Journal of Medical Entomology, XX(X), 2022, 1–5
https://doi.org/10.1093/jme/tjac013
Research

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Biological Response of *Chrysomya putoria* (Wiedemann, 1818) (Diptera: Calliphoridae) Pupae After Submersion in Freshwater

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Subject Editor: Julian Hillyer

Received 24 August 2021; Editorial decision 11 January 2022

Abstract

Forensic Entomology uses arthropods to aid in legal investigations. This study checked the biological response of *Chrysomya putoria* pupae to submersion in fresh water for up to 6 d, evaluating the critical submersion time, survival rate, and development time of the flies. Adults were collected using fish baits in two typical traps. Seven hundred and twenty fourth-generation pupae with 2 d of development were used and separated into submergence intervals: 24, 48, 72, 96, 120, and 144 h. An additional 120 pupae were used as a control. Each treatment was done in triplicate, consisting of 40 pupae distributed in four tulle-sealed test tubes containing 10 pupae each. All tubes of each treatment were co-adhered in test tube racks and were submerged in mineral water in a container with constant oxygenation, except those of the control group, which were not submerged. The tubes were removed from the water according to their respective submersion interval, until 144 h was completed. The control group had a survival rate of 90%, while the 24-h treatment had 85% and the 48-h treatment had 35.8%. The critical submersion time for pupae was 72 h, with 100% mortality by 144 h. The average development time for the control group was 3.2 d, while the 24- and 48-h treatments developed in 4.3 and 6.3 d, respectively. The longer the individuals were submerged, the lower the survival rate was, while the development time increased. The data obtained in this study have potential in applications to estimate the interval of submersion of a cadaver.

Key words: aquatic environment, Calliphoridae, forensic entomology, pupae

Forensic Entomology consists in the study of the behavior, development, ecology, and other aspects of arthropods that become vestiges may be related to criminal events, assisting in the resolutions of litigations. Its applications include lawsuits involving the presence of insects that affect the daily life and health of humans, such as insects considered zoological pests or disease vectors, as well as their appearance in stored commercial products (Linhares and Thyssen 2012). Insects are also considered bioindicators and often represent a legal asset to be preserved, whose application and repercussions are directly reflected in Environmental Legislation (Nakaza et al. 2009, Dias Filho and Palanch 2013). Forensic entomology also has medical-legal applications, mainly in the criminal field, where entomological evidences assist in solving crimes involving violent death, making it possible to relate a suspect to the crime scene, to identify both the victim and the aggressor, and to establish the approximate date of a person's death (Lord and Srevenson 1986, Dias Filho and Francez 2018).

Also, in the Forensic Medico-Legal Entomology area, it is possible to make use of insects and other arthropods that associate themselves with the occupation of corpses to estimate the Time of Colonization (TOC) and the place and under what circumstances the death may have occurred. It can also be evaluated if there was

drug use by the individuals before their death because drugs can accelerate or delay insect development, as well as be extracted from insect bodies or from their digestive tracts containing cadaveric tissues with drug residues. Moreover, it can be used to determine the displacement of the body from original crime scenes, and study cases of negligence or violence of children or the elderly (by insect colonization of poorly sanitized clothing, diapers or lesions, including myiases). Finally, it can be used to identify human genetic profiles through the ingestion of their tissues by insects, helping, for example, in the investigations of sex crimes and other types of crimes (Benecke 2001, Benecke and Lessig 2001, Introna et al. 2001, Dias Filho and Francez 2018, Chamoun et al. 2020).

The order Diptera stands out among the important forensic insects for being the largest and most frequent group associated with decomposing corpses. The main representatives of the Neotropical region are from the Calliphoridae, Sarcophagidae, Muscidae, Fanniidae, Stratiomyidae, and Mesembrinellidae families (Oliveira-Costa 2011, Alves et al. 2014).

There are numerous studies on the development of scavenger dipterofauna on outdoor corpses on the ground (Cardozo et al. 2017, Carvalho et al. 2017, Azevedo et al. 2018, Castillo et al. 2021), but few observations have been made regarding carcasses submerged in water. While studying the decomposition of partially submerged pig carcasses and their insect succession in two seasonal periods, Payne and King (1972) classified the decomposition process into six stages: Submerged Fresh, Early Floating, Floating Decay, Bloated deterioration, Floating remains, and Sunken remains, recording terrestrial insect colonization in the exposed regions of the pig at the beginning of the Submerged Fresh stage.

The family Calliphoridae has considerable forensic importance as it is generally the most abundant group found in carcasses (Nuorteva et al. 1974, Hobischak 1997, MacDonell and Anderson 1997, Barrios and Wolff 2011). *Chrysomya* Robineau-Desvoidy genus 1830 (Diptera: Calliphoridae) has a high dispersal rate and very rapid population growth that is associated with its high adaptive capacity (Guimarães et al. 1979, Baumgartner and Greenberg 1984). The species *Chrysomya putoria* (Wiedemann, 1818) is an extremely relevant forensic indicator, as this insect is one of the first to locate and colonize corpses in urban areas, actively participating in the carcass decomposition process. For that reason, it is used in the estimation of postmortem interval (Oliveira-Costa 2011).

Some studies have addressed the breathing capacity and survival of pupae of the Calliphoridae family in reduced oxygen settings (Hitchcock and Haub 1941, Keister 1953, Park and Buck 1960), but there is little research that has analysed the biological characteristics of these pupae under submerged conditions. Reigada et al. (2011) evaluated the submersion effect on the development and survival of *Chrysomya albiceps* (Wiedemann, 1819) (Diptera: Calliphoridae), *C. putoria*, and *Chrysomya megacephala* (Fabricius, 1794) (Diptera: Calliphoridae) pupae. These species were subjected to different submersion periods (0–72 h) and in different pupal ages (0–72 h). They reported white pupae (those which have been recently formed) as the most sensitive, with a decrease in the survival rate and an increase in the development time caused by an increase in the submersion time. However, the analysed period was not long enough to determine the critical submersion time, in which no survival would be observed.

Nuorteva et al. (1974) described the entomofauna in two homicide cases involving partially submerged corpses when studying the possibility of using flies from the Calliphoridae family as medical-legal indicators in Finland. Studies on the insect succession and decomposition rate of swine carcasses highlight mainly the colonization by maggots of Calliphoridae, but also by other dipteran families, such as Sarcophagidae, Muscidae and Fanniidae, as well as other aquatic insects (Hobischak 1997, MacDonell and Anderson 1997, Barrios and Wolff 2011). Silva (2011) and Lobato (2016) analysed decomposing insect species in a partially submerged swine carcass in an upland stream in the Brazilian states of Amazonas and Amapá, and observed that Calliphoridae and Sarcophagidae larvae were the main consumers of the carcass. *Chrysomya albiceps* species was the most abundant and predominant, demonstrating its importance in the aquatic environment.

Our present research aimed to verify the biological response of *C. putoria* pupae to freshwater submersion over a period of up to six days in the laboratory, seeking to gather information with potential application in forensic investigations. This study has the potential to contribute to the estimation of the submersion time of cadavers because Calliphoridae pupae are often found attached to the skin, hair, and clothing of cadavers that have been submerged after their colonization (Singh and Greenberg 1994). The influence of the submergence period on the development time and the survival rate of these dipterans was evaluated.

Materials and Methods

This research was carried out in the *Laboratório de Estudos de Dípteros (LED)* at the *Universidade Federal do Estado do Rio de Janeiro (UNIRIO)*. Adult individuals were collected on 5 August 2019, on the premises of the Rio de Janeiro Zoo (RioZoo) located at Quinta da Boa Vista, Rio de Janeiro, Brazil (–22.90581; –43.22939) to establish a stock colony. Sardines were used as attractive bait, thawed 24 h before the exposure, and disposed in two traps for muscoid diptera, as described by Mello et al. (2007). Each trap contained around 200 g of bait and were placed at 1.5 m from the ground next to dumps where they remained for 24 h.

After the collection, the specimens were taken to LED and placed in a freezer at 15°C for approximately 2 min in order to anesthetize them. A stereoscope microscope was used for identification, following the keys of Mello (2003) and Carvalho and Mello-Patiu (2008). Nearly 400 *C. putoria* adults (≅200 males and 200 females) were moved to a polyethylene cage (40 × 30 × 20 cm), with an opening in the upper surface that was covered with fabric for ventilation. Flies were fed daily with 20 ml of 50% honey solution, 20 ml of water, and chicken gizzard for ovarium maturation and oviposition stimulation. Egg masses were transferred to polyethylene containers containing chicken gizzard in a 1 g/egg proportion for the immature development. These were inserted into larger polyethylene receptacles containing sterilized wood shavings for pupation. *Chrysomya putoria* specimens from the stock colony were deposited in the Entomological collection of the 'Laboratório de Estudo de Dípteros da Universidade Federal do Estado do Rio de Janeiro', Brazil.

In total, 720 fourth-generation pupae were collected at 48 ± 3 h after the abandonment peak from the diet (Proença et al. 2014). The pupae were separated into corresponding treatments with the following submersion periods: 24, 48, 72, 96, 120, and 144 h. Three repetitions were performed for each treatment, consisting of 40 pupae divided into four test tubes and covered in tulle fabric, with 10 pupae/tube. The same procedure was adopted for the Control group, with three repetitions of 40 pupae, totalizing 120 individuals. The Control group was not subjected to submersion and was kept in sterilized wood shavings in a climatic chamber at 27°C during the day and 25°C at night, with relative humidity of 80 ± 10% and 12-h photophase.

All tubes of each submerged treatment were fitted on test tube shelves made of wire and coated with PVC (Fig. 1A), which were

Fig. 1. Experimental design. Test tubes with the pupae were separated by submersion periods (A); submersion container with oxygenation (B); and beaker with sterilized wood shavings for adult emergence (C).

submerged in mineral water in a container with constant oxygenation through a pump which promoted water circulation and consequent movement of the surface, favoring gas exchange with the atmosphere (Fig. 1B). The tubes were removed from the water at 10 am according to the respective time of each treatment until completing 144 h, i.e., in 24-h intervals.

After emersion, the pupae were placed in a beaker containing sterilized wood shavings sealed with tulle and elastic and moved to the climatic chamber at 27°C during the day and 25°C at night, with relative humidity of 80 ± 10% and 12-h photophase, with daily observations for adult quantification (Fig. 1C). The development time was counted from the day the larvae had pupated to the day they emerged as adult flies. pH and temperature of the water were measured daily at 10 am during the experiment using an aquarium thermometer and the LabconTest pH Tropical (Alcon) measurement kit. The mean water temperature was 26 ± 0. 57°C, and the pH was constant at 7.5 during all the experiment.

The Shapiro–Wilk test stated the data did not present normal distribution ($P < 0.05$). Pearson correlation was performed to evaluate the influence of pupal submersion time over the development and the survival of the flies. The Kruskal–Wallis test followed by the Nemenyi post-test were used to compare the development time and survival rates among the different submersion periods. All the statistical analyses were performed using the R Stats Software at a 5% significance level. The packages *vegan* and *PMCMR* were used.

Results

The mean values for development time and survival rates for each treatment are shown in Table 1.

Pupae survived until the second day of submersion (48 h), however with a reduction greater than 50% in the survival rate in this period. The survival rate was 0% from the third to the sixth day in all repetitions. A negative and significant correlation among the submersion period and the survival rate was observed (rho = –0.98; $P = 0.0013$), showing that the longer the submersion time, the lower the survival rate.

Pupae from the control group showed a mean development time—from the day the larvae had pupated to the day they emerged as adult flies—of 3.2 d, while the 24- and 48-h treatments showed higher mean development times of 4.3 and 6.3 d, respectively

Table 1. Submersion periods, mean survival rates, and development time of *Chrysomya putoria* pupae submerged in freshwater

Submersion period (h)	Survival rate (% ± δ)	Development time (d ± δ)
0 (controle)	90.0 ± 0.00[a]	3.2 ± 0.00[a]
24	85.0 ± 4.33[ab]	4.3 ± 0.06[ab]
48	35.8 ± 8.04[b]	6.3 ± 0.06[b]
72	0	—
96	0	—
120	0	—
144	0	—

One hundred and twenty pupae were used for each submersion period, totaling 40 pupae per trial. The values refer to the mean values of the three trials for each submersion period.

Observations followed by different letters statistically differ from each other according to the Kruskal–Wallis test, followed by the Nemenyi test, at a 5% significance level.

δ, standard deviation.

(Table 1). A positive and significant correlation was stated among the submersion and the development times (rho = 0.98; $P < 0.0001$), showing that the longer the submersion period, the longer the pupae development time.

According to the Kruskal–Wallis test, the development time is influenced by the submersion period (Kruskal–Wallis $\chi^2 = 8$, df = 2, $P = 0.02$). The Nemenyi test showed a difference in the development time length from control group individuals only after a submersion period of 48 h ($P = 0.02$). The 24-h submersion period showed no difference from either the control group or those submerged for 48 h ($P = 0.37$ for both). The same pattern is observed when analyzing the survival rate, which is also influenced by the submersion period (Kruskal–Wallis $\chi^2 = 6.83$, df = 2, $P = 0.03$). Differences from the control group are observed only after 48 h of submersion ($P = 0.037$), while the 24-h submersion period shows no difference from either the control group or the 48 h of submersion groups ($P = 0.644$ and $P = 0.261$, respectively).

Discussion

The survival rate is strongly related to the submersion period. Reigada et al. (2011) observed that the mortality rate significantly increased with the increase of the submersion period when studying *C. putoria* from 0 to 48 h of development submerged by periods of 0, 6, 24, and 72 h. Those pupae with greater development time showed higher resistance, only suffering significant reduction in the survival rate after a 48-h submersion period. Similar patterns were observed for *C. albiceps* and *C. megacephala*, with the latter being the most resistant to submersion conditions. However, they could not determine the maximum submersion period tolerated by this species in pupae over 24 h of development.

In a similar study, Souza and Keppler (2009) observed the survival capacity of *Lucilia eximia* (Wiedemann, 1819) (Diptera: Calliphoridae) pupae up to the fifth day of submersion, showing 100% mortality from the sixth day, thus presenting results with potential application to estimate the submersion postmortem interval, mainly when the pupae settlement occurs before the total submersion of the corpse. Singh and Greenberg (1994) observed 100% mortality on the fifth day of submersion when studying five Calliphoridae species. Our findings show a more accentuated response to the submersion period, with a strong decrease in the survival rate of *C. putoria* pupae after a period of 48 h and reaching total mortality after a 72-h submersion period at 26 ± 0.57°C. The development time is also related to the submersion period, as stated by Souza and Keppler (2009), when observing the development time of *Lucilia eximia* after submersion, which is another forensic indicator from the Calliphoridae family. As in our findings, the development time and the submersion period showed a positive correlation.

The observation that the submersion period affects the developmental curve of *C. putoria* pupae may be influenced by factors such as the water temperature, pH, and oxygenation, which all constitute useful data to estimate a corpse submersion period in forensic investigations. Ferraz et al. (2012) evaluated the postembryonic development of *C. putoria* raised on gizzard diet at an average temperature of 20.6°C, obtaining an average duration in the pupal phase of 4.3 d. These results diverged from our findings in the control group, probably due to the lower temperature tested by the authors when compared with our study. Dallavecchia et al. (2015) observed a mean pupae development time of 3.8 d when studying different diets for *C. megacephala* under temperatures of 25°C min./27°C max., which agrees with our findings.

The level of oxygenation could influence the survival of submerged pupae. Keister (1953) found that the O^2 consumption of *Phormia regina* (Meigen, 1826) (Diptera: Calliphoridae) follows a U-shaped curve, showing greater rates on the first and final development days, and decreasing in the intermediate period. Park and Burk (1960) observed that *P. regina* with 1–5 d of development tend to be more sensitive to hypoxia, slowing down or even stopping their development. Mądra-Bielewicz et al. (2017) tested the development limitations of *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae) and *Calliphora vomitoria* (Linnaeus, 1758) (Diptera: Calliphoridae) and found indications that blow flies have a singular survival pattern under hypoxia situations that depends on the number of developing insects, their age and the initial oxygen amount.

Another factor that could alter the survival rate and the development time of the pupae is osmotic pressure. Marshal and Wood (1990) studied the osmotic regulation of *Lucilia cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae) and found that these insects have powerful osmotic and ionic regulatory mechanisms. However, the response of each species is different, and assessments in submersion situations were not considered.

In summary, we conclude that *C. putoria* pupae can survive submersion for a period of up to 48 h at 26°C. No adult emergence was observed when pupae were submerged for longer. However, a >50% decrease in the survival rate of this species was observed when submerged for 48 h. The submersion of *C. putoria* pupae influenced the survival rate in an inversely proportional way, for which the longer the submersion period, the lower the number of adults emerged, and therefore, the survival rate. The pupae development time was also affected by the submersion period, but in a directly proportional rate; the longer the submersion period, the longer the development time. Studies on the response of Calliphoridae to submersion have the potential to be applied in criminal investigations, mainly when it is necessary to estimate the submersion period of a corpse.

Acknowledgments

We are grateful to Universidade Federal do Estado do Rio de Janeiro (UNIRIO) and Universidade Federal Rural do Rio de Janeiro (UFRRJ) for all the knowledge given, and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, grant number 88882.426021/2019-01) for their financial support.

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