Development of Chrysomya megacephala at constant temperatures within its colony range in Yangtze River Delta region of China

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ABSTRACT
Chrysomya megacephala (Fabricius, 1794) is the most abundant and predominant species which arrives and colonizes a cadaver first in most parts of China. Therefore, its growth and development patterns have great implications in the estimation of the minimum postmortem interval (PMI_{min}). In this study, C. megacephala was collected from the Yangtze River Delta region and reared at seven constant temperatures ranging from 16 °C to 34 °C. The developmental duration and accumulated degree hours, larval body length and morphological changes of C. megacephala were examined. Furthermore, we constructed three developmental models, isomorphen diagram, isomegalen diagram and thermal summation model, which can be used for estimating PMI_{min}. The developmental durations of C. megacephala at 16 °C, 19 °C, 22 °C, 25 °C, 28 °C, 31 °C and 34 °C are (794.8 ± 14.7), (533.2 ± 10.1), (377.8 ± 16.8), (280.8 ± 15.1), (218.9 ± 8.5), (190.8 ± 10.1) and (171.8 ± 6.8) h, respectively. The developmental threshold temperature D₀ is (11.41 ± 0.32) °C, and the thermal summation constant K is (3 418.7 ± 137.0) degree hours. Regression analysis was conducted to obtain equations of the variation in larval body length with time after hatching, and variation in time after hatching with body length. Moreover, our study divides the intra-pupal morphological changes of C. megacephala into 11 sub-stages, and provides the time range experienced by each sub-stage. The results of this study provide fundamental development data for the use of C. megacephala in PMI_{min} estimations.

Introduction
Among the various necrophagous insects, blowflies (Diptera: Calliphoridae) are usually the first to colonize cadavers. The developmental stages of their offspring, larval body length/weight and intra-puparial morphological changes can provide accurate estimation of the minimum postmortem interval (PMI_{min}) [1,2]. Therefore, it is particularly important to establish accurate basic developmental data for blowfly species.

The development of blowflies is relatively well characterized in forensic entomology studies [3–11]. For some of the globally distributed species, such as Calliphora vicina (Robineau-Desvoidy, 1830), Lucilia sericata (Meigen, 1826) and Phormia regina (Meigen, 1826), basic developmental data for different regions have been established using flies from different populations [12–15]. There are several developmental models that are used to estimate PMI_{min}, such as isomorphen/isomegalen diagram and thermal summation model [14,16].

Isomorphen diagram is a scatterplot that models the duration of developmental events against temperature. Isomegalen diagram is a contour plot that contains three variables: the duration after hatching, different constant temperatures and larval size. Each contour represents the larval size (body length or width or weight) indicating the developmental duration under different temperatures [2]. In the thermal summation model, the x-axis is the developmental duration and the y-axis is the developmental accumulated degree days. The thermal summation model and equation (y = ax + b) of a developmental stage can be obtained by a linear regression analysis of the relationship between the duration and accumulated degree days/hours of each developmental stage. The slope in the equation is the developmental threshold temperature (D₀) of the species, and the intercept b is the thermal summation constant K at a particular developmental stage [2,17].

Despite the large number of studies on development of blowflies, information is still lacking for populations from different geographical regions [12,18]. Furthermore, the differences in experimental feeding methods or feeding substrate may also lead to discrepancy in developmental time. Therefore, it is of great importance to establish basic development data for different regions to improve estimation of PMI_{min}.

Chrysomya megacephala (Fabricius, 1794), also known as the oriental latrine fly, is mainly distributed in Australia and the Pacific [19]. Since the 1970s, the distribution of this species has gradually expanded and now is a globally distributed species [20]. In some areas, C. megacephala has become predominant over...
the local species [21]. Previous studies have shown that *C. megacephala* is one of the first flies to arrive at a dead body [22–26], and its overly large population size makes *C. megacephala* the absolute dominant species on the corpse [27,28].

Many studies have already investigated the development of *C. megacephala*, including development at constant temperatures [19,29–32], fluctuating temperatures [28,33] as well as the effects of different feeding tissues [34] or drugs [35,36]. The results show developmental plasticity in *C. megacephala*, and that different populations have differences in development. Since China runs through the Palaearctic and Oriental regions, its complex geography may lead to divergence of different local populations. *Chrysomya megacephala* is distributed throughout China and is the most important outdoor necrophagous species [37,38]. Thus, acquisition of more precise developmental data, especially from different populations of this species, is of forensic significance. This article reports the developmental data from the Yangtze region that allows a more accurate PMI$_{\text{min}}$ estimation in this region.

**Materials and methods**

**Colony establishment**

*Chrysomya megacephala* was collected from pig carcasses placed in a field near the Forensic Autopsy Centre of Suzhou located in the centre of the Yangtze River Delta, China (31°21’ N, 120°53’ E) between 2015 and 2016. The post-feeding larvae were collected into an insect rearing box (NoA3, Huamei, 32 cm $\times$ 22 cm $\times$ 10 cm) filled with 2 cm of wet sand, along with a 3 cm $\times$ 5 cm ventilating nylon mesh window at the centre of the box. After pupation, the cap of the box was removed, and the rearing box was placed into a larger nylon insect rearing cage sized 200 cm $\times$ 100 cm $\times$ 100 cm because *C. megacephala* need more space to complete mating and oviposition based on our previous rearing experience. The rearing cage was kept at room temperature (about 25.0 °C), with 70% humidity and natural light. After eclosion, adults were fed with water and a 1:1 mixture of milk powder and sugar. Five days after eclosion, a culture dish (14 cm in diameter) containing 20 g of fresh pork was placed into the cage to induce oviposition. Eggs were collected and reared for three generations to establish a purebred colony for the subsequent study. Adult *C. megacephala* was identified using the identification key by Fan [39]. The number of adult *C. megacephala* in the cage was maintained at 3000–4000 during the study.

**Observation of intra-puparial development**

Eggs were obtained using the methods described earlier and incubated at constant temperatures of 16 °C, 22 °C, 28 °C and 34 °C with 75% humidity 12:12 light/dark cycle. Upon pupation, 10 pupae from each temperature range were sampled every 8 h until adult eclosion, and treated as described previously [40,41]. The intra-puparial morphological changes, which are pupal characteristics that are used to classify the age of a pupa, were identified and imaged with a digital vernier caliper with a precision of 0.01 mm (Shengong, Shanghai). Each experiment was repeated four times for each temperature in different incubators.

**Data analysis**

Data analysis was performed using Origin Pro 8.6. The effect of temperature on duration of development was analysed using one-way ANOVA. The relationship between the larval body length and time after hatching was examined by nonlinear regression analysis using “larval body length” as the independent variable and “time after hatching” as the dependent variable, and vice versa, in order to model the equation for
estimating the PMI$_{\text{min}}$ [4,43]. The relationship between developmental duration and accumulated degree hours (ADH) in each developmental stage and total developmental process was analysed using the revised regression model proposed by Ikemoto and Takai [17], where the slope and intercept of the linear regression equation represent the developmental threshold temperature $D_0$ and thermal summation constant $K$ of C. megacephala, respectively.

### Results

**Developmental duration and construction of isomorphen diagram**

Between 16 °C and 34 °C, the developmental duration of eggs, first instar, second instar and third instar larvae, and pupae decreases with higher temperatures, and the total developmental duration is shortened from 794.8 h at 16.0 °C to 171.8 h at 34.0 °C, indicating that the developmental duration of C. megacephala is significantly affected by temperature (Table 1). There are no significant differences in the developmental durations of larvae in the first instar stage, the second instar stage, third stage and the pupal stage between 28 °C and 31 °C, and between 31 °C and 34 °C. Nevertheless, the entire developmental process is significantly different between different temperatures.

The isomorphen diagram (Figure 1) was established based on the length of time (x-axis) for different developmental events at different constant temperatures (y-axis). In the temperature range of 16 °C–34 °C, the duration of each developmental event (hatching, first ecdysis, second ecdysis, wandering, pupation and eclosion) gradually shortens as the temperature increased, and the distance between each curve also shortened with increasing temperatures.

**Thermal summation model**

A total of six thermal summation models were constructed from the linear regression analysis of the relationship between the development duration (x-axis) and ADH (y-axis) at each developmental stage and the entire developmental process (Figure 2). The coefficient of determination ($R^2$) of the equation of each thermal summation model is ≥0.97, indicating relatively good fit of these linear models. The developmental threshold temperature and thermal summation constant of each development stage and the total development process were determined from each thermal summation model (Table 2). The developmental threshold temperature $D_0$ and the thermal summation constant $K$ of the entire developmental process is (11.41 ± 0.32) °C and (3 418.7 ± 137.0) degree hours, respectively.

**Larval body length changes and construction of isomegalen diagram**

Changes in larval body length of C. megacephala at different temperatures are shown in Figure 3, where the larval developmental rate increases rapidly with increasing temperature increased. Between 16 °C and 25 °C, the development rate is significantly different between each temperature, but the difference is less pronounced as the temperature increased to 28 °C–34 °C. The mean maximum larval body length at 16 °C, 19 °C, 22 °C, 25 °C, 28 °C, 31 °C and 34 °C are 16.2, 16.3, 17.0, 16.3, 16.5, 16.4 and 16.2 mm, respectively.

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**Table 1. Mean (±SD) development duration (h) of Chrysomya megacephala at seven constant temperatures.**

| Developmental stages (°C) | Egg       | First instar | Second instar | Third instar | Pupa      | Total duration |
|---------------------------|-----------|--------------|---------------|--------------|-----------|----------------|
| 16                        | 38.9 ± 2.1$^{\text{f}}$ | 81.3 ± 4.1$^{\text{f}}$ | 84.0 ± 3.8$^{\text{f}}$ | 248.2 ± 5.2$^{\text{f}}$ | 342.5 ± 17.2$^{\text{f}}$ | 794.8 ± 14.7$^{\text{f}}$ |
| 19                        | 27.2 ± 2.3$^{\text{f}}$ | 57.3 ± 4.0$^{\text{f}}$ | 58.7 ± 6.1$^{\text{f}}$ | 148.2 ± 14.4$^{\text{f}}$ | 241.8 ± 13.9$^{\text{f}}$ | 533.2 ± 10.1$^{\text{f}}$ |
| 22                        | 19.2 ± 1.6$^{\text{f}}$ | 35.2 ± 6.1$^{\text{f}}$ | 40.2 ± 6.1$^{\text{f}}$ | 108.6 ± 8.3$^{\text{f}}$ | 174.7 ± 8.6$^{\text{f}}$ | 377.8 ± 16.8$^{\text{f}}$ |
| 25                        | 14.8 ± 0.9$^{\text{f}}$ | 28.6 ± 2.3$^{\text{f}}$ | 32.2 ± 2.3$^{\text{f}}$ | 68.0 ± 8.3$^{\text{f}}$ | 138.8 ± 9.1$^{\text{f}}$ | 280.8 ± 15.7$^{\text{f}}$ |
| 28                        | 12.2 ± 0.9$^{\text{f}}$ | 18.0 ± 2.3$^{\text{f}}$ | 22.5 ± 4.2$^{\text{f}}$ | 61.2 ± 4.0$^{\text{f}}$ | 105.2 ± 6.1$^{\text{f}}$ | 218.9 ± 8.5$^{\text{f}}$ |
| 31                        | 10.3 ± 1.0$^{\text{f}}$ | 13.3 ± 2.3$^{\text{f}}$ | 16.2 ± 2.3$^{\text{f}}$ | 56.8 ± 3.3$^{\text{f}}$ | 94.6 ± 4.2$^{\text{f}}$ | 190.8 ± 10.1$^{\text{f}}$ |
| 34                        | 8.5 ± 0.5$^{\text{f}}$ | 11.3 ± 1.2$^{\text{f}}$ | 13.3 ± 2.3$^{\text{f}}$ | 49.6 ± 2.3$^{\text{f}}$ | 89.3 ± 4.9$^{\text{f}}$ | 171.8 ± 6.8$^{\text{f}}$ |

Note: Values within the same column followed by the same superscript letter do not differ significantly from each other based on a one-way ANOVA + LSD test at $P < 0.05$.  

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![Figure 1. Isomorphen diagram of Chrysomya megacephala.](image-url)
Figure 2. Thermal summation models of total developmental stages of *Chrysomya megacephala*. • indicates data used in the regression analysis.

Table 2. Mean (±SE) of developmental threshold temperatures ($D_0$) and thermal summation constants ($K$) for five developmental stages and the total development period of *Chrysomya megacephala*, and the coefficient of determination ($R^2$) of thermal summation models.

| Developmental stages | $K$ (degree hours) | $D_0$ (°C) | Mean | SE | Mean | SE | $R^2$ |
|----------------------|--------------------|------------|------|----|------|----|-------|
| Egg                  | 207.2              | 10.91      | 7.2  | 0.34| 10.91| 0.34| 0.99  |
| First instar         | 272.0              | 13.35      | 33.9 | 0.79| 13.35| 0.79| 0.98  |
| Second instar        | 332.8              | 12.68      | 37.3 | 0.83| 12.68| 0.83| 0.97  |
| Third instar         | 1,077.6            | 11.57      | 56.2 | 0.45| 11.57| 0.45| 0.99  |
| Pupa                 | 2,007.2            | 10.31      | 81.4 | 0.43| 10.31| 0.43| 0.99  |
| Total duration       | 3,418.7            | 11.41      | 137.0| 0.32| 11.41| 0.32| 0.99  |

Figure 3. Larval body length changes of *Chrysomya megacephala* over time after hatching at different temperatures.
The equations in Table 3 describe the changes in larval body length \((L)\) with time \((T)\) using time after hatching as the independent variable and larval body length as the dependent variable. The coefficient of determination \((R^2)\), \(F\) value and \(P\) value all suggest that the equations determined by regression analysis have a high fit for the data.

The equations in Table 4 describe the time \((T)\) changes with larval body length \((L)\) using time after hatching as the dependent variable and larval body length as the independent variable. To obtain better curves and equations, only the larval change data from hatching to peak feeding stage is modelled by regression analysis. The coefficient of determination \((R^2)\), \(F\) value and \(P\) value all suggest that the equations obtained by regression analysis have a high fit after data elimination.

The development data obtained was used to construct an isomegalen diagram (Figure 4). Using this development model, the corresponding larval age can easily be estimated based on larval body length at different temperatures up to peak feeding stage.

### Intra-puparial morphological changes over time

The pupal stage of *C. megacephala* is about 50% of the total immature stage. Aside from the first few hours after pupation, no colour or external morphological changes of the puparium can be seen to estimate the age; therefore, we observed the intra-puparial development of *C. megacephala* and categorized morphological changes into the following 11 sub-stages (A–K). Typical characteristics of each sub-stage are as follows:

A (pre-pupal stage): the formation of light coloured puparium, whose inner tissue and puparium are difficult to separate, and very easy to break during dissection. Pupa resembles a shortened larva with rough surface and yellow-white colour (Figure 5(A1)).

B (early cryptocephalic pupal stage): the colour of puparium is brown red, and the pupa and puparium can be separated, but still fragile. It still appears shortened larva-like with rough surface and yellow-white colour (Figure 5(B1)). Short legs and wing stubs emerges, and the lengths of the legs are less than one-third of the body. Light colour respiratory horns emerge in the front end of pupa (Figure 5(B2,B3)).

C (late cryptocephalic pupal stage): pupa is yellow-white colour with smooth surface. Larval-like body segmentation can still be observed (Figure 5(C1)). Legs and wings elongate, length reaches half of the body. Respiratory horns colour darkened, still locates in the front of the pupa (Figure 5(C2,C3)).

D (phanerocephalic pupal stage): pupa is yellow-white colour. A transparent membrane on the surface forms, and is easy to peel from the puparium.
Head, thorax and abdomen begin to differentiate, but the segmentation between them remains unclear. Abdomen still has larval-like body segmentation (Figure 5(D1–D3)). Legs and wings are thick and further elongate, and length of the legs exceeds more than half of the body. The mouthparts are first observed as square-shaped (Figure 5(D3)).

E (early yellow-eye stage): pupa is yellow-white colour. The segmentation into head, thorax and abdomen is clear. The abdomen is adult-liked segmented (Figure 5(G1)). The development of antennae and mouthparts is completed but not coloured (Figure 5(G2)). The legs are thin and wings are folded (Figure 5(G3)).

F (middle yellow-eye stage): pupa is yellow-white colour (Figure 5(F1)). Antennae have obvious outline. Mouthparts are elongated and narrowed (Figure 5(F2)). The legs are thinner, and wings are unfolded (Figure 5(F3)).

G (late yellow-eye stage): pupa is yellow-white colour. The abdomen is adult-liked segmented (Figure 5(G1)). The development of antennae and mouthparts is completed but not coloured (Figure 5(G2)). The legs are thin and wings are folded (Figure 5(G3)).

The abdomen is adult-liked segmented (Figure 5(G1)). The development of antennae and mouthparts is completed but not coloured (Figure 5(G2)). The legs are thin and wings are folded (Figure 5(G3)).
The time ranges of each sub-stage at different temperatures of *C. megacephala* are shown in Table 5.

### Table 5. Intra-puparial development of *Chrysomya megacephala* related to time (h) at different temperatures.

| Sub-stage | 16°C | 22°C | 28°C | 34°C |
|-----------|------|------|------|------|
|           | Min  | Max  | Min  | Max  |
| A         | 0    | 16   | 0    | 8    |
| B         | 2    | 24   | 16   | 16   |
| C         | 24   | 40   | 16   | 16   |
| D         | 32   | 64   | 16   | 16   |
| E         | 56   | 72   | 32   | 16   |
| F         | 72   | 112  | 32   | 16   |
| G         | 104  | 200  | 40   | 16   |
| H         | 176  | 224  | 40   | 16   |
| I         | 208  | 232  | 40   | 16   |
| J         | 224  | 280  | 40   | 16   |
| K         | 272  | 344  | 40   | 16   |

### Discussion

The development of *C. megacephala* has previously been reviewed by Richards and Villet [3] and Gruner et al. [21]. While in China, there are five development studies using the *C. megacephala* populations from different regions if our study is included (Table 6). Overall, *C. megacephala* from different regions have similar total development duration at same constant temperatures. However, total developmental durations of the colony in Chongqing [5] at lower temperature (16°C and 19°C) are much shorter than in Suzhou, where the Chongqing colony develops 6–8 days faster at each constant temperature. Interestingly, when comparing the results at higher temperatures (22°C–34°C), the results of present study and that of Yang et al. [5] are very similar. The difference at low temperatures is likely due to the Chongqing colony’s adaptation to low temperature, as the Chongqing colony has a lower developmental threshold temperature $D_0$. In addition, we find differences in the development duration between the colonies from China and America (Table 6). These discrepancies indicate the plasticity in developmental rates between different populations. However, studies have used different food and feeding methods, and this may also have an impact on the developmental duration. Future research is needed to rear and observe insects collected from different regions in the same laboratory using a standard method, combined with genomic analysis to determine whether the differences in developmental plasticity exist, and what genetic changes may underlie these differences.

The developmental threshold temperature $D_0$ of *C. megacephala* calculated using the revised regression model proposed by Ikemoto and Takai [17] in this study is 11.41°C. The result is similar with Richards and Villet [3], Yang et al. [5] and Gruner et al. [21], where all three studies show that $D_0$ is about 10°C. Interestingly, a development study using the *C. megacephala* colony from Guangzhou provides a different result [31]. The insects fail to complete development at 16°C, indicating that the population from the warm and humid Guangzhou area has reduced capacity to complete development at lower temperatures and that the long-term geographical isolation may affect the low-temperature tolerance.

The maximum constant temperature we exposed insects to in this study is 34°C, and *C. megacephala* can complete their developmental cycle under this temperature. Similarly, Gruner et al. [21] found that *C. megacephala* can develop at 35°C, while the insects fail to complete their development at 40°C. The results of Richards and Villet [3] show that *C. megacephala* could not complete development at 42.5°C, and the survival rate of pupae at 37.5°C is only 6.98%, indicating that 37.5°C may be close to the highest temperature that *C. megacephala* can tolerate. Taken together,
maximum resistance temperature of *C. megacephala* may be between 37.5 °C and 40 °C.

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**Compliance with Ethical Standards**

This article does not contain any studies with human participants or animals performed by any of the authors.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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