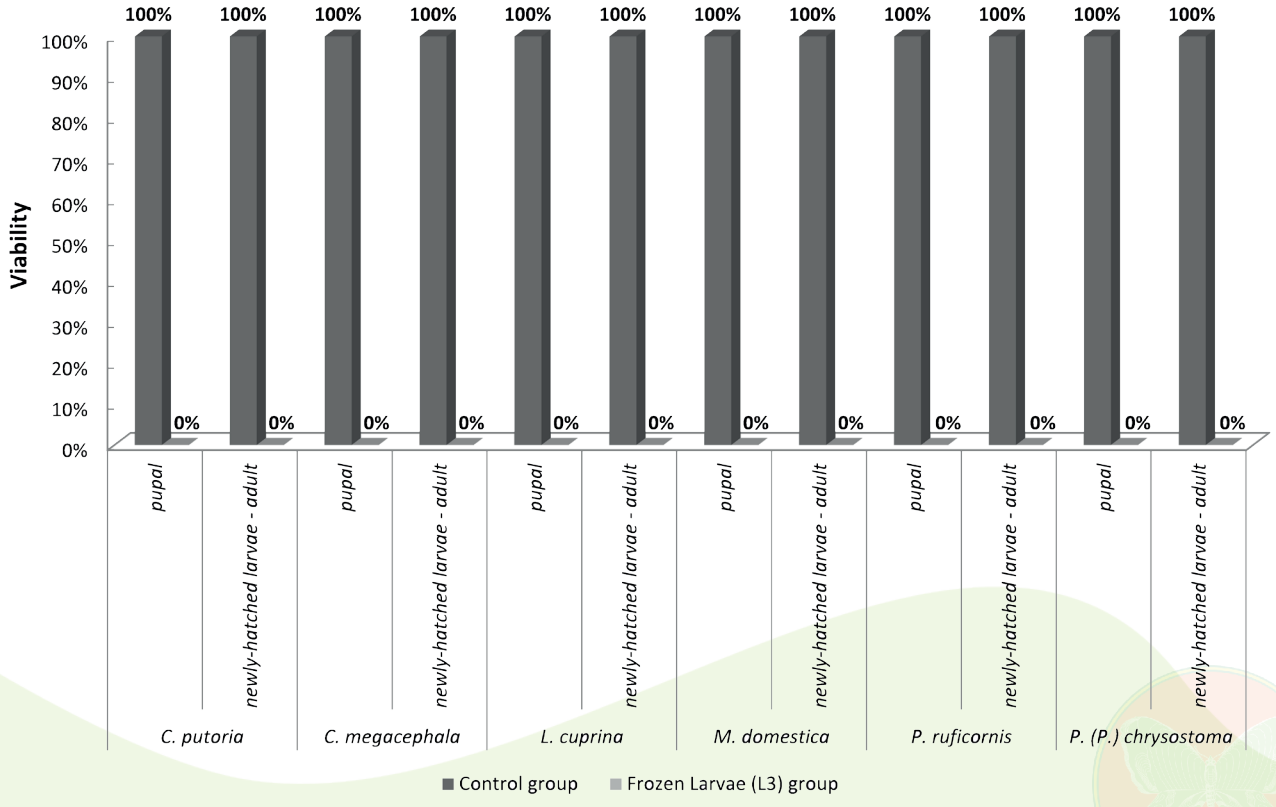

Figure 3. Pupal and newly-hatched larvae viabilities of frozen third instar larvae (L3) of *Chrysomya putoria*, *Chrysomya megacephala*, *Lucilia cuprina*, *Musca domestica*, *Parasarcophaga ruficornis* and *Peckia* (*Peckia*) *chrysostoma*.

e-ISSN 1983-0572

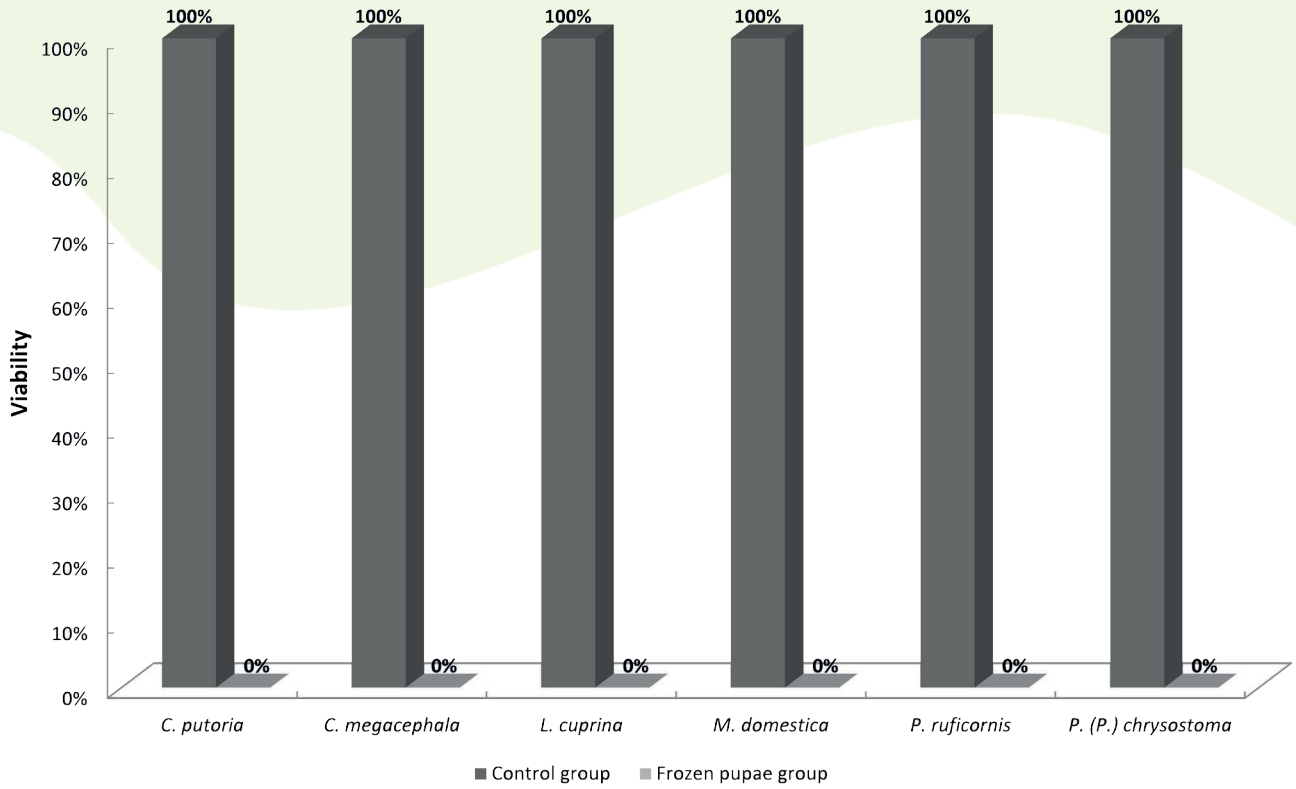

Figure 4. Newly-hatched larvae viabilitiy of frozen pupae of *Chrysomya putoria*, *Chrysomya megacephala*, *Lucilia cuprina*, *Musca domestica*, *Parasarcophaga ruficornis* and *Peckia* (*Peckia*) *chrysostoma*.

ACKNOWLEDGMENT

The authors are grateful to Paulo Vander Ferreira Santana (a teacher from the Municipality of Rio de Janeiro) for his help with reviewing the English language part of the manuscript. The authors thank to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação Oswaldo Cruz for the grants and fellowships provided.

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Suggestion citation:

Carriço C, RL Caetano, JV Barbosa & ZT Pinto, 2019. Cooling and freezing effects on development of flies of forensic importance. EntomoBrasilis, 12 (2): 57-62.
Available on: doi:10.12741/ebrasilis.v12i2.823

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