A biological timer in the fat body comprising Blimp-1, βFtz-f1 and Shade regulates pupation timing in *Drosophila melanogaster*

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**ABSTRACT**

During the development of multicellular organisms, many events occur with precise timing. In *Drosophila melanogaster*, pupation occurs about 12 h after puparium formation and its timing is believed to be determined by the release of a steroid hormone, ecdysone (E), from the prothoracic gland. Here, we demonstrate that the ecdysone-20-monoxygenase Shade determines pupation timing by converting E to 20-hydroxyecdysone (20E) in the fat body, which is the organ that senses nutritional status. The timing of *shade* expression is determined by its transcriptional activator βFtz-f1. The βFtz-f1 gene is activated after a decline in the expression of its transcriptional repressor Blimp-1, which is temporally expressed around puparium formation in response to a high titer of 20E. The expression level and stability of Blimp-1 is critical for the precise timing of pupation. Thus, we propose that Blimp-1 molecules function like sand in an hourglass in this precise developmental timer system. Furthermore, our data suggest that a biological advantage results from both the use of a transcriptional repressor for time determination and the association of developmental timing with nutritional status of the organism.

**KEY WORDS:** Biological timer, Developmental timing, Ecdysone, Metamorphosis, *Drosophila*

**INTRODUCTION**

Multicellular organisms coordinate the timing of biological events during their development and throughout their life. The most characterized systems to determine biological timing are the circadian clock (Allada and Chung, 2010) and clock-like systems (Saga, 2012). Along with the clock systems, the biological timer has been proposed to maintain a precise time period between two biological events. Although a few instances of the existence of such a timer are known (Suzuki et al., 2013), little is known about the molecular mechanisms of this biological timer, including responsive cells or organs.

During the development of insects, a pulse-release of the molting hormone ecdysone (E) from the prothoracic gland occurs several times. The E released to hemolymph is converted to 20-hydroxyecdysone (20E) in the fat body, which is the organ that senses nutritional status. The second pulse release is expected to induce pupation, the transition from a prepupa to a pupa. The transformed larva is called a prepupa at this stage. The ecdysteroid titer drops to a low level ∼2–3 h after puparium formation (APF) and it rises again for a short period at ∼10 h APF at standard rearing conditions of 25°C. This second pulse release is expected to induce pupation, the transition from a prepupa to a pupa, consistently at ∼12 h APF (Thummel, 1996), suggesting that the fly has a biological timer to measure the time until pupation.

Many transcription factors are induced and temporally expressed due to 20E during this period. Among these transcription factors, orphan nuclear receptor βFtz-f1 (Lavorgna et al., 1991; Ueda et al., 1990) is expressed after pulse exposure of 20E in the mid to late prepupal stage in almost all organs and is essential for pupation (Yamada et al., 2000). A Krüppel-type zinc finger factor Blimp-1 was found to bind the promoter of the βFtz-f1 gene (Agawa et al., 2007; Kageyama et al., 1997). The *Blimp-1* transcript is induced by 20E directly at the end of larval period and disappears soon after decline of ecdysteroid level during the early prepupal period (Agawa et al., 2007; Akagi and Ueda, 2011). Blimp-1 protein works as a repressor for the βFtz-f1 gene and contributes to determination of the expression timing of βFtz-f1 and therefore pupation timing (Agawa et al., 2007). Furthermore, ubiquitous knockdown of *Blimp-1* results in a failure to pupate (Agawa et al., 2007). These results indicate that Blimp-1 is necessary for pupation and plays an essential role to determine pupation timing. However, the mechanism of this time-measuring system is not yet understood.

Here, we describe the molecular mechanism of a precise biological timer that determines pupation timing and specify the essential role to determine pupation timing. However, the mechanism of this time-measuring system is not yet understood.

**RESULTS**

**βFtz-f1 expression determines pupation timing**

Since we have shown that the timing of both pupation and βFtz-f1 expression are delayed by prolonged expression of Blimp-1 during the prepupal stage (Agawa et al., 2007), we examined the relationship between βFtz-f1 expression and pupation timing by inducing βFtz-f1 at different times during the late prepupal period.
using the heat-inducible βftz-f1 transgenic fly line (hs-βFTZ-F1) (Murata et al., 1996). Pupation timing was significantly advanced by approximately 30 min in hs-βFTZ-F1 prepupae compared with that of the control host line when heat shock was received at 6 h APF (Fig. 1A). No significant advancement of pupation was observed when the animals were exposed to heat shock at 7 h APF or later time points (Fig. 1A and Fig. S1). To further analyze the importance of the expression timing of βftz-f1 for pupation timing, we induced βftz-f1 at various times using hs-βFTZ-F1 in the transheterozygote ftz-f1 mutant background (ftz-f1<sup>tx1</sup>/ftz-f1<sup>Cyo160</sup>), which cannot transit to the pupal stage without βftz-f1 induction (Yamada et al., 2000). None of the prepupae pupated when βftz-f1 was induced at 4 or 5 h APF. However, 75 or 100% animals were able to become pupa at 12.3 or 12.6 h APF on average, when βftz-f1 was induced at 6 or 7 h APF, respectively (Fig. 1B), which is consistent with the timing of endogenous βftz-f1 expression (Yamada et al., 2000). Furthermore, pupation timing was delayed when the induction of βftz-f1 occurred at 8 h APF or later (Fig. 1B). The length of the delay depended on the timing of the heat shock. These results indicate that the timing of βftz-f1 expression contributes to the timing of pupation. We also observed the best pupation efficiency when βftz-f1 was induced at 7 h APF; however, the efficiency was reduced depending on advanced or delayed induction of βftz-f1; no animals pupated when heat shocked at 4, 5 or 16 h APF. This observation suggests that other temporal factor(s) contribute to determine the appropriate timing for pupation.

**Timing of βftz-f1 expression determines the timing of the 20E pulse at the end of the prepupal period**

To examine the possibility that the timing of βftz-f1 expression determines the timing of the ecysteroid pulse which induces pupation (Thummel, 1996), we monitored the ecysteroid pulse by detecting expression of E75A (Eip75B–FlyBase), which is a known 20E inducible early gene. The expression of E75A was delayed about 2 h upon repress of βftz-f1 expression via ectopic expression of hs-Blimp-1 at 5 h APF (Fig. 2A) (Agawa et al., 2007). This result suggests that the timing of 20E production is determined by the timing of βftz-f1 expression. To confirm the importance of the timing of the small ecysteroid pulse at 10 h APF on pupation, we compared the onset of pupation in prepupae after injecting E or 20E at 8 h APF, prior to the endogenous ecysteroid pulse. Precocious pupation was observed in 20E-injected prepupae, but E injection caused no significant change in the onset of pupation when compared with control Ringer-injected prepupae (Fig. 2B). This result suggests that pupation timing is determined specifically by 20E, not by E.

E is converted to 20E by the E-20-monoxygenase Shade (Petryk et al., 2003). Thus, we next examined whether the expression timing of shade plays any role in the determination of the pupation timing. RT-PCR revealed that shade transcript expression decreased after puparium formation but began to increase at 8 h APF, reaching peak levels at 9 and 10 h APF (Fig. 2C). The induction of shade transcripts was abolished by ftz-f1 knockdown in hs-FTZ-F1 RNAi (Lam and Thummel, 2000) prepupae, but the expression level of shade was increased from 9 h APF in the control host line with the same heat treatment (Fig. 2D). Additionally, we observed premature expression of shade at 8 h APF by precocious induction of βftz-f1 by heat shock for 1 h at 5 h APF using the hs-βFTZ-F1 line (Fig. 2E). Together, we conclude that the expression timing of βftz-f1 determines the pupation timing through induction of Shade, which produces 20E.

**The fat body is a crucial organ for the biological timer to determine pupation timing**

We further examined which organs were responsible for this pupation timing determination pathway. Since Shade is known to be mainly expressed in the fat body (Petryk et al., 2003), which is a key tissue for post-feeding growth (Okamoto et al., 2009; Slaidina et al., 2009), we examined importance of the fat body for pupation time determination using the GALA/UAS system. Pupation was advanced by 0.5 h or delayed by 0.3 h on average, with fat body-specific shade overexpression and knockdown prepupae, respectively (Fig. 3A). Slight but significant advancement of pupation was also observed when shade was ectopically induced in other tissues, including neurons, muscle or epidermis; however, delay of pupation was not observed by induction of shade RNAi (Fig. S2). These results indicate that the expression of Shade in the fat body is important to determine pupation timing, although 20E produced in multiple organs can be sufficient for pupation if Shade is expressed at a high enough level. Next, βftz-f1 or Blimp-1 expression was manipulated in the fat body to determine their effects on fat body-specific shade induction. In order to avoid the detrimental developmental effects of gene manipulation prior to the prepupal period, we harnessed the temperature-sensitive Gal4 suppressor tub-Gal80ts. Larvae were cultured at 18°C until puparium formation, and then the culture temperature was shifted to 29°C to allow Gal4 expression only after puparium formation. Pupation timing was advanced by

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**Fig. 1. Timing of βftz-f1 expression determines pupation timing.** (A) Pupation timing was observed every 30 min after induction of βftz-f1. Heat shock was given at 33.5°C for 1 h from 6 or 9 h APF in prepupae of yw; hs-βFTZ-F1/+ and yw. (B) Pupation timing was observed every 60 min after induction of βftz-f1 in ftz-f1 mutant background. Heat shock was given at 33.5°C for 1 h at various times in prepupae of ftz-f1<sup>tx1</sup>/ftz-f1<sup>Cyo160</sup>. *P<0.05, **P<0.01 by K-S test, versus the yw control (A) and prepupae heat shocked at 7 h APF (B).
0.4 h on average in prepupae with fat body-specific \( \beta \text{ftz-f1} \) overexpression. In parallel, fat body-specific \( \beta \text{ftz-f1} \)-knockdown prepupae showed delayed pupation for 0.8 h compared with control prepupae, in addition to reduction of pupation efficiency (Fig. 3B). Similarly, fat bodies overexpressing Blimp-1 showed delayed pupation for 0.9 h and reduction of pupation efficiency, and Blimp-1 knockdown elicited an advance in pupation by 0.6 h compared with control prepupae (Fig. 3C). We did not observe this difference in pupation timing using other tissue-specific (i.e. epidermis, muscle, glia or neurons) \( \beta \text{ftz-f1} \) overexpression or knockdown lines (Fig. S3). These results indicate that the fat body is a crucial organ for the identified pupation-timing determination pathway and this pathway works as a biological timer, because both advanced and delayed pupation were induced by overexpression or knockdown of each component. In addition, increased lethality due to delayed pupation highlights the importance of this system.

**Blimp-1 stability and expression are important factors for the accurate time determination of pupation**

Blimp-1 has PEST-like sequences, which are known to modulate protein degradation, located in the middle part of the Blimp-1 protein. These sequences are consistent with the finding that Blimp-1 protein is unstable and disappears quite rapidly, which we have previously reported (Agawa et al., 2007). Thus, we established a transgenic fly line, \( hs \text{-Blimp-1APEST} \), which is able to express stable Blimp-1 upon heat treatment. While intact Blimp-1 (\( hs \text{-Blimp-1} \)) was detected at a high level until 2 h after the heat shock, truncated Blimp-1 (\( hs \text{-Blimp-1APEST} \)) was detectable for at least 6 h after the same heat treatment, indicating that the deleted region is needed for the rapid degradation of the Blimp-1 protein (Fig. 4A,B). We also confirmed that the truncated form of Blimp-1 is able to work as a repressor for \( \beta \text{ftz-f1} \). Whereas \( \beta \text{ftz-f1} \) expression was delayed about 2 h in intact Blimp-1 induced prepupae compared with the control at 5 h APF, \( \beta \text{ftz-f1} \) expression was further delayed by about 1 h in the
suggest that stability of the Blimp-1 protein is an important factor in the determination of pupation timing and that the determination mechanism of pupation is crucial for animal survival. To address the biological meaning of the instability of Blimp-1, we examined the accuracy of the pupation timing by comparing pupation timing elicited by intact and truncated Blimp-1 inductions. Prepupae induced with intact Blimp-1 showed a small window in the dispersion of pupation timing; however, truncated Blimp-1 caused a significantly wider window in the onset of pupation (Fig. 4D). These results suggest that rapid degradation of Blimp-1 due to the function of its PEST-like sequence contributes to the precise time measurement required to induce pupation.

Next, we examined whether not only the stability but also the expression level of Blimp-1 contributes to pupation timing. We used Blimp-1 mutant lines, which are homozygous lethal as they carry a P-element insertion in the first intron of the gene, in order to examine the effect of the Blimp-1 expression level on pupation timing. Precocious pupation was observed in the prepupae from two independent Blimp-1 heterozygous mutant lines, Blimp-1P14751/+ (Agawa et al., 2007) and Blimp-1P09553/+ (Fig. 4E and Fig. S4A). Furthermore, the observed precocious pupation was recovered in the prepupae of the revertant lines, Blimp-1P14751AP1/+ and Blimp-1P14751AP2/+ , which are obtained from Blimp-1P14751 by P-element excision (Fig. 4E and Fig. S4A). To see the effect of Blimp-1 gene dose on the expression timing of the Ftz-f1 gene, we examined the expression pattern of βFtz-f1 in the heterozygous Blimp-1P14751 mutant and its revertant Blimp-1P14751AP1/. βFtz-f1 expression was advanced about 0.5 h in the Blimp-1P14751 heterozygous mutant prepupae compared with the control line and the timing of βFtz-f1 expression was restored in the revertant prepupae (Fig. S4B). The importance of the Blimp-1 expression level was also indicated by induction of Blimp-1 from different copy numbers of the hs-Blimp-1ΔPEST transgene (Fig. S4C). These results indicate that the expression level of Blimp-1 is an important factor for the determination of pupation timing through the control of timing of βFtz-f1 expression.

**DISCUSSION**

Here, we show that the gene regulatory pathway consisting of Blimp-1, βFtz-f1 and shade works as a biological timer to measure a specific period during the prepupal period (Fig. 5). Our results suggest that a biological advantage results from use of a transcriptional repressor for the precise timer system. The timing of gene expression could be determined by either induction of its transcriptional activator or depletion of its transcriptional repressor. Accuracy of the induction timing of a gene depends on the rate of accumulation and depletion of its transcriptional repressor. The timing of gene expression could be determined by either induction of its transcriptional activator or depletion of its transcriptional repressor. Accuracy of the induction timing of a gene depends on the rate of accumulation and depletion of its transcriptional repressor. The timing of gene expression could be determined by either induction of its transcriptional activator or depletion of its transcriptional repressor. Accuracy of the induction timing of a gene depends on the rate of accumulation and depletion of its transcriptional repressor.
et al., 2014), suggesting the conserved importance of the degradation of Blimp-1 for the timing decision during the development.

We found that the pupation timer is composed of Blimp-1, βftz-f1 and shade; however, the delays in pupation by induction of Blimp-1 or knockdown of either βftz-f1 or shade were different (Fig. 3). The difference may be caused by the expression levels of each transgene, including the RNAi efficiency and the minimum requirement of each protein for timing determination. Other organs may also contribute to this pathway, because relatively low levels of shade expression are detected in the midgut and the Malpighian tubules in addition to the fat body (Petryk et al., 2003). This multi-organ contribution could be one of the reasons that fat body-specific knockdown of shade did not cause lethality (Fig. 3A).

Although we found that the identified timer system is crucial to determine pupation timing, several observations reveal that pupation timing is restricted only to a specific period (Fig. 1B, Fig. 2B, Fig. 3B,C and Fig. 4D), suggesting that other factor(s) provide competence for pupation. Ecdysis triggering hormone (ETH), a peptide hormone that regulates the pupation behavior, is potentially one of these factors, as βFtz-f1 is essential for release of ETH from the inka cells (Cho et al., 2014). The timing of ETH production, which is triggered by prothoracicotropic hormone (PTTH) (McBrayer et al., 2007), is an important factor for pupation; we observed that pupation timing was delayed for several hours (data not shown) in prepupae where PTTH-producing neurons are ablated. However, neither ectopic Blimp-1 induction nor βftz-f1 knockdown affected the expression of either ptth or eth transcripts (Fig. S5). It has been reported that the chromatin remodeling protein INO80 has an effect on the pupation timing by regulating the regression of ecdysone-regulated genes including βftz-f1 (Neuman et al., 2014). These results suggest the presence of other mechanisms, acting independently of our identified timer system, to restrict pupation timing.

We identified the fat body as an essential tissue necessary to drive this developmental timer system. Several reports have shown a link between nutrient status and developmental timing, and it has been suggested that the fat body is the central tissue for coordinating this link (Colombani et al., 2003; Géminard et al., 2009; Rewitz et al., 2013). Thus, we expect that the fat body incorporates the nutritional status of the animal and sends a cue for the final decision of pupation independent of the timer system.

A recent publication proposed that shade gene expression could be finely tuned by acetylation of Ftz-f1 (Borsos et al., 2015). This result supports the idea that βFtz-f1 directly regulates the shade gene. On the other hand, βFtz-f1 is expressed after decline of 20E level in almost all organs at the late embryonic stage and each larval
and pupal stage during development (Sultan et al., 2014; Yamada et al., 2000), but the upregulation of shade is limited around the high ecdysteroid period for puparium formation (Fig. 2C). These results suggest that the activation of the shade gene by βFtz-f1 is restricted only in the prepupal period. Further studies are needed to understand the regulation mechanism of the shade gene, including epigenetic regulation of the time-measuring mechanism.

In this transcriptional cascade, the mechanism for initiating the timer is crucial to drive the system. The regulatory mechanism required to determine the period of ecdysone pulse, which induces puparium formation, has been unveiled recently at the molecular level (Moeller et al., 2013; Rewitz et al., 2013). This system potentially works as a switch for the Blimp-1 timer to determine the specific period after the decline of the 20E level.

**MATERIALS AND METHODS**

**Fly culture and stocks**

Flies were raised at 25°C on 10% glucose, 8% cornmeal, 4% yeast extracts and 0.7% agar medium containing propionic acid and butyl-p-hydroxybenzoate as antifungal agents. hs-FFI-24 obtained from Dr. Carl S. Thummel (University of Utah, UT, USA) was used for hs-ftz-f1 RNAi line; ppl-Gal4 was obtained from Dr. Masayuki Miura (University of Tokyo, Tokyo, Japan); UAS-dicer; Cg-Gal4, Repo-Gal4, Mef-Gal4 and C855a-Gal4 were obtained from Dr. Naoki Okamoto (University of California, Riverside, CA, USA); and Gal80p, elav-Gal4 and 24B-Gal4 lines were obtained from Dr. Hideki Nakagoshi (Okayama University, Okayama, Japan). UAS-shd lines were obtained from Dr. Hajime Ono (Kyoto University, Kyoto, Japan) and UAS-shd-RNAi lines were obtained from National Institute of Genetics in Japan. Double homzygotic line of UAS-shd-RNAi-1 and UAS-shd-RNAi-2 was used for Fig. 3A in order to increase RNAi efficiency.

**Establishment of transgenic flies**

For UAS-Blimp-1 fly lines, a 3.1 kb EcoRI and Not fragment obtained during construction of hs-Blimp-1 gene (Agawa et al., 2007) was inserted between EcoRI and Not sites of pUAST vector. For UAS-βftz-f1 fly lines, a 2.5 kb EcoRI fragment obtained during construction of hs-βftz-f1 gene (Murata et al., 1996) was inserted into pUAST vector using the EcoRI site. Transgenic fly lines were established as previously described (Murata et al., 1996) using embryos of y′Df(1)w674 (yw) as the host strain.

**Measurement of the prepupal period**

Newly formed white prepupae were picked up every 10, 30 or 60 min and transferred to a plastic dish with moist paper. Pupation timing was observed every 10, 30 or 60 min. The Kolmogorov–Smirnov test (KS-test) was used to evaluate differences between pupation timings and determine P values. The F-test was used to evaluate the variance of pupation timings. We used the Prism software package (GraphPad Software) to carry out statistical analysis.

**Detection and quantification of transcripts**

For semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), total RNA was extracted from five animals at each time point using the NucleoSpin RNA Kit (TaKaRa). cDNAs were synthesized from the RNA using oligo(dT)15 primer (Novagen) and ReverTra Ace (Toyobo). PCR was performed in three independent biological replicates using OneTag polymerase (New England Biolabs). The following synthetic oligonucleotides were used for detecting transcripts: Blimp-1-F: 5'-GGCA-CTCTCAGAAGACATCATA; E75A-F: 5'-AGCGCAGGACAAATG, E75A-R: 5'-ACCCAGTGGGTGCAGAT; shade-F: 5'-GATGACGAGGCTGCTGGATTAC, shade-R: 5'-AGACCCGGGATCTCCCAGTAAC; ptth-F: 5'-TGAAGGATCTGGTGACCACCA; ptth-R: 5'-TTCCAGTTGCGCTCAATTGGATCC; eth-F: 5'-AGGGCAAACTTTGCCATAA, eth-R: 5'-ACCACGTTAAA- GTTCTGCTC; rp49-F: 5'-CACCAGTGCGATCATG, rp49-R: 5'-CACGTTGTGCACCCAGAAC. The reaction products were resolved on 8% Tris/borate/ethylenediaminetetraacetic acid (TBE) polyacrylamide gels and the amounts of the PCR products were quantified by LAS-4000mini Ethylenediaminetetraacetic acid (TBE) polyacrylamide gels and the amounts of the PCR products were quantified by LAS-4000mini. For semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), total RNA was extracted from five animals at each time point using the NucleoSpin RNA Kit (TaKaRa). cDNAs were synthesized from the RNA using oligo(dT)15 primer (Novagen) and ReverTra Ace (Toyobo). PCR was performed in three independent biological replicates using OneTag polymerase (New England Biolabs). The following synthetic oligonucleotides were used for detecting transcripts: Blimp-1-F: 5'-GGCA-CTCTCAGAAGACATCATA; E75A-F: 5'-AGCGCAGGACAAATG, E75A-R: 5'-ACCCAGTGGGTGCAGAT; shade-F: 5'-GATGACGAGGCTGCTGGATTAC, shade-R: 5'-AGACCCGGGATCTCCCAGTAAC; ptth-F: 5'-TGAAGGATCTGGTGACCACCA; ptth-R: 5'-TTCCAGTTGCGCTCAATTGGATCC; eth-F: 5'-AGGGCAAACTTTGCCATAA, eth-R: 5'-ACCACGTTAAA-GTTCTGCTC; rp49-F: 5'-CACCAGTGCGATCATG, rp49-R: 5'-CACGTTGTGCACCCAGAAC. The reaction products were resolved on 8% Tris/borate/ethylenediaminetetraacetic acid (TBE) polyacrylamide gels and the amounts of the PCR products were quantified by LAS-4000mini. For semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), total RNA was extracted from five animals at each time point using the NucleoSpin RNA Kit (TaKaRa). cDNAs were synthesized from the RNA using oligo(dT)15 primer (Novagen) and ReverTra Ace (Toyobo). PCR was performed in three independent biological replicates using OneTag polymerase (New England Biolabs). The following synthetic oligonucleotides were used for detecting transcripts: Blimp-1-F: 5'-GGCA-CTCTCAGAAGACATCATA; E75A-F: 5'-AGCGCAGGACAAATG, E75A-R: 5'-ACCCAGTGGGTGCAGAT; shade-F: 5'-GATGACGAGGCTGCTGGATTAC, shade-R: 5'-AGACCCGGGATCTCCCAGTAAC; ptth-F: 5'-TGAAGGATCTGGTGACCACCA; ptth-R: 5'-TTCCAGTTGCGCTCAATTGGATCC; eth-F: 5'-AGGGCAAACTTTGCCATAA, eth-R: 5'-ACCACGTTAAA-GTTCTGCTC; rp49-F: 5'-CACCAGTGCGATCATG, rp49-R: 5'-CACGTTGTGCACCCAGAAC. The reaction products were resolved on 8% Tris/borate/ethylenediaminetetraacetic acid (TBE) polyacrylamide gels and the amounts of the PCR products were quantified by LAS-4000mini. For semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), total RNA was extracted from five animals at each time point using the NucleoSpin RNA Kit (TaKaRa). cDNAs were synthesized from the RNA using oligo(dT)15 primer (Novagen) and ReverTra Ace (Toyobo). PCR was performed in three independent biological replicates using OneTag polymerase (New England Biolabs). The following synthetic oligonucleotides were used for detecting transcripts: Blimp-1-F: 5'-GGCA-CTCTCAGAAGACATCATA; E75A-F: 5'-AGCGCAGGACAAATG, E75A-R: 5'-ACCCAGTGGGTGCAGAT; shade-F: 5'-GATGACGAGGCTGCTGGATTAC, shade-R: 5'-AGACCCGGGATCTCCCAGTAAC; ptth-F: 5'-TGAAGGATCTGGTGACCACCA; ptth-R: 5'-TTCCAGTTGCGCTCAATTGGATCC; eth-F: 5'-AGGGCAAACTTTGCCATAA, eth-R: 5'-ACCACGTTAAA-GTTCTGCTC; rp49-F: 5'-CACCAGTGCGATCATG, rp49-R: 5'-CACGTTGTGCACCCAGAAC.
Competing interests
The authors declare no competing or financial interests.

Author contributions

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