Journal of Medical Entomology, XX(X), 2017, 1–7
doi: 10.1093/jme/tjx211
Research Article

Temperature and Tissue Type Impact Development of *Lucilia cuprina* (Diptera: Calliphoridae) in Sri Lanka

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Subject Editor: Guiyun Yan

Received 13 May 2017; Editorial decision 28 September 2017

Abstract

Lucilia cuprina (Wiedemann; Diptera: Calliphoridae) is a facultative ecto-parasitic fly, distributed throughout the temperate and subtropical regions of the world. This blow fly species is of medical, veterinary, and forensic importance due to it being used in maggot debridement therapy (MDT), a causative agent of myiasis, and a decomposer of vertebrate carrion. The current study examined the combined effects of temperature and tissue type on the development of *L. cuprina*. Specimens were reared on three tissue types; swine muscle, swine liver, and bovine muscle at 20°C, 25°C, 27°C, and 38°C. The optimum condition for *L. cuprina* development was for immatures reared on bovine muscle (287.4 h) followed by those reared on swine muscle (288.0 h) and swine liver (288.4 h) at 27°C. The minimum temperature threshold of *L. cuprina* was deduced to be 14°C. No significant differences in larval width and length over time were determined for the tissue type.

Key words: *Lucilia cuprina*, Calliphoridae, development temperature, tissue type, postmortem interval

Lucilia cuprina (Wiedemann; Diptera: Calliphoridae), commonly known as the Australian sheep blow fly, is originally distributed in the Afro tropical and Oriental regions. However, due to dispersal over the last few hundred years (i.e., natural and due to globalization), it is now present in warmer climates worldwide (Stevens and Wall 1996). This species is medically, veterinary, and forensically important for several reasons. *L. cuprina* causes myiasis in people, companion animals, as well as livestock (Visciarelli et al. 2007, Ahadizadeh et al. 2015, Azevedo et al. 2015). For example, *L. cuprina* is the primary agent of sheep myiasis in the Australian region with economic losses valued at several million (Stevens and Wall 1996). However, while myiasis by some *L. cuprina* populations are detrimental to animal health, other populations are useful for maggot debridement therapy (MDT) (Paul et al. 2009, Tantawi et al. 2010). *Lucilia sericata* (Meigen; Diptera: Calliphoridae) is the most common species used in MDT worldwide (Paul et al. 2009, Tantawi et al. 2010).

The importance of *L. cuprina* to forensic entomology is evident as it is found associated with human corpses in several regions of the world (Greenberg 1991). Development data for this species have a number of implications with forensic investigations, mainly to estimate the minimum post mortem interval (PMI_{min}) of death (Bernhardt et al. 2017) when assuming colonization occurred after death. Of course, in cases of myiasis, estimating time of colonization would be important for neglect and abuse investigations.

Entomologists use baseline development data of fly species to construct life tables for use in forensic investigations specifically when calculating a PMI_{min} (Bernhardt et al. 2017). These calculations are accomplished by incorporating the development data with either accumulated degree day/accumulated degree hour (ADD/ADH) methods or isomegalen/isomorphen diagrams (Schoenly et al. 2007). With ADD/ADH, development of a fly from egg to adult is predicted predominantly on the temperature conditions experienced. In the case of isomegalen/isomorphen diagrams, length and development time periods of the immature over time of the fly are diagrammatically illustrated in relation to temperature (Amendt et al. 2004). These development data are obtained by baseline studies conducted in the laboratory using nonhuman tissue due to the inability to obtain human material (Schoenly et al. 2007). Previous studies have viewed tissue from the domestic swine, *Sus scrofa*, as the most promising human surrogate due to similar internal anatomy, fat distribution, chest cavity, omnivorous diet, and lack of heavy fur (Byrd and Castner 2001, Schoenly et al. 2007).

Temperature and type of larval food have a direct effect on the development rates of fly larvae (Flores et al. 2014). Kotzé et al. (2015) recorded the development variation of *L. cuprina* larvae reared on chicken liver at six constant temperatures (18°C, 21°C, 24°C, 27°C, 30°C, and 33°C). According to this study, development was optimal near 24°C; temperatures above this threshold impacted

survival, while below this temperature development rate was retarded. Tissue type obtained from the same and different species are known to influence growth rates of larvae (Boatright and Tomberlin 2010, El-Moaty and Kheirallah 2013, Flores et al. 2014). For the hairy maggot blow fly, *Chrysomya rufifacies* (Macquart; Diptera: Calliphoridae), an accelerated growth rate was recorded when larvae were reared on equine muscle tissues compared to canine muscle and porcine muscle (Flores et al. 2014). The influence of tissue type on larval growth in *L. cuprina* was determined by Day and Wallman (2006), in which larvae were reared on three different tissue types of the same species; sheep liver, muscle, and brain at room temperature. Results of this study indicated the development rate of larvae decreased when reared on sheep liver compared to sheep muscle or brain as the group grown on liver achieved maximum body length on day 5, but larvae grown on brain and muscle achieved this on day 3. However, this study was from Australia. Thus, the level of consistency across populations and consequently application is not known. Therefore, we wanted to determine the development of a Sri Lankan strain of *L. cuprina* on three tissue types at four temperatures. Our hypothesis is neither factor will impact development.

Materials and Methods

Maintaining the Fly Colony

A colony of *L. cuprina* was commenced from a batch of maggots purchased from Mega Biotech (PVT) Ltd, Colombo; the only available local medical maggot facility where the colonies are bred from eggs laid on carcasses by wild *L. cuprina* flies. Viable healthy colonies are maintained by introducing flies periodically collected from the wild from Colombo region to minimize inbreeding (Hettiarachchi 2010). The accuracy of the species was checked using identification keys (James 1947, Spradbery 2002). Resulting adults and subsequent generations were kept in insect cages (30 × 30 × 30 cm) at room temperature and under 12:12 (L:D) h in an insectary within the Department of Zoology, University of Peradeniya. Flies were provided access to a cotton wick plugged bottle filled with sugar and milk solution (1:1 of milk and sugar with water). In order to collect eggs, a petri dish (60 × 15 mm) containing calf blood (5 ml) on tissue paper together with a piece of fish (50 g) was provided as an oviposition site. Observations were made hourly for oviposition. Eggs were harvested using fine tipped artist paint brush (size 10) and used in the experiment. Eggs were collected from different generations only up to five generations to minimize the genetic effect on the fly development.

Obtaining Tissues for the Larval Development Experiment

Approximately 2 kg of swine skeletal muscle and liver were obtained from the veterinary farm facility, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya. Fresh bovine skeletal muscle (2 kg) was purchased from a local market. All tissue types obtained for the study were fresh and free of foreign materials (e.g., antibiotics). Tissue samples were cut into100 g pieces placed in a labeled polyethylene bags, and stored in a 2°C freezer until use.

Experiment Design

Altogether three sets of larvae obtained from the same egg clutch were tested across tissue type and temperature. Larvae were obtained from the same egg clutch to maintain the genetic consistency among individuals and were separated in to three batches to minimize the intraspecific competition for provided food and maggot mass heat generated due to excessive aggregation of emerging individuals. For each larvae batch collected from the same egg clutch, three 100 g muscle samples of each tissue type were placed individually on top of 2 cm layer of saw dust obtained from chemically untreated few different wood types (grade unknown) from a timber mill in a 400 ml rearing jar. Eggs collected using a fine tipped artist paint brush was placed on each tissue type as approximately 50 eggs per container. The containers were placed in a growth chamber at 38°C, 70% relative humidity (RH) and 12:12 (L:D) h. Eggs were observed hourly until hatching. After hatching, observations were made at 4-h intervals. At each observation, the two largest larvae from each rearing jar were obtained, parboiled and preserved in 70% alcohol until length, cross width, and instar stage could be recorded. Larval length was measured with the aid of a digital microscope (Microview USB digital microscope 650X). Cross width of the last posterior segment of each individual was recorded using a vernier caliper (Baker DC20, reading: 0.02 mm, measuring range: 0–200 mm). After reaching the third larval stage, observations were made every 12-h intervals until adults' emergence. The above procedure was repeated at 20°C, 25°C, 27°C, and at 70% RH level in a climatic chamber (Pol- ekoPOL-EKO-APARATURA sp.j., Wodzislaw Slaski, Poland: Model-KK 240). Temperatures 25°C and 27°C represented room temperatures and 38°C was to represent the maggot mass temperature inside a bandaged wound (McGuiness et al. 2004, Simmons et al. 2014).We used 20°C as a means to estimate the minimum threshold temperature for larval development.

Data Analysis

The time taken to achieve each instar stage for each temperature and tissue type combination was determined. Data were analyzed with an analysis of covariance (ANCOVA) using Minitab 14 statistical software. Another Analysis of covariance was performed using the same statistical software to determine the level of significance of the effect of tissue type and temperature on length and cross width of larvae over time ($P < 0.05$).

Minimum threshold temperature for development of *L. cuprina* larvae was deduced by linear approximation estimation method which 1/total days to develop (from hatching to adulthood) was plotted against the temperature for each tissue type. The abscissas of these graphs resemble the minimum threshold temperature. Isomegalen/isomorphen diagrams were constructed by the development data of *L. cuprina* developed under swine muscle as the swine muscle provides the similar conditions of the human flesh hence it is important for forensic investigations. All the graphs of this study formed by the sigma plot VER 10 (Systat software, San Jose, CA).

Results

Minimum time needed for *L. cuprina* to complete each life stage was determined (Table 1 and Fig. 1). The quickest development (minimum total time taken to reach the adult stage from egg stage) was observed for specimens reared on bovine muscle followed by swine muscle and swine liver under three temperature regimes; 20°C, 25°C, and 27°C. But at 38°C, this observation was reversed as the slowest rate was recorded in bovine muscle followed by swine muscle and swine liver. ANCOVA was performed to determine tissue type did not impact development ($P = 0.98$, $F = 0.01$).

All graphs represent (Fig. 2A–D) the larval length change along the time showed the same variation pattern as a fastened length increase can be seen in the beginning across 1st instar to 3rd instar stage and after reaching the peak length at a certain point of the

Table 1. Minimum time (h) taken for the development of *L. cuprina* reared on swine liver, swine muscle, and bovine muscle at 20°C, 25°C, 27°C, 38°C and 70% RH

	Bovine muscle				Swine muscle				Swine liver			
Stage	20°C	25°C	27°C	38°C	20°C	25°C	27°C	38°C	20°C	25°C	27°C	38°C
Eggs	18.30	18.00	14.40	4.00	18.30	18.00	17.00	5.20	24.40	18.00	14.40	06.00
1st instar	20.40	20.30	15.40	13.30	14.00	16.30	13.00	10.40	32.20	20.30	16.40	12.30
2nd instar	24.30	20.00	16.00	8.30	28.30	27.30	16.00	09.00	23.40	14.00	17.00	7.30
3rd instar	217.00	103.30	66.30	168.30	195	101.30	71.00	168.00	223.30	138.00	67.30	165.00
Pupa	332.00	209.30	175.30	216.00	359.10	209.30	171.00	216.00	335.10	207.00	173.30	216.00
Total	612.00	371.30	287.40	410.30	615.10	372.20	288.00	409.00	638.40	397.30	288.40	407.00

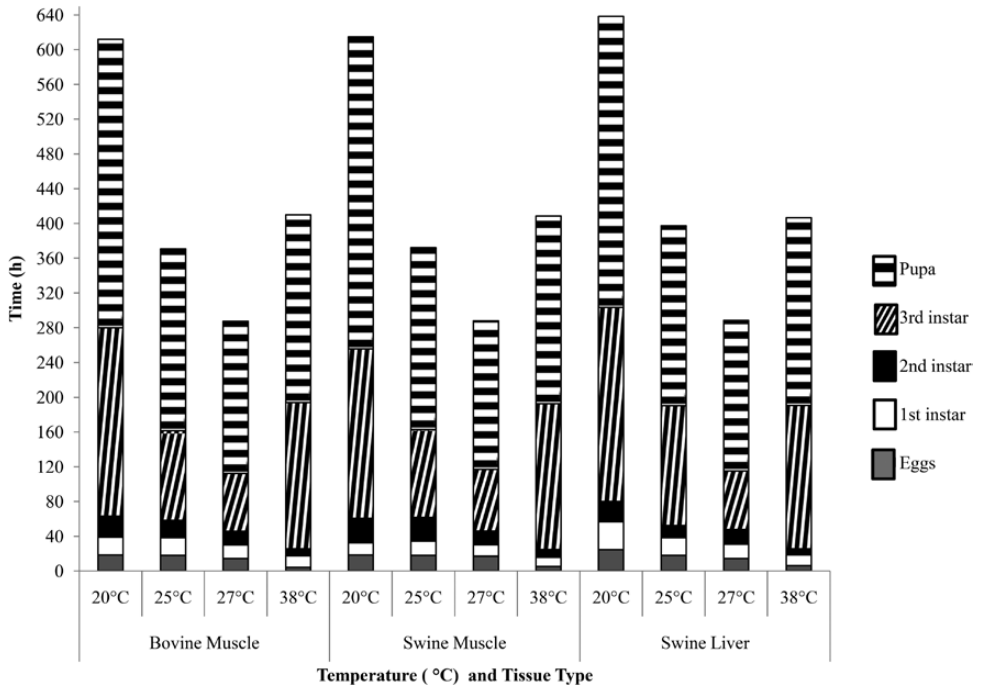

Fig. 1. Development time variations in each instar stage of *L. cuprina* reared on swine liver, swine muscle, and bovine muscle at different temperatures.

3rd instar, a slight decrease in length was observed when larvae enter the non-feeding wandering stage.

Of all the studied temperatures, highest individual length for the 1st instar was recorded at 38°C in bovine muscle (6.2 mm) and the smallest larval length for the same instar stage was recorded at 20°C in swine liver (1.4 mm). In 2nd and 3rd instars, the smallest individual lengths were observed at 20°C in swine muscle and bovine muscle, respectively (2nd instar: 3.25 mm, 3rd instar: 5.05 mm). However, the greatest larval individual lengths of these stages were recorded in swine muscle as the 2nd instar at 25°C (11.5 mm) and 3rd instar at 38°C (14.1 mm), respectively (Fig. 2). ANCOVA indicated larval length was not impacted by tissue type ($P = 0.567$, $F = 0.68$). But, there was a significant difference between larval length with temperatures ($P \leq 0.0001$, $F = 9.52$) and time ($P \leq 0.0001$, $F = 193.35$).

The greatest individual width for 1st and 3rd instars was recorded at 38°C when reared on bovine muscle (1st instar: 1.09 mm, 3rd instar: 2.57 mm) respectively, but in 2nd instar stage this was recorded for larvae exposed to 25°C when reared on swine muscle (2.15 mm). Whereas the lowest cross width of all these instar stages was observed at 20°C as 1st instar in swine liver (0.30 mm), 2nd instar in swine muscle (0.39 mm) and 3rd instar in bovine muscle (0.85 mm) (Fig. 3A–D). The larval cross width differed significantly with the different temperatures ($P \leq 0.0001$, $F = 19.55$) and time ($P \leq 0.0001$, $F = 365.53$). But, such kind of difference was not observed between the tissue types ($P = 0.93$, $F = 0.15$).

The linear approximation estimation method was showed the cross of X axis by each regression line (constructed for development curves of three muscle types) at ~14°C. Therefore the 14°C was considered as the minimum threshold temperature of this species (Fig. 4A–C).

Change in mean larval length of *L. cuprina* over time when fed swine muscle at each temperature is showed in Fig. 5. The rate of length variation increases with increasing temperature. Similar results were recorded for the remaining two muscle types; swine liver and bovine muscle.

The two graphs (Figs. 6 and 7) represent the isomegalen and isomorphen diagrams of *L. cuprina* and in both graphs, time from egg eclosion to adult emergence was plotted against the temperature. The isomegalen diagram was constructed to show the identical variation of each unit length (mm) of *L. cuprina* along their larval life stages at four different temperatures. This diagram was formed based on the data presented in the growth curves of *L. cuprina* larvae developed in swine muscle (Fig. 6).

Based on the development time periods of each life stages of *L. cuprina*, following isomorphen diagram was constructed to show

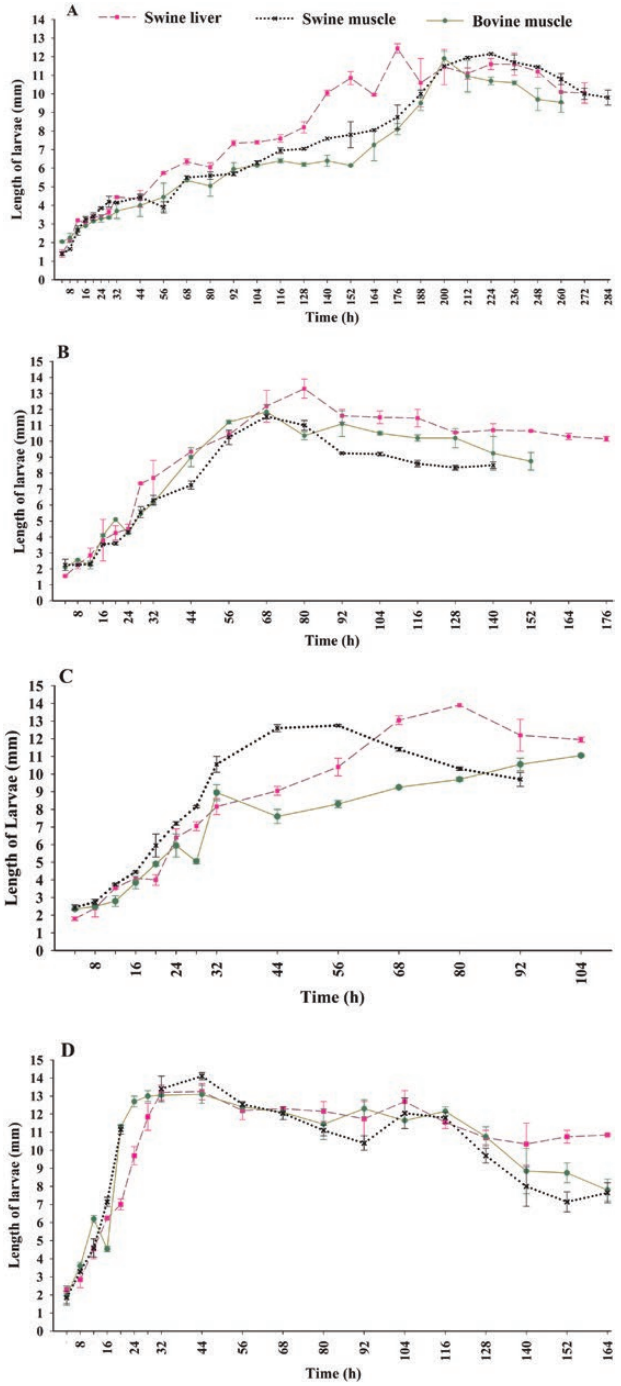

Fig. 2. Length change in *L. cuprina* larvae reared on swine liver, swine muscle, and bovine muscle under 70% RH, 12:12 (L:D) h and at (A) 20°C, (B) 25°C, (C) 27°C, and (D) 38°C.

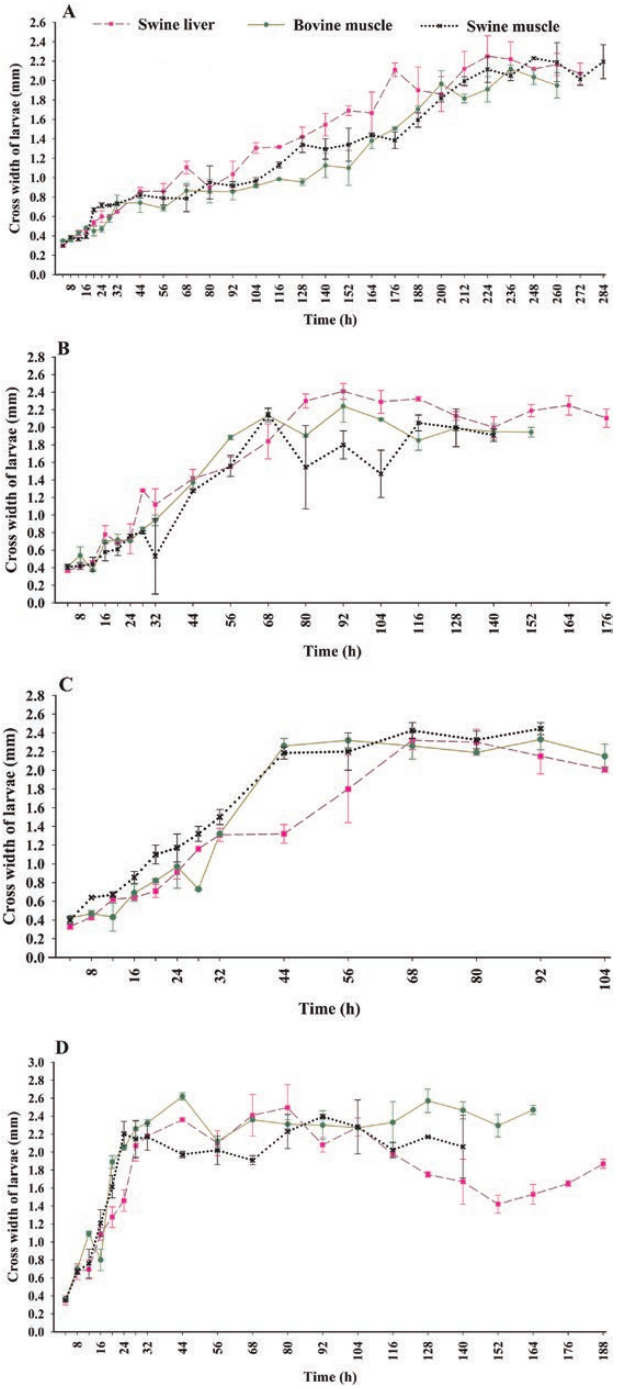

Fig. 3. Cross-width change for *L. cuprina* larvae reared on swine liver, swine muscle, and bovine muscle under 70% RH, 12:12 (L:D) h and at (A) 20°C, (B) 25°C, (C) 27°C, and (D) 38°C.

the development time variation of larvle to an adult of *L. cuprina* at three temperatures. The areas between those lines represent the identical morphological stages at various temperatures (Fig. 7).

Discussion

Development rate of *L. cuprina* was impacted by tissue type fed to larvae and temperature experienced. Of the four studied temperatures, three (20°C, 25°C, 27°C) showed similar result as the highest rate of development (minimum total time taken by eggs to attain adulthood) was observed in bovine muscle followed by swine muscle and swine liver and at remaining temperature level (38°C) this observation was vice versa. The factors related to tissue types such as moisture level, energy amount, nutritional composition, the distribution pattern of fat and connective tissues may have caused these development variations. During the present study, generally, swine liver showed the lowest development may be due to its less energy amount (134 Kcal/ 100 g) and less fat content (3.65 g/ 100 g) compared to the bovine and swine muscle as these two muscle types

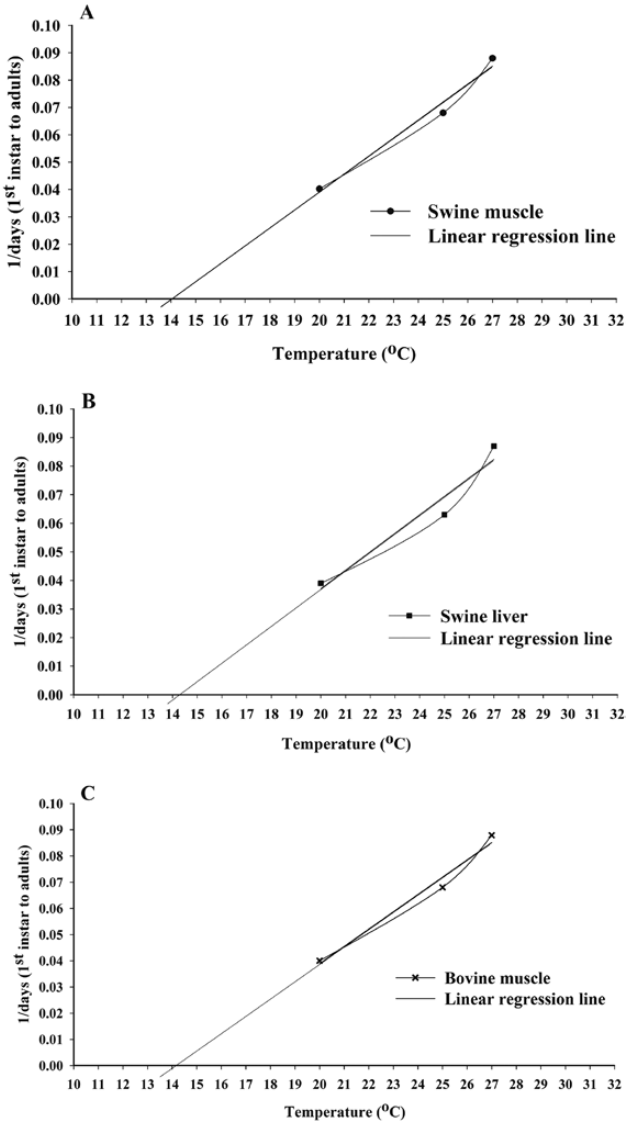

Fig. 4. Linear regression lines for *L.cuprina* larvae reared on (A) swine muscle, (B) swine liver, and (C) bovine muscle at~14°C, 70% RH and 12:12 (L:D) h.

contained higher level of fat (beef: 12.73 g/ 100 g, swine: 12.59 g/ 100 g) and energy (beef: 198 Kcal/ 100 g, swine: 212 Kcal/ 100 g) (United States Department of Agriculture 2017). The results show the importance of using swine muscle tissues compared to muscles from other animals to conduct development studies of flies causing human myiasis.

Before this study, only two studies have been conducted on *L. cuprina* by giving priority to its development time periods. The study conducted by Day and Wallman (2006) in Australia recorded that at 24°C, 1st, 2nd, 3rd instar and pupal stages were reached within 48 h, 72 h, 96 h, and 144 h, respectively. A study conducted in Pretoria, South Africa showed a different result as 26.2 h, 56.2 h, 105.6 h, and 165.0 h to reach the above stages, respectively (Flores et al. 2014). In the present study, at 25°C, a different result was seen; less time to reach the 1st and 2nd instar (19.3 h and 20.4 h) and more time were taken to reach the remaining two stages (114.2 h and 208.5 h). Except for the variations in experimented temperatures, these development variations may have occurred due to several factors such as regional differences, genetic differences among studied colonies, larval mass temperature, the quantity of given food sources and intra-specific competition among individuals (Day and Wallman 2006). In addition, the differences in the experimental setup such as pupation medium, sizes of containers, types of incubators etc. may have caused development variations in *L. cuprina*.

Temperature impacted maximum length and width of *L. cuprina* larvae. Generally, for 1st and 2nd instars, maximum measures were recorded when reared at 27°C; however, for the 3rd instar, maximum measurements occurred at 38°C. These results are in partial agreement with previous research with this species. Kotzé et al. (2015) recorded the length variation of *L. cuprina* within the range of 20–33°C and the maximum length was observed at 27°C. Although in this study, the greatest length and cross width of each instar stage were observed under different temperatures and tissue types, the lowest values for such parameters were recorded under the same tissue–temperature combination (20°C in swine liver). In early studies similar results were observed as in the liver, growth of fly larvae was delayed regardless of the obtained animal due to two reasons; structurally it is denser than other organs and it accumulates toxic substance which detrimental to larval growth (Day and Wallman 2006).

In this study, the minimum threshold temperature evaluated for *L. cuprina* in all the tissue types was averaged at 14°C. This was a

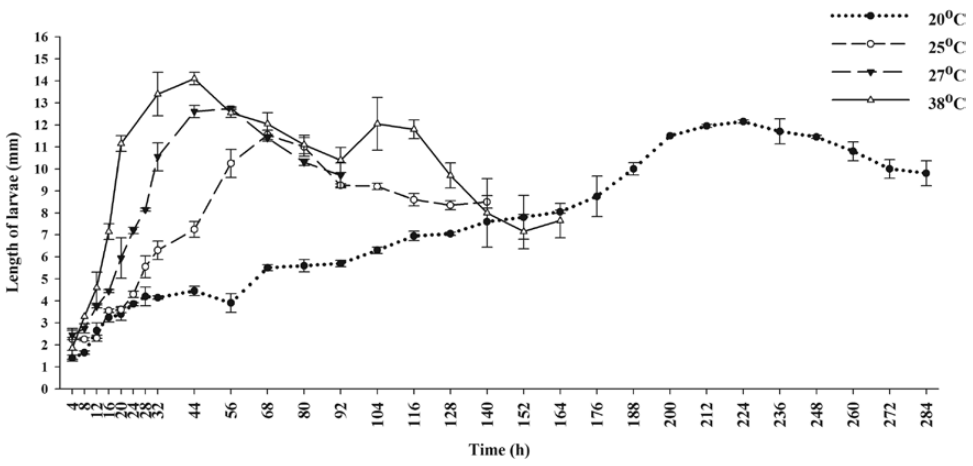

Fig. 5. Growth curves of *L. cuprina* larvae reared on swine muscle at four temperature regimes (20°C, 25°C, 27°C, and 38°C).

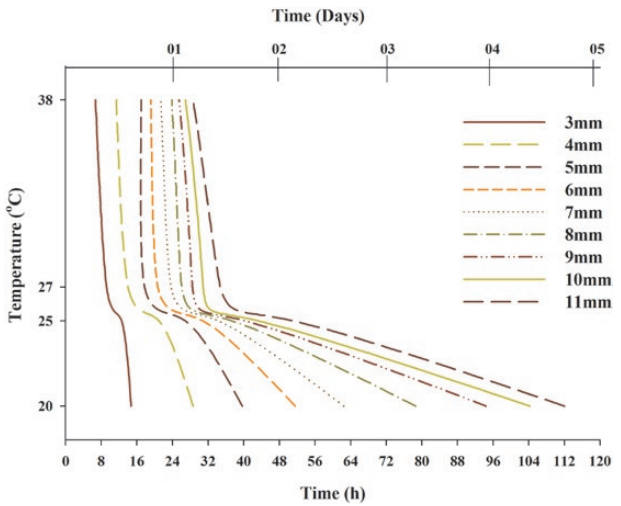

Fig. 6. Isomegalen diagram of *L. cuprina* larvae reared at four different temperatures (20°C, 25°C, 27°C, 38°C), time is plotted against the temperature to represent the identical larval length change along the studied temperatures.

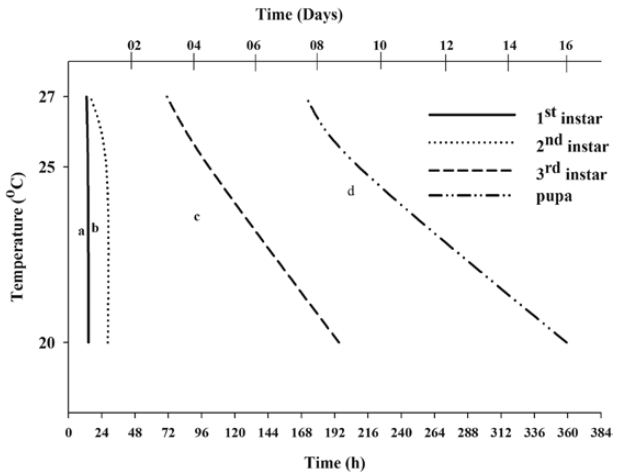

Fig. 7. Isomorphen diagram of *L. cuprina* development at three different temperatures (20°C, 25°C, 27°C). Areas between line represent identical morphological stages at various temperatures (a = 1st instar, b = 2nd instar, c = 3rd instar, d = pupa).

slightly deviated value compared to the values given by Kotzé et al. (2015) as 12.2°C and Grassberger and Reiter (2001) as 13.3°C. However, in these previous studies, minimum threshold temperature has been calculated based on the medians for all four developmental events (first ecdysis, second ecdysis, the onset of wandering, and the onset of pupation). Although in the present study, this was calculated by a different method; linear approximation estimation method proposed by Gennard (2007), this may be also occurred due to the regional differences of *L. cuprina*.

In this study, the minimum time periods required for egg hatching to adult emergence at 20°C (25.5 d), 25°C (15.45 d), and 27°C (11.97 d) were observed in bovine muscle and at 38°C (16.94 d) this was recorded in swine liver. In Ash and Greenberg's study (1975) these development time periods were recorded as 30.3 d at 19°C, 12.8 d at 27°C, and 10.4 d at 35°C for *L. cuprina*. In these both studies, time taken to complete all the life stages at 27°C lay between the same range (11–13 d). Also, both studies clearly showed the hindrance of development at lower temperatures close to the minimum threshold temperature level. But at 35°C median number of days obtained to complete life stages was lower than 38°C of this study. This may be due to that 38°C is closer to the upper threshold temperature of *L. cuprina* as upper threshold temperature also hindrance the fly development.

Acknowledgments

We would like to thank the National Research Council, Sri Lanka (Grant no. 15-084) for providing financial support for this research.

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