

Development of *Sarcophaga dux* (Diptera: Sarcophagidae) at constant temperatures and differential gene expression for age estimation of intrapuparial

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Research

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Abstract

Background

Sarcophaga dux (Diptera: Sarcophagidae) is a necrophagous flesh fly with potential forensic value in estimating minimum postmortem interval (PMI_{min}). The basic developmental data and precise intrapuparial age estimates are significant for PMI_{min} estimation of application entomological data in legal medicine.

Methods

The development parameters of *S. dux* at seven constant temperatures from 16°C to 34°C were investigated by rearing using pig lung in the artificial climate box. The appropriate reference genes and intrapuparial differentially expressed genes (DEGs) of *S. dux* at constant temperatures 34, 25 and 16°C were selected and analyzed using RT-qPCR for more precisely age estimations.

Results

The developmental durations of *S. dux* at 16, 19, 22, 25, 28, 31 and 34°C from larviposition to adult eclosion were 1478.6±18.3, 726.1±15.8, 538.5±0.9, 394.1±9.5, 375.6±10.8, 284.1±7.3, and 252.5±6.1 h, respectively. The thermal summation constant of *S. dux* was 5341.71±249.29 degree hours, and the developmental threshold temperature was 12.266±0.35°C. The most reliable reference genes under intrapuparial and different temperature in our study were: *GST1* and *18S rRNA* for 34°C group, *GST1* and *RPL49* for 25 °C group, and *18S rRNA* and *28S rRNA* for 16 °C group. The four differential expression genes (*Hsp60*, *A-alpha*, *ARP*, *RPL8*) can be used to more precisely intrapuparial age estimation of *S. dux*.

Conclusions

The basic developmental data of *S. dux* at constant temperatures, such as body length changing of the larva period, accumulated degree hours and duration of development, to establish developmental models that can be used to estimate the PMI_{min}. The selection and evaluation of appropriate reference genes at different experimental conditions are primary with RT-qPCR. The differentially expressed gene can contribute to more accuracy age estimations of *S. dux* intrapuparial. The result from this study can make contributes to the use of *S. dux* for estimating PMI_{min}.

Background

Postmortem interval (PMI) estimation is crucial to investigation of cases. Forensic entomology may be more accurate in determining PMI than other technical methods and may be the most effective method at present, especially for decomposed corpses with intervals more than 72h [1, 2, 3]. Therefore, necrophagous insects correlated with the decomposed corpses have overwhelming superiority of PMI estimation in forensic investigations [4, 5]. The common types of carrion-related arthropods mainly belong to the Diptera order, including Sarcophagidae, Calliphoridae, and Muscidae families in general [6, 7].

The research of developmental patterns of the necrophilic insects on the corpse represents a method to calculate a reliable estimation of the PMI using entomological evidence [8, 9]. The rate of development under controlled temperatures and various parameters including the larval size (body length, width or weight), duration of development, and developmental accumulated temperature can indicate the age of the necrophagous insects, can be used to directly reflect the minimum PMI (PMI_{min}) that the corpse [10, 11, 12, 13, 14, 15]. Thus, collecting precise basic developmental data of forensically important insects are significant for improving the accuracy calculation of PMI_{min} [16, 17, 18]. The development models of the thermal summation models, the isomegalen and isomorphen diagrams were employed, to establish precise developmental data, and determine the age of insect [19, 20, 21, 22].

The precisely determination developmental age of necrophagous insects from the crime scenes is a crucial step for PMI_{min} estimation. However, in the actual case, we found that at low temperatures, the intrapuparial stage of some flies species can last up to 50 % of the immature stage or even several weeks, and the aging estimation of this period is increasingly difficult because anatomical and morphological changes are not visible [7, 8]. Metamorphosis is a complete set of developmental processes controlled by a transcriptional hierarchy that coordinates the activities of hundreds of genes [23]. The analysis of differentially expressed genes (DEGs) is one of the basic methods to understand the formation process of the metamorphosis of forensically relevant intrapuparial, which represent a potentially method of predicting intrapuparial age when the pupae is the oldest entomological evidence on corpse [24, 25, 26, 27, 28, 29]. In addition, the analysis expression profiles of DEGs have been used to estimation age of *Calliphora vicina* [24], *Lucilia sericata* [27] (Diptera: Calliphoridae) by Boehme et al and Tarone and Foran. Several studies have shown that in gene expression studies, selection and evaluation of appropriate reference genes are essential with RT-qPCR because the expression of some reference genes may be different in different experimental conditions [30, 31, 32, 33].

Sarcophaga (Liosarcophaga) dux Thomson, 1869 (Diptera: Sarcophagidae), a synanthropic flesh fly species in many parts of the world, is of medical importance as a vector of disease or parasitic, myiasis-producing agent [34], and as forensically important species with the necrophagous habits, it was found to colonize corpses of a male infant in Malaysia [35]. This species widely distributed around the world, such as Oriental region. Previous entomology investigations on *S. dux* have focused on the description of important morphological features in each developmental stage [34], molecular identification analysis by mitochondrial cytochrome oxidase gene subunits I and II (COI and COII) sequences [36], the human death investigations in forensic entomology [34]. However, there are still lacking a detailed investigation of *S. dux* development. Collecting precise basic data on the growth and development in specific regions and the analysis of DEGs for a more precise intrapuparial age estimations of insect species has been emphasized as a way to ensure accurate calculation of PMI_{min} [18, 24, 37].

In the present study, the adult of *S. dux* were collected from a pig carcasses and established a laboratory colony, the developmental data of *S. dux* at 16, 19, 22, 25, 28, 31 and 34°C from larviposition to adult emergence such as accumulated temperature, larval body length and developmental duration were collected and measured to establish developmental models for estimation the PMI_{min}. In addition, the ten candidate reference genes of *S. dux* including 60S acidic ribosomal protein P0 (*RPLP0*), 28S ribosomal RNA (*28S rRNA*), cAMP-dependent protein kinase (*PKA*), 18S ribosomal RNA (*18S rRNA*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Glutathione S-transferases 1 (*GST1*), α -tubulin (*α -TUB*), Elongation factor 1-alpha (*EF1a*), 40S ribosomal protein S3 (*RPS3*), ribosomal protein L49 (*RPL49*) were sequenced and firstly evaluated the stability of gene expression normalization at different temperatures and intrapuparial developmental ages. The four DEGs of *S. dux* including heat shock proteins 60 (*Hsp60*), sarcocystatin A-alpha (*A-alpha*), 60S acidic ribosomal protein 8 (*RPL8*), acidic ribosomal protein (*ARP*) were used to intrapuparial age estimations. This work provides an important basic developmental data and a more precise intrapuparial stage age estimates way of *S. dux*, improves the value of this insect of PMI_{min} estimation in forensic investigations.

Methods

Establishment of laboratory colony

Sarcophaga dux was obtained from pig carcasses placed in a deserted yard near a reservoir in Changsha city (28°12'N, 112°58'E), Hunan province, China, in March 2018. About 20 adult flies were collected, and species determination was performed by forensic entomologist using the adult identification key [38], and confirmed again by sequencing the COI (Genbank No. MN543093): F: 5-GGTCAACAAATCATAAAGATATTGG-3' and R: 5-RAAACTTCAGGRTGACCAAAGAATCA-3. These adult *S. dux* were maintained in an insect-rearing cage of size 35×35×35cm, in forensic entomology laboratory with about 70% humidity, average temperature of 25°C, natural light. The sugar and milk powder (1:1 mixture) and fresh water were provided for adult flies by the dish (12 cm diameter). The fresh pig lung as larviposition medium was placed in the cages, the larvae (between 500 and 1000 individuals) were collected within 4 hours, and reared in a biomimetic appliance of rearing larvae, the sufficient amount of fresh pig lung were provided until pupation. After eclosion, the adult of *S. dux* were reared once again five generations to obtain a purebred laboratory colony and increase the number of adult flies.

Collection of Developmental Data

The proper amount of fresh pig lung was provided to induce larviposition, about 2,000 to 3,000 larvae were collected within 2h, the larvae was placed in a rearing boxes containing pig lung, and transfer to the artificial climate box (LRH-250-GSI, Taihong Co., Ltd, Shaoguan, China), with the experimental conditions of 75% humidity and photoperiod of 12D∶12L, and 16, 19, 22, 25, 28, 31 and 34°C constant temperatures. The fresh pig lung was provided to meet the development needs of the larvae until pupation. Larvae were observed and collected once every 8 h, and 10 larvae were randomly taken from rearing boxes at each examination time, and immediately kill larvae with boiling water for at least 60 s, then stored in 80% ethanol at room temperature [39, 40]. The posterior spiracle slits was observed using a stereoscopic microscope (Motic SMZ-168) to determine the larval instar [41]. After stored 24 h of each larval using tubes, the body length was measured with a digital vernier caliper (Mahr, 16EWRi, Germany), to avoid the variation caused by the preservative solution, due to the increase of the larvae body length of 80% ethanol preserved occurred mainly in the first 12 h, and remained basically constant after 24 h [16]. The time of each developmental stage, including larval, pupation, and eclosion, were recorded. At each constant temperature, five replicates were performed for the study of development parameters.

Data analysis of Developmental

The development data was analyzed by the software Origin Pro 8.6 (SCR: 015636), the one-way ANOVA was used for analyzing the influence of different temperatures on duration of development. The relationship between development time after larviposition and the body length of larval was analyzed by nonlinear regression, to obtain a fitted equation for the PMI_{min} estimation [11]. The Ikemoto and Takai proposed the revised regression model was adopted [42]. The relationship between accumulated degree hours (ADH) and developmental duration was analyzed by the linear regression. The development threshold temperature (D_0) and thermal summation constant (K) were determined using the slope of the linear regression equation and the y-intercept, respectively.

Gene expression study

Collection of intrapuparial samples

During the pupae stage at 16, 25 and 34°C constant temperatures, the intrapuparial age of *S. dux* was set to “zero” when approximately 50% pupae were observed, and sampled firstly from the individuals at this point, then intrapuparial samples were collected once every 24 h at 25 and 34°C, every 48 h intervals at 16°C due to pupae development lasted longer at this temperature, 8-10 intrapuparial were randomly taken at each examination time until 50% adult eclosion [43], and immediately kill with liquid nitrogen, then stored in 5 mL cryovial at -80°C for the subsequent gene expression study. The experiment at each temperature was repeated three times. Pupa development lasted 48 days, 12 days and 7 days at 16°C, 25°C and 34°C, respectively.

Total RNA Isolation and cDNA Synthesis

The TaKaRa MiniBEST Universal RNA Extraction Kit (TAKARA BIO INC) is used to the extraction of each intrapuparial samples RNA according to the instruction. The *Evo-M-MLVRT* Kit for qPCR with gDNA Clean (Accurate Biotechnology (Hunan) Co., Ltd. China) is used to the synthesis of complementary DNA (cDNA) from Total RNA (10ng-5µg) following the instructions of manufacturer. The reaction condition: 37°C for 15 min, 85°C for 5 s, and -20°C stored for the RT-qPCR.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

The candidate reference genes and DEGs involved were selected and downloaded from the NCBI based on the description of relevant literature of diptera insects [24, 25, 26, 27, 44, 45, 46, 47, 48, 49, 50]. These genes of *S. dux* were amplified and sequenced, and uploading to GenBank BLAST, compared with other insect species, which to verify their identity and confirmed was correctly detected. The RT-qPCR primers of these genes were prepared by the Primer Premier 5, the sensitivity and efficiency were checked by the relative standard curve using the 10-fold concentration dilution method and melting curve [43]. The characteristics of candidate gene and the primer were shown in Table 1. The RT-qPCR reactions were carried out using the SYBR[®] Green Premix *Pro Taq* HS qPCR Kit (Accurate Biotechnology (Hunan) Co., Ltd. China), and operated on a 7500 Real-Time PCR System (Applied Biosystems). Amplification conditions: 95°C 30 s, 40 cycles followed 95°C 5 s; 60°C 30 s. All the samples were measured independently three times.

Data Analysis of Reference Genes and DEGs

The raw qPCR data was analyzed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems), and the cycle threshold value was automatically determined. The reliability and number of candidate reference genes at different temperatures conditions was comprehensively evaluated by geNorm software program [51], which widely used to establish suitable reference genes for qPCR data normalization.

The relative quantification (RQ) of the DEGs was calculated using the $2^{-\Delta\Delta C_t}$ method and standardized against the reference genes selected [43]. Fold change values of the DEGs (FC i.e. relative expression of gene) was normalized by the log-transform to base 2 [$\log_{FC} = \log_2(RQ)$], and the age zero of *S. dux* intrapuparial was taken as a reference time to calculate the relative change in gene expression of other Intra-puparial at each temperature regime.

The effects of both the temperatures and ageing for the expression level of the Intra-puparial DEGs of *S. dux* were calculated by a two-way ANOVA. The normal distribution of relative expression of DEGs was conducted by the Kolmogorov–Smirnov and Q–Q plots. The relationship between LogFC values of the DEGs under different temperature conditions and Intra-puparial ageing of *S. dux* was analyzed through regression to obtain equations for estimating the PMin. All statistical analysis was conducted by GraphPad Prism 6 (SCR: 002798), and OriginPro version 8.6 (SCR: 015636), SPSS version 22.0 (SCR: 002865).

Results

Development Duration and Isomorphen Diagram

Between 16 and 34°C, *S. dux* can complete the development process from larviposition to eclosion, in this range of temperature, the durations of each developmental phase decreases with temperature increasing. The total developmental time was 1478.6 h and 252.5 h at 16 and 34°C (Table 2).

The isomorphen diagram was completed using the development time of each life stages and different temperatures conditions (Fig. 1). The time of all developmental duration gradually shortened with the increasing of growth temperature in 16–34°C.

Thermal Summation Models

The thermal summation models of all developmental process, including total developmental duration, were plotted according to the development time and accumulated degree hours using linear regression analysis (Fig. 2). The thermal summation constants (K) and the developmental threshold temperatures (D_0) of *S. dux* during the whole development process were calculated by the thermal summation model, which were 5341.71 ± 249.29 degree hours and $12.266 \pm 0.35^\circ\text{C}$, respectively (Table 3).

Changes in Larval Body Length and Isomegalen Diagram

The relationship between body length changing of the larva period and development time at different temperatures was shown in Fig. 3. In 16–34°C temperature, with the temperature increasing, the developmental rate of larvae increasing. The equations describing the body length changing of the larva and development time after larviposition were plotted through a regression analysis. The determination coefficient (R^2) of the equation at each temperature was greater than 0.95, which indicated that the development data was fit well using the regression analysis (Table 4). The isomegalen diagram show the relationships between the changes of the larval body length after larviposition to peak (3-16cm) and development time under different temperatures (Fig. 4).

Total RNA Quality

The concentration of each intrapuparial samples RNA was quantitated spectrophotometrically (NanoDrop 2000, Thermo, USA). The 1% agarose gel electrophoresis was used to the detection of RNA integrity. The correlation coefficient and PCR efficiency are shown in Table 1.

Stability of Reference Gene Expression

The reliability of reference genes at 34°C, 25°C and 16°C temperatures conditions of different Intra-puparial samples was calculated by geNorm methods, and the results was shown in Supplementary Material 1 and Fig. 5. The lower ranking indicateds more stable expression. The minimum number of candidate reference genes were determined using the pair-wise variation value (V) by the geNorm, $V_{2/3}$ below 0.15 indicated that two reference genes would be suitable in this experiment condition (Fig. 6), and the most reliable reference genes for this research of *S. dux* pupae at different constant temperatures were: GST1 and 18S rRNA for 34°C group, GST1 and RPL49 for 25 °C group, and 18S rRNA and 28S rRNA for 16 °C group.

Overall and Detailed Expression Profiles of the DEG

We selected and validated the expression profiles of the four DEGs (*Hsp60*, *A-alpha*, *ARP*, *RPL8*), which have potential represented developmental-specific and statistical significance for accurately predicting intrapuparial age of *S. dux*. The overall expression profiles of four DEGs at three constant temperatures were averaged, and plotted against the developmental percentage of *S. dux* Intra-puparial (Fig. 7). The results shown that the expression profiles of *Hsp60* increased at the puparial development process—the expression level of *A-alpha* first increased steadily until reached its maximum, and then tended to increase steadily until completion puparial development—and the expression profiles of *ARP* has an opposite tendency with *A-alpha*, the expression level of *RPL8* were in a state of fluctuation. The simulation equations of fourth-order polynomials were used to describe the relationship between the overall expression profiles of four DEGs at three constant temperatures and developmental percentage of Intra-puparial, which conducted using a nonlinear regression analysis (Table 5).

The simulation equations (Table 6) and curves (Fig. 8) describing the detailed gene expression and Intra-puparial age at constant 34, 25 and 16°C were obtained using a nonlinear regression analysis, which adopting the differential gene expression levels as the independent variable and Intra-puparial age at each temperature as the dependent variable. The influence of rearing temperature and Intra-puparial age on the expression of DEGs was tested by a two-way ANOVA analysis (Supplementary Material 2), the result showed that the interaction of age×temperature has a significant influence on the expression profiles of *S. dux* Intra-puparial DEGs.

Discussion

Although many studies have emphasized the importance of *S. dux* in forensic science, the development durationre data available is limited. In this study, the basic developmental data of *S. dux* at seven constant temperatures from 16°C to 34°C was reported, and the age indication index to use *S. dux* for estimation PMImin including body length changing of the larva period, accumulated degree hours and duration of development was provided. The duration of development of *S. dux* from larviposition to adult eclosion at 16, 19, 22, 25, 28, 31 and 34°C were 1478.6 ± 18.3 , 726.1 ± 15.8 , 538.5 ± 0.9 , 394.1 ± 9.5 , 375.6 ± 10.8 , 284.1 ± 7.3 , and 252.5 ± 6.1 h, respectively. However, the research of the developmental durations of *S. dux* in Saudi Arabia showed that the time from first instar to adult emergence was 1243.2, 792, 600, 393.6 and 362.4 h at 16, 20, 24, 28, 32 and 36°C, respectively [34]. In addition, the developmental durations of this species from the larvae to pupariation were 72-96h in ranging between 27.1–29.8°C in Thailand [52], 168h at 29.5°C in Guam [53], 196-244h at 25°C in South Africa [54], and 114, 108, 86h at 25, 28, 31°C in our study. The developmental time of second instar of *S. dux* that we measured (19h) at constant temperatures 28°C is similar with the reported (19h) by Kumara et al. (2013) at fluctuation temperature $28.9 \pm 1.2^\circ\text{C}$ in Malaysia [55], but faster development at third instar and post-feeding and slower development at pupa stages compared to Kumara et al. (2013) experiments.

The three development models of isomegalen diagram, isomorphen diagram, and thermal summation model were established basis on the developmental data obtained for facilitating the estimation of PMI_{min}. The application range of isomorphen diagram widespread, and can calculate the time of different stages of development at various temperatures, but does not include the variability between events [7]. The isomegalen diagram can clearly to calculate the relationships between the changes of the body length of larval after larviposition to peak and development time under different temperatures [11, 12, 13]. The thermal summation model is more applicable to fluctuating temperatures environments [12, 14]. The developmental threshold temperatures (D_0) of *S. dux* at total developmental process in our study were calculated were $12.266 \pm 0.35^\circ\text{C}$ using thermal summation model, and this species has been recorded in Nepal at altitudes of 2,000 m, indicated they are well adapted to low temperatures and high altitudes [56].

The biological and abiotic factors can affect the developmental process of necrophagous insects, such as, Maggots hardly reared individually because of the gregarious behavior [57]. Too much food will lead to delayed development and even larvae death[58], and starvation can result adults smaller and early larvae pupariation [59]. The different laboratory equipment and conditions, including photoperiod and humidity impact the development process, but temperature is still main factor affecting growth and development of insect [12, 16, 17], thus, evaluated the above factors and detailed data recording and careful experimental design to establish a development duration of fly should be emphasized.

RT-qPCR is a reliable and sensitive technology to explore the DEGs in many complex biological progresses [60]. However, the key to obtain accurate target gene results with stable standard gene is normalization, and the normalization of qPCR data requires the appropriate reference genes [61]. The studies have suggests that in different environmental conditions or developmental stages, the stably reference genes are differently [62]. Moreover, standardizing experimental shown that two or more reference genes will be sufficient than single reference gene [63]. In this study, the stability of ten candidate reference genes at different pupae developmental stages and constant temperatures of *S. dux* was firstly evaluated for gene expression research.

During fly metamorphosis, the analysis of differentially expressed genes (DEGs) may provide a way to estimate age of forensically important fly in death investigations [64]. DEGs have the advantage in objective data. Importantly, its method is easier to master than morphology. Therefore, it would be beneficial to have an alternative way which could be carried out by a standard forensic laboratory, leading to produce more standardized results. Until now, the forensically relevant species involved in DEGs studies including *C. vicina*, *L. sericata*, and *S. peregrine*, the DEGs including *bcd*, *sll*, *cs*, *ace*, *hsp90*, *hsp 60*, *usp*, *ecr*, *rop-1*, *w*, *15_2*, *2014192*, *actin*, *arylphorin receptor* [24, 25, 26, 27, 28, 29, 43]. There are lots of other forensically important species need to be investigated.

In this study, the expression level of *S. dux* Intra-puparial four DEGs, including *Hsp60*, *RPL8*, *A-alpha*, *ARP* were firstly evaluated at 34°C , 25°C and 16°C constant temperatures for Intra-puparial age estimation. The results indicated that for the same DEGs, the expression profiles at three temperature conditions were similar, and with the increase of temperature, puparial development time was shortened, which led to the faster change of gene expression. The research show that in the early embryonic and pupa stage, the sarcocystatin A (*A-alpha*) gene was activated, indicating that sarcocystatin A was involved in the morphogenesis of the Sarcophaga larvae and adult structures [65], and the expression profiles of *A-alpha* has the potentially used to more precisely age estimation of *S. dux* intra-puparial in our study. The potential of *hsp 60* gene used to estimate the puparia age of fly has been proved by Tarone and Foran (2011) and this study [27]. In the ideal of circumstances, the best-suited gene for indicating insect age ought to exhibit the continuous increase or decrease trend in expression level and development time, resulting in the expression profiles would match with the sole corresponding development time. But in fact, it is hard to find out the genes that accord with this criterion because the insects change dramatically during pupal stage. Therefore, combining DEGs is an effective way for precisely age estimation of intra-puparial.

Recent studies have shown that geographically variation can affect the rates of insect developmental, such as *C. vicina* and *L. sericata* [66, 67, 68]. The geographically separate populations may result a different discoveries of the same species. Therefore, the precisely PMI_{min} estimation at specific crime scene by necrophagous sarcophagids requires collection the development data from different geographical regions in the world. Furthermore, the effect of geographic variation on DEGs expression profile is still unclear and needs further study.

Conclusions

The basic developmental data and precise intrapuparial age estimates are significant for PMI_{min} estimation of application *S. dux* in crime investigations. In present study, the development parameters of *S. dux* at seven constant temperatures from 16°C to 34°C in body length changing of the larva period, accumulated degree hours and duration of development were investigated, the appropriate reference genes in intrapuparial and different temperature of *S. dux* were selected for the accuracy of RT-qPCR, the four differential expression genes (*Hsp60*, *A-alpha*, *ARP*, *RPL8*) has the potentially used to more precisely age estimation of *S. dux* intra-puparial.

Abbreviations

PMI_{min}: minimum postmortem interval

PMI: postmortem interval

DEGs: differentially expressed genes

RT-qPCR: Quantitative real time polymerase chain reaction

COI: cytochrome oxidase gene subunits I

COII: cytochrome oxidase gene subunits II

RPLP0: 60S acidic ribosomal protein P0

28S rRNA: 28S ribosomal RNA

PKA: cAMP-dependent protein kinase

18S rRNA: 18S ribosomal RNA

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GST1: Glutathione S-transferases 1

α -TUB: α -tubulin

EF1 α : Elongation factor 1-alpha

RPS3: 40S ribosomal protein S3

RPL49: ribosomal protein L49

Hsp60: heat shock proteins 60

A-alpha: sarcocystatin A-alpha

RPL8: 60S acidic ribosomal protein 8

ARP: acidic ribosomal protein

ADH: accumulated degree hours

RQ: relative quantification

Declarations

Conflict of interest

The authors have no relevant conflicts of interest to declare.

Ethics approval and consent to participate

Not applicable

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Availability of data and materials

The data supporting the conclusions of this article are included within the article, and species determination was confirmed by sequencing the COI (Genbank No. MN543093).

References

1. Ren L, Shang Y, Chen W, Meng F, Cai J, Zhu G, et al. A brief review of forensically important flesh flies (Diptera: Sarcophagidae). *Forensic sciences research*. 2018;3 1:16-26; doi: 10.1080/20961790.2018.1432099.
2. Smith K. *A manual of forensic entomology*. Trustees of the British Museum. Natural History and Cornell University Press, London. 1986;205; doi: 10.2307/505635.
3. Byrd JH. *Forensic entomology: the utility of arthropods in legal investigations*. CRC press; 2002.
4. Amendt J, Campobasso CP, Gaudry E, Reiter C, LeBlanc HN, Hall MJ. Best practice in forensic entomology—standards and guidelines. *International journal of legal medicine*. 2007;121 2:90-104; doi: 10.1007/s00414-006-0086-x.
5. Tomberlin JK, Benbow ME. *Forensic entomology: international dimensions and frontiers*. CRC Press; 2015.
6. Wang J, Li Z, Chen Y, Chen Q, Yin X. The succession and development of insects on pig carcasses and their significances in estimating PMI in south China. *Forensic Science International*. 2008;179 1:11-8; doi: 10.1016/j.forsciint.2008.04.014.
7. Amendt J, Richards C, Campobasso CP, Zehner R, Hall MJ. *Forensic entomology: applications and limitations*. Forensic science, medicine, and pathology. 2011;7 4:379-92; doi: 10.1007/s12024-010-9209-2.
8. Wang Y, Wang J, Zhang Y, Tao L, Wang M. Forensically important *Boettcherisca peregrina* (Diptera: Sarcophagidae) in China: development pattern and significance for estimating postmortem interval. *Journal of medical entomology*. 2017;54 6:1491-7; doi: 10.1093/jme/tjx139.
9. Yang Y, Lyu Z, Li X, Li K, Yao L, Wan L. Development of *Hemipyrellia ligurriens* (Wiedemann) (Diptera: Calliphoridae) at constant temperatures: Applications in estimating postmortem interval. *Forensic science international*. 2015;253:48-54; doi: 10.1016/j.forsciint.2015.05.006.
10. Niederegger S, Pastuschek J, Mall G. Preliminary studies of the influence of fluctuating temperatures on the development of various forensically relevant flies. *Forensic Science International*. 2010;199 1-3:72-8; doi: 10.1016/j.forsciint.2010.03.015.
11. Wang Y, Li L, Wang J, Wang M, Yang L, Tao L, et al. Development of the green bottle fly *Lucilia illustris* at constant temperatures. *Forensic science international*. 2016;267:136-44; doi: 10.1016/j.forsciint.2016.07.019.
12. Zhang Y, Wang Y, Sun J, Hu G, Wang M, Amendt J, et al. Temperature-dependent development of the blow fly *Chrysomya pinguis* and its significance in estimating postmortem interval. *Royal Society open science*. 2019;6 9:190003; doi: 10.4172/2157-7145.1000238.
13. Wang Y, Yang L, Zhang Y, Tao L, Wang J. Development of *Musca domestica* at constant temperatures and the first case report of its application for estimating the minimum postmortem interval. *Forensic science international*. 2018;285:172-80; doi: 10.1016/j.forsciint.2018.02.004.
14. Hu G, Wang Y, Sun Y, Zhang Y, Wang M, Wang J. Development of *Chrysomya ruffifacies* (Diptera: Calliphoridae) at Constant Temperatures Within its Colony Range in Yangtze River Delta Region of China. *Journal of Medical Entomology*. 2019; doi: 10.1016/j.forsciint.2016.04.033.
15. Yang L, Wang Y, Li L, Wang J, Wang M, Zhang Y, et al. Temperature-dependent Development of *Parasarcophaga similis* (Meade 1876) and its Significance in Estimating Postmortem Interval. *Journal of forensic sciences*. 2017;62 5:1234-43; doi: 10.1111/1556-4029.13389.
16. Lecheta MC, Moura MO. Estimating the Age of Forensically Useful Blowfly, *Sarconesia chlorogaster* (Diptera: Calliphoridae), Using Larval Length and Weight. *Journal of medical entomology*. 2019;56 4:915-20; doi: 10.1093/jme/tjz028.
17. Wang Y, Hu G, Zhang Y, Wang M, Amendt J, Wang J. Development of *Muscina stabulans* at constant temperatures with implications for minimum postmortem interval estimation. *Forensic science international*. 2019;298:71-9; doi: 10.1016/j.forsciint.2019.02.051.
18. Chen W, Yang L, Ren L, Shang Y, Wang S, Guo Y. Impact of Constant Versus Fluctuating Temperatures on the Development and Life History Parameters of *Aldrichina grahami* (Diptera: Calliphoridae). *Insects*. 2019;10 7:184; doi: 10.3390/insects10070184.
19. Grassberger M, Reiter C. Effect of temperature on *Lucilia sericata* (Diptera: Calliphoridae) development with special reference to the isomegalen-and isomorphen-diagram. *Forensic Science International*. 2001;120 1-2:32-6; doi: 10.1016/s0379-0738(01)00413-3.
20. Ridgeway J, Midgley J, Collett I, Villet M. Advantages of using development models of the carrion beetles *Thanatophilus micans* (Fabricius) and *T. mutilatus* (Castelneau) (Coleoptera: Silphidae) for estimating minimum post mortem intervals, verified with case data. *International journal of legal medicine*. 2014;128 1:207-20; doi: 10.1007/s00414-013-0865-0.
21. Richards C, Villet M. Data quality in thermal summation development models for forensically important blowflies. *Medical and veterinary entomology*. 2009;23 3:269-76; doi: 10.1111/j.1365-2915.2009.00819.x.
22. Velásquez Y, Vilorio AL. Effects of temperature on the development of the Neotropical carrion beetle *Oxelytrum discicolle* (Brullé, 1840) (Coleoptera: Silphidae). *Forensic Science International*. 2009;185 1-3:107-9; doi: 10.1016/j.forsciint.2008.12.020.
23. White KP, Rifkin SA, Hurban P, Hogness DS. Microarray analysis of *Drosophila* development during metamorphosis. *Science*. 1999;286 5447:2179-84; doi: 10.1126/science.286.5447.2179.
24. Boehme P, Spahn P, Amendt J, Zehner R. Differential gene expression during metamorphosis: a promising approach for age estimation of forensically important *Calliphora vicina* pupae (Diptera: Calliphoridae). *International journal of legal medicine*. 2013;127 1:243-9; doi: 10.1007/s00414-012-0699-1.

25. Boehme P, Spahn P, Amendt J, Zehner R. The analysis of temporal gene expression to estimate the age of forensically important blow fly pupae: results from three blind studies. *International journal of legal medicine*. 2014;128 3:565-73; doi: 10.1007/s00414-013-0922-8.
26. Zehner R, Amendt J, Boehme P. Gene expression analysis as a tool for age estimation of blowfly pupae. *Forensic Science International: Genetics Supplement Series*. 2009;2 1:292-3; doi: 10.1016/j.fsigss.2009.08.008.
27. Tarone AM, Foran DR. Gene expression during blow fly development: improving the precision of age estimates in forensic entomology. *Journal of Forensic Sciences*. 2011;56:S112-S22; doi: 10.1111/j.1556-4029.2010.01632.x.
28. Abdel-Ghaffer H, Abd-Elmottelb M, Zaky A. Age-dependent gene expression of blow fly *Lucilia cuprina* (Diptera: Calliphoridae) during egg development improving age estimation in forensic entomology. *Journal of Forensic Research*. 2014;5 5:1; doi: 10.4172/2157-7145.1000238.
29. Tarone AM, Jennings KC, Foran DR. Aging blow fly eggs using gene expression: a feasibility study. *Journal of forensic sciences*. 2007;52 6:1350-4; doi: 10.1111/j.1556-4029.2007.00587.x.
30. Ibanez F, Tamborindeguy C. Selection of reference genes for expression analysis in the potato psyllid, *Bactericera cockerelli*. *Insect molecular biology*. 2016;25 3:227-38; doi: 10.1111/imb.12219.
31. Zhang L, Zhang QL, Wang XT, Yang XZ, Li XP, Yuan ML. Selection of reference genes for qRT-PCR and expression analysis of high-altitude-related genes in grassland caterpillars (Lepidoptera: Gynaephora) along an altitude gradient. *Ecology and evolution*. 2017;7 21:9054-65; doi: 10.1002/ece3.3431.
32. Zhang S, An S, Li Z, Wu F, Yang Q, Liu Y, et al. Identification and validation of reference genes for normalization of gene expression analysis using qRT-PCR in *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Gene*. 2015;555 2:393-402; doi: 10.1016/j.gene.2014.11.038.
33. Arya SK, Jain G, Upadhyay SK, Singh H, Dixit S, Verma PC. Reference genes validation in *Phenacoccus solenopsis* under various biotic and abiotic stress conditions. *Scientific reports*. 2017;7 1:13520; doi: 10.1038/s41598-017-13925-9.
34. Sukontason KL, Sanit S, Klong-Klaew T, Tomberlin JK, Sukontason K. *Sarcophaga dux* (Diptera: Sarcophagidae): A flesh fly species of medical importance. *Biological research*. 2014;47 1:14; doi: 10.1186/0717-6287-47-14.
35. Zuha RM, Baba MHJS. *Sarcophaga dux* Thomson (Diptera: Sarcophagidae) recovered from wrapped body of an infant: its evidential value. 2017;21 2.
36. Tan SH, Rizman-Idid M, Mohd-Aris E, Kurahashi H, Mohamed Z. DNA-based characterisation and classification of forensically important flesh flies (Diptera: Sarcophagidae) in Malaysia. *Forensic Science International*. 2010;199 1-3:43-9; doi: 10.1016/j.forsciint.2010.02.034.
37. Brown K, Thorne A, Harvey M. *Calliphora vicina* (Diptera: Calliphoridae) pupae: a timeline of external morphological development and a new age and PMI estimation tool. *International journal of legal medicine*. 2015;129 4:835-50; doi: 10.1007/s00414-014-1068-z.
38. Fan ZD. Key to the common flies of China, 1st ed. . Science Publishing House, Beijing, China. 1992.
39. Adams ZJ, Hall MJ. Methods used for the killing and preservation of blowfly larvae, and their effect on post-mortem larval length. *Forensic Science International*. 2003;138 1-3:50-61; doi: 10.1016/j.forsciint.2003.08.010.
40. Bugelli V, Campobasso CP, Verhoff MA, Amendt J. Effects of different storage and measuring methods on larval length values for the blow flies (Diptera: Calliphoridae) *Lucilia sericata* and *Calliphora vicina*. *Science & Justice*. 2017;57 3:159-64; doi: 10.1016/j.scijus.2016.10.008.
41. Kotzé Z, Villet MH, Weldon CW. Effect of temperature on development of the blowfly, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae). *International journal of legal medicine*. 2015;129 5:1155-62; doi: 10.1007/s00414-015-1153-y.
42. Ikemoto T, Takai K. A new linearized formula for the law of total effective temperature and the evaluation of line-fitting methods with both variables subject to error. *Environmental Entomology*. 2000;29 4:671-82.
43. Shang Y, Ren L, Yang L, Wang S, Chen W, Dong J, et al. Differential Gene Expression for Age Estimation of Forensically Important *Sarcophaga peregrina* (Diptera: Sarcophagidae) Intrapuparial. *Journal of medical entomology*. 2019; doi: 10.1016/j.trstmh.2010.07.005.
44. Baumann A, Lehmann R, Beckert A, Vilcinskas A, Franta Z. Selection and evaluation of tissue specific reference genes in *Lucilia sericata* during an immune challenge. *PloS one*. 2015;10 8:e0135093; doi: 10.1371/journal.pone.0135093.
45. Kim JY, Lim HY, Shin SE, Cha HK, Seo J-H, Kim S-K, et al. Comprehensive transcriptome analysis of *Sarcophaga peregrina*, a forensically important fly species. *Scientific data*. 2018;5:180220; doi: 10.1016/j.trstmh.2010.07.005.
46. Shen G-M, Jiang H-B, Wang X-N, Wang J-J. Evaluation of endogenous references for gene expression profiling in different tissues of the oriental fruit fly *Bactrocera dorsalis* (Diptera: Tephritidae). *BMC molecular biology*. 2010;11 1:76; doi: 10.1186/1471-2199-11-76.
47. Bagnall N, Kotze A. Evaluation of reference genes for real-time PCR quantification of gene expression in the Australian sheep blowfly, *Lucilia cuprina*. *Medical and veterinary entomology*. 2010;24 2:176-81; doi: 10.1111/j.1365-2915.2010.00866.x.
48. Zhai Y, Lin, Q., Zhou, X., Zhang, X., Liu, T., & Yu, Y. . Identification and validation of reference genes for quantitative real-time PCR in *Drosophila sukuzii* (Diptera: Drosophilidae). *PLoS One*. 2014; 9(9), e106800.; doi: 10.1371/journal.pone.0106800.
49. Wang X, Xiong M, Wang J, Lei C, Zhu F. Reference gene stability of a synanthropic fly, *Chrysomya megacephala*. *Parasites & vectors*. 2015;8 1:565; doi: 10.1186/s13071-015-1175-9.

50. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research*. 2001;29 9:e45-e; doi: 10.1093/nar/29.9.e45.
51. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology*. 2002;3 7:research0034. 1; doi: 10.1186/gb-2002-3-7-research0034.
52. Sukontason K, Sukontason KL, Piangjai S, Boonchu N, Chaiwong T, Ngern-klun R, et al. Antennal sensilla of some forensically important flies in families Calliphoridae, Sarcophagidae and Muscidae. *Micron*. 2004;35 8:671-9; doi: 10.1016/j.micron.2004.05.005.
53. Bohart GE, Gressitt JL. *Filth-inhabiting flies of Guam*. vol. 204: Citeseer; 1951.
54. Aspoas B. Comparative micromorphology of third instar larvae and the breeding biology of some *Afrotropical Sarcophaga* (Diptera: Sarcophagidae). *Medical and Veterinary Entomology*. 1991;5 4:437-45; doi: 10.1111/j.1365-2915.1991.tb00572.x.
55. Kumara T, Abu Hassan A, Che Salmah M, Bhupinder S. Larval growth of *Liosarcophaga dux* Thompson (Diptera: Sarcophagidae) under uncontrolled indoor temperatures in Malaysia. *Southeast Asian J Trop Med Public Health*. 2013;44 2:182-7; doi: 10.1186/1475-2875-12-84.
56. SUGIYAMA E, SHINONAGA S, KANO R. Sarcophagine flies from Nepal with the description of a new species (Diptera: Sarcophagidae). *Medical Entomology and Zoology*. 1988;39 4:355-62; doi: 10.7601/mez.41.81.
57. Charabidze D, Bourel B, Gosset D. Larval-mass effect: characterisation of heat emission by necrophagous blowflies (Diptera: Calliphoridae) larval aggregates. *Forensic science international*. 2011;211 1-3:61-6; doi: 10.1016/j.forsciint.2011.04.016.
58. Nability P, Higley LG, Heng-Moss T. Light-induced variability in development of forensically important blow fly *Phormia regina* (Diptera: Calliphoridae). *Journal of medical entomology*. 2007;44 2:351-8; doi: 10.1603/0022-2585(2007)44[351:lvidof]2.0.co;2.
59. Greenberg B. Flies as forensic indicators. *Journal of Medical Entomology*. 1991;28 5:565-77; doi: 10.1093/jmedent/28.5.565.
60. Scharlaken B, de Graaf DC, Goossens K, Brunain M, Peelman LJ, Jacobs FJ. Reference gene selection for insect expression studies using quantitative real-time PCR: The head of the honeybee, *Apis mellifera*, after a bacterial challenge. *Journal of insect Science*. 2008;8 1:33; doi: 10.1673/031.008.3301.
61. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of molecular endocrinology*. 2000;25 2:169-93; doi: 10.1677/jme.0.0250169.
62. Nagy NA, Németh Z, Juhász E, Póliska S, Rácz R, Kosztolányi A, et al. Evaluation of potential reference genes for real-time qPCR analysis in a biparental beetle, *Lethrus apterus* (Coleoptera: Geotrupidae). *PeerJ*. 2017;5:e4047; doi: 10.7717/peerj.4047.
63. Pan H, Yang X, Bidne K, Hellmich RL, Siegfried BD, Zhou X. Selection of reference genes for RT-qPCR analysis in the monarch butterfly, *Danaus plexippus* (L.), a migrating bio-indicator. *PloS one*. 2015;10 6:e0129482; doi: 10.1371/journal.pone.0129482.
64. Fremdt H, Amendt J, Zehner R. Diapause-specific gene expression in *Calliphora vicina* (Diptera: Calliphoridae)—a useful diagnostic tool for forensic entomology. *International journal of legal medicine*. 2014;128 6:1001-11; doi: 10.1007/s00414-013-0920-x.
65. Saito H, Suzuki T, Ueno K, Kubo T, Natori S. Molecular cloning of cDNA for sarcocystatin A and analysis of the expression of the sarcocystatin A gene during development of *Sarcophaga peregrina*. *Biochemistry*. 1989;28 4:1749-55; doi: 10.1021/bi00430a049.
66. Grassberger M, Reiter C. Effect of temperature on development of the forensically important holarctic blow fly *Protophormia terraenovae* (Robineau-Desvoidy)(Diptera: Calliphoridae). *Forensic Science International*. 2002;128 3:177-82; doi: 10.1016/s0379-0738(01)00413-3.
67. Hwang C, Turner B. Small-scaled geographical variation in life-history traits of the blowfly *Calliphora vicina* between rural and urban populations. *Entomologia Experimentalis et Applicata*. 2009;132 3:218-24; doi: 10.1111/j.1570-7458.2009.00891.x.
68. Tarone A, Picard C, Spiegelman C, Foran D. Population and temperature effects on *Lucilia sericata* (Diptera: Calliphoridae) body size and minimum development time. *Journal of Medical Entomology*. 2011;48 5:1062-8; doi: 10.1603/ME11004.

Tables

Table 1. Primers used for RT-qPCR analysis.

	Gene name	Abbreviation	Accession number	Primer sequences (5'→3') ^a	PCR products (bp)	T _m (°C)	Primer efficiency (%)	R ² ^b	
Candidate reference genes	<i>Glyceraldehyde-3-phosphate dehydrogenase-1</i>	<i>GAPDH</i>	MK861149	F: CCACCCAAAAGACCGTTGAC R: ACCAGTTGAGGCAGGAATGA	89	57.4	95.4	0.937	
	<i>18S ribosomal RNA</i>	<i>18S</i>	MK861148	F: TGTTGCGGTTAAAACGTTCCG R: ACTCCACCGGTAATACGCTT	108	53.4	97.3	0.967	
	<i>28S ribosomal RNA</i>	<i>28S</i>	MK861150	F: TAAATAGTACGTGAAAACCTAGAGGT R: AAAATAATTTGAATAAACTTACGCATC	197	56.7	98.4	0.981	
	<i>Elongation factor 1-alpha</i>	<i>EF1a</i>	MK861143	F: CGTGTGCAAACGGTGTCTT R: ACGGACTTGACTTCAGTGGT	80	55.4	98.7	0.978	
	<i>ribosomal protein L49</i>	<i>RPL49</i>	MK861145	F: CCAGTCGGATCGGTATGCTA R: GGTACGCTTGTTGGAACCAT	130	57.4	96.4	0.963	
	<i>alpha-tubulin</i>	<i>α-TUB</i>	MK861154	F: ACAGTGGGTGGTTGGTAGTT R: CCATTGCCACCATCAAGACC	94	55.4	98.3	0.978	
	<i>cAMP-dependent protein kinase</i>	<i>PKA</i>	MK861155	F: GGTAGCCCTGTGAGTCGATT R: CTGCGCAAAGTAGTCTGTTT	145	57.4	96.5	0.985	
	<i>60S acidic ribosomal protein P0</i>	<i>RPLP0</i>	MK861147	F: GCATTGCCAACGGTTTCAAG R: TTGCTGGGGTCTTGATGTA	103	55.4	97.4	0.943	
	<i>Glutathione S-transferases 1</i>	<i>GST1</i>	MK861144	F: CGTTTGTACTTCGACATGGGT R: CATTCTGGATCAGCAGGGG	93	55.6	99.5	0.967	
	<i>40S ribosomal protein S3</i>	<i>RPS3</i>	MK861153	F: CTTCGAACCTGGCCGTATTG R: AAAACACCATAGCAGGCACG	135	57.4	97.8	0.986	
	the DEGs	<i>heat shock proteins 60</i>	<i>Hsp60</i>	MK861146	F: TGCTAATCAACAACGCAAACC R: GTTGATTTTCGGTTGTCCCCA	147	53.7	98.9	0.956
		<i>sarcocystatin A-alpha</i>	<i>A-alpha</i>	MK861151	F: GTTGTCCCAGCGAAGTACAG R: TCACCAGCTGCTAACTTCGA	85	57.4	97.5	0.962
		<i>acidic ribosomal protein</i>	<i>ARP</i>	MK861152	F: AGTTGTCTCTATGCCCGTT R: TTCTTCCTTCTTGCCGGCAT	92	55.4	98.7	0.993
		<i>60S acidic ribosomal protein 8</i>	<i>RPL8</i>	MK861156	F: ATTTGAAGGGTGGCTTTGCCG R: TGGCTGTAGTTCACTTCCGT	121	55.4	99.2	0.972

^aF and R refer to forward and reverse primers, respectively;

^bRegression coefficient of the qPCR reaction

Table 2 Mean (±SD) development duration (h) of *S. dux* at seven constant temperatures.

Developmental stages	First-instar	Second-instar	Third-instar	Wandering	Pupariation	Total duration
16°C	62.6±2.3	63.6±1.5	119.3±1.2	81.3±3.1	1151.6±24.5	1478.6±18.3
19°C	46.1±2.0	42.6±4.6	67.3±5.0	51.3±4.2	518.6±16.1	726.1±15.8
22°C	40.1±2.3	24.8±2.8	48.1±4.8	44.6±3.1	380.6±3.4	538.5±0.9
25°C	30.3±2.9	20.16±3.4	29.3±4.6	35.6±4.7	279.6±8.5	394.1±9.5
28°C	27.1±4.3	19.3±4.2	26.1±5.1	34.3±5.8	267.6±4.0	375.6±10.8
31°C	21.1±4.1	17.6±5.9	21.6±4.9	26.3±4.0	197.3±6.4	284.1±7.3
34°C	18.3±4.9	14.1±5.5	18.6±3.8	24.6±5.2	176.6±4.2	252.5±6.1

Table 3. Mean (\pm SE) of developmental threshold temperatures (D_0) and thermal summation constants (K) for five developmental stages and the total development period of *S. dux*, and the coefficient of determination (R^2) of thermal summation models.

Developmental stages	K (degree hours)		D_0 ($^{\circ}$ C)		R^2
	Mean	SE	Mean	SE	
1st instar	499.27	32.25	10.4	0.8486	0.94177
2nd instar	314.38	24.56	11.11	0.73667	0.97419
3rd instar	397.3844	19.18	12.819	0.33134	0.99601
wandering	623.77	41.28	11.0477	0.8918	0.93058
pupa	3633.43	187.22	12.728	0.35379	0.99538
Total duration	5341.71	249.298	12.266	0.35538	0.9949

Table 4. Simulation equations, P values, degrees of freedom (df), and coefficients of determination (R^2) of the relationship between the body length (L) (mm) of *S. dux* larvae and the time after larviposition (T) (d) at seven constant temperatures.

Temperature ($^{\circ}$ C)	Equation	df	P	R^2
16	$L = 2.88187 - 0.07372T + 2.23133E-4T^2 - 1.38926E-6T^3 + 7.39917E-10T^4$	36	<0.001	0.99317
19	$L = 3.51402 - 3.15827E-4T + 0.00239T^2 - 1.48681E-5T^3 + 1.81862E-8T^4$	19	<0.001	0.99211
22	$L = 2.79057 - 0.07658T + 0.00178T^2 - 1.42788E-5T^3 + 1.62403E-8T^4$	16	<0.001	0.99891
25	$L = 3.75416 - 0.0496T + 0.00682T^2 - 6.90763E-5T^3 + 1.80623E-7T^4$	12	<0.001	0.99609
28	$L = 2.75538 - 0.01529T + 0.00753T^2 - 8.72071E-5T^3 + 2.01283E-7T^4$	8	<0.001	0.99609
31	$L = 2.9976 - 0.04967T + 0.01587T^2 - 2.54573E-4T^3 + 1.05944E-6T^4$	6	<0.001	0.99768
34	$L = 2.10532 - 0.07471T + 0.01761T^2 - 3.66136E-4T^3 + 1.90078E-6T^4$	5	<0.001	0.99268

Table 5. Simulation equations, F values, P values, and coefficient of determination (R^2) of the relationship between the overall differential gene expression level of *S. dux* pupae (y) (logFC) and the percentage (x) (%) of *S. dux* pupal development at all temperatures

Gene	Simulation equation	F	P	R^2
<i>hsp60</i>	$y = -0.23795 - 0.24969x + 0.01143x^2 - 1.97656E-4x^3 + 9.97241E-7x^4$	11.26125	<0.001	0.96613
<i>A-alpha</i>	$y = -0.68392 - 0.28431x + 0.00877x^2 - 7.93449E-5x^3 + 2.58888E-7x^4$	16.55372	<0.001	0.95274
<i>ARP</i>	$y = 0.53898 - 0.36275x + 0.01302x^2 - 1.34292E-4x^3 + 4.57392E-7x^4$	59.76327	<0.001	0.85917
<i>RPL8</i>	$y = 1.30288 - 0.58663x + 0.02512x^2 - 3.41269E-4x^3 + 1.44213E-6x^4$	30.67204	<0.001	0.8792

Table 6. Simulation equations, F values, P values, and coefficient of determination (R^2) of the relationship between the differential gene expression level of *S. dux* pupae (y) (Mean of logFC) and the ageing (x) (d) of *S. dux* pupae at different constant temperatures (34, 25 and 16 $^{\circ}$ C)

Gene	Temperature ($^{\circ}$ C)	Simulation equation	F	P	R^2
<i>Hsp60</i>	16	$y = -0.05753 - 0.50637x + 0.05349x^2 - 0.002x^3 + 2.14218E-5x^4$	1.99062	<0.001	0.98765
	25	$y = -0.24316 - 1.94192x + 0.67709x^2 - 0.09898x^3 + 0.00425x^4$	1.92938	<0.001	0.97419
	34	$y = -0.13562 - 3.05418x + 1.69969x^2 - 0.36752x^3 + 0.0232x^4$	1.06334	<0.001	0.97412
<i>A-alpha</i>	16	$y = -0.37123 - 0.46395x + 0.02757x^2 - 4.33067E-4x^3 + 2.54602E-6x^4$	5.65103	<0.001	0.96598
	25	$y = -0.19736 - 1.93418x + 0.46961x^2 - 0.02895x^3 + 5.59423E-4x^4$	2.65593	<0.001	0.96214
	34	$y = -0.43167 - 4.4724x + 2.11088x^2 - 0.29623x^3 + 0.0142x^4$	4.88929	<0.006	0.881
<i>ARP</i>	16	$y = 0.16863 - 0.50794x + 0.02986x^2 - 4.33602E-4x^3 + 1.87505E-6x^4$	10.99039	<0.001	0.93851
	25	$y = 0.38444 - 3.66669x + 1.26313x^2 - 0.12062x^3 + 0.00367x^4$	6.55433	<0.001	0.93545
	34	$y = 0.25255 - 5.69497x + 3.09229x^2 - 0.47139x^3 + 0.0229x^4$	2.75375	<0.001	0.93616
<i>RPL8</i>	16	$y = -0.88725 - 1.07948x + 0.09457x^2 - 0.00263x^3 + 2.26449E-5x^4$	12.54842	<0.001	0.98138
	25	$y = -0.66096 - 4.79975x + 1.84907x^2 - 0.21486x^3 + 0.00767x^4$	8.22417	<0.001	0.98248
	34	$y = -0.19148 - 5.52171x + 3.31988x^2 - 0.59173x^3 + 0.03223x^4$	1.39029	<0.002	0.98108

Figures

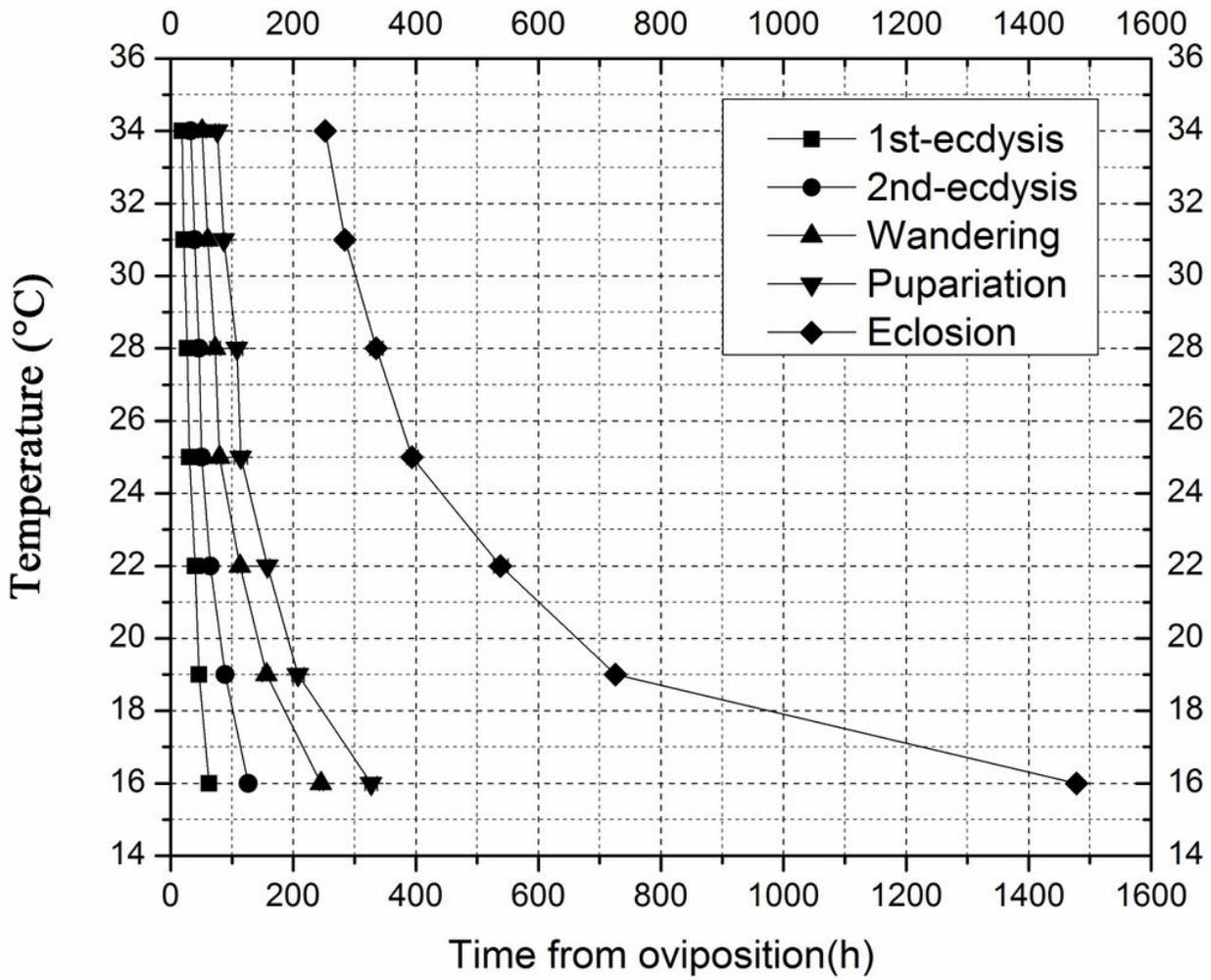


Figure 1

Isomorphen diagram of *Sarcophaga dux*. The duration of each development milestone (first-ecdysis, second-ecdysis, wandering, pupation, and eclosion) was plotted with the time from larviposition to the onset of each milestone. Each curve corresponds to a developmental milestone, and the error bar is the standard deviation of each milestone.

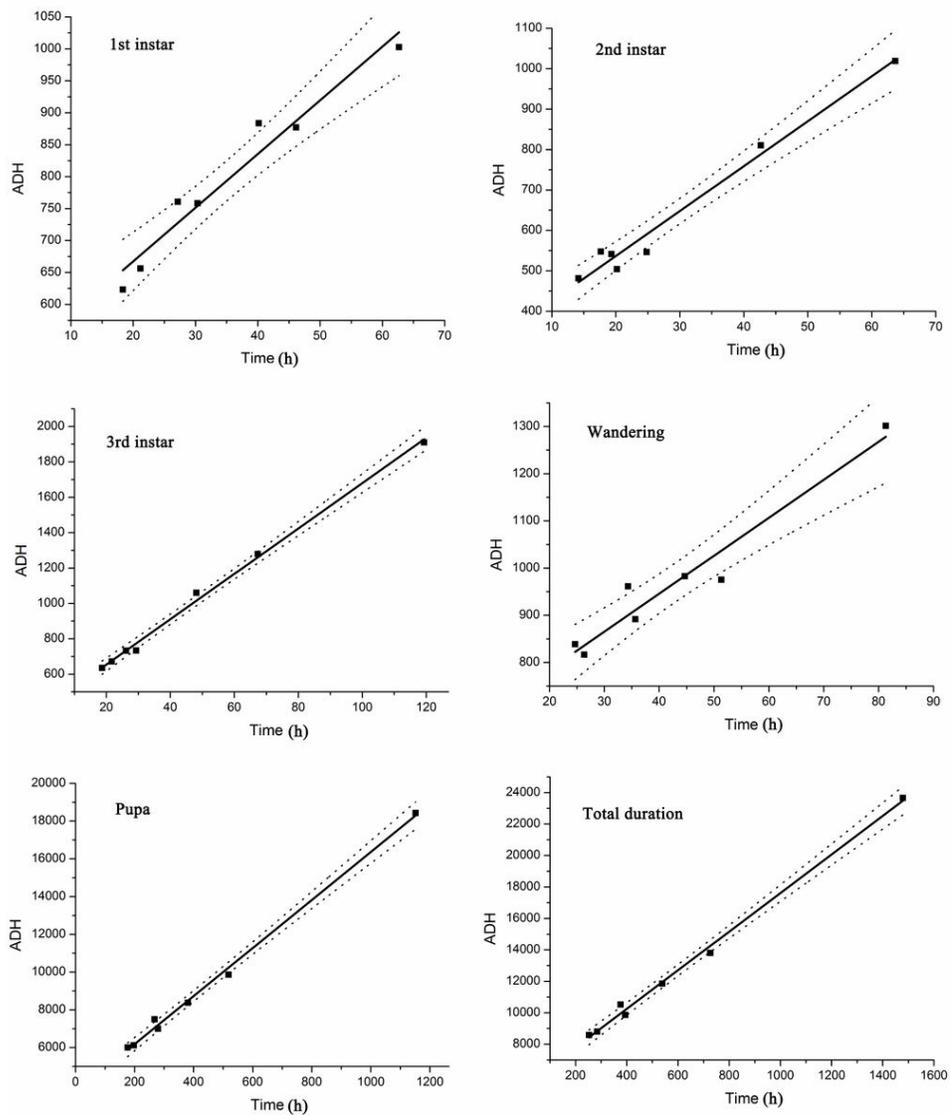


Figure 2

Thermal summation models of five developmental stages and total development duration of *Sarcophaga dux*, the solid line represents the regression line. Dashed line represents 95% confidence interval.

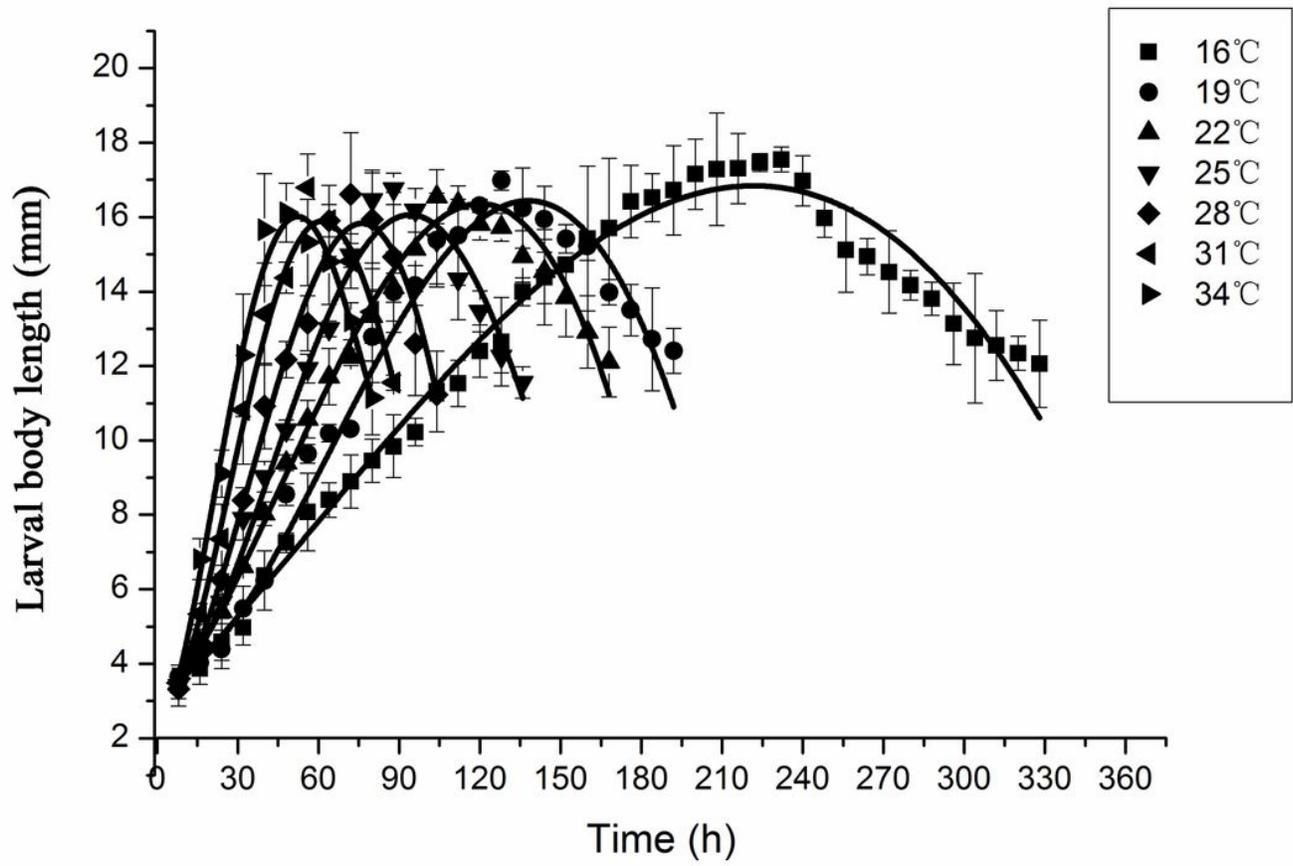


Figure 3

Changes in *Sarcophaga dux* larval body length (mm) over time (h) under different constant temperatures. The vertical bars represent the standard deviation.

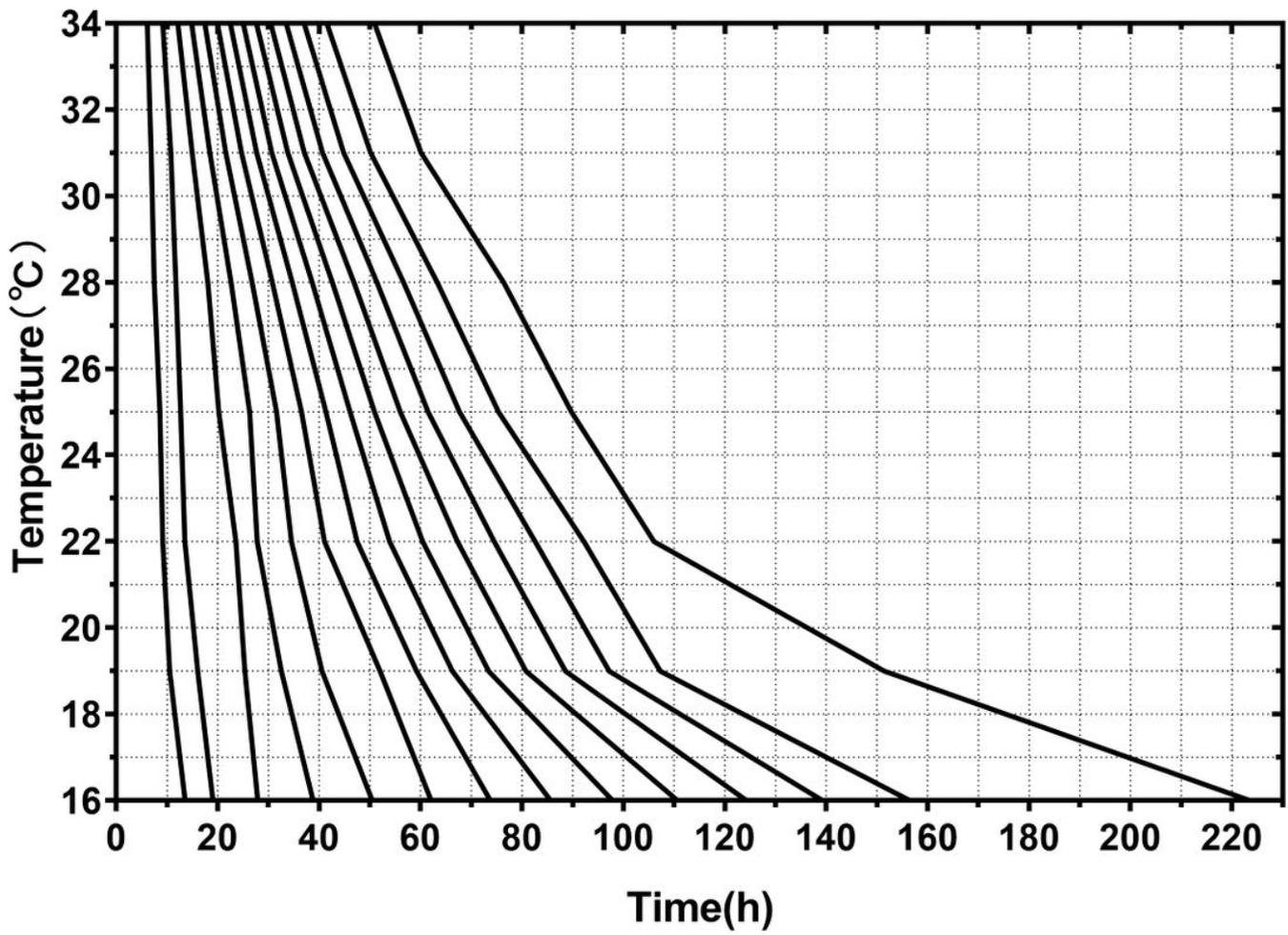


Figure 4

Isomegalen diagram of *Sarcophaga dux* larvae from larviposition to peak feeding stage. Time (h) is plotted against temperature (°C), where each line represents developmental larval length in mm 3–16, size indicated by number at the lower left of each contour.

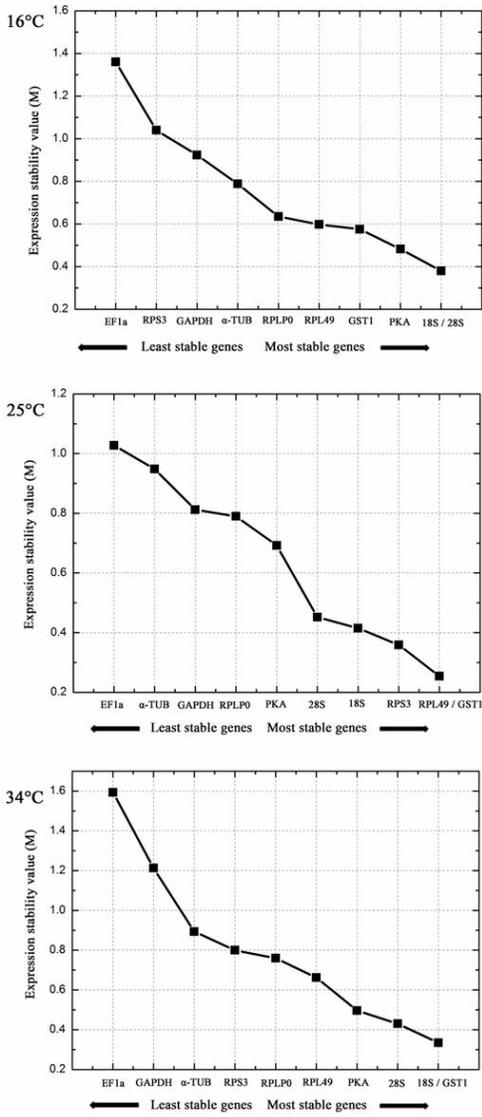


Figure 5

Stability of the candidate reference genes of *S. dux* Intra-puparial at different constant temperatures (34°C, 25°C and 16°C) calculated by geNorm. A lower expression stability measure (M) values indicates more stable expression.

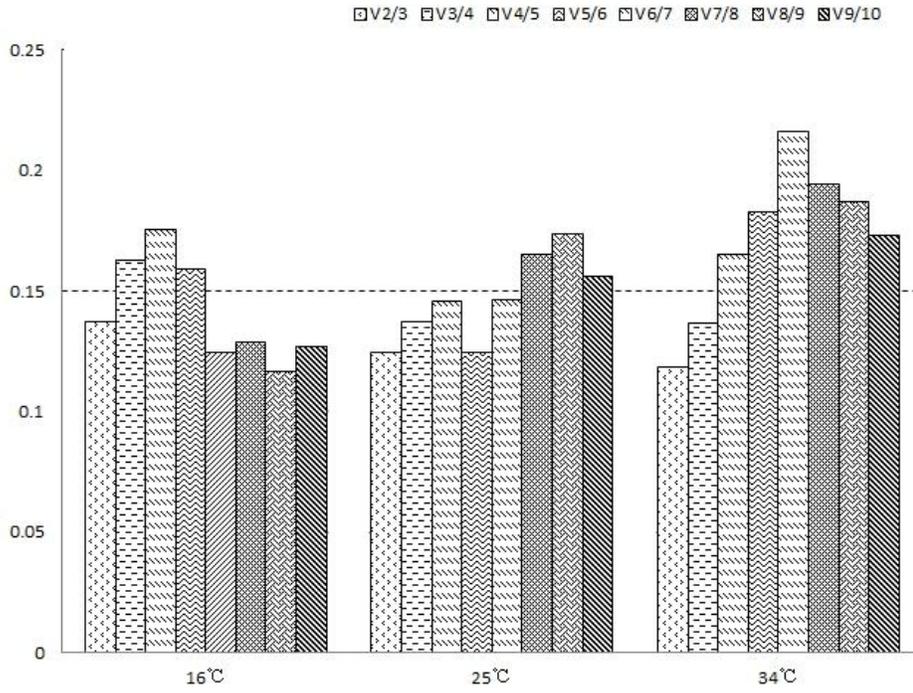


Figure 6

The optimal number of reference genes for normalization by geNorm analysis. Average pairwise variations (V) were calculated by geNorm between the normalization factors NFn and $NFn+1$ to indicate whether inclusion of an extra reference gene would add to the stability of the normalization factor. Values < 0.15 indicate that additional genes are not required for the normalization of gene expression.

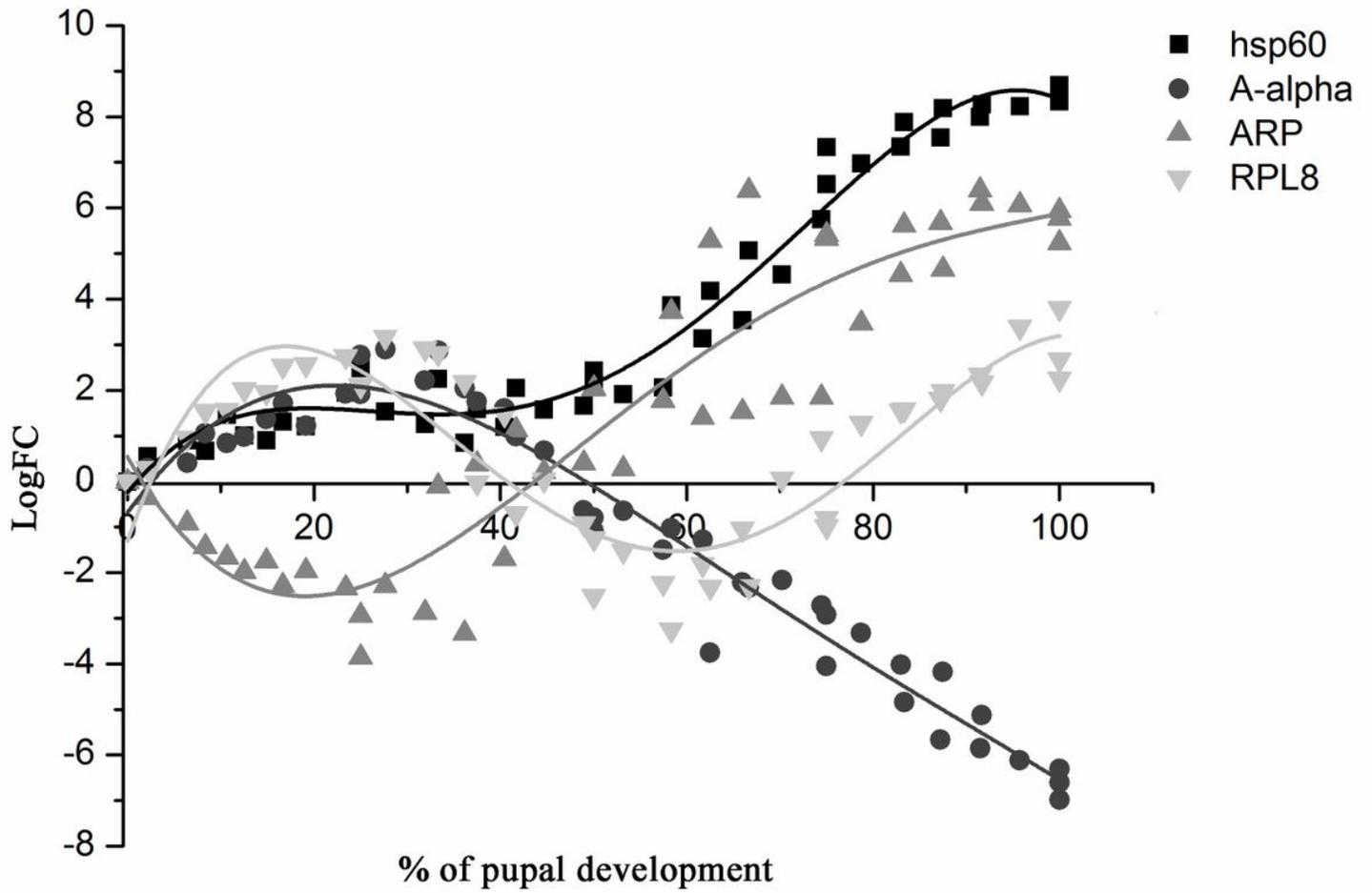


Figure 7

The overall differential gene expression levels of *S. dux* Intra-pupal. LogFC values of four DEGs on three temperatures were averaged and plotted against the percentage of Intra-pupal development. The data for regression were fit using the fourth-order polynomials. The dots represent the mean values.

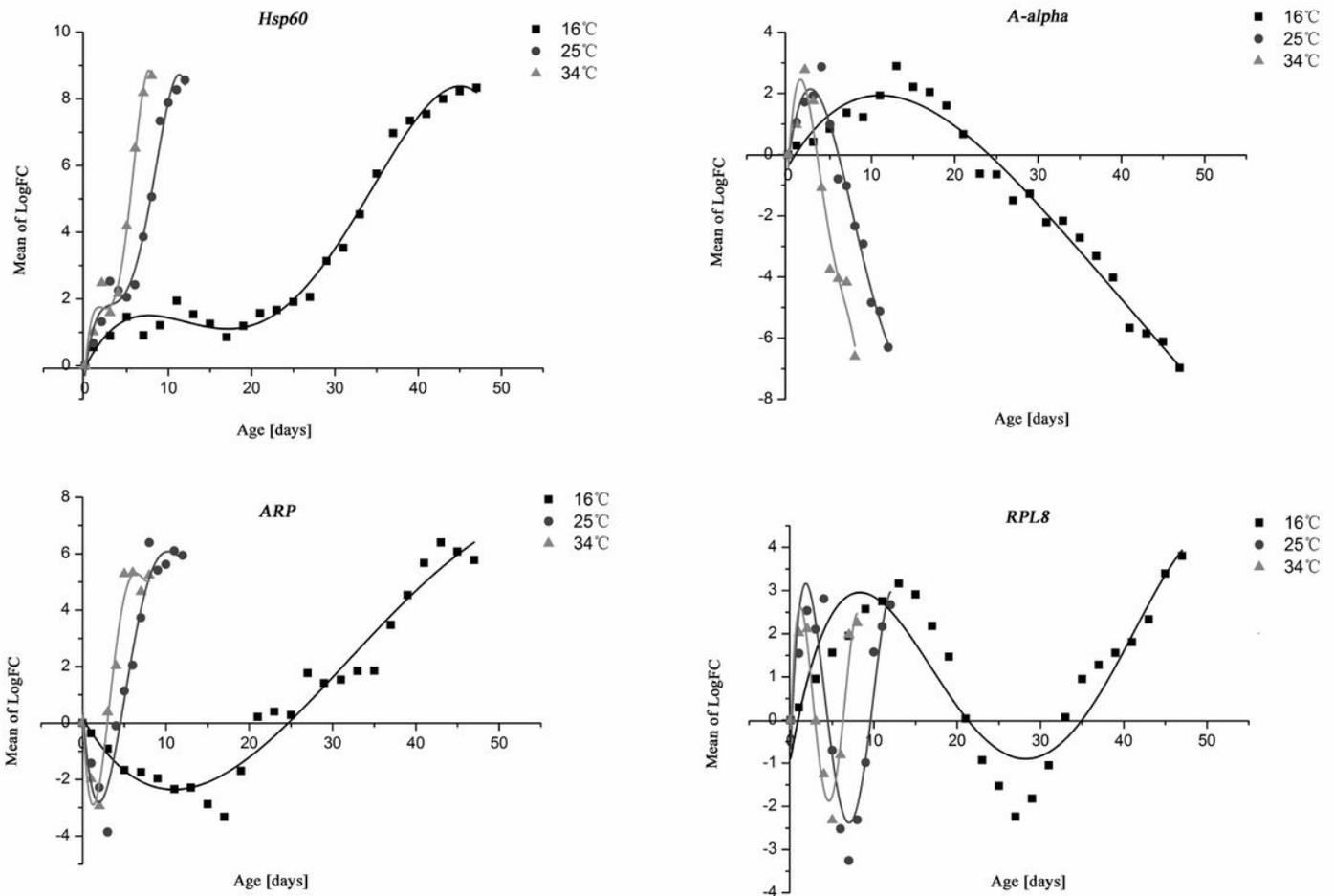


Figure 8

The detailed interaction plots of gene expression against Intra-pupariial age (days) at three constant temperatures (34°C, 25°C and 16°C). For each temperature-dependent experiment, LogFC values were averaged. The data for regression were fit using fourth-order polynomials.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMaterial1.xlsx](#)
- [SupplementaryMaterial2.xlsx](#)