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The impact of chilling on selected attributes of the blowfly, *Calliphora vicina* (Robineau-Desvoidy, 1830) (Diptera: Calliphoridae), under laboratory conditions

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Abstract: The purpose of this study was to evaluate the effects of four different chilling periods, varying from 24 to 120 h, on the mean survival rate, larval and pupal development durations and the pupal and adult weight of *Calliphora vicina* (Robineau-Desvoidy) (Diptera: Calliphoridae) under laboratory conditions at 4 °C in 2018 at Ondokuz Mayıs University. Chilling reduced the survival rate of the pupae of all developmental stages. The adult eclosion rate of first instar larvae and pupae was lower, especially after 72 and 120 h chilling periods, but it increased for the second instar, third instar and postfeeding larvae with increasing chilling period. In addition, the larval development period was increased by increased chilling period for all developmental stages except for postfeeding larvae for 120 h. Pupal and adult weight were also affected by chilling period, and the postfeeding larval and pupal stages were the most intolerant groups to chilling. Overall, the effects of chilling on the life history parameters of *C. vicina* were substantial and should be considered to provide a more reliable estimate of postmortem interval.

Key words: *Calliphora vicina,* chilling period, development duration, survival rate, weight

1. Introduction

Forensic entomology, which is the use of insects for legal proceedings, is becoming increasingly valuable in criminal investigations (Kökdener, 2016). Insects can be used to help solve a crime, with the most important contribution of forensic entomology being the determination of approximate elapsed time since death (Anderson and VanLaerhoven, 1996; Amendt et al., 2004; Amendt et al., 2007). Furthermore, insects provide valuable clues in the determination of the location of death and postmortem transfer, recognition of wound sites, cause and manner of death, chemical and drug intoxication, the abuse and neglect of children, and other aspects of forensic investigation (Goff and Flynn, 1991; Introna et al., 1998; Sumodan, 2002). In deaths that occurred more than 72 h earlier, forensic entomology is the most accurate, and frequently the only approach, for the estimation of a minimum postmortem interval (min PMI) (Warren and Anderson, 2013; Ahmed and Samson, 2016). Determination of the minimum postmortem interval is based on the estimation of the time of insect colonization (TOC) (Sanford, 2017).

There are two entomological approaches available for estimating the postmortem interval. The first approach focuses on the predictable succession pattern of human colonization by insects and other arthropods (Schoenly and Reid, 1987; Amendt et al., 2007). The succession approach involves the analysis of the pattern of necrophagous insect colonization of the body or carrion and use of ecologically based techniques for postmortem interval estimation. Forensic entomologists employ the accumulated degree days (ADD) and accumulated degree hours (ADH) methods to estimate the time of insect colonization. The second method involves the estimation of the age of the oldest individuals, typically primary blowfly larvae, on the body (Ames and Turner, 2003). The application of the ADH method requires developmental data for the particular species collected from the corpse (Sanford, 2017). Decomposition begins several minutes after death and the body becomes attractive to insects. Blowflies have a strong sense of smell and remarkable flight capacity and therefore can locate a corpse very quickly after death occurs (Pellitero and Saloña Bordas, 2007; Dekeirsschieter et al., 2009). This behaviour makes them the most frequently available and effective insects for minimum postmortem interval estimation. Female blowflies lay their eggs on the corpse and the offspring of blowflies then pass through three larval stages and pupate before the adult emerges (Donovan et al., 2006; Magni et al., 2016).

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Evidence related to insects is collected from the victim's remains at the crime scene by police or crime scene officers. Sometimes collecting these evidences directly from the corpse is not possible. In that case, the corpse and the insects associated with it should be stored in a morgue cooler at approximately 4 °C before autopsy. The length of time that the insects were in the morgue cooler and the impact of cooler temperature on maggot growth and development should be considered for the subsequent estimation of a correct minimum postmortem interval.

The aim of the current study, therefore, was to determine whether the chilling duration in the cooler affects the survival, development and weight of different developmental stages of *Calliphora vicina* (Robineau-Desvoidy) (Diptera: Calliphoridae). It was hypothesized that some life history traits will be affected by exposure to cold at different developmental stages. *C. vicina* was selected for the experiments because it is the most common blowfly species in Turkey (Açıkgöz et al., 2017) and it can be collected from October to July in Samsun (Kökdener, 2012).

2. Materials and methods

C. vicina individuals were obtained from two different locations in Samsun, Turkey, namely the Taflan area and Ondokuz Mayıs University campus, in 2017, and since then reared at the Animal Physiology Research Laboratory of Ondokuz Mayıs University in Samsun while new wild type blowflies are added to the colonies. About 300 adult flies were maintained in ten rearing cages (30 × 30 × 40 cm) at 23 °C, 60% relative humidity (RH) and 12:12 h (L:D) photoperiod.

The flies were fed with granulated sugar and water ad libitum. Newly emerged females were offered fresh chicken liver to stimulate the development of their ovaries. When eggs were required, 20 g portions of fresh chicken liver were placed in the rearing cages as oviposition sites. The colonies were checked regularly and the liver was removed when sufficient eggs had been laid. Each piece of liver with eggs was placed in a plastic box (15 × 10 × 7 cm) and kept at 23 °C and 75% RH in a SANYO incubator (SANYO Electric Co., Ltd., Osaka, Japan). A Leica dissecting microscope (Leica Microsystems, Wetzlar, Germany) was used to determine their developmental stages from specific larval structures, i.e. the anterior and posterior spiracles. The most distinctive feature for separating the larvae of different instars of calliphorid flies such as *C. vicina* is the structure of the posterior spiracles; two spiracular slits are visible in the second stage larvae, whereas the third larval stage has three slits.

The effects of temperature on the length of the development period, weight and survival of five different developmental stages (first, second and third stage larvae, postfeeding larvae and pupa) of *C. vicina* were determined by placing them in a refrigerator, 24, 48, 72 and 120 h. Specifically, after the eggs hatched, 25 randomly selected individuals from each developmental stage were removed and placed in, small polystyrene boxes containing 20 g of chicken liver. For the postfeeding and pupal groups, chicken liver was not provided. Each box was subjected to constant chilling in a refrigerator at 4 ± 0.5 °C. After the chilling period, the various stages were returned to the incubator at 23 ± 1 °C until the adults emerged. All stages were checked twice daily and survival rate, larval and the pupal development period and pupal and adult weight were recorded. Each experiment was repeated 5 times.

2.1. Statistical analysis

The effects of length of chilling period on the development period, weight and survival of *C. vicina* were analyzed separately for each developmental stage. Data were subjected to one-way analysis of variance (ANOVA) in the SPSS program (Version 22.0; IBM Corp., Armonk, NY, USA). Normality of the data was determined by the Kolmogorov–Smirnov test. We used a Student–Newman–Keuls (SNK) posthoc test with significance level of 0.05 to find out the differences among the treatments levels and to clarify which of the means were significantly different from each other.

3. Results and discussion

In this study, five different life stages of *C. vicina* were chilled at 4 ± 0.5 °C for 24, 48, 72 or 120 h and then returned to an ambient temperature of 23 °C until the adults emerged. The survival rates were determined as the percentages of individuals reaching the pupal and adult stages (Figure 1).

The percentage of individuals of all larval stages reaching the pupal stage declined significantly with increasing chilling period (F_{L1} = 3.167, df = 3, p = 0.000; F_{L2} = 220.257, df = 3, p = 0.000; F_{L3} = 57.481, df = 3, p = 0.000; F_{PF} = 93.757, df = 3, p = 0.000) (Figure 1). The same pattern however, was not observed for the adult eclosion percentages since different developmental stages showed different responses to chilling. For instance, the adult eclosion rate of first stage larvae and pupae declined significantly (F_{L1} = 41.265, df = 3, p = 0.000; F_{P} = 70.25, df = 3, p = 0.000) while the second and third stage and postfeeding larvae had significantly increased adult eclosion rates as the chilling period increased (F_{L2} = 74.545, df = 3, p = 0.000; F_{L3} = 158.391, df = 3, p = 0.000; F_{PF} = 113.667, df = 3, p = 0.000) (Figure 1).

In a previous study, Alipour et al. (2018) assessed the effects of long-term cold preservation on the viability of *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae) pupae. They found that the percentage of individuals reaching adulthood was decreased with increasing storage

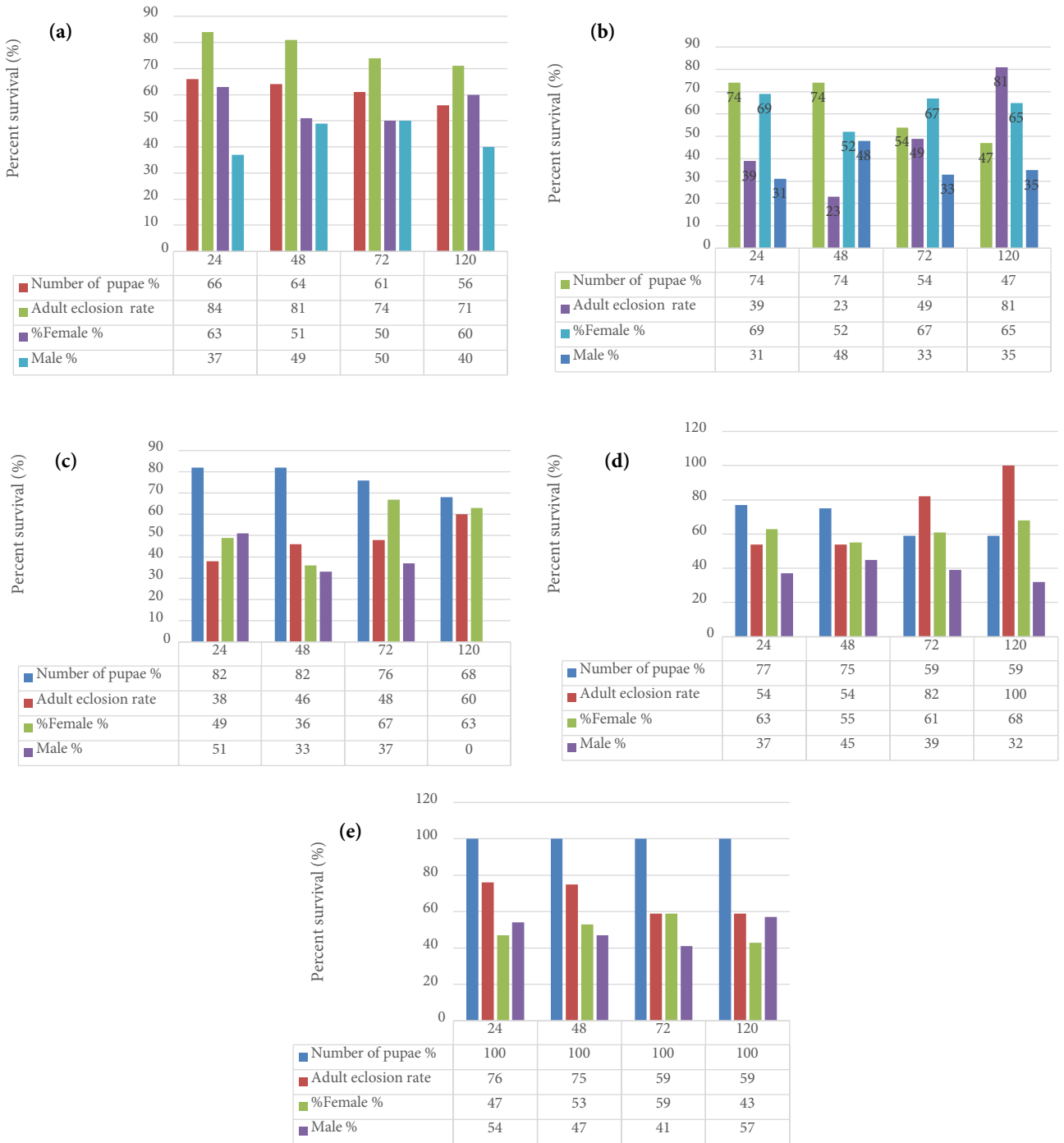

Figure 1. Effects of chilling period (24 h, 48 h, 72 h, 120 h) on the percentage number of individuals reaching pupae and adulthood stage of *Calliphora vicina* (a: 1st instar larva, b: 2nd instar larva, c: 3rd instar larva, d: Postfeeding larva, e: Pupa).

time at 4 ºC. Similarly, Bucher et al. (1948) demonstrated that F1 generation of house flies had lower hatching, larval survival and pupation when their puparia were exposed to 6 ºC for 10 days. In another study, Leopold (2000) compared the effects of short-term chilling on the survival of house fly embryos. The same author reported that survival and vitality of embryos were significantly correlated with the length of the storage period and the age of the embryo. Recently, Carriço et al. (2019) compared the effects of cooling and freezing on the development of the third stage larvae and pupae of various forensically important flies. The authors reported that the proportions

of *Chrysomya megacephala* (Fabricius, 1794), *Chrysomya putoria* (Wiedemann, 1830), *Lucilia cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae) and *Musca domestica* Linnaeus, 1758 (Diptera: Muscidae) pupae that survived a 24-h cooling period was not different from the controls, but decreased for *Peckia ruficornis* (Fabricius, 1794) and *Peckia chrysostoma* (Wiedemann, 1830) (Diptera: Sarcophagidae). They also reported a significant reduction in adult emergence for all of these fly species after 24 h of cooling at 3 °C. These results have emphasized that cold tolerance of insects varies with species, developmental stages, temperature and length of cold exposure. In addition, insects' ecophysiological characteristics such as hibernal quiescence and diapause may play an important role in their cold tolerance.

However, our findings contradict the results of Johl and Anderson (1996)'s. They reported that no mortalities occurred in any immature stages of *C. vicina*, including eggs and 1st stage larvae, when they were chilled to 3 °C for 24 h. As noted in various studies, inherent biogeographical variation between populations, humidity, population density and other intrinsic factors can cause such differences (Saunders and Hayward, 1998; Salimi et al., 2018).

The times required for the development of the three larval stages and pupae of *C.vicina* after different chilling periods are provided in Figure 2.

The mean larval periods for the first larval stage (F_{L1} = 189.086, df = 3, p = 0.000), second larval stage (F_{L2} = 561.095, df = 3, p = 0.000), third larval stage (F_{L3} = 110.344, df = 3, p = 0.000) and postfeeding larval stage (F_{PF} = 144.48, df = 3, p = 0.000) increased as the chilling period increased (Figure 2a). The time for pupal development did not change markedly for the first stage larvae (F_{L1} = 0.245, df = 3, p = 0.865) and postfeeding larvae (F_{PF} = 1.887, df = 3, p = 0.139) but it was significantly prolonged in the second stage larvae (F_{L2} = 13.543, df = 3, p = 0.000), third stage larvae (F_{L3} = 12.706, df = 3, p = 0.000) and pupae

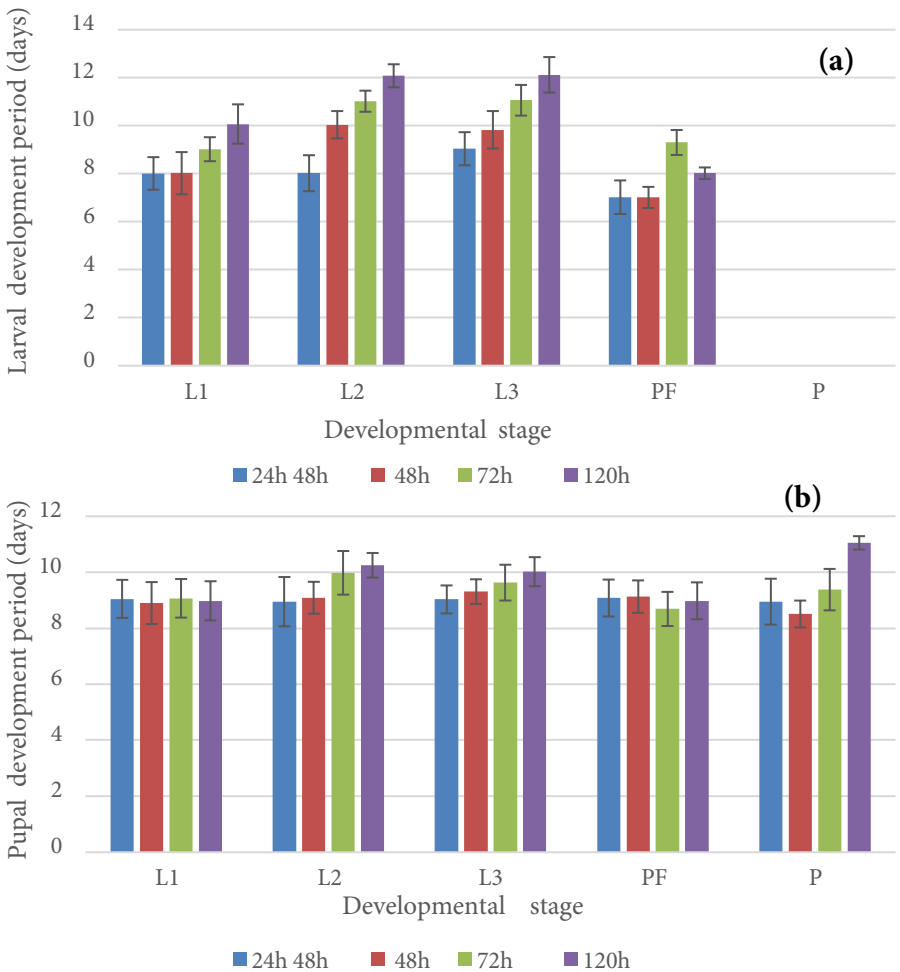

Figure 2. Effect of chilling period on the larval (a) and pupal (b) development durations of *Calliphora vicina*.

(F_p = 64.470, df = 3, p = 0.000), especially after the 72 h and 120 h chilling periods (Figure 2b). It is obvious that cooling considerably affects the larval development of *C. vicina*. Our findings also showed that the different developmental stages showed different levels of sensitivity to the length of cooling. Myskowiak and Doums (2002) reported similar differences for the different developmental stages of *Protophormia terraenovae* (Robineau-Desvoidy, 1830) (Diptera: Calliphoridae) that were exposed to a temperature of 4 ºC for a period varying from 1 to 10 days. Specifically they found that first larval and prepupal stage of *Protophormia terraenovae* (Robineau-Desvoidy, 1830) (Diptera: Calliphoridae) decreased the development durations as refrigeration period increased, while second larval and pupal stages prolonged development durations over time. Johl and Anderson (1996) also reported that the chilling of different life stages of *C. vicina* for 24 h at 3 ºC caused about one day retardation in adult emergence. It is not clear what causes the variation between different species and developmental stages, when exposed to chilling, but it might be related to the differences in the cellular, morphological and behavioural adaptations that protect them against the harmful effects of cold temperatures. The changes in pupal and adult weight are shown for each developmental stage of *C. vicina* and different chilling periods in Figure 3.

Accordingly the effects of chilling on pupal weight varied according to the developmental stage. The mean

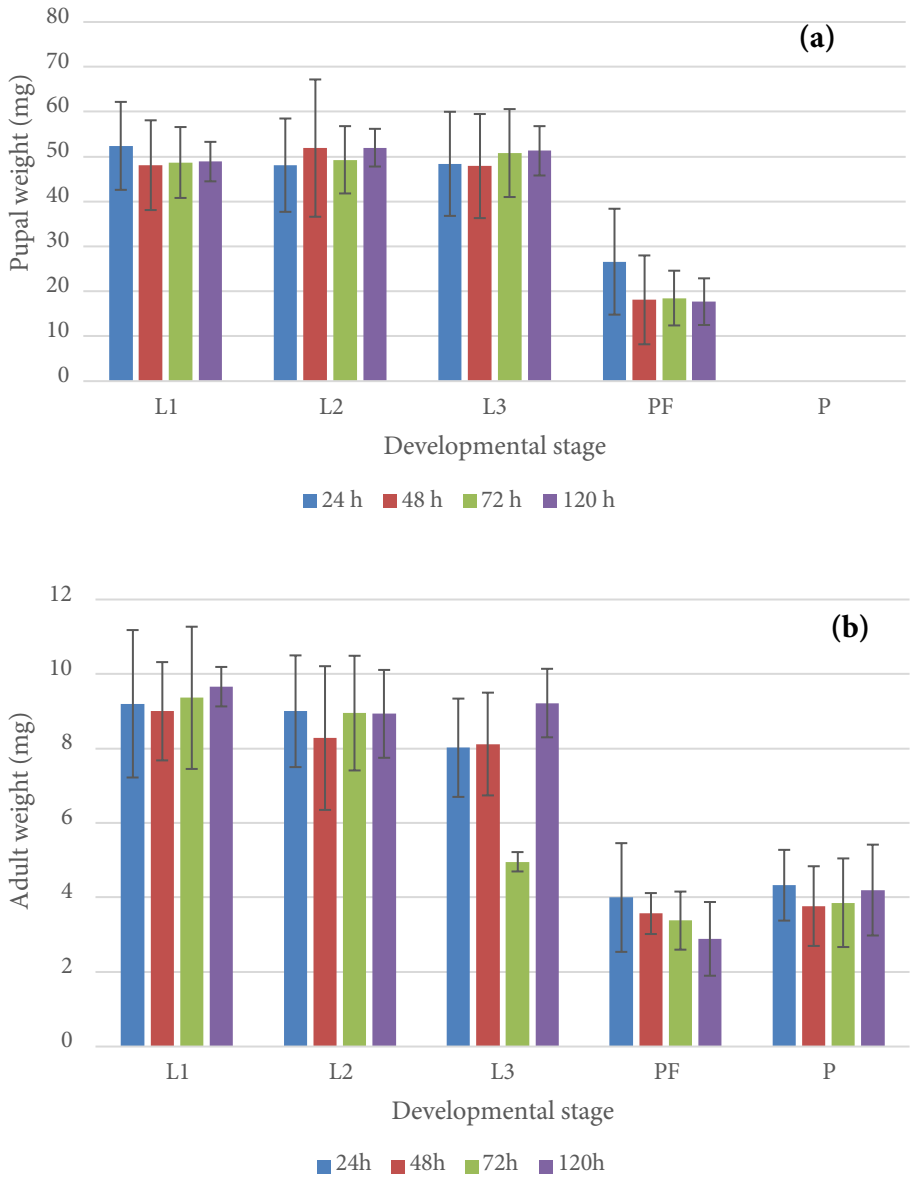

Figure 3. Effect of chilling period on pupal (a) and adult (b) weight of *Calliphora vicina*.

pupal weight of first instar larvae (F_{L1} = 2.640, df = 3, p = 0.052) and second instar larvae (F_{L2} = 2.355, df = 3, p = 0.072) did not change significantly but there was a significant increase for third instar larvae (F_{L3} = 4.150, df = 3, p = 0.007) and a significant reduction for postfeeding larvae (F_{PF} = 3.066, df = 3, p = 0.028) (Figure 3a). In addition, the mean adult weight of first instar larvae (F_{L1} = 1.708, df = 3, p = 0.166) and second instar larvae (F_{L2} = 1.443, df = 3, p = 0.233) did not change significantly with chilling period. However, the adult weights of third instar larvae (F_{L3} = 65.52, df = 3, p = 0.007), postfeeding larvae (F_{PF} = 13.242, df = 3, p = 0.000) and pupae (F_{P} = 6.360, df = 3, p = 0.000) were significantly different.

Data indicated that pupal weight of third instar larvae showed a significant increase at 72 and 120 h of chilling periods. A similar increase was also determined at 120 h for adult weight of this developmental stage. However, chilling had a negative effect on the pupal and adult weights of both the postfeeding larval and pupal stages. It is not surprising since intense developmental activities occurring throughout these two developmental periods.

Similar results have also been reported for *M. domestica* (Leopold et al., 1998) and *P. terraenovae* (Myskowiak and Doums, 2002).

Results from this study indicate that chilling period can strongly affect the survival rate, development and weight of a blowfly species of forensic importance and that there is differential sensitivity in tolerance to chilling between different developmental stages. These points are important in determining the developmental rate of the species. Therefore, the chilling period to which at corpse and the insects associated with it was exposed should be considered in both criminal and suspicious cases for the estimation of minimum postmortem interval. If chilling is not considered, the minimum postmortem interval can be incorrectly estimated.

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