Universidade Federal do Rio de Janeiro

EFEITOS DA REFRIGERAÇÃO NO DESENVOLVIMENTO DE DÍPTEROS CALIFORÍDEOS DE IMPORTÂNCIA FORENSE

Larissa Thans Carneiro

2025

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Tese de Doutorado apresentada ao Programa de Pós-graduação em Ciências Biológicas (Zoologia), Museu Nacional, da Universidade Federal do Rio de Janeiro, como parte dos requisitos necessários à obtenção do título de Doutora em Ciências Biológicas (Zoologia).

Orientadora: Márcia Souto Couri
Co-orientadora: Janyra Oliveira-Costa

Rio de Janeiro
Abril de 2025

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“Quando em meu peito rebentar-se a fibra.
Que o espírito enlaça à dor vivente.
Não derramem por mim nem uma lágrima
Em pálpebra demente.
E nem desfolhem na matéria impura
A flor do vale que adormece ao vento:
Não quero que uma nota de alegria
Se cale por meu triste passamento.
Eu deixo a vida como deixa o tédio
Do deserto, o poente caminheiro
- Como as horas de um longo pesadelo
Que se desfaz ao dobre de um sineiro;
Como o desterro de minh’alma errante.
Onde fogo insensato a consumia:
Só levo uma saudade – é desses tempos
Que amorosa ilusão embelecia.”

Lembrança de Morrer
Àlvares de Azevedo

RESUMO GERAL

EFEITOS DA REFRIGERAÇÃO NO DESENVOLVIMENTO DE DÍPTEROS CALIFORÍDEOS DE IMPORTÂNCIA FORENSE

Larissa Thans Carneiro

Orientador (as): Márcia Souto Couri e Janyra Oliveira-Costa

Resumo da Tese de Doutorado submetida ao Programa de Pós-graduação em Ciências Biológicas (Zoologia), Museu Nacional, da Universidade Federal do Rio de Janeiro – UFRJ, como parte dos requisitos necessários à obtenção do título de Doutor em Zoologia.

RESUMO

O presente estudo investigou os efeitos da refrigeração contínua e descontínua no desenvolvimento e sobrevivência de dípteros califorídeos de importância forense. O objetivo principal foi avaliar como a exposição a baixas temperaturas, tanto de forma constante quanto intermitente, afeta os diferentes estágios de desenvolvimento dessas moscas (ovos, larvas de primeiro, segundo e terceiro instares e pupas) e quais as implicações para a estimativa do intervalo pós-morte (IPM). No Capítulo I, foram realizados experimentos com refrigeração contínua (4 ± 2 ºC) em quatro espécies forensicamente relevantes: *Chrysomya megacephala*, *Chrysomya putoria*, *Hemilucilia segmentaria* e *Lucilia cuprina*. Os resultados demonstraram um retardo significativo ou interrupção do desenvolvimento, com alta mortalidade de ovos e larvas de primeiro instar, enquanto larvas de terceiro instar apresentaram maior tolerância, entrando em quiescência. O Capítulo II avaliou os efeitos da refrigeração descontínua em larvas de terceiro instar das mesmas espécies, com ciclos alternados de frio e reaquecimento. Observou-se que a refrigeração descontínua também induziu quiescência, levando a um aumento considerável no tempo total de desenvolvimento e redução na sobrevivência. No Capítulo III, o desenvolvimento larval foi avaliado em cadáveres humanos refrigerados (4 ± 2 ºC) no Instituto Médico Legal Afrânio Peixoto, com coleta de larvas de diversas espécies, incluindo *Chrysomya albiceps*, *C. megacephala*, *Peckia (Peckia) chrysostoma*, *H. segmentaria* e *Synthesiomyia nudiseta*. Os resultados

confirmaram um retardo significativo no desenvolvimento larval sob refrigeração, afetando a estimativa do dia de oviposição e alertando para imprecisões no IPM mínimo. Conclui-se que a refrigeração é um fator crítico que interfere significativamente no desenvolvimento de dípteros califorídeos, impactando a acurácia da estimativa do IPM. Há uma necessidade premente de mais pesquisas sobre a biologia térmica dessas espécies em baixas temperaturas, especialmente para espécies tropicais e subtropicais, para aprimorar metodologias.

Palavras-chave:ciências forenses, entomologia forense, baixas temperaturas, intervalo pós-morte, moscas varejeiras.

Rio de Janeiro

Abril de 2025

GENERAL ABSTRACT

EFFECTS OF REFRIGERATION ON THE DEVELOPMENT OF CALLIPHORID DIPTERANS OF FORENSIC IMPORTANCE

Larissa Thans Carneiro

Orientador (as): Márcia Souto Couri e Janyra Oliveira-Costa

Abstract da Tese de Doutorado submetida ao Programa de Pós-graduação em Ciências Biológicas (Zoologia), Museu Nacional, da Universidade Federal do Rio de Janeiro - UFRJ como parte dos requisitos necessários à obtenção do título de Doutor em Zoologia.

ABSTRACT

This study investigated the effects of continuous and discontinuous refrigeration on the development and survival of forensically important calliphorid dipterans. The primary objective was to assess how exposure to low temperatures, both constant and intermittent, affects the different developmental stages of these flies (eggs, 1st, 2nd, and 3rd instar larvae, and pupae) and the implications for estimating the post-mortem interval (PMI). In Chapter I, experiments were conducted with continuous refrigeration (4 ± 2 ºC) on four forensically relevant species: *Chrysomya megacephala*, *Chrysomya putoria*, *Hemilucilia segmentaria*, and *Lucilia cuprina*. The results demonstrated a significant delay or cessation of development, with high mortality in eggs and first instar larvae, while third instar larvae showed greater tolerance, entering quiescence. Chapter II evaluated the effects of discontinuous refrigeration on third instar larvae of the same species, with alternating cycles of cold and rewarming. It was observed that discontinuous refrigeration also induced quiescence, leading to a considerable increase in total development time and a reduction in survival. In Chapter III, larval development was assessed on refrigerated human corpses (4 ± 2 ºC) at the Afrânio Peixoto Forensic Medical Institute, with the collection of larvae from various species, including *Chrysomya albiceps*, *C. megacephala*, *Peckia (Peckia) chrysostoma*, *H. segmentaria*, and *Synthesiomyia nudiseta*. The results confirmed a significant delay in larval development under refrigeration, affecting the estimation of the oviposition day and highlighting potential inaccuracies in the minimum PMI. The study

concludes that refrigeration is a critical factor that significantly interferes with the development of calliphorid dipterans, impacting the accuracy of PMI estimation. There is a pressing need for further research on the thermal biology of these species at low temperatures, especially for tropical and subtropical species, to improve methodologies.

Key-words: Forensic entomology, forensic science, postmortem interval, blow flies, low temperatures.

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SUMÁRIO

INTRODUÇÃO GERAL

Utilizada em ocorrências com morte, a entomologia forense tem auxiliado na estimativa do IPM - intervalo mínimo pós-morte (Amendt *et al*. 2004; Pujol-Luz *et al*. 2008). O IPM corresponde ao período de tempo entre a ocorrência da morte e o momento em que o corpo é encontrado (Pujol-Luz *et al*. 2008; Zurawsk *et al*. 2009; Berg & Benbow 2013). Nestes casos, larvas de moscas que colonizam um cadáver em decomposição podem ser coletadas e se tornam evidências tão importantes quanto outros vestígios encontrados no local do crime (Catts & Goff 1992), podendo o tempo de colonização indicar o IPM mínimo (Oliveira-Costa 2024).

A estimativa do IPM, por métodos entomológicos, se baseia em padrões conhecidos de sucessão de insetos (IPM máximo) e do estágio de desenvolvimento dos imaturos coletados num corpo em decomposição (IPM mínimo) (Oliveira-Costa 2011). Portanto, o conhecimento dos fatores que podem interferir na decomposição, na colonização do cadáver e no desenvolvimento dos insetos associados a ele é de suma importância para o estabelecimento do IPM com maior acurácia (Stamper *et al*. 2009; Mohr & Tomberlin 2014).

A temperatura é uma chave extremamente importante na determinação da idade de insetos, afetando diretamente seu desenvolvimento e, consequentemente, a estimativa do IPM mínimo (Catts & Goff 1992). O modelo mais aceito para o cálculo do tempo de colonização é o linear, no qual são relacionados o tempo transcorrido para o desenvolvimento do inseto e a temperatura a que o inseto foi submetido (Oliveira-Costa 2011). As taxas de desenvolvimento de várias espécies, inclusive das moscas (Diptera: Calliphoridae) ocorrem de acordo com uma curva assimétrica em forma de “S”, na qual a velocidade de desenvolvimento é de baixa a nula sob baixas temperaturas, aumentando

linearmente em temperaturas médias e tornando a abrandar sob temperaturas elevadas até atingir um limiar letal (Higley & Haskell 2009).

É essencial determinar com precisão as temperaturas a que os estágios imaturos foram expostos durante o seu desenvolvimento, dentro ou fora de um corpo (Charabidze & Hedouin 2019). Os entomologistas forenses devem utilizar dados de temperatura confiáveis para estimar o tempo de colonização ao se basear na fase de desenvolvimento dos insetos (Amendt *et al*. 2007). No entanto, é considerado praticamente impossível fazer a determinação exata da temperatura que efetivamente prevaleceu durante o desenvolvimento dos insetos, devido a fatores que implicam na diferença microclimática entre diferentes regiões geográficas. Características do local onde o corpo se encontra também afetam a aferição da temperatura, como ele estar dentro de um imóvel, no interior de um carro, em local externo na área rural, em uma floresta, dentre outros (Hofer *et al*. 2017). A coleta dos dados climatológicos, geralmente, é feita em estações meteorológicas as quais, muitas vezes, encontram-se distantes do local onde um corpo é encontrado, fornecendo dados não condizentes devido a diferença microclimática de cada região (Charabidze & Hedouin 2019).

Quando um corpo é encontrado, durante investigações criminais iniciais, larvas de insetos colonizadores são coletadas, pelos peritos competentes, na própria cena do crime e levadas, logo em seguida, para serem analisadas no laboratório. Entretanto, há casos em que um corpo, ainda contendo insetos saprófagos, pode ficar refrigerado por um tempo considerável, de vários dias, até que seja realizada a necropsia (Huntington *et al*. 2007). Também pode acontecer de não ser possível realizar um transporte rápido das larvas dos insetos para o laboratório onde serão estudadas, e, nessas situações, elas podem ser acondicionadas em refrigeradores (ou em coolers com gelo gel), até sua chegada ao laboratório (Myskowiak & Doums 2002; Magnin 2016).

Tal procedimento de expor os insetos a baixas temperaturas irá afetar seu desenvolvimento, porém ainda não se sabe ao certo o quão afetado ele será, uma vez que existem poucas pesquisas a respeito do desenvolvimento de cada estágio de vida do inseto (ovo, instares larvais, pupa) quando este é mantido em ambientes com baixas temperaturas por várias horas ou dias (Myskowiak & Doums 2002; Huntington *et al*. 2007). Este período sob refrigeração acarreta consequências que, muitas vezes, são negligenciadas ao se estimar o tempo de colonização. A temperatura de refrigeração, raramente, é precisa ou estritamente controlada, gerando erros e desvios no cálculo do tempo de colonização que, dificilmente, conseguem ser corrigidos (Thevan *et al*. 2010; Archer 2004; Charabidze & Hedouin 2019). Sendo assim, as investigações criminais são demasiadamente prejudicadas, uma vez que não se consegue fazer uma estimativa confiável de tempo mínimo de morte.

A partir do exposto, o presente estudo teve o intuito de avaliar como o desenvolvimento e a sobrevivência são afetados quando moscas (em diferentes estágios de desenvolvimento: ovos; larvas de primeiro, segundo e terceiro instares e pupas) são expostas a condições contínuas ou descontínuas de refrigeração, sob condições controladas em laboratório e também sob as condições de refrigeração nas quais cadáveres humanos foram mantidos no necrotério do Instituto Médico Legal Afrânio Peixoto, Rio de Janeiro, durante o processo de realização dos exames periciais e necroscópicos.

Os resultados desta pesquisa estão expressos em três artigos, conforme a seguir.

CAPÍTULO I:

Effects of continuous refrigeration on forensically important immatures of the Calliphoridae (Diptera)

O artigo intitulado “Effects of continuous refrigeration on forensically important immatures of the Calliphoridae (Diptera)” apresenta resultados e informações obtidos através da realização de experimentos que avaliaram o desenvolvimento, sob baixas temperaturas de forma contínua, das espécies *Chrysomya megacephala* (Fabricius, 1794). *Chrysomya putoria* (Wiedemann, 1818). *Hemilucilia segmentaria* (Fabricius, 1805) e *Lucilia cuprina* (Fabricius, 1775) (Diptera: Calliphoridae); avaliando também qual a implicação disso para as estimativas do intervalo pós-morte. Os autores do artigo são Larissa Thans Carneiro, aluna de Doutorado do Programa de Pós-graduação em Ciências Biológicas (Zoologia) do Museu Nacional/UFRJ; Janyra Oliveira-Costa, professora, pesquisadora, perita criminal oficial e chefe do Laboratório de Entomologia Forense do Instituto Médico Legal Afrânio Peixoto – Polícia Civil do Estado do Rio de Janeiro e Márcia Souto Couri, professora e pesquisadora do Departamento de Entomologia do Museu Nacional - Universidade Federal do Rio de Janeiro (MN/UFRJ). O artigo será submetido em abril de 2025 para publicação no “Journal of Forensic Sciences”, um periódico oficial da American Academy of Forensic Sciences, com classificação “A4” no Qualis Periódicos e Fator de Impacto “1.5”, referente a última avaliação realizada no ano de 2023 pela Journal Citation Reports (Clarivate Analytics). O periódico publica uma variedade de tópicos como antropologia, criminalística, ciências digitais e multimídia, engenharia e ciências aplicadas, ciência da enfermagem forense, jurisprudência, odontologia, patologia/biologia, psiquiatria e ciência comportamental, documentos questionados e toxicologia.

Effects of continuous refrigeration on forensically important immatures of the Calliphoridae (Diptera)

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ABSTRACT

This study investigated the effects of continuous refrigeration (4 ± 2 ºC and 50% ± 5% RH) on the development and survival of immature stages (eggs, 1st, 2nd, and 3rd instar larvae, and pupae) of four forensically important blowfly species (*Chrysomya megacephala*, *Chrysomya putoria*, *Hemilucilia segmentaria*, and *Lucilia cuprina*) compared to a control group (27 ± 2 ºC and 50% ± 5% RH). Groups of approximately 500 individuals at each stage were exposed to refrigeration and the control treatment, with periodic verifications to assess development and survival. Statistical analysis (two-way ANOVA and Šidák post-hoc test) revealed significant differences ($P<0.0001$) in development time and survival between the groups. Eggs and 1st instar larvae did not survive longer than 36 hours under refrigeration. Third instar larvae showed greater resistance, with *L. cuprina* surviving for up to 612 hours, entering quiescence without molting. The results highlight the significant impact of refrigeration on the development of these warm-climate species, warning against potential errors in time of colonization (TOC) estimations and, consequently, in post-mortem interval (PMI) if refrigeration effects are not considered. The research emphasizes the need for species-specific data and caution when applying linear development models in forensic entomology, especially in cases involving refrigeration.

Keywords: Forensic entomology, temperature, postmortem interval, blow flies.

RESUMO

Este estudo investigou os efeitos da refrigeração contínua (4 ± 2 ºC e 50% ± 5% UR) sobre o desenvolvimento e a sobrevivência de fases imaturas (ovos, larvas de 1º, 2º e 3º instar e pupas) de quatro espécies de moscas varejeiras forensicamente importantes (*Chrysomya megacephala*, *Chrysomya putoria*, *Hemilucilia segmentaria* e *Lucilia cuprina*) em comparação com um grupo controle (27 ± 2 ºC e 70% ± 5% UR). Grupos de aproximadamente 500 indivíduos em cada estágio foram expostos à refrigeração e ao tratamento controle, com verificações periódicas para avaliar o desenvolvimento e a sobrevivência. A análise estatística (ANOVA two-way e teste post-hoc de Šidák) revelou diferenças significativas ($P<0.0001$) no tempo de desenvolvimento e na sobrevivência entre os grupos. Os ovos e as larvas de 1º instar não sobreviveram por mais de 36 horas sob refrigeração. As larvas de 3º instar demonstraram maior resistência, com *L. cuprina* sobrevivendo por até 612 horas, entrando em quiescência sem mudar de instar. Os resultados destacam o impacto significativo da refrigeração no desenvolvimento dessas espécies de clima quente, alertando para potenciais erros na estimativa do tempo de colonização (TOC) e, consequentemente, do intervalo pós-morte (IPM) se os efeitos da refrigeração não forem considerados. A pesquisa enfatiza a necessidade de dados específicos para cada espécie e cautela ao aplicar modelos lineares de desenvolvimento em entomologia forense, especialmente em casos envolvendo refrigeração.

Palavras-chave: Entomologia forense, temperatura, intervalo pós-morte, moscas varejeiras.

1| INTRODUCTION

For at least 170 years, forensic entomology, when applied to death cases, has been essential for determining the PMI - post-mortem interval [1, 2]. The PMI refers to the time interval between the victim's death and the moment when the body is discovered [2–4]. In this context, fly larvae that develop on a decaying body can be collected and constitute evidence as significant as any other vestiges present at the crime scene [5].

However, for about 20 years now, there has been a worldwide consensus that, in fact, forensic entomology estimates the time of colonization (TOC) by insects on corpses, which helps determine the minimum post-mortem interval (PMI). After death, a variety of diptera are quickly attracted to the body due to specific odors. Oviposition on the corpse marks the beginning of a "biological clock", which forensic entomologists use to estimate the PMI by analyzing the development of immature stages and the community of insects present [6]. Estimating the time since insect colonization provides a minimum PMI, used to infer the time of death. The use of forensic entomology to estimate the TOC, rather than the PMI directly, recognizes that insects colonize a body after death, and the time required for this colonization must be considered in the PMI estimation [6, 7].

Currently, during initial criminal investigations, when a body is found, specialized forensic experts collect the larvae of colonizing insects directly at the crime scene and quickly transport them to the Forensic Entomology laboratory for analysis. However, there are situations in which the body, still containing saprophagous insects, can remain refrigerated for several days prior to the autopsy [8]. In addition, it may not be possible to immediately transport the insect larvae to the laboratory; in these cases, the specimens are stored in refrigerators (or coolers with ice) until they reach the laboratory [9, 10].

Exposing insects to low temperatures has an impact on their development, but the degree of this interference is still not entirely clear, as there is little research into how each insect life stage (egg, larval instars and pupa) is affected when kept in cold environments for several hours or days [8, 9]. This period of time under refrigeration has negative consequences that are often underestimated when inferring the time of colonization (TOC). The refrigeration temperature is rarely exact or strictly controlled, which results in errors and deviations in the calculation of the TOC, which are difficult to correct [11–13]. Therefore, criminal investigations are seriously compromised, as a reliable estimate of the time of death becomes unfeasible.

Based on the above, this study investigated the development of immature stages of forensically important flies subjected to refrigeration conditions, analyzing how exposure to low temperatures influences the life cycle of these insects. The experiments aimed to simulate the refrigeration of insects used in forensic investigations before reaching the forensic entomology laboratory, as forensic evidence collection protocols recommend refrigeration of these specimens, and medico-legal protocols recommend refrigerating bodies when autopsies cannot be performed within a few hours after death. We sought to assess how development and survival are affected when the immatures of flies from the Calliphoridae family (at different stages of development: eggs; first, second and third instar larvae and pupae) are exposed to continuous refrigeration under controlled conditions in the laboratory.

2| MATERIALS AND METHODS

2.1 Insect breeding and maintenance

A stock colony of the species *Chrysomya megacephala* (Fabricius, 1794). *Chrysomya putoria* (Wiedemann, 1818). *Hemilucilia segmentaria* (Fabricius, 1805) and *Lucilia cuprina* (Fabricius, 1775) (Diptera: Calliphoridae) was established in the laboratory. The colony was maintained and reared according to the methodology recommended by Queiroz & Azevedo (1991) [14]. Specimens were collected to establish the colony at the Instituto Médico Legal Afrânio Peixoto (IMLAP), located in the city of Rio de Janeiro, Brazil - according to the methodology proposed by Mello et al. (2007) [15].

2.2 Exposure of immature insects to low temperatures continuously

Groups of approximately 500 specimens, at different stages of development - eggs, 1st, 2nd and 3rd instar larvae (named L1, L2 and L3, respectively) - were packed separately in polyethylene containers (700 mL) containing around 500g of ground beef as a food substrate (1g/larva ratio). these were inserted into larger jars (1L) containing 2cm of sterilized sand where the errant maggots would stay after abandoning the diet (Figure 1: A – E). The pupae were placed in polyethylene containers (700 mL) containing only 2 cm of sterilized sand (Figure 1: F). Subsequently, the immatures were kept in a climate chamber for bioassays (BOD – New Lab, model NL-161-01) under 4 ± 2 ºC and relative humidity (RH) of 50 ± 5 % until they had completed their development (Figure 1: G). In total, 6 containers were used: 3 for the insects maintained under low temperatures continuously and 3 for the control group, which remained in the BOD (New Lab, model NL-41-02) at 27 ± 2ºC and a RH of 70 ± 5%. Both treatments were kept under a 12-hour photoperiod (photophase-scotophase), starting at 6 a.m.. In the control group experiment, the

development of the insects was verified every 2 hours; from the 3rd larval instar onwards, the verifications were made every 12 hours. In immatures kept under continuous refrigeration, verifications were made every 12 hours. During each verification, 30 specimens from each stage of development were randomly removed from their containers and analyzed under a stereoscopic microscope (Feldmann Wild Leitz, model FWL – SMZ 7.5) to measure their biometrics.

The effect of refrigeration on insect development and biometry was analyzed using a two-way analysis of variance (ANOVA two-way), since one way represented the experiments under refrigeration and the other the control treatments, obtaining a comparison between them; the Šidák correction was also carried out as a post-hoc test to confirm significant differences. A 95% significance level was used for both tests, i.e. "P" values <0.05 were considered statistically significant. The Microsoft Excel program (version 2019) was used to analyze the raw data and the other analyses were carried out using GraphPad Prism software (version 10.0.2).

3| RESULTS

When exposed to continuous refrigeration (4 ± 2 ºC and RH of 50 ± 5%), after a period of approximately 12 to 14 h, the eggs of the species observed *(C. megacephala, C. putoria, H. segmentaria and L. cuprina*) were already non-viable. In the control treatments (27 ± 2 ºC and RH of 70 ± 5%). more than 90% of the eggs remained viable for around 12 to 16 h, with eclosion occurring after this period (Tables 1-4 and Figure 2 A-D). Under refrigeration, L1 larvae and pupae of all species also did not survive for more than 36 h (Tables 1-4 and Figure 2 A-D).

The two-way analysis of variance (ANOVA) indicated significant differences ($P<0.0001$) in the average development time and survival of the immatures exposed to continuous refrigeration in relation to the control treatments, with the greatest difference between the L2 larvae of *C. putoria* (Table 2, Figures 2B; 3 G and H). *H. segmentaria* (Table 3; Figures 2C; 4I and J) and *L. cuprina* (Table 4; Figures 2D; 4K and L); and, above all, L3 larvae of all the species studied in this work, which was confirmed by the Šidák correction post-hoc test.

When kept at low temperature, none of the immatures "moulted", but the L3 larvae resisted for a considerable time: 6% of the individuals of *C. megacephala* and 5% of *H. segmentaria* survived for around 216 h (Figures 3E and 4I); 4% of *C. putoria* for 312 h (Figure 3G); 11% of *L. cuprina* for 612h (Figure 4K). In contrast, in the control treatments, the immatures showed reduced development time and much higher survival rates compared to the experiments under continuous refrigeration, with around 98 to 76% of the individuals still alive in each of the different instars (Figures 3F and H; 4J and L).

For all species, the Sidak post-hoc test also indicated that the average development time of each of the stages exposed to refrigeration also differed significantly from the average time of the stages in the control treatments (i.e. L1 under refrigeration versus L1 in the control treatments; L2 under refrigeration versus L2 in the control treatments and so on. The values can be seen in tables 1 to 4.

4| DISCUSSION

To accurately estimate the Post-Mortem Interval (PMI), it is essential to obtain precise temperature measurements to which developing insects were exposed at the crime scene, as temperature is one of the most critical factors affecting the development of necrophagous insects [13, 16, 17]. The growth rates of species such as blowflies (Diptera: Calliphoridae) can be represented as an asymmetrical "S" shaped curve: development is almost zero at low temperatures, increases linearly at medium temperatures, and decreases at high temperatures, up to a lethal point [13, 16]. However, it is common for bodies to be stored in refrigeration until the time of the autopsy, before insect samples are collected, which can have a significant impact on PMI calculations. Refrigeration can affect the TOC, the development of fly larvae and, consequently, PMI estimates [11, 18]. Simulations of a refrigeration period at 4°C, varying from 24 to 240 hours, for example, have shown that the time of exposure to low temperatures influences the rate of development after cooling [18]. The handling of cadavers and the data collected are as important as data collection at the crime scene. Therefore, it is crucial to consider the effects of refrigeration when estimating the PMI based on entomological evidence

Our results indicate that refrigeration significantly delays the development of immature insects and can even halt it, which is consistent with findings from previous studies [8–11, 17, 19, 20]. However, the majority of studies on the effects of temperature on insect development focus on species adapted to cold climates. In contrast, our work evaluates the impact of refrigeration on species such as *Chrysomya putoria*, *Chrysomya megacephala*, *Hemilucilia segmentaria*, and *Lucilia cuprina*, which are more commonly found in warm climate regions. This is significant because the response of these species to refrigeration may differ from that of species adapted to cold, making our results particularly relevant for

forensic investigations in tropical and subtropical regions. Even within the same species, different genetic populations may exhibit varying levels of cold tolerance [17]. When working with species from warm climate regions, one may potentially uncover genetic variations that influence the capacity to adapt to refrigeration episodes. This could have significant implications for the accuracy of PMI estimates, as the response to cold may vary depending on the geographic origin of the insect population.

In addition to the delay in development, the present study also examines the survival rate of refrigerated larvae. This information is crucial, as the mere fact that a larva survives refrigeration does not imply that its development has not been affected. By combining data on development and survival, we provide a more comprehensive understanding of the impact of refrigeration on insect populations. At low temperatures, none of the juvenile forms underwent molting; however, the L3 larvae persisted for a considerable duration. Myskowiak & Doums (2002) and Magni et al. (2016) [9,10] also analyzed the effects of refrigeration on insect development and its implications for the estimation of the post-mortem interval in forensic investigations. However, our study differs by focusing on different species, with a methodology adapted to the procedures carried out by Brazilian law enforcement agencies and the limited technological resources available. Additionally, we examined further variables, such as survival rate and the effect of refrigeration on different stages of larval development, thus offering new perspectives and contributions to the field of forensic entomology.

It was observed that eggs became unviable within a few hours of refrigeration exposure (between 12–14 h), likely due to rapid desiccation at low temperatures, as reported by Magni et al. (2016) [10]. Lee (1991) and Sømme (1982) [19, 21] also found that eggs, immature stages, and small-sized insects generally supercool at lower temperatures

compared to adults or larger individuals. This phenomenon is related not only to size but also to body weight and water volume in insects. L1 larvae of all species did not survive, possibly because, even when aggregated, they could not generate enough metabolic heat to warm themselves and stay alive [10, 11, 13]. This reinforces the importance of considering larval mass and thermoregulatory effects in forensic investigations. The larval mass plays a crucial role in maintaining temperature and larval development, especially in environments with unfavorable temperatures. Aggregated larvae generate metabolic heat, raising the internal temperature of the mass and creating a microclimate more favorable to development [10]. This thermoregulation phenomenon is particularly important for first instar larvae (L1), which have a lower capacity to generate heat individually. Aggregation allows larvae to overcome physiological limitations and maintain active metabolism, prolonging their survival in refrigerated conditions. Similarly, it is believed that pupae were also unable to maintain functional metabolism at low temperatures. Additionally, a recent study demonstrates that exposure to low temperatures causes physiological damage and neuromuscular dysfunction in insects [22].

The present research faced logistical challenges in replicating large larval masses, which may have limited the ability to observe the thermoregulatory effect at its maximum potency. Creating large larval masses requires a significant volume of food substrate and rigorous control of environmental conditions, such as humidity and ventilation, to avoid the accumulation of metabolic waste and the proliferation of pathogenic microorganisms. The absence of large larval masses in our experiments may have led to an underestimation of the thermoregulation potential of the studied species and, consequently, to an incomplete understanding of their response to refrigeration. Other problems faced were the lack of space, material and more climate chambers for bioassays to conduct the experiments. In real

forensic cases, bodies are often found wrapped in removal bags or with larvae colonizing natural orifices, such as the mouth, nose, and ears. These situations create microclimates that can significantly influence the temperature and humidity around the larvae. Removal bags can isolate the body from the external environment, slowing cooling and allowing larvae to maintain higher temperatures for longer. Natural orifices offer protection against desiccation and can house dense larval masses, with high metabolic activity and thermoregulation. The absence of these microclimates in our experiments may have exacerbated the negative effects of refrigeration and limited the generalization of the results to real forensic scenarios.

It was observed that L2 larvae, and especially L3 larvae, remained in a state of quiescence until their energy reserves were depleted, resulting in their death. This phenomenon, also reported by Myskowiak & Doums (2002) [9] in studies with *Protophormia terraenovae*, demonstrates that refrigeration does not prevent larval survival, but induces a state of temporary metabolic inactivity, extending the time window in which colonization can be estimated. The ability of larger larvae to tolerate refrigeration allows the body to remain at low temperatures for a longer period before examination, which requires consideration of both time and temperature when estimating the PMI. The refrigeration time directly influences the consumption of the larvae's energy reserves, while the temperature affects the metabolic rate during quiescence. Thus, the prolonged survival of larger larvae can mask the actual colonization time, impacting the accuracy of the PMI. Myskowiak & Doums (2002) [9] found that exposing different instars to ten days of refrigeration can lead to errors of more than 6 hours in the PMI estimation. It is important to note that species relevance also plays a crucial role, as different fly species exhibit different levels of cold tolerance. To mitigate these errors, it is essential to collect accurate data on the time and temperature of refrigeration, the identification of the larval species, the stage of

development, and the size of the larval mass, if applicable. The application of development models and corrective calculations, such as Monte Carlo simulation, as proposed by Hill et al. (2020) [7], can assist in adjusting the PMI estimation. taking into account the effects of refrigeration on larval development and allowing for a more accurate interpretation of entomological data in forensic contexts.

L3 larvae exhibited greater resistance to refrigeration, with *Lucilia cuprina* showing the highest survival capacity, remaining viable for up to 612 hours. This higher tolerance may be explained by the greater amount of energy reserves accumulated at this larval stage [13], allowing them to survive longer under adverse conditions [11, 20]. However, it is important to note that, although L3 larvae survive for an extended period, their development is halted, and no molting occurs, reinforcing the hypothesis of a state of quiescence or cold-induced diapause.

The induction of diapause or quiescence in larvae exposed to low temperatures, as observed in this study, has significant implications for the estimation of the Post-Mortem Interval (PMI). Linear methods for calculating the PMI, which assume a constant development rate in relation to temperature, can lead to inaccurate estimates when larval development is interrupted by cold episodes [17]. This interruption in development must be considered when estimating the PMI, as it can generate errors in predictions based on these models. Linear Models vs. Development Interruptions: Linear models for PMI estimation often do not incorporate the complexity of insect physiological responses to temperature variations, such as entering diapause or quiescence. Lower Development Threshold Temperature: Accurate determination of the lower development threshold temperature (minimum threshold) for each species it’s very important. The lack of accurate data on these thresholds can lead to erroneous interpretations of the effects of cold episodes on larval

development [17]. The comparison with control treatments maintained at 27 ± 2 ºC clearly demonstrates the importance of temperature in the "normal" development of flies. The control groups showed faster development and significantly higher survival rates at all stages of development. These results reinforce that refrigeration directly interferes with larval development and that PMI calculations must consider the refrigeration time and conditions, as emphasized by Ikemoto & Egami (2013) and Charabidze & Hedouin (2018) [13, 23]. The body cooling rate is also an important factor. Bodies without larval infestation cool at a rate of about 1–2 ◦C per hour, while bodies with larval infestation cool at a lower rate, about 0.12 ºC/hour, and it can take up to 100 hours to reach the minimum temperature of the refrigerated environment [24]. The time it takes for larvae to reach the lower development threshold temperature after refrigeration must be considered in the PMI calculation. Therefore, research that aims to identify the real minimum threshold of different species is fundamental to assess the real effects of cold episodes and improve the accuracy of PMI estimations as Ames & Turner (2003) has already pointed out [17].

Additionally, this study reveals variations in refrigeration tolerance among different species. *L. cuprina* exhibited greater cold resistance in the L3 stage compared to *Chrysomya megacephala*, *Chrysomya putoria*, and *Hemilucilia segmentaria*. These interspecific differences may be attributed to physiological and behavioral adaptations specific to each species [20, 22]. such as the ability to generate and retain heat within larval aggregations [10, 11, 13]. These variations highlight the need for species-specific studies in forensic entomology.

The forensic implications of these findings are crucial for entomological practice. Refrigeration is a common practice at crime scenes and in medical examiner offices to delay cadaveric decomposition [7]. However, as demonstrated in this study and others [7, 9, 11,

13, 20, 25], refrigeration can lead to significant errors in PMI estimation. Fluctuating temperatures in refrigerated chambers, lack of strict control, absence of precise measurements, and variations in storage conditions make correcting PMI estimates difficult. Therefore, it is essential to assess the temperature at which bodies are kept, the developmental stage, and the species involved [7, 13]. Additionally, the duration of storage in a refrigerator can also affect species development [9].

The use of thermal summation models to calculate PMI can be inaccurate when temperature is not constant, as in refrigerated environments [Ikemoto & Egami 2013. Hill et al 2020]. The use of accumulated degree-day (ADD) methods may not be suitable for simulating development conditions in refrigerators, as these methods assume that development occurs at a constant rate at temperatures above the minimum threshold. However, under low-temperature conditions, development may be halted or drastically reduced, leading to PMI underestimations [6, 7].

Considering variations in crime scene temperatures and refrigerated environments is also highly important. Studies have shown that ambient temperatures can vary significantly depending on location, sun exposure, and other environmental factors [13, 25]. Body temperature may also vary depending on body mass, decomposition stage, and environmental conditions. Therefore, it is essential to record local and body temperatures to improve PMI estimation accuracy [13, 25]. Moreover, the presence of larval masses can generate metabolic heat, creating a microenvironment warmer than the surrounding environment, thereby accelerating larval development. The intensity of this effect varies with larval mass size and the species involved. Consequently, larval mass temperature should also be considered in PMI estimation [10,11, 13, 26].

In summary, our study demonstrated that continuous refrigeration negatively affects the development of flies in the Calliphoridae family and that the L3 larval stage shows greater tolerance to refrigeration. Refrigeration induces a state of diapause/quiescence that halts larval development and eventually leads to death. The different responses of each species to refrigeration emphasize the need for species-specific studies in forensic entomology. The results highlight the importance of considering the temperature and refrigeration history of samples in PMI estimation. It is essential for forensic entomologists to record storage conditions and refrigeration duration to minimize errors in PMI estimation. Standardizing body and insect sample storage conditions and using precise, case-relevant temperature data are crucial for improving forensic investigation accuracy. Additional studies are needed to investigate the effects of refrigeration at different insect developmental stages, including the combination of different temperatures and the impact of storage conditions on PMI estimation.

Future studies should focus on replicating the conditions found at crime scenes, including the use of corpse removal bags and the simulation of natural orifice colonization. The use of experimental models with different sizes of larval masses and continuous monitoring of temperature and humidity in the created microclimates will allow for a more accurate understanding of the impact of refrigeration on larval development in relevant forensic contexts. Furthermore, the analysis of the expression of genes related to thermoregulation and cold tolerance may provide insights into the molecular mechanisms involved in the adaptation of larvae to low-temperature environments as also proposed by Ullah et al. (2024).

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FIGURES

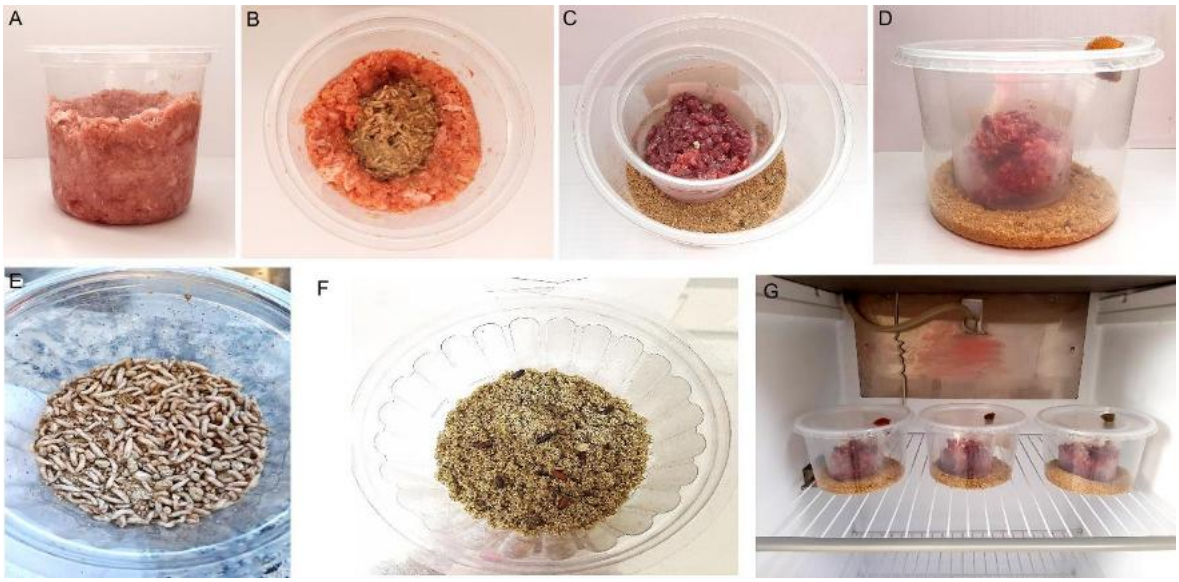

Figure 1. A - D: Containers in which the immatures (eggs and larvae) were placed, along with the food substrate. E and F: Containers in which errant larvae (3rd instar) and pupae were placed with sterilized sand. G: Containers inside the climate chamber (BOD).

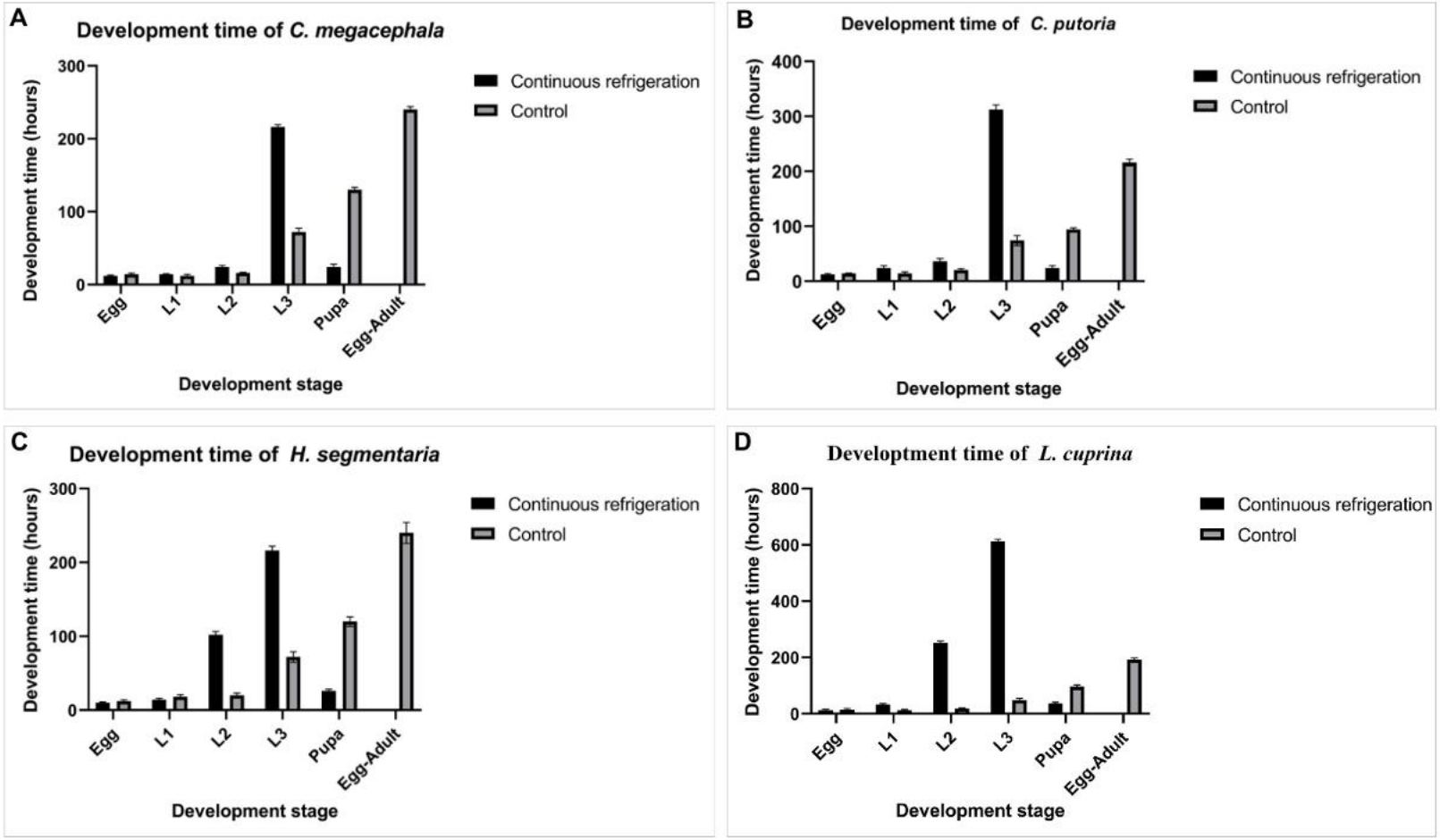

Figure 2. Comparison of average development time between treatments under continuous refrigeration and control. A: development time of *C. megacephala*. B: development time of *C. putoria*. C: development time of *H. segmentaria*. D: development time of *L. cuprina*.

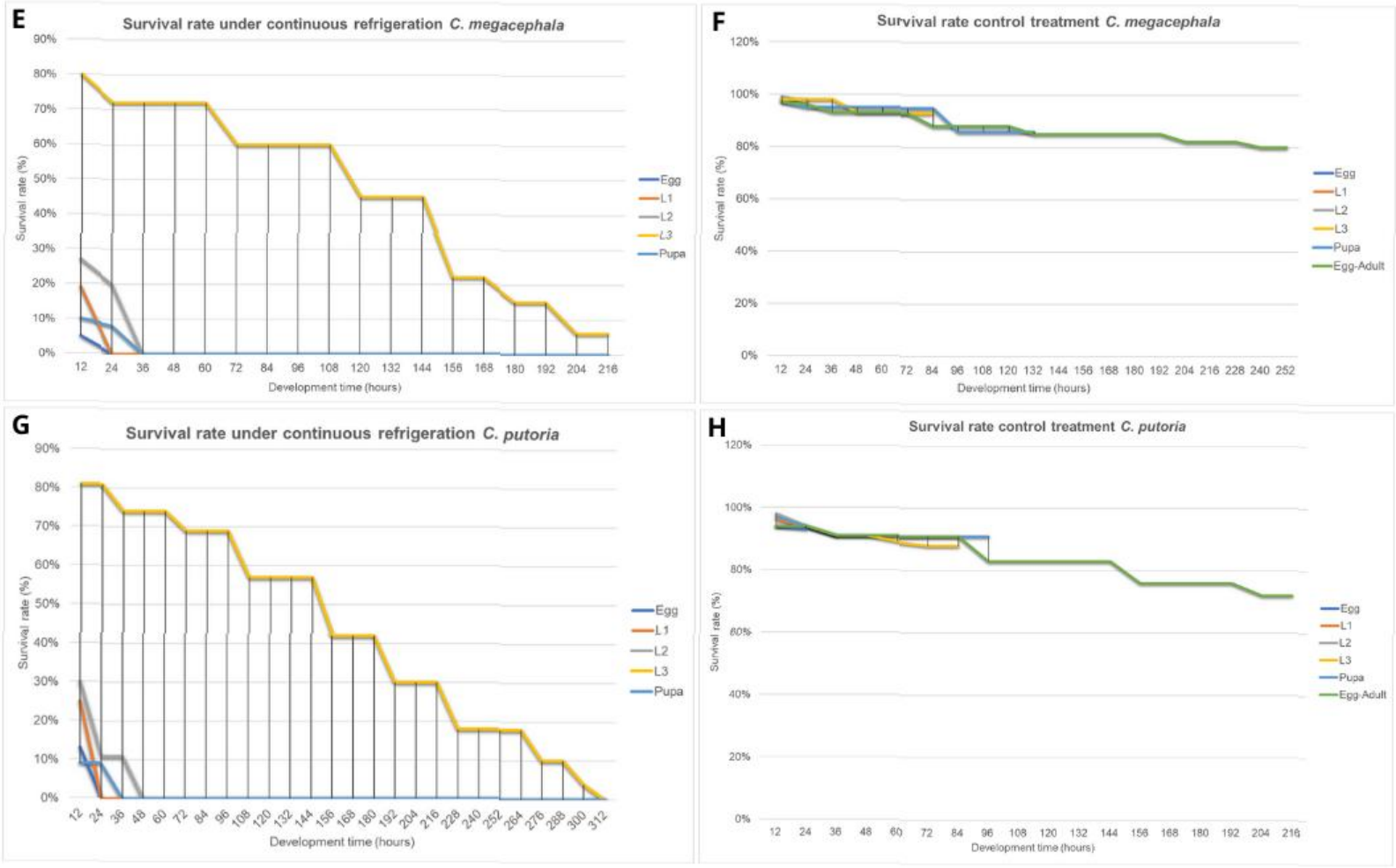

Figure 3. E and G: Survival rate of *C. megacephla* and *C. putoria* under continuous refrigeration (4 ± 2 ºC). F and H: Survival rate of *C. megacephla* and *C. putoria* in the control treatment (27 ± 2ºC and RH of 70 ± 5%).

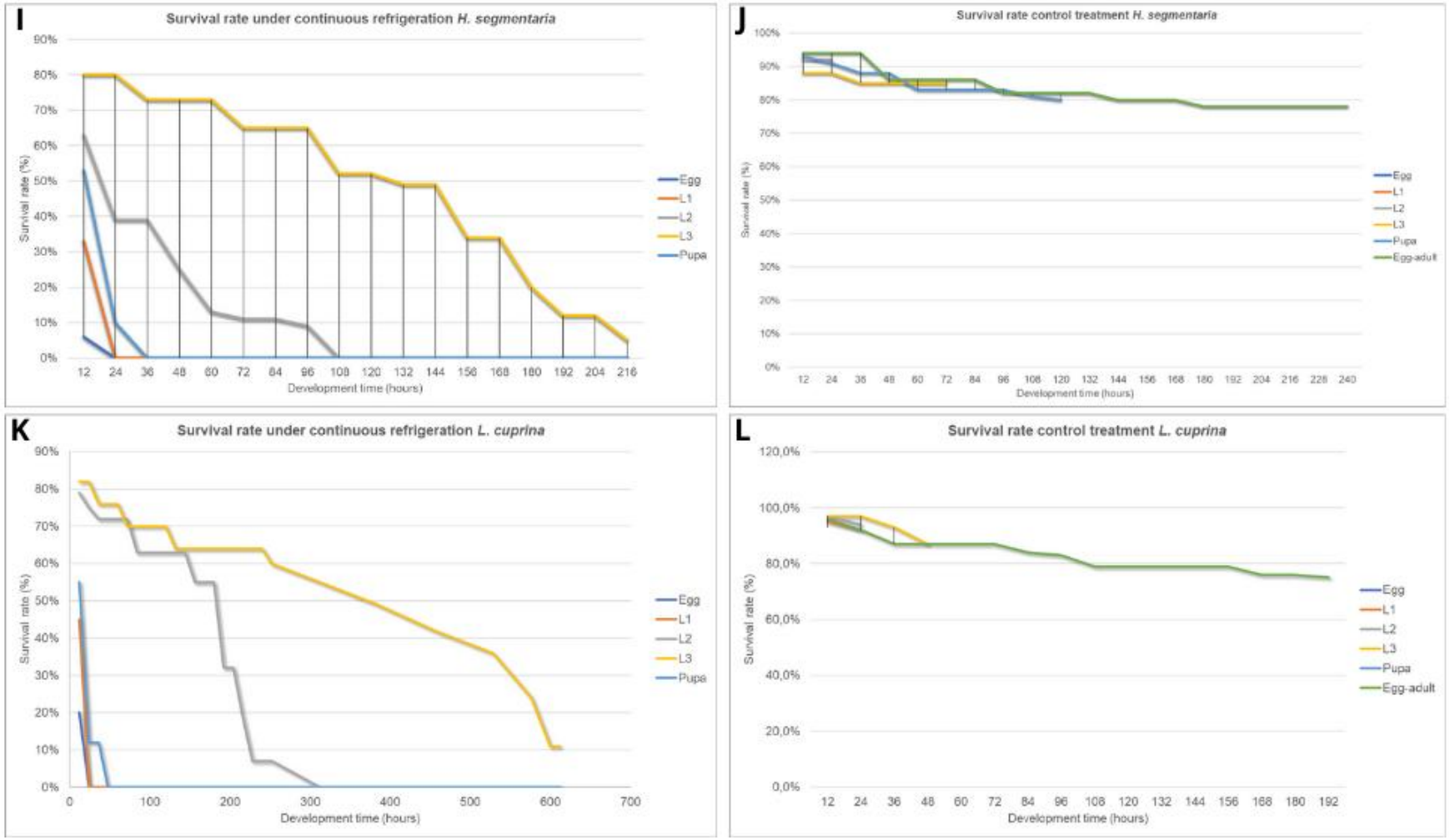

Figure 4. I and K: Survival rate of *Hemilucilia segmentaria* and *Lucilia cuprina* under continuous refrigeration (4 ± 2 ºC). J and L: Survival rate of *H. segmentaria* and *L. cuprina* in the control treatment (27 ± 2ºC and RH of 70 ± 5%).

TABLES

Table 1. Average development time (in hours ± standard deviation) of the immature stages of *Chrysomya megacephala* that were exposed to continuous refrigeration (4 ± 2 ºC and 50 ± 5% RH) and the control treatment (27 ± 2 ºC and 70 ± 5% RH). The F-statistic, P-values and the percentage of total variation corresponding to the two-way ANOVA analysis are also presented.

Development Stage	Time (Hours)		ANOVA two-way	F-valor	P-valor	% of total variation
	Continuous refrigeration [a]	Control [b]				
Egg	12 ± 1.15 †	14 ± 2.00	Interaction	479082	P<0.0001	53.03
1st larval instar	14 ± 1.10 †	12 ± 2.00	Development stage	387110	P<0.0001	42.85
2nd larval instar	24 ± 2.30 †	16 ± 1.20	Development time	180225	P<0.0001	3.990
3rd larval instar	216 ± 3.46 †	72 ± 5.29				
Pupa	24 ± 4.16 †	130 ± 3.50				
Egg-adult	_	244 ± 4.20				

†Indicates that the immatures did not survive beyond the time period informed. there was no change of instar.

[a] and [b] Indicates that the average development time of all instars differed significantly between the treatments under continuous refrigeration and the control group according to the two-way ANOVA and Šidák post-hoc test with 95% of confidence.

Table 2. Average development time (in hours ± standard deviation) of the immature stages of *Chrysomya putoria* that were exposed to continuous refrigeration (4 ± 2 ºC and 50 ± 5% RH) and the control treatment (27 ± 2 ºC and 70 ± 5% RH). The F-statistic, P-values and the percentage of total variation corresponding to the two-way ANOVA analysis are also presented.

	Time (Hours)		ANOVA two-way	F-valor	P-valor	% of total variation
Development stage	**Continuos refrigeration [a]**	**Control [b]**				
Egg	12 ± 2.31 †	14 ± 1.15	Interaction	225753	P<0.0001	52.56
1st larval instar	24 ± 4.00 †	14 ± 3.05	Development stage	202375	P<0.0001	47.12
2nd larval instar	36 ± 5.03 †	20 ± 3.10	Development time	999.1	P<0.0001	0.04652
3rd larval instar	312 ± 8.72 †	74 ± 9.02				
Pupa	24 ± 4.16 †	94 ± 3.06				
Egg-adult	_	216 ± 6.11				

†Indicates that the immatures did not survive beyond the time period informed, there was no change of instar.

[a] and [b] Indicates that the average development time of all instars differed significantly between the treatments under continuous refrigeration and the control group according to the two-way ANOVA and Šidák post-hoc test with 95% of confidence.

Table 3. Average development time (in hours ± standard deviation) of the immature stages of *Hemilucilia segmentaria* that were exposed to continuous refrigeration (4 ± 2 ºC and 50 ± 5% RH) and the control treatment (27 ± 2 ºC and 70 ± 5% RH). The F-statistic, P-values and the percentage of total variation corresponding to the two-way ANOVA analysis are also presented.

	Time (Hours)		ANOVA two-way	F-valor	P-valor	% of total variation
Development Stage	**Continuous refrigeration [a]**	**Control [b]**				
Egg	10 ± 1.15 †	12 ± 2.00	Interaction	144847	P<0.0001	60.16
1st larval instar	14 ± 2.00 †	18 ± 3.06	Development stage	91306	P<0.0001	37.92
2nd larval instar	102 ± 4.62 †	20 ± 3.10	Development time	17097	P<0.0001	1.420
3rd larval instar	216 ± 6.11†	72 ± 7.02				
Pupa	26 ± 2.31 †	120 ± 6.43				
Egg-adult	_	242 ± 14.04				

†Indicates that the immatures did not survive beyond the time period informed, there was no change of instar.

[a] and [b] Indicates that the average development time of all instars differed significantly between the treatments under continuous refrigeration and the control group according to the two-way ANOVA and Šidák post-hoc test with 95% of confidence.

Table 4. Average development time (in hours ± standard deviation) of the immature stages of *Lucilia cuprina* that were exposed to continuous refrigeration (4 ± 2 ºC and 50 ± 5% RH) and the control treatment (27 ± 2 ºC and 70 ± 5% RH). The F-statistic, P-values and the percentage of total variation corresponding to the two-way ANOVA analysis are also presented.

Development Stage	Time (Hours)		ANOVA two-way	F-valor	P-valor	% of total variation
	Continuous refrigeration [a]	Control [b]				
Egg	12 ± 3.46 †	14 ± 4.60	Interaction	629570	P<0.0001	52.45
1st larval instar	32 ± 5.00 †	12 ± 3.06	Development stage	476945	P<0.0001	39.73
2nd larval instar	252 ± 6.11 †	18 ± 3.10	Development time	463483	P<0.0001	7.722
3rd larval instar	612 ± 8.33 †	48 ± 7.21				
Pupa	36 ± 4.62 †	96 ± 7.02				
Egg-adult	_	188 ± 6.10				

†Indicates that the immatures did not survive beyond the time period informed, there was no change of instar.

[a] and [b] Indicates that the average development time of all instars differed significantly between the treatments under continuous refrigeration and the control group according to the two-way ANOVA and Šidák post-hoc test with 95% of confidence.

CAPÍTULO II:

Discontinuous Cooling Effects on Forensically Significant Calliphoridae Larvae

O artigo intitulado “Discontinuous Cooling Effects on Forensically Significant Calliphoridae Larvae” apresenta resultados e informações obtidos através da realização de experimentos que avaliaram o desenvolvimento, sob baixas temperaturas de forma descontínua, das espécies *Chrysomya megacephala* (Fabricius, 1794). *Chrysomya putoria* (Wiedemann, 1818). *Hemilucilia segmentaria* (Fabricius, 1805) e *Lucilia cuprina* (Fabricius, 1775) (Diptera: Calliphoridae); avaliando também qual a implicação disso para as estimativas do intervalo pós-morte. Os autores do artigo são Larissa Thans Carneiro, aluna do Programa de Pós-graduação em Zoologia do Museu Nacional/UFRJ; Janyra Oliveira-Costa, professora, pesquisadora, perita criminal oficial e chefe do Laboratório de Entomologia Forense do Instituto Médico Legal Afrânio Peixoto – Polícia Civil do Estado do Rio de Janeiro e Márcia Souto Couri, professora e pesquisadora do Departamento de Entomologia do Museu Nacional - Universidade Federal do Rio de Janeiro (MN/UFRJ). O artigo será submetido no mês de julho de 2025 para publicação no “Journal of Forensic Sciences”. um periódico oficial da American Academy of Forensic Sciences, com classificação “A4” no Qualis Periódicos e Fator de Impacto “1.5”. referente a última avaliação realizada no ano de 2023 pela Journal Citation Reports (Clarivate Analytics). O periódico publica uma variedade de tópicos como antropologia, criminalística, ciências digitais e multimídia, engenharia e ciências aplicadas, ciência da enfermagem forense, jurisprudência, odontologia, patologia/biologia, psiquiatria e ciência comportamental, documentos questionados e toxicologia.

Discontinuous Cooling Effects on Forensically Significant Calliphoridae Larvae

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ABSTRACT

Forensic entomology plays a crucial role in estimating the postmortem interval (PMI) by analyzing insect colonization on decomposing bodies. Refrigeration is a common variable in forensic casework, applied to both human remains before autopsy and collected larvae before analysis, which can significantly alter the development of necrophagous insects and, consequently, the accuracy of PMI estimations. While the effects of continuous refrigeration have been examined, the impact of discontinuous refrigeration, characterized by alternating periods of cold exposure and rewarming, remains less understood. This study investigated the effects of discontinuous refrigeration on the development and survival of third-instar larvae of forensically important Calliphoridae species under controlled laboratory conditions. Larval groups were subjected to refrigeration cycles at 4 ± 2 ºC for different periods (up to the survival limit) and subsequently returned to 27 ± 2 ºC until they completed development, comparing them to a control group maintained at 27 ± 2 ºC. The results demonstrated that discontinuous refrigeration induced quiescence in the larvae, with development resuming after rewarming. A significant increase ($P<0.0001$) in total development time (egg to adult) and a sharp reduction in survival were observed in all refrigerated treatments compared to the control. For instance, *Chrysomya megacephala* larvae subjected to discontinuous refrigeration exhibited a total development time ranging from 275.33 to 634 hours, with only 25% survival, whereas the control took an average of 249.33 hours with 85% survival. This study highlights the significant impact of discontinuous refrigeration on the development of forensic insects, emphasizing the need to consider these variable conditions to improve the accuracy of PMI estimations in forensic investigations.

Keywords: Forensic entomology, blowflies, postmortem interval, larval development.

RESUMO

A entomologia forense desempenha um papel crucial na estimativa do intervalo pós-morte (IPM), analisando a colonização de insetos em corpos em decomposição. A refrigeração é uma variável comum em casos forenses, aplicada tanto aos restos mortais antes da autópsia quanto às larvas coletadas antes da análise, podendo alterar significativamente o desenvolvimento de insetos necrofágos e, consequentemente, a precisão das estimativas de IPM. Embora os efeitos da refrigeração contínua já tenham sido examinados, o impacto da refrigeração descontínua, caracterizada por períodos alternados de frio e reaquecimento, permanece menos compreendido. Este estudo investigou os efeitos da refrigeração descontínua no desenvolvimento e sobrevivência de larvas de terceiro instar de espécies de Calliphoridae de importância forense sob condições controladas de laboratório. Grupos de larvas foram submetidos a ciclos de refrigeração a 4 ± 2 ºC por diferentes períodos (até o limite de sobrevivência) e subsequentemente retornados a 27 ± 2 ºC até completarem o desenvolvimento, comparando-os a um grupo controle mantido a 27 ± 2 ºC. Os resultados demonstraram que a refrigeração descontínua induziu quiescência nas larvas, com o desenvolvimento sendo retomado após o reaquecimento. Observou-se um aumento significativo ($P<0.0001$) no tempo total de desenvolvimento (ovo a adulto) e uma redução acentuada na sobrevivência em todos os tratamentos refrigerados em comparação com o controle. Por exemplo, larvas de *Chrysomya megacephala* submetidas à refrigeração descontínua apresentaram um tempo total de desenvolvimento variando de 275.33 a 634 horas, com apenas 25% de sobrevivência, enquanto o controle levou em média 249.33 horas com 85% de sobrevivência. Este estudo destaca o impacto significativo da refrigeração descontínua no desenvolvimento de insetos forenses, enfatizando a necessidade de considerar essas condições variáveis para aprimorar a precisão das estimativas de IPM em investigações forenses.

Palavras-chave: Entomologia forense, moscas varejeiras, intervalo pós-morte, desenvolvimento larval.

1| INTRODUCTION

Forensic entomology plays a crucial role in estimating the postmortem interval (PMI) by analyzing insect colonization on decomposing bodies [1, 2]. The presence and development of necrophagous fly larvae provide essential evidence for determining the time of colonization (TOC) and, therefore, the minimum PMI [3, 4, 5]. However, various external factors influence insect development and, consequently, the accuracy of PMI estimates. Among these factors, refrigeration is a common yet often overlooked variable that can significantly alter the growth rate and survival of forensically relevant species [6, 7, 8].

In forensic casework, human remains are frequently refrigerated prior to autopsy, which can delay decomposition and insect activity. Additionally, collected larvae may also be stored in refrigeration before analysis [9, 10]. The impact of continuous refrigeration on immature necrophagous flies has been previously examined, revealing significant developmental delays and increased mortality rates, which can lead to errors in PMI estimation [11]. However, less is known about the effects of discontinuous refrigeration, where insects experience alternating periods of cold exposure and rewarming, which may influence their development differently compared to continuous refrigeration.

Fluctuating temperature conditions can affect insect physiology in distinct ways, potentially inducing quiescence or slowing metabolic processes without completely halting development [12, 13, 14]. Unlike continuous refrigeration, where insects remain at a stable low temperature for an extended period, intermittent exposure to refrigeration may allow for partial recovery between cold episodes, altering survival rates and growth dynamics [10, 15]. Understanding these effects is critical for refining PMI calculations, especially in cases where bodies or larvae are stored under variable temperature conditions before forensic analysis.

This study aims to investigate the effects of discontinuous refrigeration on the development and survival of third-instar larvae of forensic Calliphoridae species under controlled laboratory conditions. By simulating intermittent cooling and rewarming cycles, we seek to determine how these

fluctuations impact larval development thereby improving the accuracy of PMI estimates in forensic investigations.

2| MATERIALS AND METHODS

2.1 Insect breeding and maintenance

Specimens were collected to establish the colony at the Instituto Médico Legal Afrânio Peixoto (IMLAP). located in the city of Rio de Janeiro, Brazil, according to the methodology proposed by Mello et al. (2007) [16]. A stock colony of the species *Chrysomya megacephala* (Fabricius, 1794). *Chrysomya putoria* (Wiedemann, 1818). *Hemilucilia segmentaria* (Fabricius, 1805). and *Lucilia cuprina* (Fabricius, 1775) (Diptera: Calliphoridae) was established in the laboratory. The colony was maintained and reared according to the methodology recommended by Queiroz & Azevedo (1991) [17].

2.2 Exposure of immature insects to low temperatures discontinuously

Groups of ~500 individuals in third larval instar (named L3) of development were placed separately in 700 mL polyethylene containers with approximately 500g of ground beef as a food substrate (1g/larva ratio). These containers were then inserted into larger 1L jars containing 2cm of sterilized sand, where the wandering larvae would stay after abandoning the diet (Figure 1: A–D).

Initially, the immatures were being reared in climate chamber for bioassays (BOD - New Lab, model NL-41-02) at 27 ± 2 ºC and relative humidity (RH) of 70 ± 5% and were then exposed separately to refrigeration in another BOD (New Lab, model NL-161-01) under 4 ± 2 ºC and RH of

50 ± 5% for different periods of 24h, 48h, 72h (separately, in different treatments), as well as so on, until the insects reached their maximum survival period under refrigeration. After this, they were returned to the BOD at 27 ± 2 ºC and RH of 70 ± 5% until they had completed their development. In each treatment, 6 containers were used: 3 containers for the refrigeration experiments and 3 containers for the control treatment, which always remained in a climate chamber for bioassays at 27 ± 2 ºC and RH of 70 ± 5%.

All treatments were kept under a 12-hour photoperiod (photophase-scotophase) starting at 6 a.m. The development of the insects was monitored every 12 hours when insects were kept under refrigeration. When the insects were not under refrigeration or during the control treatment, verifications were made every 2 hours; from the 3rd instar onwards, the immatures were also observed every 12 hours. To measure the biometry of the imatures, 30 individuals were randomly removed from their containers and analyzed under a stereoscopic microscope (Feldmann Wild Leitz, model FWL – SMZ 7.5). Afterwards, the individuals were returned to their respective containers.

To analyze the effect of refrigeration on insect development and biometry, a two-way analysis of variance (ANOVA two-way) was conducted, comparing experiments under refrigeration with control treatments. The Šidák correction was applied as a post-hoc test to confirm significant differences. A 95% significance level was used for both tests, with “P” values <0.05 considered statistically significant. Microsoft Excel (version 2019) was used to analyze the raw data, while additional analyses were carried out using GraphPad Prism software (version 10.0.2).

3| RESULTS

In both experiments that involved subjecting the immatures to continuous and cyclical refrigeration, it was observed that the larvae remained in a state of quiescence until their energy reserves were exhausted, leading to death, However, when the insects were subsequently exposed to

27ºC ± 2ºC and a relative humidity of 70 ± 5%, development resumed, and the immatures of all the species studied were able to molt and reach the adult stage. It was also found that the longer the exposure time to low temperatures, the longer the total development time of the species, from egg to adult stage. Figures 2, 3, 4, and 5 illustrate the development time of the immatures until emergence based on the duration of refrigeration and the corresponding survival rates.

Overall, in all experimental groups, statistical analyses (two-way ANOVA and Šidák post-hoc test) revealed significant differences ($P<0.0001$), in the third-instar larvae (L3) and total development time (from egg to adult) when comparing the control treatment to the discontinuous refrigeration treatments. Tables 1–4 provide a summary of the development times for each species and the corresponding statistical values.

Third-instar larvae of *Chrysomya megacephala* tolerated refrigeration for up to 192 hours. In the control treatment, the average development time for this instar was approximately 76 hours, whereas under refrigeration followed by rewarming, it ranged from 96 to 288 hours. Regarding total development time (from egg to adult), the control group exhibited an average duration of approximately 249.33 hours, with 85% of individuals reaching adulthood. In the intermittent refrigeration treatments, development time ranged from 275.33 to 634 hours, depending on the refrigeration duration (24 to 192 hours), with only 25% of individuals surviving to adulthood (Table 1, Figure 2E, F).

Third-instar larvae of *Chrysomya putoria* endured refrigeration for up to 240 hours. In the control group, the average development time for this instar was approximately 80 hours, while under refrigeration and subsequent rewarming, it ranged from 92 to 436 hours. The total development time in the control treatment was around 228 hours, with 72% of individuals reaching adulthood. In the intermittent refrigeration treatments, this period varied from 259.33 to 966 hours, depending on the refrigeration duration (24 to 240 hours), with only 6% of individuals reaching adulthood (Table 2, Figure 3G, H).

Third-instar larvae of *Hemilucilia segmentaria* withstood refrigeration for up to 144 hours. Their average development time in the control group was approximately 107.33 hours, while under refrigeration followed by rewarming, it ranged from 128 to 296 hours. Total development time in the control treatment averaged 252 hours, with 80% of individuals reaching adulthood. In the intermittent refrigeration treatments, this period varied from 306 to 632.67 hours, depending on the refrigeration duration (24 to 144 hours), with 16% of individuals surviving to adulthood (Table 3, Figure 4I, J).

Third-instar larvae of *Lucilia cuprina* tolerated refrigeration for up to 312 hours. The control group exhibited an average development time of approximately 56 hours for this instar, whereas under refrigeration and subsequent rewarming, it ranged from 64 to 576 hours. Total development time in the control treatment was around 203.33 hours, with 75% of individuals reaching adulthood. Under intermittent refrigeration, development time varied from 210.67 to 1147.33 hours, depending on refrigeration duration (24 to 312 hours), with 13% of individuals surviving to adulthood (Table 4, Figure 5K, L). Notably, the development time in the treatment exposed to 24 hours of refrigeration followed by rewarming did not differ significantly from the control group, according to the Šidák post-hoc test with 95% confidence.

4| DISCUSSION

The present study contributes valuable insights into the impact of discontinuous refrigeration on the development and survival of third-instar larvae of forensically important Calliphoridae species. Our findings, obtained under controlled laboratory conditions simulating intermittent cooling and rewarming cycles, offer a nuanced understanding compared to studies focusing solely on continuous refrigeration [6, 7, 10, 11, 14, 18]. While continuous refrigeration has been shown to delay development and reduce survival in immature stages of various blow fly species, our investigation into discontinuous temperatures provides a more realistic scenario reflecting potential real-world conditions such as power outages or when bodies are refrigerated for a few days before necropsy and the collection of entomological evidence.

Chrysomya putoria, *Hemilucilia segmentaria*, and *Lucilia cuprina* exhibit specific responses to discontinuous refrigeration expands on the knowledge derived from studies on continuous cooling [11] and constant temperatures [18, 19, 20]. Previous research has highlighted the species-specific nature of thermal responses in blow flies [21], with species from the same genus often displaying different developmental trajectories under varying temperature regimes. For instance, the lower survival of L1 larvae and pupae under continuous refrigeration across all species in Alonso et al. (2015) and Carneiro et al. (2025) [11, 21] contrasts with the resilience observed in later larval stages in our discontinuous cooling experiments (as implied by the study's aim to determine the impact on larval development). This difference may be attributed to the developmental stage and the ability of more mature larvae to withstand short periods of stress an [9].

The species examined in our study are of significant forensic relevance in Brazil due to their necrophagous and synanthropic behavior [21], and the establishment of our colonies in Rio de Janeiro provides a geographically relevant context. Studies on other Calliphoridae species, such as *Calliphora vicina* and *Protophormia terraenovae* (Robineau-Desvoidy, 1830), have explored the effects of refrigeration, often showing developmental delays and varying survival rates [9, 15]. For example, Myskowiak & Doums (2002) [9] reported developmental delays but maintained viability in *P.terraenovae* larvae exposed to ten days of continuous refrigeration. Our focus on species prevalent in warmer climates, as opposed to those adapted to colder regions, adds a critical dimension to understanding the impact of cold exposure in forensic investigations in subtropical and tropical areas.

It is crucial to emphasize that the species studied in the present work (*C. megacephala, C. putoria, H. segmentaria*, and *L. cuprina*) are adapted to warmer climates than species such as *Ca. vicina* or *P. terraenovae* [9, 12], which are frequently used in studies on the effects of low temperatures in temperate regions. Research on *Ca. vicina* by Ames & Turner (2003) [12] showed significant differences in development depending on the larval stage at which a continuous cold exposure occurred. Our results on intermittent refrigeration in tropical/subtropical species demonstrated a different pattern of tolerance and developmental delay compared to these cold-climate

species under similar temperature regimes (adjusted for each species); in our study, more specifically, the longer the insects remain under refrigeration, the more the total development time lags behind the refrigeration time itself. This reinforces the need for species-specific studies that take geographic distribution into account.

Our results on intermittent refrigeration may also indicate greater resilience of third-instar larvae (L3) to temperature fluctuations. For instance, Carriço et al. (2019) [18] observed that cooled L3 larvae of *Chrysomya putoria* were still able to pupate, although with low adult emergence and reproductive incapacity. In our experiments with intermittent refrigeration, we observed considerable survival rates and subsequent development of L3 larvae compared to the study by Carriço et al. (2019) and other studies that did not clearly report survival rates [6, 9, 12, 14], which would be expected under continuous refrigeration depending on the exposure duration. This highlights the importance of considering temperature patterns rather than just the total duration of cold exposure.

It is worth mentioning that factors such as larval aggregation (studied by Magni et al., 2016) [10] and the presence of the body in specific environments (microclimates, as discussed by Charabidze & Hedouin, 2018 and Hofer et al., 2017) [8, 22] may interact with the effects of intermittent refrigeration. For example, a large larval mass can generate metabolic heat, partially counteracting periods of cold exposure. However, in our study, the larvae didn't always remain in a larval mass, remaining scattered or isolated in the food substrate, or when they were in a larval mass, the temperature of the mass didn't differ by more than 3ºC in relation to the refrigeration temperature. Perhaps this small variation wasn't enough for the larvae to develop, but it was enough to keep them alive for a while.

The implications of our findings for TOC and, consequently, for Post-Mortem Interval (PMI) estimations are considerable. The common practice of storing bodies in refrigeration prior to autopsy necessitates a thorough understanding of how cold exposure affects insect development [14, 23]. Our simulation of intermittent cooling and rewarming reveals that the developmental impact might differ from that under constant cold temperatures, potentially leading to more complex deviations in PMI

estimations if only continuous refrigeration models are considered. As suggested by Lutz et al. (2021), the thermal history of maggots on a body, including cooling periods, is a crucial factor in accurate PMImin estimation [23]. Our work underscores the need to account for the specific patterns of temperature fluctuation, not just the duration and minimum temperature of cooling. A differential key of Myskowiak & Doums (2002), Ames & Turner (2003) [9, 12] and our researches lies in the simulation of discontinuous refrigeration cycles, which more accurately reflects certain real-life scenarios encountered in forensic casework. While other studies have examined the effects of constant low temperatures [6, 7, 10, 11, 14, 18] or the impact of single refrigeration periods followed by development at constant temperatures [24], our approach provides a more dynamic perspective. This is particularly relevant as temperature loggers placed inside body storage bags have shown that the temperature experienced by larvae can be significantly higher and more variable than the set temperature of a walk-in cooler as pointed out by Lutz et al (2021) [23]. Perhaps a "limitation" in our study was not using a methodology that simulated body storage bags. This might have favored larval development, as it would likely create a more suitable microclimate. Therefore, we suggest that future studies should consider large larval masses and the use of body storage bags.

Furthermore, our focus on third-instar larvae, often the stage collected for TOC estimation, allows for direct assessment of how discontinuous cooling affects their subsequent development. The survival and developmental rate of these larvae after exposure to fluctuating cold temperatures will directly influence the interpretation of entomological evidence. Inferring the PMI based on entomological evidence relies heavily on the accuracy of developmental data in relation to temperature. Our experiments demonstrated that discontinuous refrigeration leads to a developmental pattern different from that observed under continuous refrigeration or constant temperatures (whether low or high). This means that existing PMI models, which are primarily based on stable temperature scenarios or continuous refrigeration, may result in inaccurate estimates in cases where temperature fluctuations have occurred. We emphasize the need to incorporate the complexity of intermittent

thermal histories into PMI estimation models, especially in regions where the studied species are dominant.

In conclusion, our study provides a significant contribution to the field by specifically investigating the effects of discontinuous refrigeration on key forensic indicator species in Brazil. By comparing our results with existing literature on continuous cooling and constant temperature development, we highlight the importance of considering realistic temperature fluctuations when interpreting entomological evidence from refrigerated bodies because sometimes the body's chain of custody may involve periods of refrigeration interruption, to conduct additional analyses necessary in the course of the investigations. This research emphasizes the need for further studies that explore the complex interplay of temperature dynamics and insect development to improve the accuracy of PMI estimations in forensic investigations worldwide.

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FIGURES

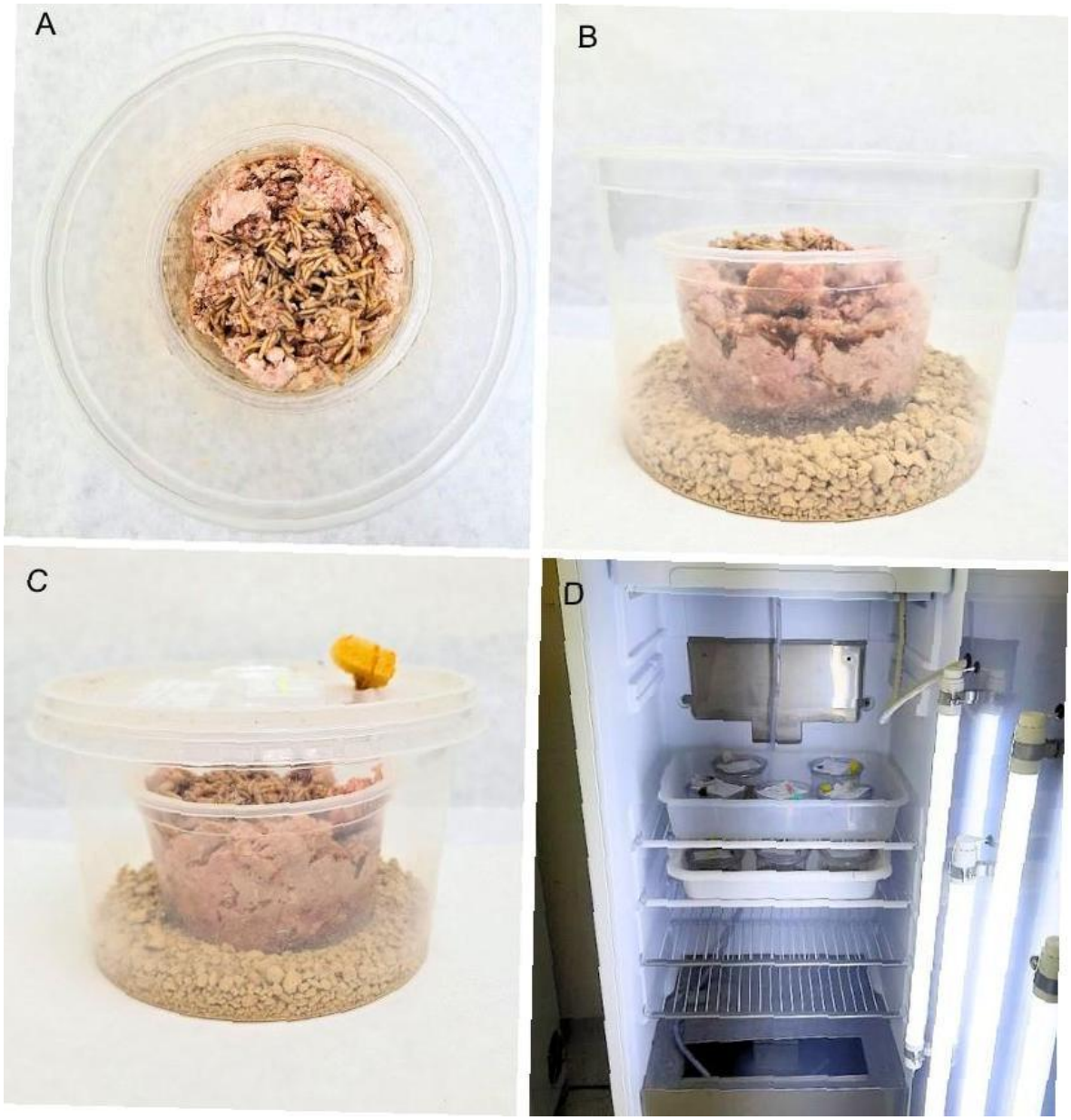


Figure 1. A–C : Containers in which the immatures were placed, along with the food substrate and a larger container containing sterilized sand that would be used for errant larvae that abandoned the diet. D: Containers in the bioassay climate chamber.

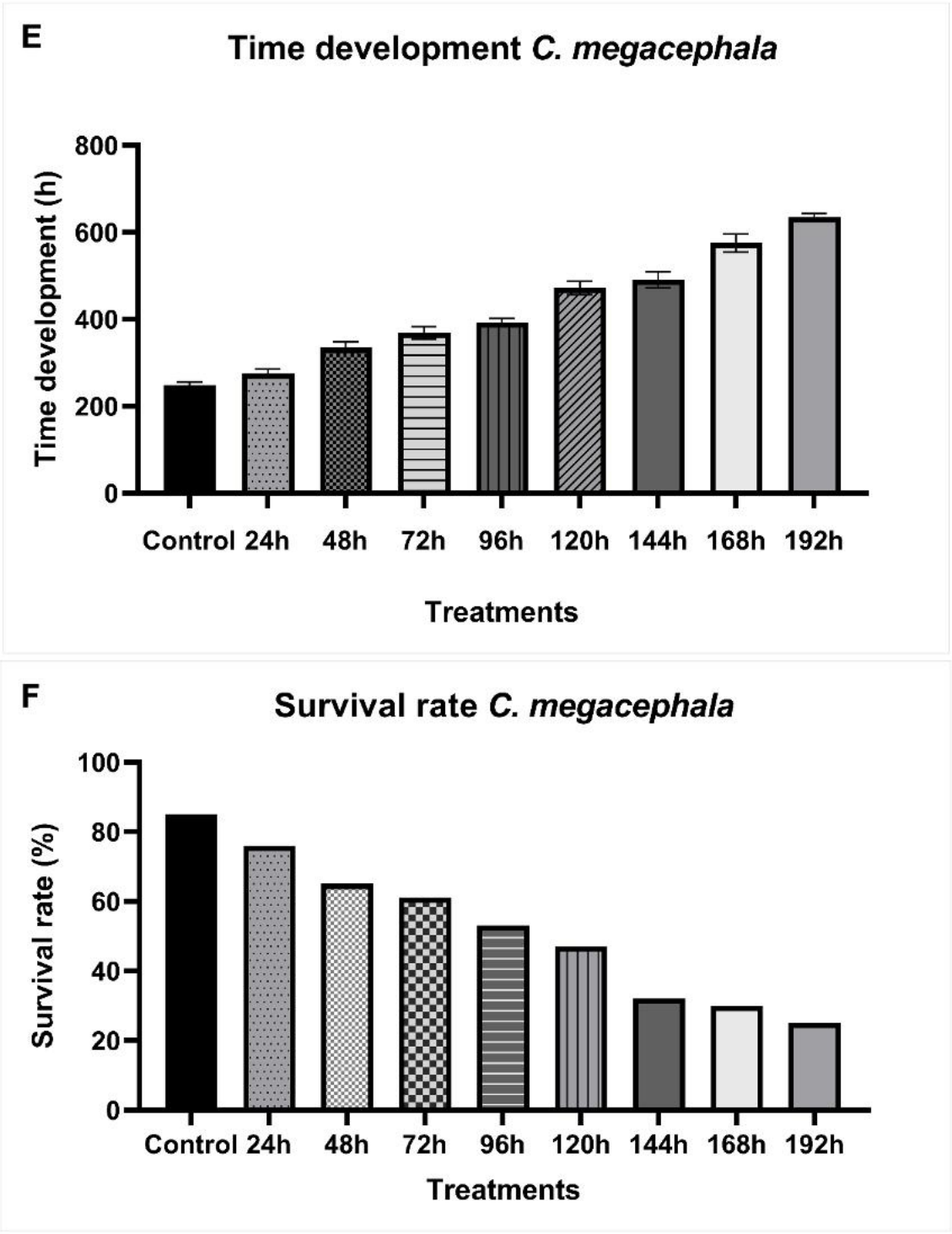


Figure 2. E: development time of *Chrysomya megacephala* (egg–adult) during control treatment and other treatments under refrigeration (4 ± 2 ºC) and subsequent exposure to 27 ± 2ºC and RH of 70 ± 5% (discontinuous refrigeration). F: survival rate of *C. megacephala* (egg–adult) during control treatment and other treatments under discontinuous refrigeration.

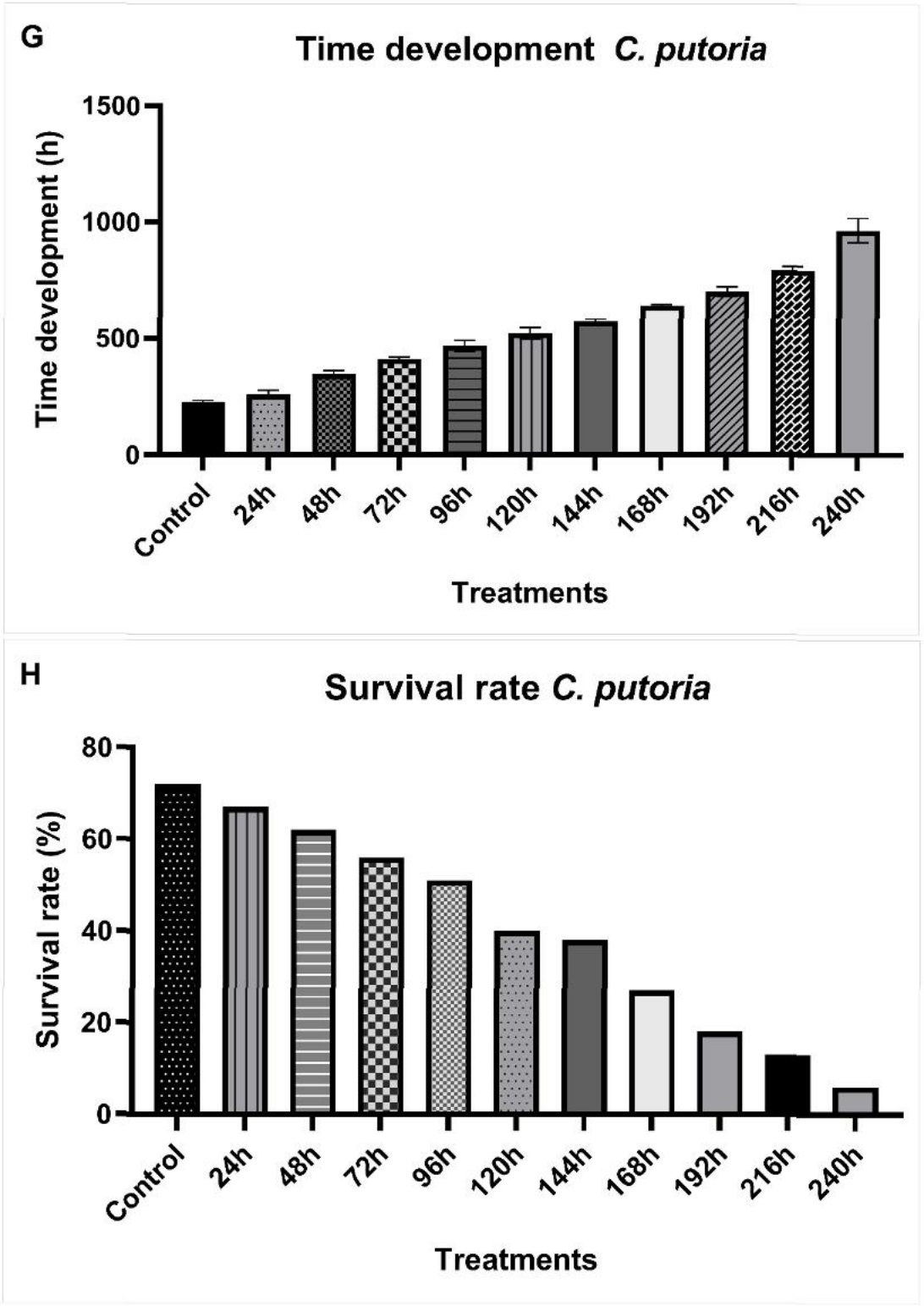


Figure 3. G: development time of *Chrysomya putoria* (egg–adult) during control treatment and other treatments under refrigeration (4 ± 2 ºC) and subsequent exposure to 27 ± 2ºC and RH of 70 ± 5% (discontinuous refrigeration). H: survival rate of *C. putoria* (egg–adult) during control treatment and other treatments under discontinuous refrigeration.

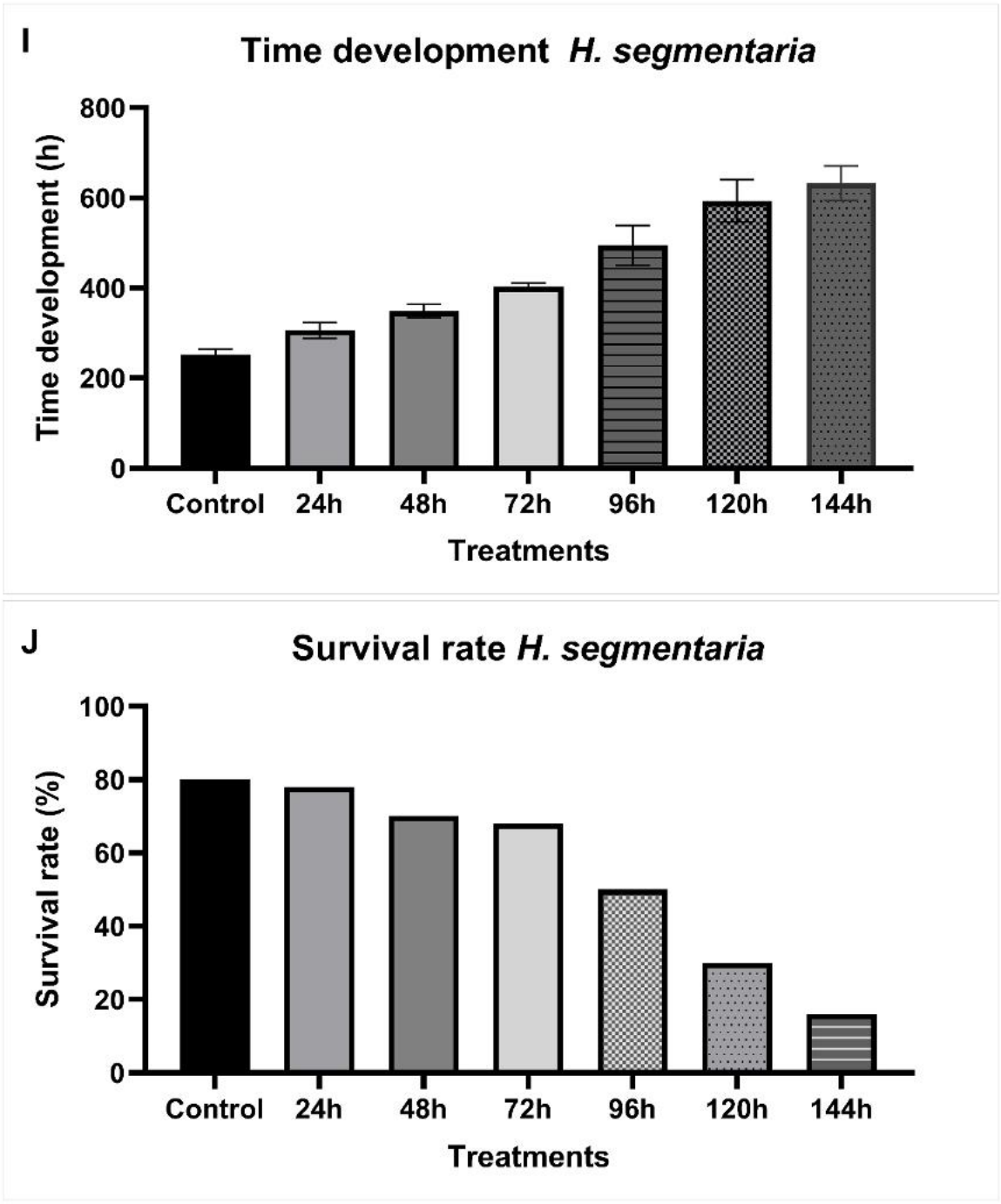
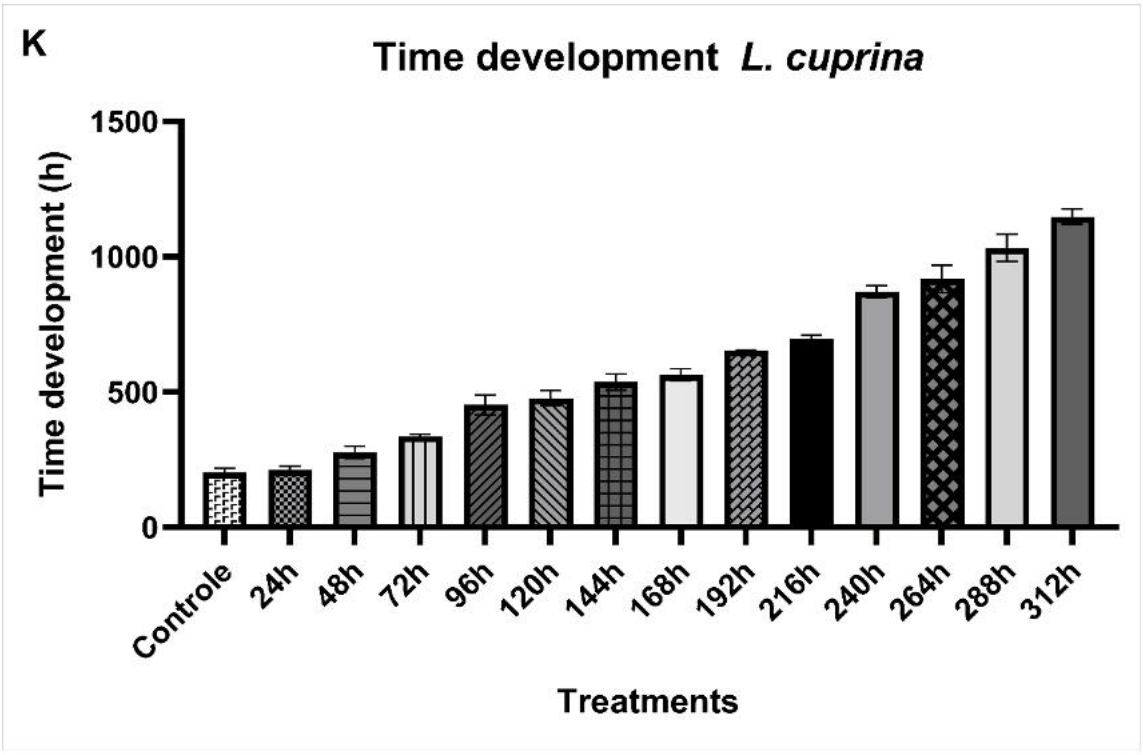


Figure 4. I: development time of *Hemilucilia segmentaria* (egg–adult) during control treatment and other treatments under refrigeration (4 ± 2 ºC) and subsequent exposure to 27 ± 2ºC and RH of 70 ± 5% (discontinuous refrigeration). J: survival rate of *Hemilucilia segmentaria* (egg–adult) during control treatment and other treatments under discontinuous refrigeration.



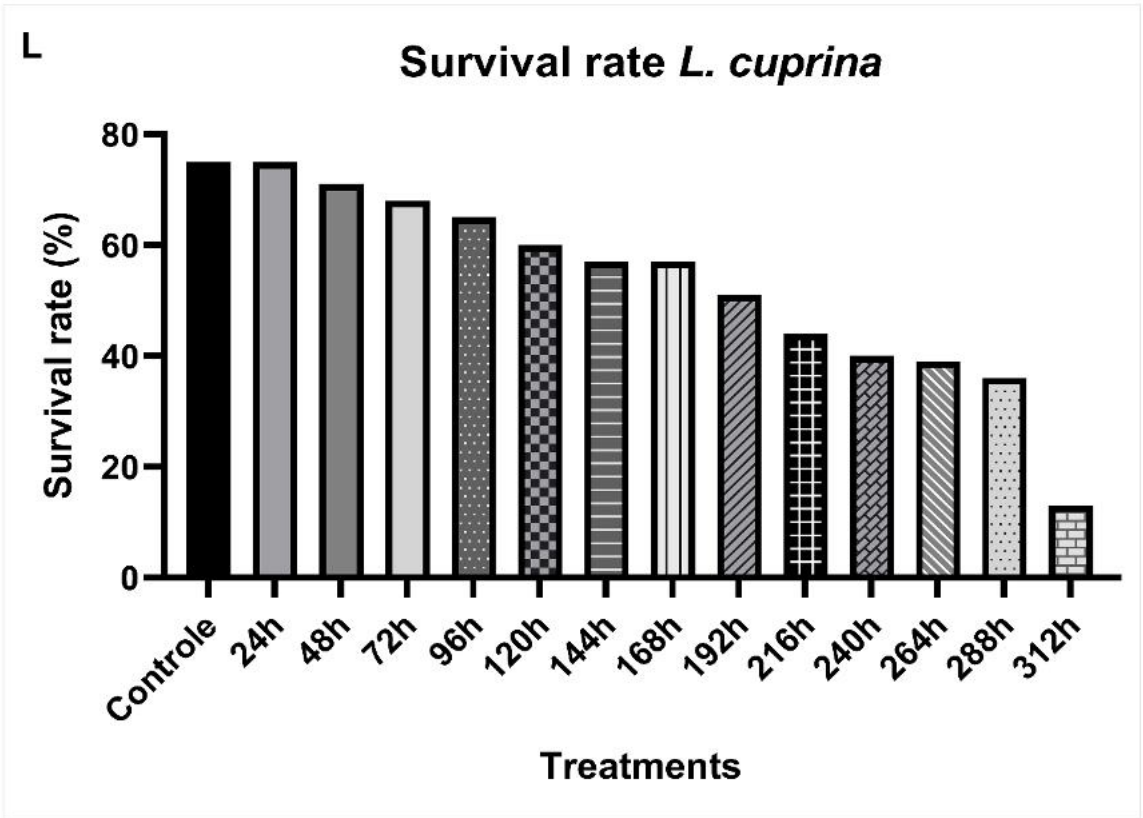


Figure 5. K: development time of *Lucilia cuprina* (egg–adult) during control treatment and other treatments under refrigeration (4 ± 2 ºC) and subsequent exposure to 27 ± 2ºC and RH of 70 ± 5% (discontinuous refrigeration). L: survival rate of *Lucilia cuprina* (egg–adult) during control treatment and other treatments under discontinuous refrigeration.

TABLES

Table 1. Average development time (in hours ± standard deviation) of *Chrysomya megacephala* during the control treatment (27 ± 2 ºC and 70 ± 5% RH) and during the treatments of refrigeration (4 ± 2 ºC and 50 ± 5% RH) and subsequent exposure to 27 ± 2 ºC and 70 ± 5% RH of the 3rd instar larvae. The F-statistic, P-values and the percentage of total variation corresponding to the two-way ANOVA analysis are also presented.

Treatment	Egg	L1	L2	L3 [a]	Pupa [b]	Egg-adult [c]	ANOVA two-way	F-valor	P-valor	% of total variation
Control	12 ± 0.00	11.33 ± 1.5	18 ± 5.29	76 ± 6.93	132 ± 0.00	249.33 ± 6.30	Interaction	18346	<0.0001	8.410
24h	12.67 ± 2.31	14.67 ± 1.15	16 ± 2.00	96 ± 12.00	136 ± 6.93	275.33 ± 10.07	Development stage	1478157	<0.0001	84.70
48h	12 ± 0.00	17.33 ± 2.31	18 ± 0.00	136 ± 18.33	152 ± 13.86	335.33 ± 12.22	Development time *	71802	<0.0001	6.583
72h	14 ± 2.00	12.67 ± 2.31	22 ± 3.46	152 ± 6.93	168 ± 12.00	368.67 ± 14.19				
96h	10.67 ± 1.15	12.67 ± 1.15	14 ± 0.00	160 ± 13.86	196 ± 18.33	393.33 ± 8.08				
120h	13.33 ± 2.31	14.67 ± 1.15	16 ± 0.00	212 ± 13.86	216 ± 0.00	472 ± 15.62				
144h	12 ± 0.00	14 ±2.00	14 ± 0.00	232 ± 6.93	218.67 ± 12.86	490.67 ± 18.15				
168h	13.33 ± 1.15	14 ± 0.00	16 ± 3.46	264 ± 12	268 ± 18.33	575.33 ± 20.82				
192h	12 ± 2.00	15.33 ± 1.15	14.67 ± 4.16	288 ± 12.00	304 ± 6.93	634 ± 9.17				

*Development time for each treatment (control vs. hours of exposition to discontinuous refrigeration of the other treatments)

[a] Indicates that there was a significant difference in the development times of the L3 larvae between the control treatment and the other treatments under discontinuous refrigeration (according Šidák post-hoc test with 95% of confidence).

[b] Indicates that there was a significant difference in the development times of the Pupae between the control treatment and the other treatments under discontinuous refrigeration (according Šidák post-hoc test with 95% of confidence).

[c] Indicates that there was a significant difference in total development times (Egg-adult) between the control treatment and the other treatments under discontinuous refrigeration (according Šidák post-hoc test with 95% of confidence).

Table 2. Average development time (in hours ± standard deviation) of *Chrysomya putoria* during the control treatment (27 ± 2 ºC and 70 ± 5% RH) and during the treatments of refrigeration (4 ± 2 ºC and 50 ± 5% RH) and subsequent exposure to 27 ± 2 ºC and 70 ± 5% RH of the 3rd instar larvae. The F-statistic, P-values and the percentage of total variation corresponding to the two-way ANOVA analysis are also presented.

Treatment	Egg	L1	L2	L3 [a]	Pupa [b]	Egg-adult [c]	ANOVA two-way	F-valor	P-valor	% of total variation
Control	15.33 ± 1.15	14 ± 0.00	22.67 ± 1.15	80 ± 6.93	96 ± 0.00	228 ± 5.29	Interaction	29618	<0.0001	13.89
24h	14.67 ± 1.15	20 ± 0.00	20.67 ± 1.15	92 ± 11.14	112 ± 6.93	259.33 ± 18.04	Development stage	1596110	<0.0001	74.85
48h	12 ± 0.00	24.67 ± 3.06	22 ± 2.00	132 ± 0.00	156 ± 12.00	346.67 ± 14.74	Development time *	116809	<0.0001	10.96
72h	14 ± 0.00	21.33 ± 1.15	24 ± 2.00	172 ± 6.93	180 ± 0.00	411.33 ± 8.08				
96h	16 ± 2.00	12.67 ± 2.31	20 ± 0.00	208 ± 6.93	212 ± 13.86	468.67 ± 23.69				
120h	14 ± 2.00	18.67 ± 1.15	23.33 ± 2.31	244 ± 13.86	221.33 ± 16.65	521.33 ± 25.01				
144h	14.67 ± 3.06	17.33 ± 2.31	18 ± 0.00	254.67 ± 16.17	268.67 ± 12.70	573.33 ± 10.26				
168h	11.33 ± 1.15	20.67 ± 2.31	24 ± 0.00	312 ± 0.00	272 ± 6.93	640 ± 5.29				
192h	17.33 ± 4.62	12 ± 0.00	16 ± 0.00	308 ± 13.86	348 ± 12.00	701.33 ± 20.53				
216h	22 ± 0.00	18 ± 0.00	16.67 ± 1.15	352 ± 6.93	384 ± 12.00	792.67 ± 18.15				
240h	16 ± 0.00	18 ± 2.00	20 ± 0.00	436 ± 42.14	476 ± 18.15	966 ± 52.12				

*Development time for each treatment (control vs. hours of exposition to discontinuous refrigeration of the other treatments).

[a] Indicates that there was a significant difference in the development times of the L3 larvae between the control treatment and the other treatments under discontinuous refrigeration (according Šidák post-hoc test with 95% of confidence).

[b] Indicates that there was a significant difference in the development times of the Pupae between the control treatment and the other treatments under discontinuous refrigeration (according Šidák post-hoc test with 95% of confidence).

[c] Indicates that there was a significant difference in total development times (Egg-adult) between the control treatment and the other treatments under discontinuous refrigeration (according Šidák post-hoc test with 95% of confidence).

Table 3. Average development time (in hours ± standard deviation) of *Hemilucilia segmentaria* during the control treatment (27 ± 2 ºC and 70 ± 5% RH) and during the treatments of refrigeration (4 ± 2 ºC and 50 ± 5% RH) and subsequent exposure to 27 ± 2 ºC and 70 ± 5% RH of the 3rd instar larvae. The F-statistic, P-values and the percentage of total variation corresponding to the two-way ANOVA analysis are also presented.

Treatment	Egg	L1	L2	L3 [a]	Pupa [b]	Egg-adult [c]	ANOVA two-way	F-valor	P-valor	% of total variation
Control	10.67 ± 1.15	12 ± 0.00	12.67 ± 1.15	107.33 ± 1.15	112 ± 6.93	252 ± 12	Interaction	6323	<0.0001	8.821
24h	11.33 ± 1.15	12 ± 0.00	14.67 ± 3.06	128 ± 18.33	140 ± 6.93	306 ± 17.44	Development stage	357565	<0.0001	83.14
48h	14 ± 2.00	13.33 ± 2.31	14 ± 5.29	152 ± 6.93	156 ± 12.00	349.33 ± 15.28	Development time *	25321	<0.0001	7.065
72h	12 ± 0.00	10 ± 0.00	17.33 ± 1.15	180 ± 0.00	184 ± 6.93	403.33 ± 7.57				
96h	9.33 ± 2.31	14 ± 0.00	15.33 ± 2.31	224 ± 18.33	232 ± 30.20	494.67 ± 44.60				
120h	12.67 ± 3.31	16.67 ± 1.15	12 ± 0.00	272 ± 6.93	280 ± 42.14	593.33 ± 47.09				
144h	10 ± 0.00	12.67 ± 3.06	14 ± 3.46	296 ± 36.66	300 ± 0.00	632.67 ± 38.07				

*Development time for each treatment (control vs. hours of exposition to discontinuous refrigeration of the other treatments). [a] Indicates that there was a significant difference in the development times of the L3 larvae between the control treatment and the other treatments under discontinuous refrigeration (according Šidák post-hoc test with 95% of confidence). [b] Indicates that there was a significant difference in the development times of the Pupae between the control treatment and the other treatments under discontinuous refrigeration (according Šidák post-hoc test with 95% of confidence). [c] Indicates that there was a significant difference in total development times (Egg-adult) between the control treatment and the other treatments under discontinuous refrigeration (according Šidák post-hoc test with 95% of confidence).

Table 4. Average development time (in hours ± standard deviation) of *Lucilia cuprina* during the control treatment (27 ± 2 ºC and 70 ± 5% RH) and during the treatments of refrigeration (4 ± 2 ºC and 50 ± 5% RH) and subsequent exposure to 27 ± 2 ºC and 70 ± 5% RH of the 3rd instar larvae. The F-statistic, P-values and the percentage of total variation corresponding to the two-way ANOVA analysis are also presented.

Treatment	Egg	L1	L2	L3 a	Pupa b	Egg-adult c	ANOVA two-way	F-valor	P-valor	% of total variation
Control	16 ± 0,00	12 ± 0,00	15,33 ± 2,31	56 ± 6,93**	104 ± 6,93**	203,33 ± 15,14**	Interaction	2071	<0,0001	16.80
24h	12,67 ± 1,15	12 ± 0,00	14 ± 0,00	64 ± 13,86	108 ± 0,00	210,67 ± 14,47	Development stage	103475	<0,0001	64.55
48h	12 ± 0,00	12,67 ± 1,15	12 ± 0,00	112 ± 13,86	128 ± 27,71	276,67 ± 23,01	Development time *	8271	<0,0001	13.42
72h	16 ± 2,00	14 ± 0,00	13,33 ± 1,15	136 ± 6,93	156 ± 0,00	335,33 ± 7,57				
96h	14 ± 0,00	14 ± 3,46	18,67 ± 1,15	180 ± 20,78	225,33 ± 36,95	452 ± 36				
120h	18,67 ± 1,15	11,33 ± 1,15	18 ± 0,00	209,33 ± 22,74	220 ± 6,93	477,33 ± 28,10				
144h	12 ± 0,00	14 ± 0,00	12 ± 2,00	247,33 ± 29,28	252 ± 0,00	537,33 ± 30,09				
168h	10 ± 2,00	16 ± 2,00	10 ± 2,00	240 ± 12,00	288 ± 12,00	564 ± 21,63				
192h	12,67 ± 1,15	14,67 ± 4,16	18 ± 3,46	292 ± 13,86	316 ± 6,93	653,33 ± 1,15				
216h	18 ± 0,00	10,00 ± 0,00	10,67 ± 2,31	344 ± 13,86	312 ± 0,00	694,67 ± 15,14				
240h	11,33 ± 4,16	19,33 ± 1,15	12 ± 0,00	396 ± 0,00	432 ± 20,78	870,67 ± 21,94				
264h	10 ± 00	12 ± 0,00	15,33 ± 4,62	392 ± 27,71	488 ± 42,14	917,33 ± 50,96				
288h	14 ± 0,00	14 ± 2,00	20 ± 0,00	480 ± 52,31	504 ± 0,00	1032 ± 50,71				
312h	13,33 ± 2,31	16 ± 0,00	22 ± 2,00	576 ± 0,00	520 ± 24,98	1147,33 ± 28,02				

*Development time for each treatment (control vs. hours of exposition to discontinuous refrigeration of the other treatments).

** The development time of the treatment exposed to 24 hours of refrigeration and subsequent heating did not differ significantly from the control treatment according to the Šidák post-hoc test with 95% of confidence. [a] Indicates that there was a significant difference in the development times of the L3 larvae between the control treatment and the other treatments under discontinuous refrigeration (according Šidák post-hoc test with 95% of confidence). [b] Indicates that there was a significant difference in the development times of the Pupae between the control treatment and the other treatments under discontinuous refrigeration (according Šidák post-hoc test with 95% of confidence). [c] Indicates that there was a significant difference in total development times (Egg-adult) between the control treatment and the other treatments under discontinuous refrigeration (according Šidák post-hoc test with 95% of confidence).

CAPÍTULO III:

Evaluating Dipteran Larvae Development on Refrigerated Corpses: Implications on Post-Mortem Interval Determination

O artigo intitulado “Evaluating Dipteran Larvae Development on Refrigerated Corpses: Implications on Post-Mortem Interval Determination” apresenta resultados e informações obtidos através da avaliação do desenvolvimento de larvas de dípteros que estavam colonizando, sob condições de refrigeração, cadáveres humanos mantidos no necrotério do Instituto Médico Legal Afrânio Peixoto durante o processo de realização dos exames periciais e necroscópicos. Também foi discutida qual a implicação disso para as estimativas do intervalo pós-morte. Os autores do artigo são Larissa Thans Carneiro, aluna do Programa de Pós-graduação em Zoologia do Museu Nacional/UFRJ; Janyra Oliveira-Costa, professora, pesquisadora, perita criminal oficial e chefe do Laboratório de Entomologia Forense do Instituto Médico Legal Afrânio Peixoto – Polícia Civil do Estado do Rio de Janeiro e Márcia Souto Couri, professora e pesquisadora do Departamento de Entomologia do Museu Nacional - Universidade Federal do Rio de Janeiro (MN/UFRJ). O artigo será submetido no mês de agosto de 2025 para publicação no “Forensic Science International”, com classificação “A2” no Qualis Periódicos e Fator de Impacto “2.2”, referente a última avaliação realizada no ano de 2023 pela Journal Citation Reports (Clarivate Analytics). O periódico se dedica a publicação das contribuições mais inovadoras e influentes das ciências forenses. Os campos incluem: patologia forense e histoquímica, química, bioquímica e toxicologia, biologia, sorologia, odontologia, psiquiatria, antropologia, ciência forense digital, ciências físicas, armas de fogo e exame de documentos, bem como investigações de valor para a saúde pública em que a ciência e a medicina interagem com a lei.

Evaluating Dipteran Larvae Development on Refrigerated Corpses: Implications on Post-Mortem Interval Determination

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ABSTRACT

The estimation of the minimum PMI relies on the relationship between insect development and environmental temperature. The accumulated degree-day (ADD) method is common, but cadaver refrigeration can affect its accuracy. This study investigated the effects of refrigeration on the development of immature flies under morgue conditions in Rio de Janeiro, Brazil. Larvae of different species (*Chrysomya albiceps*, *Chrysomya megacephala*, *Peckia (Peckia) chrysostoma*, *Hemilucilia segmentaria*, *Synthesiomyia nudiseta*) were collected from refrigerated cadavers (4 ± 7 ºC) and reared under controlled conditions (27 ± 2 ºC). Development time was monitored, and PMImin was estimated using the ADD method, comparing refrigerated and control groups. A significant delay in larval development under refrigeration was observed for all studied species ($p < 0.0001$ for L3 comparisons between control and refrigeration in *C. megacephala* and *C. albiceps*; $p < 0.0001$ for L2 and L3 in *P. chrysostoma*; $p < 0.0001$ for L3 in *H. segmentaria*; $p < 0.0001$ for L3 in *S. nudiseta*). The estimated day of oviposition was delayed by 1 to 2 days for specimens refrigerated for more than 24 hours. Refrigeration significantly affects the development of forensically important larvae, impacting PMImin estimation. The scarcity of developmental data at low temperatures for tropical and subtropical species limits the accurate application of the ADD method in prolonged refrigeration cases. This study highlights the need for further research on the thermal biology of forensic insects at temperatures relevant to forensic refrigeration in Brazil.

Keywords: Forensic entomology, corpse storage, time of colonization, blow flies.

RESUMO

A estimativa do IPMm depende da relação entre o desenvolvimento de insetos e a temperatura ambiental. O método de graus-dia acumulados (GDA) é comum, mas a refrigeração de cadáveres pode afetar a precisão. Investigamos os efeitos da refrigeração no desenvolvimento de moscas imaturas em condições de morgue no Rio de Janeiro. Foram coletadas larvas de diferentes espécies (*Chrysomya albiceps*, *C. megacephala*, *Peckia (Peckia) chrysostoma*, *Hemilucilia segmentaria*, e *Synthesiomyia nudiseta*) de cadáveres refrigerados (4 ± 2 ºC) e criadas em condições controladas (27 ± 2 ºC). O tempo de desenvolvimento foi monitorado, e o IPMm foi estimado usando o método GDA, comparando os grupos refrigerados e controle. Observou-se um retardo significativo no desenvolvimento larval sob refrigeração para todas as espécies estudadas ($p < 0.0001$ para as comparações L3 entre controle e refrigeração em *C. megacephala* e *C. albiceps*; $p < 0.0001$ para L2 e L3 em *P. chrysostoma*; $p < 0.0001$ para L3 em *H. segmentaria*; $p < 0.0001$ para L3 em *S. nudiseta*). A estimativa do dia de oviposição foi atrasada em 1 a 2 dias para espécimes refrigerados por mais de 24 horas. A refrigeração afeta significativamente o desenvolvimento de larvas de importância forense, impactando a estimativa do IPMm. A escassez de dados de desenvolvimento em baixas temperaturas para espécies tropicais e subtropicais limita a aplicação precisa do método GDA em casos de refrigeração prolongada. Este estudo destaca a necessidade de mais pesquisas sobre a biologia térmica de insetos forenses em temperaturas relevantes para a refrigeração forense no Brasil.

Palavras-chave: Entomologia forense, acondicionamento de corpos, tempo de colonização, moscas varejeiras.

1| INTRODUCTION

The development of insects is associated with abiotic factors, particularly the environmental temperature to which they are exposed [1, 2]. This relationship allows the development time of insects to be used as an indicator to estimate the minimum postmortem interval (minPMI) especially in violent death situations [3, 4]. The time of death can be inferred from the colonization time of the body by insects, providing a minimum PMI [3, 4]. One of the most common methods to estimate the time since death involves using accumulated degree-days (ADD) or accumulated degree-hours (ADH) [4, 5]. This method employs the amount of physiological energy required for development, assuming a linear regression that predicts development based on different temperatures over the immature insect stages [2, 3]. The average development per hour or day is accumulated, yielding a combined effect of temperature and time, known as the thermal summation model [2]. Consequently, this method requires precise temperature and time records to ensure PMI estimation accuracy.

During the collection of entomological evidence, whether at the time of autopsy [6, 7] or after [8], several factors must be considered, such as the temperature at the location of death, the time the body was removed from this site, and the storage conditions of the body prior to autopsy, including refrigeration time and temperature [1]. It is also important to record when the body was removed from the refrigerator. However, such information is often overlooked, which may compromise PMI analyses.

Exposure to low temperatures and the duration of this exposure can influence insect development, directly impacting in time of colonization (TOC) and minimum PMI estimation [2, 6–10]. However, little is known about the true impact of this exposure, as few studies have addressed this topic. One study suggested that the cooling period might alter

the development of larvae in some fly species, causing them to become inactive. This behavioral change, triggered by adverse conditions, may indicate a state of diapause (Myskowiak and Doums 9). Diapause, essential for the survival of many insect species during winter, possibly occurs during the refrigeration of cadavers colonized by dipteran larvae, especially if the refrigeration temperatures fall below the minimum thermal limit required for insect metabolism, affecting their development [11].

According to Huntington et al. (2007) [7], when cadavers are kept in body bags, bacterial activity generates heat, creating a warmer microclimate. In this scenario, larvae kept in morgue refrigerators may remain active instead of entering diapause, influencing the minimum PMI estimation due to the temperature difference between the refrigerator and the microclimate within the bag. Another hypothesis, suggested by Thevan et al. (2010) [8], is that larval development does not cease within so-called larval masses, where the metabolic heat generated by the aggregated larvae also creates a microclimate, allowing some degree of development even under refrigeration.

Given these challenges, the present study aims to evaluate the effects of refrigeration exposure on the development and survival of immature flies under the refrigeration conditions of the Instituto Médico Legal Afrânio Peixoto Morgue morgue (in Rio de Janeiro, Brazil), where human cadavers are stored before and during forensic and necroscopic examinations.

2| MATERIALS AND METHODS

During forensic and necroscopic examinations, the development of fly immatures (of the species that were found) in the larval stage was observed colonizing “unclaimed” or not identified cadavers stored in the refrigerated chamber (at 4 ± 2 ºC and relative humidity - RH of 50 ± 10%) (Mortech, Walk-In Mortuary Racks Storage System, capacity for 80

bodies) at the Instituto Médico Legal Afrânio Peixoto (IMLAP), located in Rio de Janeiro, Brazil. Insects in the larval stage as second and third instar larvae (called L2 and L3, respectively), in three groups of 10 individuals (N=30) were also collected as part of the control treatment, kept at 27 ± 2 ºC and RH of 70 ± 5% in a bioassay chamber (New Lab, model NL-41-02) in the forensic entomology laboratory of the same institute (for further clarification, in Brazil, a corpse is considered unclaimed when there is no documentation or when, despite being identified, there is no information about relatives or legal guardians).

The development of the insects on the refrigerated bodies was assessed every 12 hours, while the control insects were observed every 2 hours; from the 3rd instar onwards they were observed every 12h. During evaluations, three groups of 10 individuals (N=30) were randomly sampled from the cadavers and examined under a stereoscopic microscope (Feldmann Wild Leitz, model FWL – SMZ 7.5) to measure their biometric data.

In the control treatment, groups of about 30 immatures in various developmental stages were placed separately in 700 mL polyethylene containers containing ground beef as a food substrate at a ratio of 1g/larva. These were set into larger 1L containers with a 2 cm layer of sterilized sand - following the methodology of Carneiro et al. (2025a) [12], where wandering larvae could migrate after leaving the diet. The photoperiod in this treatment was set to 12 hours (light/dark cycle).

Additional recorded data included the arrival time of the cadavers at the IMLAP, date of specimen collection, temperature data from the weather stations closest to where the corpses were discovered (Instituto Nacional de Meteorologia - INMET: https://portal.inmet.gov.br/) and the duration of their refrigeration in order to try to calculate the accumulated degree day (ADD) and infer the possible post-mortem interval of the bodies based on the entomological evidence encountered.

The formulas informed by Oliveira-Costa (2011) and which were originally described by Byrd & Castner (2010) [13] was used to calculate the ADD:

$$ADD = (development\ temperature\ - base\ temperature) \times development\ time$$

The calculations were made for the oldest immature instar collected in each case. According to Hill et al. (2020) [14], to calculate the ADD of insects that have been refrigerated, the basal temperature must be assumed to be zero. It is also necessary to average the temperature for the cooling periods. To do this, it is enough to make an average using the temperatures measured in the days before the body was found in a particular place and the temperatures under refrigeration. For example, let's assume that the temperatures measured in the 5 days before a body was found were: 26ºC; 27ºC; 27.5ºC; 28ºC and 28.2ºC. And the temperatures of a 3-day refrigeration period (the time a body usually remains in the cadaveric refrigerator before necroscopic examination) were: 4ºC; 4.5ºC and 5ºC. Adding these values up and dividing by 8 gives an average temperature of 18.75ºC. Therefore, in order to calculate the PMI from the ADD, it is advisable to look for studies that have reared the insects at this temperature (or close to it).The effect of refrigeration on insect development and biometric measurements was analyzed using two-way analysis of variance (ANOVA), with one factor representing the insects under morgue refrigeration and the other factor representing the control treatments, enabling comparison between these groups. Significance was determined with “P” values <0.05. The raw data was analyzed using the Microsoft Excel program (version 2019) other analyses were carried out with the assistance of GraphPad Prism software (version 10.0.2).

3| RESULTS

Case reports

Case I

The corpse of a brown-skinned man, approximately 180 cm in length and around 60 years old, in an advanced state of decomposition (bloated stage), was found inside a residence in the northern zone of the municipality of Rio de Janeiro, Brazil. According to the police incident report, the body was discovered on July 10, 2022, and was sent to the Instituto Médico Legal Afrânio Peixoto (IMLAP), arriving there at 5:40 PM on the same day for necropsy. The cause of death was classified by the responsible forensic doctor as undetermined due to the advanced state of decomposition (Figure 1). Larvae were collected from the body on July 11, 2022, at around 8:00 AM.

L3 larvae of the species *Chrysomya albiceps* (Wiedmann, 1819) and *Chrysomya megacephala* (Fabricius, 1794) (Diptera: Calliphoridae) were found. In the control treatment, *C. megacephala* larvae remained in the third instar for approximately 90 hours, and the average development time from L3 to adulthood was 204 hours. In contrast, *C. albiceps* larvae remained in the third instar for approximately 82 hours, with an average development time to adulthood of 175.33 hours. Statistical tests (two-way ANOVA and Šidák post-hoc test with 95% confidence) indicated a significant difference in the development time of the third-instar larvae that remained under refrigeration compared to the larvae in the control treatment (Tables 1 and 2). During this period the temperature of the refrigerator was approximately 4 ± 2 °C.

The PMI estimation was performed using the accumulated degree-days (ADD) method for the larval stage of the oldest collected specimen (L3), calculated, based on the average temperature collected from the weather station closest to where the body was found (INMET Vila Militar); to do this, data was collected from the days preceding the corpse

discovery at the reported location. For *C. megacephala* the data were calculated based on Rabêlo et al. (2011) [15] at 26°C for the individuals that have not been exposed to refrigeration. For immatures under refrigeration, the average temperatures were 21.4 ºC, 17.92 ºC, 15.6 ºC and 13.94ºC for the individuals kept in the morgue refrigerator for 24h, 48h, 72h and 96h, respectively. That way, the data was calculated based on Velez & Wolf (2008) at 23ºC, Yang et al. (2016) at 19 ºC, Gruner et al. (2016) at 16 ºC and Ngando et al. (2024) at 15 ºC [16–19], which reared the insects at the temperatures closest to the averages mentioned.

Similarly, for *C. abiceps* that have not been refrigerated the data were calculated based on Queiroz et al. (1997) at 27°C [20]. For immatures under refrigeration, the data was calculated based on Kordshouli et al. (2021) at 23 ºC [21] and Richards et al. (2008) at 17.5 ºC [22] (for individuals refrigerated for 24h and 48h); after 72 hours of refrigeration, it was not possible to perform the calculations due to the lack of biological data for 15.6 ºC onwards. It cannot be guaranteed that the specimens found correspond to the first oviposition on the cadaver, as the forensic investigators did not verify the presence of open puparia at the crime scene.

According to the Higley & Haskel (2010) [11], when there are no studies providing information on the basal temperature of insects, it is advisable to consider it to be 10ºC for species from tropical and subtropical regions. Therefore, for some calculations, the basal temperature was considered to be 10ºC for *C. megacephala* and 12,78 ºC for the larvae exposed to 48h based on Yang et al. (2016) [17]. For *C. albiceps*, calculations were made using basal temperatures of 15.04ºC, as reported by Queiroz (1996) [23] and 13ºC according to Richards et al. (2007) for the larval stages.

The Accumulated Degree-Day (ADD) was calculated based on the formulas informed in methodology. Based on the calculations for the unrefrigerated individuals of *C.*

megacephala, the larval specimens collected would have originated from egg-laying on July 9th. For *C. albiceps*, it would be July 8th. It was also noted that, for *C. megacephala* larvae, exposure to 24 hours of refrigeration did not affect the estimated minimum PMI. However, after 48 hours of refrigeration, the estimates were affected, indicating that the eggs would have been laid on July 8th or July 10th, depending on the basal temperature used for the calculations (Chart 1).

For *C. albiceps* that were not refrigerated, the day the eggs were laid was estimated as July 8th. For larvae refrigerated for 24 hours, the estimated day was July 9 and, for 48 hours of refrigeration, the estimate was July 10 (Chart 2).

Case II

The body of a white-skinned man, approximately 175 cm in length, of medium build, appearing to be in good nutritional condition, and with an estimated age in the fifth decade of life, was found on a public road lined with sidewalks, abundant vegetation, and devoid of residential buildings, in the West Zone of Rio de Janeiro, Brazil, on February 14, 2022, at 4:00 PM.

At the scene, the forensic investigator reported that the body was in a generalized state of rigor mortis and exhibited contused, incised-contused wounds and abrasions distributed across the back, arms, and legs (suspected homicide). The body arrived at IMLAP only at 4:00 AM on February 15, already in the early gas formation stage of decomposition. After the autopsy, the forensic doctor determined that the cause of death was trauma to the thorax and limbs, with internal hemorrhage. The instrument that caused the death was a blunt force object, and the crime was classified as cruel, as the multiplicity of injuries indicated a deliberate intent to inflict physical suffering on the victim (Figure 2).

Larvae were collected from the body on the same day, February 15, at around 8:00 AM. L3 larvae of *Peckia (Peckia) chrysostoma* (Wiedemann, 1830) (Diptera: Sarcophagidae) were found. This body remained in the morgue's refrigerator for 18 days. Around 30 larvae were collected and reared in the laboratory as part of the control treatment. The development of the remaining larvae under refrigeration was evaluated directly in the morgue's refrigerator. During this period, the larvae remained in L2 for approximately 152 hours and in L3 for 432 hours. They did not molt and did not complete their development to the adult stage. Throughout this period, the refrigerator temperature was recorded at approximately 4 ± 2°C.

In the control treatment, the larvae remained in the second instar for approximately 19 hours and in the third instar for 64 hours, with the average development time from L2 to adulthood being 275 hours. Statistical tests (two-way ANOVA and Šidák post-hoc test with 95% confidence) indicated a significant difference in the development time of the third-instar larvae that remained under refrigeration compared to the larvae in the control treatment (Table 3).

The minimum PMI estimation was performed using the Accumulated Degree-Day (ADD) method, based on the average temperature at the crime scene in the days prior to the body's discovery (approximately 26,6 °C according to the nearest meteorological station - INMET Jacarepaguá station). The data were calculated retrospectively, estimating the possible date of egg laying on the body, based on the developmental stage of the oldest immature specimen collected (L3), according to data provided by Ferraz (1995) at 27ºC (value closest to the average temperature of the place where the corpse was found) for the individuals not refrigerated and by Ferreira et al. (2024) at 25 ºC for individuals who were exposed to refrigeration for 24h (value closest to the average temperature estimated for the location where the corpse was, together with the refrigeration time, 20.2ºC)[24, 25].

Calculations could not be made for individuals that had been under refrigeration for more than 24 hours, as there are no studies about the biology of the species at temperatures below 20 ºC. There was no report of empty puparia at the location where the body was found. For the estimates, a basal temperature of 10°C was considered as indicated by Higley & Haskel (2010) [11] for species from tropical and subtropical regions. For the ADD calculation, the formula cited in methodology was used. Based on these calculations, the larvae of these dipterans originated from oviposition on February 12 for individuals who were not refrigerated and on February 13 for the individuals that were refrigerated for 24 hours (Chart 3).

Case III

On January 26, 2024, at 4:10 PM, the body of a white-skinned man, approximately 170 cm in length, around 80 years old, of normolinear physique, in an advanced state of decomposition (colliquative stage, with the face beginning the process of skeletonization) and without clothing, was found in the riverbed, in a forested area in the West Zone of Rio de Janeiro, Brazil.

The forensic investigator who attended the case reported that it was not possible to determine whether the location examined was where the victim died or if the body had been transported there. Thus, the body was sent to the Afrânio Peixoto Forensic Medical Institute (IMLAP), arriving at 7:22 PM on the same day for autopsy. Due to the advanced stage of decomposition, it was not possible to determine the nature of some injuries (whether they resulted from violence or from the activity of insects colonizing the body). Therefore, the forensic doctor classified the cause of death as indeterminate (Figure 3).

Larvae were collected from the body on January 27, at around 8:00 AM. Large larval masses (in the third instar of development) were found, forming aggregations on the face and neck, with a temperature of approximately 30°C. The larvae were identified as belonging to the species *Hemilucilia segmentaria* (Fabricius, 1805) (Diptera: Calliphoridae), which is typical of forested areas with lower anthropogenic influence.

The body remained in the morgue's refrigerator for 15 days. Around 30 larvae were collected and reared in the laboratory. The development of the remaining larvae under refrigeration was evaluated directly in the morgue's refrigerator. During this period, the larvae remained in L3 for approximately 300 hours, meaning they did not molt and did not complete their development to the adult stage. Throughout this period, the refrigerator temperature was recorded at approximately 4 ± 2°C. In the control treatment, *H. segmentaria* larvae remained in the third instar for approximately 104 hours, and the average development time from L3 to adulthood was 224 hours. Statistical tests (two-way ANOVA and Šidák post-hoc test with 95% confidence) indicated a significant difference in the development time of the third-instar larvae that remained under refrigeration compared to the larvae in the control treatment (Table 4).

The minimum PMI estimation was performed using the Accumulated Degree-Day (ADD) method for the larval stage (oldest collected specimens – L3), calculated based on the average temperature at the body recovery site (around 24.7ºC), recorded by the nearest meteorological station (INMET Jacarepaguá station). For individuals who were not exposed to refrigeration, the data were calculated based on Thyssen (2005) at 25°C [26], as this was the closest rearing temperature to the average temperature recorded in the days prior to body discovery. For immatures under refrigeration, the average temperatures were 21.3 ºC, 18.8 ºC, 17 ºC, 15.4 ºC and 11 ºC for the individuals kept in the morgue refrigerator for 24h, 48h, 72h, 96h and 240h respectively. That way, the data was calculated also based on Thyssen

(2005) at 20ºC, 15ºC and 10ºC who reared the insects at the temperatures closest to the averages mentioned. For the calculations, basal temperature of 10°C is considered [11]

At the scene, no search for pupae was conducted, but the forensic doctor reported the presence of pupae in the autopsy report. However, only third-instar larvae were collected. It is important to note that if pupae were indeed present on the body, they should have been collected, as they represent the oldest immature stage. Consequently, the colonization time estimation was underestimated, as it was based only on the end of the third-instar larval stage. Based on the calculations for individuals not refrigerated, the larvae of these dipterans originated from oviposition on 23 January 2024. For specimens refrigerated for 24h, the laying date was estimated at January 24; January 25 to those refrigerated for 48h and 72h; January 26 to those refrigerated for 96h and so on, leading to differences of 1 to 2 days in the estimate up to about 240h of refrigeration, in which the calculations indicated that the eggs would have been laid on February 4, about 13 days later than the non-refrigerated individuals (Chart 4). It was not possible to calculate the minimum PMI for individuals refrigerated for more than 240h due to the absence of biology studies that raised the species at less than 10ºC (average temperature calculated by considering the days under refrigeration together with the days in which the corpse was found).

Case IV

An unidentified-skinned body, normolinear physique, measuring approximately 165 cm in length, estimated to be between 60 and 70 years old, in an advanced state of decomposition (colliquative stage), was found inside his apartment in the South Zone of Rio de Janeiro, Brazil. According to the police incident report, the body was discovered on June

3, 2024, at 10:03 PM, and was sent to the Afrânio Peixoto Forensic Medical Institute (IMLAP), arriving there at 10:42 PM on the same day for autopsy. The forensic pathologist classified the cause of death as indeterminate due to the advanced stage of decomposition (Figure 4).

Larvae were collected from the body on June 4, at around 8:30 AM. L3 larvae of the species *Synthesiomyia nudiseta* were found. This body remained in the morgue's refrigerator for 5 days (no larval mass was present). Around 30 larvae were collected and reared in the laboratory as part of the control treatment. The development of the remaining larvae under refrigeration was evaluated directly in the morgue's refrigerator. During this period, the larvae remained in L3 for approximately 108 hours, meaning they did not molt and did not complete their development to the adult stage. Throughout this period, the refrigerator temperature was recorded at 4 ± 2°C. In the control treatment, *S. nudiseta* larvae remained in the third instar for approximately 200 hours, and the average development time from L3 to adulthood was 512 hours. Statistical tests (two-way ANOVA and Šidák post-hoc test with 95% confidence) indicated a significant difference in the development time of third-instar larvae that remained under refrigeration compared to the larvae in the control treatment (Table 5).

The PMI estimation was performed using the Accumulated Degree-Day (ADD) method, based on the average temperature recorded (approximately 25.2 ºC) at the nearest meteorological station (INMET Copacabana station) in the days preceding the body's discovery. The data were calculated for the larval stage of the oldest collected specimen (L3) based on Krüger et al. (2002) at 26°C for the larvae that were not kept under refrigeration [27]. For specimens under refrigeration, the average temperatures were 21.5 ºC, 19.2 ºC 17.3ºC and 15.8 ºC when they kept in the morgue refrigerator for 24h, 48h, 72h, 96h . That way, the data was calculated for the total larval stage based on Velásquez et al. (2013) [28]

at 20ºC and 15ºC, who reared the insects at the temperatures closest to the averages mentioned.A basal temperature of 10 ºC was considered for *S. nudiseta* [11]. It cannot be guaranteed that the specimens found correspond to the first oviposition on the cadaver, as the forensic investigators did not verify the presence of open puparia at the location of the corpse.

Based on the calculations for non-refrigerated individuals, the larvae of these dipterans originated from oviposition on May 27, 2024. According to the calculations for refrigerated individuals, the eggs were laid on May 29th for individuals refrigerated for 24 and 48h, day 30 for individuals refrigerated for 72 and day 31 for individuals refrigerated for 96h. As there are no studies that present data only for the total duration of the 3rd larval instar (the oldest stage of the specimens collected from the corpse) or of the molt between larval instars, for the refrigerated individuals, the calculation was underestimated, as it was only possible to use the total development time of the larval stage for the calculations. It was not possible to calculate the minimum PMI for individuals refrigerated for more than 96h due to the absence of biology studies that raised the species at less than 15ºC (Chart 5).

4| DISCUSSION

The accuracy of the minimum Post-Mortem Interval (PMImin) estimation in forensic entomology is critically dependent on the precise calculation of insect development time, a process known to be highly influenced by temperature [29–31]. The Accumulated Degree-Day (ADD) method is a commonly utilized approach to quantify the thermal energy required for insect development [14]. However, the traditional application of ADD, often relying on

linear relationships between temperature and development rate, becomes unreliable when insects are exposed to temperatures near or below their developmental basal temperature, such as during refrigeration [1].

In the present study, even when exposing the larvae to refrigeration, the calculation based on the larval instar collected from the bodies did not always result in significant differences. For example, for individuals of the species *Chrysomya megacephala* refrigerated for up to 24 hours, the minimum PMI estimate was not drastically different from the estimate for individuals that were not refrigerated. However, for the other species, this estimate showed greater discrepancies. Carneiro et al. (2025b) [32] also observed that, for some Calliphoridae species, exposure to low temperatures for short periods did not have a significantly negative impact on insect development. We also noticed that when insects were refrigerated for more than 24 hours, as the exposure time increased, the estimated day of oviposition was delayed by approximately 1 to 2 days. This highlights the significant impact of refrigeration on the early stages of insect development and subsequent PMI estimations. Furthermore, it was noted that variations in PMI calculations can arise depending on the specific ADD methodology and the basal temperature utilized. Different species have different thermal requirements, and using an inappropriate basal temperature can lead to inaccurate results [33].

A significant limitation encountered in this work is the scarcity of research on the thermal requirements of forensically important tropical and subtropical Calliphoridae species reared at temperatures below 10°C. Consequently, it was not feasible to accurately apply ADD-based calculations for the majority of individuals that remained refrigerated for more than 96 hours. This knowledge gap underscores the need for species-specific developmental data at lower temperatures, particularly given the prevalence of refrigeration in forensic casework. Unfortunately, conducting dedicated experiments to determine the

specific thermal requirements for the insect species encountered in this study was beyond its scope. However, we strongly recommend and advocate for future investigations focused on elucidating these crucial parameters. It is possible that the nonlinear effects of continuous and discontinuous refrigeration on insect development may require the creation of a new calculation or formula for estimating ADD and the minimum PMI, potentially establishing new baseline temperature thresholds that more accurately reflect development under such conditions.

This study also sought to determine whether cadavers stored under refrigeration would offer a significant microclimate and enhanced thermal protection to colonizing larvae, thereby maintaining larval mass temperature and promoting development. However, our findings indicate that in practice, the cadavers did not provide substantial insulation to the larvae against the low temperatures of the cadaver refrigeration unit, leading to significant alterations in their development and survival, consistent with other studies [12, 34]. Perhaps a "flaw" in our study was not conducting the experiments with the cadavers inside body bags. This might have favored the creation of a more suitable microclimate.

Furthermore, it is essential to emphasize the necessity of accurately measuring the temperature of cadavers during their transportation. In regions like Rio de Janeiro, where this study was conducted, and elsewhere, cadavers may be transported for extended periods, often exceeding 12 hours, before arriving at the morgue for examination. Ideally, refrigerated transport vehicles should be used; however, many lack this capability. This poses a considerable concern, as elevated temperatures within non-refrigerated vehicles, particularly in warm climates, can significantly influence the development of colonizing insects. Global standards in forensic entomology also recommend the use of dataloggers to record temperature at the crime scene in the days leading up to the body's discovery, providing a more reliable temperature history [30]. However, law enforcement and forensic agencies

often face financial limitations that restrict the availability of sufficient dataloggers. Additionally, security concerns and high crime rates in certain areas can impede the deployment and retrieval of such equipment. As a result, practitioners often resort to using ambient temperature data from the nearest meteorological station, which can introduce further inaccuracies in PMI estimations derived from entomological evidence. As highlighted by Lutz and Amendt (2020) [35], comparing on-site data with meteorological station data is a minimum requirement, and the limitations of temperature reconstruction should always be discussed.

The challenges highlighted in this study underscore the critical importance of adhering to established best practice standards and guidelines in forensic entomology to ensure the reliable use of insect evidence in legal investigations [1]. The European Association for Forensic Entomology (EAFE) has developed comprehensive protocols emphasizing meticulous collection, preservation, and analysis of entomological evidence [1]. The increasing recognition of forensic entomology in judicial proceedings worldwide necessitates high-quality, scientifically sound findings [31]. Research has increasingly focused on the effects of cold temperatures on the development and survival of forensically relevant insects [12, 36, 37]. Studies investigating both continuous and discontinuous refrigeration have demonstrated significant delays in development, reduced survival rates, and the induction of quiescence in certain larval stages [12, 31]. These findings emphasize the potential for substantial errors in estimating the time of colonization (TOC) and, consequently, the minimum PMI if the impact of refrigeration is not thoroughly accounted for.

In conclusion, this study emphasizes the complexities introduced by refrigeration in forensic entomology caseworks in Brazil. The limited insulation provided by cadavers against refrigeration temperatures, potential temperature fluctuations during transport, and

the inherent limitations in obtaining precise environmental temperature data underscore the need for caution when applying linear development models in cases involving refrigerated bodies. Further research is crucial to enhance our understanding of the thermal biology of forensically important insect species in Brazil, particularly their responses to realistic temperature fluctuations, and to develop more robust methodologies for PMI estimation in cases where refrigeration is a factor. This will ultimately contribute to greater accuracy and reliability of entomological evidence in forensic investigations.

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FIGURES

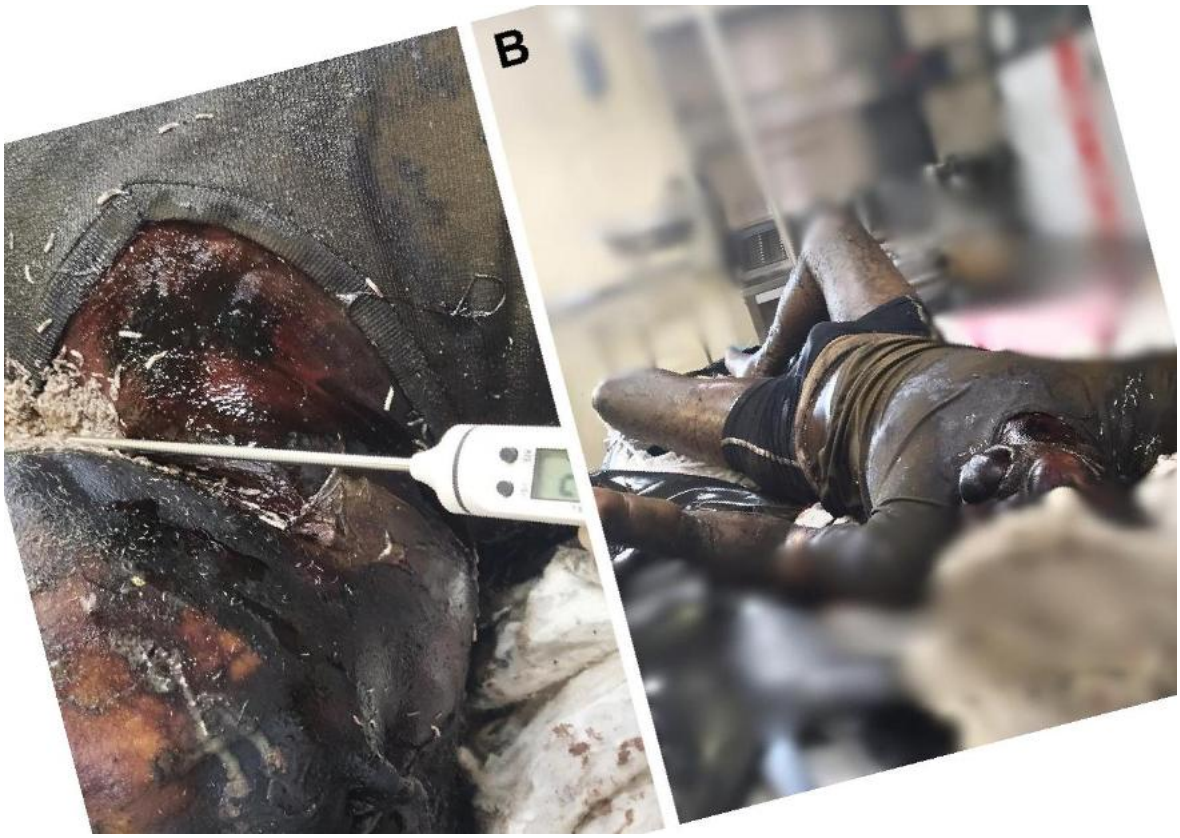

Figure 1. Corpse reported in case I. A: Larval mass found in the neck region when the insects were collected during the necroscopic examination. B: General view of the corpse in dorsal decubitus at the time of necropsy and larvae collection at IMLAP.

Figure 2. Corpse reported in case II. A: General view of the body in ventral decubitus at the time of necropsy and larvae collection at IMLAP. B: larvae collected from the head and face (there was no larval mass). C: general view of the body in dorsal decubitus at the time of necropsy and collection of larvae at IMLAP. D: place where the body was found in the prone position (image: Polícia Civil do Estado do Rio de Janeiro) E: body in the place where it was found at the time of the perinecroscopic examination (image:Policia Civil do Estado do Rio de Janeiro).

Figura 3. Corpse reported in case III. A: general view of the corpse in the dorsal decubitus position where it was found. B: body at the scene with masses of larvae scattered mainly in the facial and pectoral regions. C: body at IMLAP when the larvae were collected from the face and pectoral region.

Figura 4. Corpse reported in case IV. cadaver in ventral decubitus at the time of necropsy at IMLAP and collection of larvae in the head and neck region.

TABLES

Table 1. Case I. Average development time (in hours ± standard deviation) of the immature stages of *Chrysomya megacephala* that were colonizing the corpse in the refrigerator (4 ± 3ºC and 50 ± 10% RH) and the control treatment (27 ± 2 ºC and 70 ± 5% RH). The F-statistic, P-values and the percentage of total variation corresponding to the two-way ANOVA analysis are also presented.

	Time (Hours)		ANOVA two-way	F-valor	P-valor	% of total variation
Development stage	**Control**	**Refrigeration**				
Egg	NA	NA	Interaction	113024	<0.0001	55.78
1st larval instar	NA	NA	Development stage	79541	<0.0001	39.26
2nd larval instar	NA	NA	Development time	19939	<0.0001	4.92
3rd larval instar	90 ± 2.00[a]	744 ± 12.00[b]				
Pupa	108 ± 0.00	NA				
L3-adult	204 ± 5.29	NA				

[a] and [b] Indicates that the average development time of 3rd larval instar differed significantly between the treatments under continuous refrigeration and the control group according to Šidák post-hoc test with 95% of confidence

NA: not applicable

Table 2. Case I. Average development time (in hours ± standard deviation) of the immature stages of *Chrysomya albiceps* that were colonizing the corpse in the refrigerator (4 ± 3ºC and 50 ± 10% RH) and the control treatment (27 ± 2 ºC and 70 ± 5% RH). The F-statistic, P-values and the percentage of total variation corresponding to the two-way ANOVA analysis are also presented.

	Time (Hours)		ANOVA two-way	F-valor	P-valor	% of total variation
Development stage	**Control**	**Refrigeration**				
Egg	NA	NA	Interaction	128508	<0.0001	53.30
1st larval instar	NA	NA	Development stage	97019	<0.0001	40.24
2nd larval instar	NA	NA	Development time	30978	<0.0001	6.42
3rd larval instar	82 ± 0.00	740 ± 18.33				
Pupa	94 ± 2.00	NA				
L3-adult	175.33 ± 1.15	NA				

[a] and [b] Indicates that the average development time of 3rd larval instar differed significantly between the treatments under continuous refrigeration and the control group according to Šidák post-hoc test with 95% of confidence

NA: not applicable

Table 3. Case II. Average development time (in hours ± standard deviation) of the immature stages of *Peckia (Peckia) chrysostoma* that were colonizing the corpse in the refrigerator (4 ± 2 ºC and 50 ± 10% RH) and the control treatment (27 ± 2 ºC and 70 ± 5% RH). The F-statistic, P-values and the percentage of total variation corresponding to the two-way ANOVA analysis are also presented.

Development stage	Time (Hours)		ANOVA two-way	F-valor	P-valor	% of total variation
	Control	Refrigeration				
Egg	NA	NA	Interaction	7360	<0.0001	79.28
1st larval instar	NA	NA	Development stage	1838	<0.0001	19.8
2nd larval instar	19 ± 1^{a}	152 ± 18.33^{b}	Development time	24.05	<0.0001	0.08363
3rd larval instar	64 ± 18.33^{c}	432 ± 12.00^{d}				
Pupa	192 ± 24.00	NA				
L2-adult	275 ± 7.00	NA				

[a, b, c] and [d] Indicates that the average development time of 2nd and 3rd larval instar differed significantly between the treatments under continuous refrigeration and the control group according to Šidák post-hoc test with 95% of confidence. NA: not applicable.

Table 4. Case III. Average development time (in hours ± standard deviation) of the immature stages of *Hemilucilia segmentaria* that were colonizing the corpse in the refrigerator (4 ± 2 ºC and 50 ± 10% RH) and the control treatment (27 ± 2 ºC and 70 ± 5% RH). The F-statistic, P-values and the percentage of total variation corresponding to the two-way ANOVA analysis are also presented.

Development stage	Time (Hours)		ANOVA two-way	F-valor	P-valor	% of total variation
	Control	Refrigeration				
Egg	NA	NA	Interaction	1121	<0.0001	63.08
1st larval instar	NA	NA	Development stage	483.9	<0.0001	27.22
2nd larval instar	NA	NA	Development time	171.1	<0.0001	4.813
3rd larval instar	104 ± 13.86[a]	300 ± 48.00[b]				
Pupa	120 ± 24	NA				
L3-adult	224 ± 27.71	NA				

[a] and [b] Indicates that the average development time of 3rd larval instar differed significantly between the treatments under continuous refrigeration and the control group according to Šidák post-hoc test with 95% of confidence NA: not applicable.

Table 5. Case IV. Average development time (in hours ± standard deviation) of the immature stages of *Synthesiomyia nudiseta* that were colonizing the corpse in the refrigerator (4 ± 2 ºC and 50 ± 10% RH) and the control treatment (27 ± 2 ºC and 70 ± 5% RH). The F-statistic, P-values and the percentage of total variation corresponding to the two-way ANOVA analysis are also presented.

Development stage	Time (Hours)		ANOVA two-way	F-valor	P-valor	% of total variation
	Control	Refrigeration				
Egg	NA	NA	Interaction	2364	<0.0001	22.15
1st larval instar	NA	NA	Development stage	728.8	<0.0001	6.83
2nd larval instar	NA	NA	Development time	14983	<0.0001	70.2
3rd larval instar	200 ± 30.20[a]	108 ± 12.00[b]				
Pupa	312 ± 24.00	NA				
L3-adult	512 ± 6.93	NA				

[a] and [b] Indicates that the average development time of 3rd larval instar differed significantly between the treatments under continuous refrigeration and the control group according to Šidák post-hoc test with 95% of confidence NA: not applicable.

CHARTS

Chart 1. Case I. Calculation of minimum PMI estimates from the larval period of *Chrysomya megacephala*.

A

Date	Environmental temperature	DD	ADD
10/jul	27,5	17,5	24
09/jul	27,3	17,3	6,5
08/jul	28,3		

B

Date	Environmental temperature	DD	ADD
11/jul	4	4	35,8
10/jul	27,5	17,5	31,8
09/jul	27,3	17,3	14,3
08/jul	28,3	18,3	

C

Date	Environmental temperature	DD	ADD
12/jul	4	4	31
11/jul	4	4	27
10/jul	27,5	17,5	23
09/jul	27,3	17,3	5,5
08/jul	28,3	18,3	

D

Date	Environmental temperature	DD	ADD
12/jul	4	4	21,44
11/jul	4	4	17,44
10/jul	27,5	17,5	13,44
09/jul	27,3	17,3	
08/jul	28,3	18,3	

E

Date	Environmental temperature	DD	ADD
13/jul	4	4	50,77
12/jul	4	4	46,77
11/jul	4	4	42,77
10/jul	27,5	17,5	38,77
09/jul	27,3	17,3	21,27
08/jul	28,3	18,3	3,97

F

Date	Environmental temperature	DD	ADD
14/jul	4	4	41,68
13/jul	4	4	37,68
12/jul	4	4	33,68
11/jul	4	4	29,68
10/jul	27,5	17,5	25,68
09/jul	27,3	17,3	8,18
08/jul	28,3	18,3	

A: Not refrigerated. Data calculated based on Rabêlo et al. (2011) at 26 ºC [15]. B: Refrigerated for 24h. Data calculated based on Velez & Wolf (2008) at 23 ºC [16]. C: Refrigerated for 48h. Data calculated based on Yang et al. (2016) at 19 ºC. D: Refrigerated for 48h. Data calculated based on Yang et al. (2016) at 19 ºC and base temperature = 12,78 ºC [17]. E: Refrigerated for 72h. Data calculated based on Gruner et al. (2016) at 16 ºC [18]. F: refrigerated for 96h. Data calculated based on Ngando et al. (2024) at 15 ºC [19]. Environmental temperature= Environmental temperature, in ºC, collected from the nearest weather station to where the corpse was found (INMET Vila Militar station) and the temperature under refrigeration. Base temperature = 10ºC according to Higley & Haskell (2010) [11] for estimates in A, B, C, E and F. Base temperature = 12,72ºC according to Yang et al. (2016) for estimating in D [17]. ADD = Accumulated degree day - basal temperature. DD = Degree day.

Chart 2. Case I. Calculation of minimum PMI estimates from the larval period of *Chrysomya albiceps*.

A

Date	Environmental temperature	DD	ADD
10/jul	27,5	12,46	25,83
09/jul	27,3	12,26	13,37
08/jul	28,3	13,26	1,11

B

Date	Environmental temperature	DD	ADD
10/jul	27,5	14,5	31,63
09/jul	27,3	14,3	17,12
08/jul	28,3	15,3	2,82

C

Date	Environmental temperature	DD	ADD
11/jul	4	4	24,25
10/jul	27,5	12,45	20,25
09/jul	27,3		7,79
08/jul	28,3		

D

Date	Environmental temperature	DD	ADD
11/jul	4	4	24,25
10/jul	27,5	14,5	20,25
09/jul	27,3	14,3	5,75
08/jul	28,3		

E

Date	Environmental temperature	DD	ADD
12/jul	4	4	17,93
11/jul	4	4	13,93
10/jul	27,5	12,46	9,93
09/jul	27,3		
08/jul	28,3		

F

Date	Environmental temperature	DD	ADD
12/jul	4	4	32,81
11/jul	4	4	28,81
10/jul	27,5	14,5	24,81
09/jul	27,3	14,3	10,31
08/jul	28,3		

A: Not refrigerated. Data calculated based on Queiroz et al. (1997) at 27 ºC and base temperature of 15.04 ºC[20]. B: Not refrigerated. Data calculated based on Queiroz et al. (1997) at 27 ºC using base temperature of 13 ºC proposed by Richards et al. (2008) [22]. C: Refrigerated for 24h. Data calculated based on Kordshouli et al (2021) at 23 ºC and base temperature of 15.04 ºC [21]. D: Refrigerated for 24h. Data calculated based on Kordshouli et al (2021) at 23 ºC and base temperature of 13 ºC. E: Refrigerated for 48h. Data calculated based on Richards et al. (2008) at 17,5 ºC and a base temperature of 15.04 ºC. F: Refrigerated for 48h. Data calculated based on Richards et al. (2008) at 17,5 ºC and a base temperature of 13 ºC. Environmental temperature= Environmental temperature, in ºC, collected from the nearest weather station to where the corpse was found (INMET Vila Militar station) and the temperature under refrigeration. Base temperature = 15.04 according to Queiroz (1996) [23] and 13 ºC according to Richards et al. (2007). ADD = Accumulated degree day - basal temperature. DD = Degree day.

Chart 3. Case II. Calculation of minimum PMI estimates from the larval period of *Peckia (Peckia) chrysostoma*.

A

Date	Environmental temperature	DD	ADD
14/fev	30,1	20,1	44,2
13/fev	24,8	14,8	24,1
12/fev	24,5	14,5	9,3

B

Date	Environmental temperature	DD	ADD
15/fev	4	4	27,25
14/fev	30,1	20,1	23,25
13/fev	24,8	14,8	3,15

A: Not refrigerated; data calculated based on Ferraz (1995) at 27ºC [24]. B: Refrigerated for 24h; data calculated based on Ferreira et al. (2024) at 25ºC [25]. Base temperature= 10ºC according to Higley & Haskell (2010) [11]. Environmental temperature= Environmental temperature, in ºC, collected from the nearest weather station to where the corpse was found (INMET Grand Meier) and the temperature under refrigeration. ADD = Accumulated degree day - basal temperature. DD = Degree day.

Chart 4. Case III. Calculation of minimum PMI estimates from the larval period of *Hemilucilia segmentaria*.

A

Date	Environmental temperature	Larval mass temperature	DD	ADD
27/jan	–	30	10	58.8
26/jan	23.2 °C		13.2	48.8
25/jan	24 °C		14	35.6
24/jan	25.6 °C		15.6	21.6
23/jan	23.7		13.7	6

B

Date	Environmental temperature	Larval mass temperature	DD	ADD
28/jan	4		4	48
27/jan	–	30	10	44
26/jan	23.2		13.2	34
25/jan	24		14	20.8
24/jan	25.6		15.6	6.8
23/jan	23.7		23.7	

C

Date	Environmental temperature	Larval mass temperature	DD	ADD
29/jan	3.7		3.7	37.4
28/jan	4		4	33.7
27/jan	–	30	10	29.7
26/jan	23.2 °C		13.2	19.7
25/jan	24 °C		14	6.5
24/jan	25.6 °C		15.6	

D

Date	Environmental temperature	Larval mass temperature	DD	ADD
30/jan	4.2		4.2	42
29/jan	3.7		3.7	37.8
28/jan	4		4	34.1
27/jan	–	30	10	30.1
26/jan	23.2 °C		13.2	20.1
25/jan	24 °C		14	6.9
24/jan	25.6 °C		15.6	

E

Date	Environmental temperature	Larval mass temperature	DD	ADD
31/jan	3.2		3.2	32.4
30/jan	4.2		4.2	29.2
29/jan	3.7		3.7	25
28/jan	4		4	21.3
27/jan	–	30	10	17.3
26/jan	23.2 °C		13.2	7.3
25/jan	24 °C		14	

F

Date	Environmental temperature	Larval mass temperature	DD	ADD
06/fev	3		3	8.25
05/fev	4		4	5.25
04/fev	6.2		6.2	1.25
03/fev	4.3		4.3	
02/fev	5		5	
01/fev	3		3	
31/jan	3.2		3.2	
30/jan	4.2		4.2	
29/jan	3.7		3.7	
28/jan	4		4	
27/jan	–	30	10	
26/jan	23.2 °C		13.2	
25/jan	24 °C		14	
24/jan	25.6 °C		15.6	
23/jan	23.7		23.7	

A: Not refrigerated. B: Refrigerated for 24h. C: Refrigerated for 48h D: Refrigerated for 72h E: Refrigerated for 96h. F: Refrigerated for 240h. Data calculated based on Thyssen (2005) at 25ºC (for A), 20ºC (for B and C) 15ºC (for D and E) and 10ºC (for F) [26]. Base temperature= 10ºC according to Higley & Haskell (2010) [11]. Environmental temperature= Environmental temperature, in ºC, collected from the nearest weather station to where the corpse was found (INMET Jacarepaguá) and the temperature under refrigeration. ADD = Accumulated degree day - basal temperature. DD = Degree day. Larval mass temperature= temperature of the larval mass measured when the specimens were collected

Chart 5. Case IV. Calculation of minimum PMI estimates from the larval period of *Synthesiomyia nudiseta*.

A

Date	Environmental temperature	DD	ADD
04/jun	25.6	15.6	129.2
03/jun	20.4	10.4	113.6
02/jun	23.7	13.7	103.2
01/jun	28.2	18.2	89.5
31/mai	25.4	15.4	71.3
30/mai	27.8	17.8	55.9
29/mai	26	16	38.1
28/mai	25.8	15.8	22.1
27/mai	26.2	16.2	6.3

B

Date	Environmental temperature	DD	ADD
05/jun	3.6	3.6	105.6
04/jun	25.6	15.6	102
03/jun	20.4	10.4	86.4
02/jun	23.7	13.7	76
01/jun	28.2	18.2	62.3
31/mai	25.4	15.4	44.1
30/mai	27.8	17.8	28.7
29/mai	26	16	10.9
28/mai	25.8	15.8	

C

Date	Environmental temperature	DD	ADD
06/jun	5.2	5.2	105.6
05/jun	3.6	3.6	100.4
04/jun	25.6	15.6	96.8
03/jun	20.4	10.4	81.2
02/jun	23.7	13.7	70.8
01/jun	28.2	18.2	57.1
31/mai	25.4	15.4	38.9
30/mai	27.8	17.8	23.5
29/mai	26	16	5.7

D

Date	Environmental temperature	DD	ADD
07/jun	4.4	4.4	86.7
06/jun	5.2	5.2	82.3
05/jun	3.6	3.6	77.1
04/jun	25.6	15.6	73.5
03/jun	20.4	10.4	57.9
02/jun	23.7	13.7	47.5
01/jun	28.2	18.2	33.8
31/mai	25.4	15.4	15.6
30/mai	27.8	17.8	0.2
29/mai	26	16	

E

Date	Environmental temperature	DD	ADD
08/jun	3.2	3.2	86.7
07/jun	4.4	4.4	83.5
06/jun	5.2	5.2	79.1
05/jun	3.6	3.6	73.9
04/jun	25.6	15.6	70.3
03/jun	20.4	10.4	54.7
02/jun	23.7	13.7	44.3
01/jun	28.2	18.2	30.6
31/mai	25.4	15.4	12.4
30/mai	27.8	17.8	

A: Not refrigerated; data calculated based on Kruger et al. (2002) at 26ºC [27]. B: Refrigerated for 24h. C: Refrigerated for 48h D: Refrigerated for 72h E: Refrigerated for 96h. Data calculated based on Velásquez et al. (2013) at 20ºC (for B and C) and 15ºC (for D and E) [28]. Base temperature= 10ºC according to Higley & Haskell (2010) [11]. Environmental temperature= Environmental temperature, in ºC, collected from the nearest weather station to where the corpse was found (INMET Copacabana station) and the temperature under refrigeration. ADD = Accumulated degree day - basal temperature. DD = Degree day.

CONCLUSÕES GERAIS

A refrigeração, tanto contínua quanto descontínua, impacta significativamente o desenvolvimento e a sobrevivência de dípteros califorídeos de importância forense, com implicações diretas na estimativa do tempo de colonização (TOC) e, consequentemente, no intervalo pós-morte mínimo (IPMmin). Os estudos conduzidos em laboratório, detalhados nos Capítulos I e II, demonstraram que a refrigeração contínua retarda ou até mesmo interrompe o desenvolvimento dos estágios imaturos (ovos, larvas e pupas) das espécies *Chrysomya megacephala*, *Chrysomya putoria*, *Hemilucilia segmentaria* e *Lucilia cuprina*. Foi observado que os ovos e larvas de primeiro instar são particularmente sensíveis, com baixa sobrevivência sob refrigeração. Por outro lado, as larvas de terceiro instar apresentaram maior tolerância, entrando em quiescência. A pesquisa enfatizou a necessidade de dados específicos para cada espécie e a cautela ao aplicar modelos lineares de desenvolvimento em casos envolvendo refrigeração.

A investigação sobre os efeitos da refrigeração descontínua (Capítulo II) em larvas de terceiro instar revelou que períodos alternados de frio e reaquecimento também induzem quiescência, resultando em um aumento significativo no tempo total de desenvolvimento (ovo a adulto) e uma redução na sobrevivência. Estes achados sublinham a importância de considerar os padrões reais de flutuação de temperatura em investigações forenses.

A avaliação do desenvolvimento larval em cadáveres humanos refrigerados no Instituto Médico Legal Afrânio Peixoto (Capítulo III) corroborou os resultados laboratoriais, demonstrando um retardo significativo no desenvolvimento larval de diferentes espécies (*Chrysomya albiceps*, C*hrysomya megacephala*, *Peckia (Peckia) chrysostoma*, *Hemilucilia segmentaria* e *Synthesiomyia nudiseta*) sob condições de morgue. A estimativa do dia de

oviposição foi atrasada em casos de refrigeração prolongada, alertando para potenciais erros na determinação do IPMmin se os efeitos da refrigeração não forem considerados.

Uma limitação importante identificada nesta pesquisa é a escassez de dados de desenvolvimento em baixas temperaturas para espécies de importância forense tropicais e subtropicais. Esta lacuna no conhecimento dificulta a aplicação precisa de métodos como o de graus-dia acumulados (GDA) em casos que envolvem refrigeração por períodos extensos.

Em suma, o presente estudo demonstra de forma abrangente que a refrigeração é um fator crítico que interfere no desenvolvimento de dípteros califorídeos de importância forense e, consequentemente, na estimativa do IPMmin. Os resultados obtidos ressaltam a necessidade de incorporar os efeitos da refrigeração nas metodologias de estimativa do IPM, considerando as condições específicas de temperatura e a espécie envolvida. Além disso, enfatiza-se a urgência de futuras pesquisas que investiguem a biologia térmica de insetos forenses em temperaturas relevantes para a refrigeração, principalmente no Brasil, a fim de aprimorar a acurácia e a confiabilidade da evidência entomológica em investigações criminais.

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