Introduction

The number of larval instars in insects varies greatly across insect taxa, and can even vary at the intraspecific level [1,2,3]. In general, phylogenetically higher insects tend to have fewer larval instars (three to eight) compared to species from basal lineages, such as Ephemeroptera, Odonata and Plecoptera (more than ten) [1,2,3]. In many species, the number of larval instars is affected by genetic and environmental factors, such as temperature, nutritional conditions, photoperiod, humidity, injuries, and sex [1,2]. The variation in the number of larval instars in the insect lifecycle is generally considered to be an adaptive response to diverse environmental conditions in order to ensure the attainment of a threshold-size for metamorphosis [1,2,3,4].

The silkworm Bombyx mori, a classic model organism for endocrinology, has been reared by humans for thousands of years, and more than 1,000 strains are currently maintained [5,6,7]. Among these, several “moltinism” strains have been identified that exhibit variations in the number of larval molts; however, none of them have been characterized molecularly. Here we report the identification and characterization of the gene responsible for the moltinism (mod) mutation that causes precocious metamorphosis in this model insect will lead to a greater understanding of the molecular basis of the hormonal control of development and metamorphosis.

Abstract

Insect molting and metamorphosis are intricately governed by two hormones, ecdysteroids and juvenile hormones (JHs). JHs prevent precocious metamorphosis and allow the larva to undergo multiple rounds of molting until it attains the proper size for metamorphosis. In the silkworm, Bombyx mori, several “moltinism” mutations have been identified that exhibit variations in the number of larval molts; however, none of them have been characterized molecularly. Here we report the identification and characterization of the gene responsible for the dimolting (mod) mutant that undergoes precocious metamorphosis with fewer larval–pupal molts. We show that the mod mutation results in complete loss of JHs in the larval hemolymph and that the mutant phenotype can be rescued by topical application of a JH analog. We performed positional cloning of mod and found a null mutation in the cytochrome P450 gene CYP15C1 in the mod allele. We also demonstrated that CYP15C1 is specifically expressed in the corpus allatum, an endocrine organ that synthesizes and secretes JHs. Furthermore, a biochemical experiment showed that CYP15C1 epoxidizes farnesoic acid to JH acid in a highly stereospecific manner. Precocious metamorphosis of mod larvae was rescued when the wild-type allele of CYP15C1 was expressed in transgenic mod larvae using the GAL4/UAS system. Our data therefore reveal that CYP15C1 is the gene responsible for the mod mutation and is essential for JH biosynthesis. Remarkably, precocious larval-pupal transition in mod larvae does not occur in the first or second instar, suggesting that authentic epoxidized JHs are not essential in very young larvae of B. mori. Our identification of a JH-deficient mutant in this model insect will lead to a greater understanding of the molecular basis of the hormonal control of development and metamorphosis.

Citation: Daimon T, Kozaki T, Niwa R, Kobayashi I, Furuta K, et al. (2012) Precocious Metamorphosis in the Juvenile Hormone–Deficient Mutant of the Silkworm, Bombyx mori. PLoS Genet 8(3): e1002486. doi:10.1371/journal.pgen.1002486

Editor: David L. Stern, Janelia Farm Research Campus, Howard Hughes Medical Institute, United States of America

Received October 10, 2011; Accepted December 1, 2011; Published March 8, 2012

Copyright: © 2012 Daimon et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by Grants-in-Aid for Scientific Research (Nos. 20688003, 22128004, and 23688008) and by Special Coordination Funds for Promoting Science and Technology from MEXT, the Program for Promotion of Basic Research Activities for Innovative Biosciences (PRO-BRAIN), MAFF-NIAS (Agrigenome Research Program), JST (Professional Program for Agricultural Bioinformatics), and National Bioresource Project, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: shinoda@affrc.go.jp
* Current address: Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Matsue, Japan
The number of larval instars in insects varies greatly across insect taxa and can even vary at the intraspecific level. However, little is known about how the number of larval instars is fixed in each species or modified by the environment. The silkworm, *Bombyx mori*, provides a unique bioresource for investigating this question, as there are several “moltingism” strains that exhibit variations in the number of larval molts. The present study describes the first positional cloning of a moltingism gene. We performed genetic and biochemical analyses on the *dimolting (mod)* mutant, which shows precocious metamorphosis with fewer larval–larval molts. We found that *mod* is a juvenile hormone (JH)–deficient mutant that is unable to synthesize JH, a hormone that prevents precocious metamorphosis and allows the larvae to undergo multiple rounds of larval–larval molts. This JH–deficient mutation is the first described to date in any insect species and, therefore, the mod strain will serve as a useful model for elucidating the molecular mechanism of JH action. Remarkably, precocious larval–pupal transition in *mod* larvae does not occur in the first or second instar, suggesting that morphostatic action of JH is not necessary for young larvae of *B. mori*.

In the silkworm, premature metamorphosis can be induced by the loss of or low levels of JH signaling, which can occur due to the surgical removal of the CA [17] or to overexpression of the JH-degrading enzyme [16]. We therefore hypothesized that precocious metamorphosis in the *mod* strain was caused by the prevention of JH biosynthesis or signaling. To examine this hypothesis, we first determined whether the *mod* phenotype could be rescued by treatment with methoprene, a JH analogue. We topicaly applied several doses of methoprene to newly-molted third or fourth instar *mod* larvae and found that a fourth larval molting was induced by the treatment (Figure 1E). Fifth instar larvae that had undergone fourth larval ecysis grew normally, began to spin after ~6 days, and eventually metamorphosed to pupae and adults that were normal and fertile. This result suggests that JH reception and subsequent JH signaling is normal in the *mod* strain. Therefore, we next compared the JH titers in the hemolymph of third instar larvae of *mod* and p50T at 24 h after molting to the third instar. JHs were extracted from the hemolymph and their methoxyhydrin derivatives were analyzed by liquid chromatography-mass spectrometry (LC-MS). We detected JH I and JH II in the hemolymph of p50T, whereas the JH titer in the hemolymph of the mod strain was below the detectable level (Figure 1F). These results indicate that the *mod* strain is a JH-deficient mutant in which complete (or almost complete) loss of JH caused precocious metamorphosis.

### Positional cloning of the *mod* locus

To identify the gene responsible for the *mod* locus, we performed positional cloning using backcross 1 progeny (BC1) obtained from crossing females of the *mod* strain (t011 strain, see http://www.shigen.nig.ac.jp/silkwormbase/index.jsp) with F1 heterozygote males of *mod* and p50T strains (see Figure S1). We mapped the *mod* locus within ~400 kb region on the scaffold Bm_scaf16 (chromosome 11) [8] using 792 BC1 individuals. Twenty-five genes were predicted to be present within this region. Among them, we focused on *BGBMG0271708*, a gene encodes a cytochrome P450 monooxygenase. Based on sequence homology and phylogenetic analysis (Figure 2B), the gene was designated as *CYP15C1*. We found that *CYP15C1* shares high homology with the *CYP15A1* of the cockroach *Diplotettus punctatus*, which is involved in JH biosynthesis in CA of the cockroach [18]. Given that the *mod* phenotype is a result of the loss of the JH titer (Figure 1F), we speculated that the *mod* phenotype is due to the loss of function of *CYP15C1*. To examine this possibility, we first determined the
Figure 1. Characterization of the mod mutant. (A) Precocious metamorphosis observed in mod larvae. (left panel) Lateral and dorsal views and (middle panel) a magnified view of a larval-pupal intermediate. In intermediate animals, the new head capsule of the next instar (fifth) is formed (arrowhead). Beneath the old cuticles (asterisk), a new exoskeleton with larval eye spot markings (arrows) and brown-colored pupal cuticles are formed. (Right panel) Late-maturing trimolters form small cocoons and are able to develop into small but normal adults with normal fertility. (B) The developmental profiles of two batches of mod larvae (t011 strain). All of the larvae underwent precocious metamorphosis in the fourth instar, and no dimolters or tetramolters were observed. Larvae could be classified into two groups (early- and late-maturing trimolters) on the basis of the timing of onset of spinning. The numbers in parentheses indicate the sex of the moths (male/female). (C) Timing of the onset of spinning in mod (red, n = 178) and p50T (black, n = 28) strains after final larval molting. As highlighted by the grey ellipses, spinning was induced at two distinct timings in the mod strain, unlike the p50T strain. (D) Comparison of timings of the onset of spinning among early- and late-maturing trimolters of the mod strain and normal strain larvae that had been allatectomized (CAX) at the beginning of the fourth instar. Data on CAX larvae are from [17]; these larvae were reared at relatively low temperatures (23.0–25.5 °C), which delays the timing of the onset of spinning to some extent. (E) Methoprene treatment of Juvenile Hormone–Deficient Mutant Silkworm.
nucleotide sequence of the full-length CYP15C1 cDNA from p50T and mod strains. We identified a 66-bp deletion in the mod allele that introduces a premature stop codon in the coding region of CYP15C1 (Figure 2C–2E). This deletion seemed to produce a functionally null mutation in CYP15C1, since a heme-binding motif, which is essential for enzymatic activities in P450s [19], was eliminated in the mod allele (Figure 2D). This result indicates that CYP15C1 is a strong candidate for the mod locus. Therefore, we further characterized CYP15C1 and its gene product.

Temporal and spatial expression of CYP15C1

The strict regulation of JH biosynthesis in CA is critical for the successful development and reproduction of insects [14,15,20]. We next examined the spatial expression pattern of CYP15C1 mRNA. We examined 12 tissues at four different developmental stages and found that CYP15C1 mRNA was highly specific to the corpus cardiacum (CC)-CA complex (Figure 3A). A whole mount in situ hybridization experiment in the brain (Br)-CC-CA complex (Figure 3B and Figure S2) showed that the signal for CYP15C1 was strictly limited to CA, where JH is synthesized, and could not be detected in the brain or CC. These results showed a close spatial correlation between CYP15C1 expression and JH biosynthesis.

Next, we carried out a detailed analysis of the temporal expression pattern of CYP15C1 in the CC-CA complex and compared it to that of the gene for JHA methyltransferase (JHAMT), a key enzyme that acts in the final step of the JH biosynthetic pathway in CA [21]. CYP15C1 mRNA was constitutively expressed in CA from the first instar larval to adult stages (Figure 3D), even when JH is not synthesized (Figure 3C) [20]; no apparent differences in levels of CYP15C1 mRNA were observed between males and females during pupal and adult stages (Figure 3D). In contrast, the temporal expression pattern of JHAMT correlates well with the JH synthetic activity of CA (Figure 3D and Figure S2). JHAMT transcript completely disappeared by day 4 of the fifth instar when CA ceased production of JH (see Figure 3C). It reappeared from the mid-pupal stage and increased to a very high level in the female CA. This was consistent with the temporal profile of JH biosynthesis activity in CA as this occurs only in females during the pupal and adult stages [20]. Taken together, our results strongly indicate that CYP15C1 is involved in JH biosynthesis in CA, but does not appear to act as a rate-limiting factor for JH biosynthesis.

Enzymatic properties of CYP15C1

The cockroach CYP15A1, the ortholog of B. mori CYP15C1, catalyzes the epoxidation of (2E,6E)-methyl farnesoate (MF) to JH III [18]. Although biochemical studies predicted the presence of FA epoxidase in the CA of the lepidopteran insect Manduca sexta [22,23], the corresponding gene has not been identified to date. Therefore we examined the enzymatic activity of B. mori CYP15C1 against two plausible substrates, FA and MF. First, we employed a transient expression system using Drosophila S2 cells. When S2 cells expressing CYP15C1 were incubated with medium containing FA, a major HPLC peak was generated that had the same retention time (15.1 min) as standard JH III acid (JHA III) (Figure 4A, middle). This peak did not appear when S2 cells expressing GFP were used (Figure 4A, bottom). The ESI-MS spectrum of this peak gave an [M-H]− at m/z 251, consistent with the C15H23O3 formula of JHA III, confirming that CYP15C1 catalyzes the conversion of FA to JHA III. The enzymatic properties of CYP15C1 were further examined in a stable S9 cell line (S9/BmCYP15C1) that constitutively expresses CYP15C1. When the S9/BmCYP15C1 cells were cultured in medium containing FA, significant levels of JHA III were detected; in contrast, JHA III production was difficult to detect when original S9 cells were used (Table S2, Exp.1). When S9/BmCYP15C1 cells were cultured in medium containing MF, JH III generation was detected at low levels. However, a similar level of JH III production was also detected in the original S9 cells when they were cultured in the same medium (Table S2, Exp.1). These results suggest that JH III production observed in S9/BmCYP15C1 might be due to the presence of endogenous P450 epoxides in S9 cells, which have been reported previously to have lower substrate specificity and stereospecificity [18,24]. The addition of the JH esterase inhibitor 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP) did not increase production of JHA III (Table S2, Exp. 2), indicating that the degradation of JH III by intrinsic JH esterases in the cells was negligible. Therefore, we were able to estimate the conversion ratio of FA and MF to JH III by CYP15C1. This showed that CYP15C1 exhibited at least 18-fold higher activity for FA than MF (Table S2, Exp. 1), a result that is consistent with previous biochemical studies on lepidopteran FA epoxidase in CA.

To further examine the stereospecificity of CYP15C1, the JHA III generated by S9/CYP15C1 was chemically methylated and analyzed by a Chiral-HPLC. The methylated product had a major (R)-JH III and a minor (S)-JH III peak (R:S = 97:3) (Figure 4B). These results show that B. mori CYP15C1 encodes a functional P450 epoxide that preferentially converts FA to JHA III rather than MF to JH III, and does so in a highly (R)-enantioselective manner (Figure 4C).

Transgenic rescue experiments using the GAL4/UAS system

To obtain direct evidence that CYP15C1 is responsible for the mod mutation, we performed transgenic rescue experiments using the GAL4/UAS system [25]. We generated transgenic silkworm lines carrying the UAS-CYP15C1 transgene with the eye-specific 3xP3-EGFP marker [26]. The UAS-CYP15C1 transgene was driven using a silkworm enhancer trap line ET14 in which GAL4 was strongly expressed in CA (Figure 5A), although weak expression was also detected in peripheral tissues including fat bodies and the midgut [9,27]. As these lines were generated using the standard Shiro-C (w-; +/mod) strain, we changed the genetic background to w-1/w-1; mod/mod by crossing to the mod strain. The resultant w-1; mod, ET14/+ females were then crossed with w-1; mod, UAS-CYP15C1/+ males to determine whether the mod phenotype could be rescued by CYP15C1 overexpression. We used two independent UAS-CYP15C1 lines with ET-14 (Figure 5B). In both UAS-CYP15C1 lines, CYP15C1 overexpression efficiently prevented precocious metamorphosis and 97.1% of the larvae (34/35 in total) underwent the fourth larval molt to become fifth instar larvae (Figure 5B and 5C). Only one larva (1/35) became a late-maturing trimolter, but neither dimolters nor early-maturing
Figure 2. Positional cloning of the mod locus. (A) Physical map showing the outcome of the linkage analysis using 792 BC1 individuals. The mod locus was narrowed to the genomic region flanked by the PCR markers M2 and Q, as indicated by the orange arrows. Putative genes predicted by the Gene model program [8,9] are shown below the map, and CYP15C1 (BGIBMGA011708) is shown in red. For more details refer to Figure S1. (B) A phylogenetic tree showing the relationship of CYP15C1 and other related P450 genes. The rootless tree was constructed based on the entire amino acid sequence by the neighbor-joining method using the ClustalX program [55]. Sequences were retrieved from public databases, and the species names are abbreviated as follows: Aedes, A. aegypti; Anopheles, A. gambiae; Apis, A. mellifera; Bombyx, B. mori; Diploptera, D. punctata; Drosophila, D. melanogaster; and Tribolium, T. castaneum. The scale bar indicates the number of amino acid substitutions per site. Note that CYP15 was not found in D. melanogaster. (C) The genomic structure of CYP15C1 in the wild-type (p50T) strain. White box, grey box, and a black bar indicate untranslated, coding, and intronic regions, respectively. (D) Transcripts of CYP15C1 from p50T and mod strains. A 68-bp deletion was found in CYP15C1 of the mod strain, and this deletion introduced a premature stop codon as indicated in red. Heme-binding motifs of P450s [19] are indicated in orange. (E) Genomic PCR showing the presence of the 68-bp deletion in CYP15C1 from the mod strain. PCR primers (Table S1) that flank the deletion are shown by arrows in (C).

doi:10.1371/journal.pgen.1002486.g002
Figure 3. Temporal and spatial expression of CYP15C1. (A) qRT-PCR analysis of the spatial expression of CYP15C1 in the silkworm strain Kinshu x Showa. "CYP15C1/rp49" on the vertical axis indicates the level of CYP15C1 mRNA normalized to that of internal rp49 mRNA. RNAs were collected from larvae on day 1 of the fourth instar (4th D1), fourth instar larvae showing head capsule slippage (4th HCS), larvae on day 2 of the fifth instar (5th D2), and larvae on day 1 after the onset of spinning (Spin+1). CC-CA, corpus cardiacum-corpus allatum complex; PG, prothoracic gland; Br,
trimolters appeared. This result was in contrast to what was observed in control larvae or larvae carrying either the GAL4 or UAS construct alone: approximately half of the larvae became dimolters and the remainder became trimolters, while no larvae became tetramolters. We also measured the JH titer in the hemolymph (Figure 5D). As expected, the JH titers in control, ET14, and UAS larvae were below the detectable limit. In contrast, we were able to detect JH I and JH II in the hemolymph of mod larvae carrying both ET14 and UAS-CYP15C1 constructs. Taken together, these results provide direct evidence that CYP15C1 is responsible for the mod mutation and is essential for JH biosynthesis.

**Discussion**

In this study, we identified and characterized the gene responsible for the mod locus that causes precocious larval-pupal metamorphosis in *B. mori*. The data we present here have two important implications. First, we provide direct genetic evidence for the significance of P450 epoxidase in the late step of the JH biosynthetic pathway, whose expression is essential for normal growth and metamorphosis. Second, we show that the mod strain is a JH-deficient mutant strain carrying a null allele of CYP15C1, in which developmental abnormalities are mostly limited to larval-pupal transitions and are not observed before the second larval molt.

**Biochemical and physiological function of CYP15C1**

JH III is the most common JH in many insect orders, although its ethyl-branched homologs (JH I and II) are the major JHs in the order Lepidoptera [22,28]. Biochemical studies have shown that in the late steps of JH biosynthesis in many insect species, including cockroaches and locusts, FA is first methylated to MF and then epoxidized to JH III in CA [22]. However, the final two steps of JH biosynthesis are reversed in Lepidoptera: ethyl-branched homologs of FA (homo-FAs) are first epoxidized and the resultant

**Figure 4. Enzymatic properties of *B. mori* CYP15C1.** (A) Enzymatic activity against FA. Medium containing FA was incubated with *Drosophila* S2 cells transiently expressing CYP15C1 (middle) or GFP (bottom), and analyzed by HPLC. Standard JHA III (top). Arrows indicate peaks of JHA III. (B) Stereospecificity. JHA III generated from FA by Sf9 cells stably expressing CYP15C1 (sF9/CYP15C1) was chemically methylated and analyzed by a Chiral-HPLC. R and S indicate peaks of (R)- and (S)-JH III enantiomers, respectively. The R/S ratio of standard racemic JH III (top) was 50:50, while that of CYP15C1-produced JH III (bottom) was 97:3. (C) The late JH biosynthetic step in *B. mori*, in which major JHs in the hemolymph are JH I and II [28]. Ethyl-branched farnesyl diphosphates (homo-FPPs) are converted to homo-FAs, epoxidized to JHAs by the cytochrome P450 epoxidase CYP15C1 (this study), and then methylated by the JHA methyltransferase (JHAMT) [21]. JH I: R1 = R2 = C2H5, JH II: R1 = C2H5, R2 = CH3.

doi:10.1371/journal.pgen.1002486.g004
JHAs (i.e., JHA I and II) are then methylated to the authentic JHs (i.e., JH I and II) [22]. This study showed that B. mori CYP15C1 epoxidizes FA to JHA III in a highly stereospecific manner. CYP15C1 might also epoxidize MF to JH III, but in a far less efficient manner (Table S2). Given that B. mori JHAMT can methylate both FA and JHAs with similar efficiencies [21], our data clearly demonstrate the major JH biosynthetic pathway in B. mori: homo-FAs are first epoxidized to JHAs by CYP15C1, and then methylated to JHs by JHAMT (Figure 4C and Figure 6A). Interestingly, D. punctata CYP15A1 does not convert FA to JHA III [18]. Thus, the difference in specificity of CYP15 to the substrates FA and MF may determine the order of the final steps of JH biosynthesis in insects.

The expression of most early JH biosynthetic enzyme genes and JHAMT in B. mori is limited to the CA and shows dynamic developmental fluctuations [20,21,29]. In particular, the temporal expression profile of JHAMT correlates well with JH biosynthetic activity in B. mori [20,21,30,31] and in the Eri silkworm Samia cynthia ricini [32], indicating that JHAMT is a key regulatory gene whose transcriptional control is critical for the regulation of JH biosynthesis in Lepidoptera. Here, we found that expression of CYP15C1 was also limited in CA but in a different pattern to other JH biosynthesis genes in that it was constitutively expressed from larval to adult stages. This result suggests that the transcriptional regulation of CYP15C1 is less important than JHAMT for the temporal regulation of JH production in B. mori. CA of the

---

**Figure 5. Transgenic rescue of mod.** (A) Visualization of GAL4 expression in CA of the enhancer trap line ET14 carrying the UAS-GFP construct. GFP expression (green) is limited to CA (arrowhead). Red fluorescence in the optic nerve is due to DsRed2 expression driven by the 3xP3 promoter [26]. Br, brain; SOG, suboesophageal ganglion; and CA, corpus allatum. (B) Developmental profiles of binary GAL4/UAS transgenic lines. Male moths with a w-1; mod background and carrying UAS-CYP15C1 were crossed with w-1; mod female moths carrying ET14, and their progenies were analyzed. Tetramolters appeared in GAL4/UAS transgenic lines, but not in nonbinary lines. (C) Images of pupae and moths of GAL4/UAS transgenic lines. Larvae carrying both ET14 and UAS-CYP15 constructs entered the fifth larval instar and eventually formed larger adults. Control animals did not carry transgenic vectors. (D) Measurement of the JH titer in the hemolymph of GAL4/UAS transgenic lines on the w-1; mod background. Hemolymph was collected from fourth instar larvae at 24 h after molting and analyzed. JH was detected only in GAL4/UAS lines, but not in nonbinary lines. ND, not detected.

doi:10.1371/journal.pgen.1002486.g005
silkworm ceases JH biosynthesis by day 3 of the last (fifth) instar [20]; however, it is speculated that CA synthesizes and secretes JHAs during the following prepupal period. Our data indicate that this endocrine switch can be explained by constitutive CYP15C1 expression and the shut-off of JHAMT expression in CA (Figure 6A). During the larval-pupal transition, homo-FAs are constantly converted to JHAs (predominantly JHA I and II in Lepidoptera). When JHAMT is expressed in CA, JHAs are further converted to JHs, and released from CA, thereby preventing precocious metamorphosis. When JHAMT expression is shut off (e.g., in the prepupal stage), JHAs are likely to be released from CA. In CA of the mod strain, homo-FAs are not converted to JHAs because of the loss of CYP15C1, but instead, homo-FAs are converted to ethyl-branched homologs of MF (homo-MFs, i.e., unepoxidized JH I and II) by JHAMT. The loss of CYP15C1 does not allow the conversion of homo-MFs to the authentic JHs. Therefore, neither JHs is synthesized nor released from CA of the mod strain, thereby causing precocious metamorphosis. The synthesized homo-MFs might be released from CA of the mod strain, similar to that of higher dipteran insects [57]. JH I: R1 = R2 = C2H5, JH II: R1 = C2H5, R2 = CH3.

doi:10.1371/journal.pgen.1002486.g006

Figure 6. A model for JH biosynthetic pathway in the CA of wt and mod silkworms. (A) In the B. mori CA, constitutive CYP15C1 expression allows the consistent conversion of homo-FAs to JHAs (predominantly JHA I and II in Lepidoptera). When JHAMT is expressed in CA, JHAs are further converted to JHs, and released from CA, thereby preventing precocious metamorphosis. When JHAMT expression is shut off (e.g., in the prepupal stage), JHAs are likely to be released from CA. (B) In CA of the mod strain, homo-FAs are not converted to JHAs because of the loss of CYP15C1, but instead, homo-FAs are converted to ethyl-branched homologs of MF (homo-MFs, i.e., unepoxidized JH I and II) by JHAMT. The loss of CYP15C1 does not allow the conversion of homo-MFs to the authentic JHs. Therefore, neither JHs is synthesized nor released from CA of the mod strain, thereby causing precocious metamorphosis. The synthesized homo-MFs might be released from CA of the mod strain, similar to that of higher dipteran insects [57]. JH I: R1 = R2 = C2H5, JH II: R1 = C2H5, R2 = CH3.

doi:10.1371/journal.pgen.1002486.g006

silkworm ceases JH biosynthesis by day 3 of the last (fifth) instar [20]; however, it is speculated that CA synthesizes and secretes JHAs during the following prepupal period. Our data indicate that this endocrine switch can be explained by constitutive CYP15C1 expression and the shut-off of JHAMT expression in CA (Figure 6A). During the larval-pupal transition, homo-FAs are constantly converted to JHAs (predominantly JHA I and II in Lepidoptera). When JHAMT is expressed in CA, JHAs are further converted to JHs, and released from CA, thereby preventing precocious metamorphosis. When JHAMT expression is shut off (e.g., in the prepupal stage), JHAs are likely to be released from CA. In CA of the mod strain, homo-FAs are not converted to JHAs because of the loss of CYP15C1, but instead, homo-FAs are converted to ethyl-branched homologs of MF (homo-MFs, i.e., unepoxidized JH I and II) by JHAMT. The loss of CYP15C1 does not allow the conversion of homo-MFs to the authentic JHs. Therefore, neither JHs is synthesized nor released from CA of the mod strain, thereby causing precocious metamorphosis. The synthesized homo-MFs might be released from CA of the mod strain, similar to that of higher dipteran insects [57]. JH I: R1 = R2 = C2H5, JH II: R1 = C2H5, R2 = CH3.

doi:10.1371/journal.pgen.1002486.g006

Figure 6. A model for JH biosynthetic pathway in the CA of wt and mod silkworms. (A) In the B. mori CA, constitutive CYP15C1 expression allows the consistent conversion of homo-FAs to JHAs (predominantly JHA I and II in Lepidoptera). When JHAMT is expressed in CA, JHAs are further converted to JHs, and released from CA, thereby preventing precocious metamorphosis. When JHAMT expression is shut off (e.g., in the prepupal stage), JHAs are likely to be released from CA. (B) In CA of the mod strain, homo-FAs are not converted to JHAs because of the loss of CYP15C1, but instead, homo-FAs are converted to ethyl-branched homologs of MF (homo-MFs, i.e., unepoxidized JH I and II) by JHAMT. The loss of CYP15C1 does not allow the conversion of homo-MFs to the authentic JHs. Therefore, neither JHs is synthesized nor released from CA of the mod strain, thereby causing precocious metamorphosis. The synthesized homo-MFs might be released from CA of the mod strain, similar to that of higher dipteran insects [57]. JH I: R1 = R2 = C2H5, JH II: R1 = C2H5, R2 = CH3.

doi:10.1371/journal.pgen.1002486.g006

Figure 6. A model for JH biosynthetic pathway in the CA of wt and mod silkworms. (A) In the B. mori CA, constitutive CYP15C1 expression allows the consistent conversion of homo-FAs to JHAs (predominantly JHA I and II in Lepidoptera). When JHAMT is expressed in CA, JHAs are further converted to JHs, and released from CA, thereby preventing precocious metamorphosis. When JHAMT expression is shut off (e.g., in the prepupal stage), JHAs are likely to be released from CA. (B) In CA of the mod strain, homo-FAs are not converted to JHAs because of the loss of CYP15C1, but instead, homo-FAs are converted to ethyl-branched homologs of MF (homo-MFs, i.e., unepoxidized JH I and II) by JHAMT. The loss of CYP15C1 does not allow the conversion of homo-MFs to the authentic JHs. Therefore, neither JHs is synthesized nor released from CA of the mod strain, thereby causing precocious metamorphosis. The synthesized homo-MFs might be released from CA of the mod strain, similar to that of higher dipteran insects [57]. JH I: R1 = R2 = C2H5, JH II: R1 = C2H5, R2 = CH3.

doi:10.1371/journal.pgen.1002486.g006

Precocious pupation in mod larvae

Our results consistently indicate that the mod strain is a JH-deficient mutant that is unable to synthesize JHs in CA. One unique characteristic of the precocious pupation in the mod strain is the variation in the timing of the onset of spinning (Figure 1). The
feeding period in early-maturing trimolters was unusually short (50 h after molting) compared with that observed in surgical allatectomy of newly molted fourth instar larvae. In the latter larvae, the feeding period was comparable in length to that of the late-maturing trimolters [e.g., ~130 h [17]] and no timing segregation was observed [17]. In addition, most of the early-maturing trimolters displayed a larval-pupal intermediate phenotype and eventually died, unlike allatectomized larvae, most of which successfully developed into small but normal pupae [17].

One explanation for this phenomenon is that the early-maturing trimolters were destined to undergo larval molting to the fifth instar after a prolonged fourth instar, similar to allatectomized trimolters were destined to undergo larval molting to the fifth instar, which successfully developed into small but normal pupae [17].

In addition, most of the early-maturing trimolters on day 2 usually resulted in the formation of larval-pupal intermediates. One possible explanation for this mixed phenotype is that metamorphosis in the mod strain is induced in the presence of homo-MFs (unepoxidized JH I and II), presumed products instead of epoxidized JH I and II in CA of the mod strain (see Figure 1B). MF is known as a crustacean JH and has recently been reported to have JH activity in D. melanogaster [38,39]. Therefore, MF and its homologs might have JH-like activity but not able to substitute for authentic (epoxidized) JHs in the physiology of the silkworm. Alternatively, other P450 epoxidases in B. mori that have low substrate specificity and stereospecificity, like CYP9E1 [18] and CYP6A1 [24] in other insects, might substitute for the absence of CYP15C1 in peripheral tissues of mod larvae, and such locally-synthesized JHs may prevent precocious metamorphosis in the first and second instar larvae carrying the mod mutation. Further studies are needed to elucidate the mechanism for this unique characteristic of the mod strain.

We found that the precocious phenotype was more severe in the w; mod strain compared to that in t011, a genetic stock of the mod strain. We rarely observed dimolter larval in the t011 stock (Figure 1B). However, in the original manuscript in 1956, it was reported that 28–92% of mod larvae became dimolters [11]. This difference might have developed as a consequence of unintended artificial selection during stock maintenance that favored broods producing trimolters in higher proportions, as it is difficult to obtain sufficient number of eggs using dimolter moths [11,12]. Thus, we speculate that the present t011 stock may be genetically fixed to produce mostly trimolters, and that this attribute can be varied by outcrossing to other strains.

In the silkworm, premature metamorphosis can be induced by surgical removal of JH-producing CA (allatectomy) [17], by application of an imidazole-based insect growth regulator KK-42 [40] or an anti-juvenile hormone agent KF-138 [41,42], or by continuous overexpression of the JH-degrading enzyme, JH esterase [16]. In any case, however, premature pupation is not induced in larvae younger than the third instar. In agreement with these studies, we did not observe precocious pupation in first or second instar mod larvae, nor did we observe apparent developmental abnormalities during these early instars. Therefore, our data support the hypothesis that there are two physiological phases in the life of silkworm larvae [16]: the JH-independent phase (first and second instar) in which JH does not have a morphogenetic function; and, the JH-dependent phase (third instar and thereafter) in which the morphostatic action of JH is required to prolong the larval stage until the attainment of the appropriate body size for metamorphosis. Given that most generally the minimum number of the larval instar in insects is three [1,2], our data further imply that insect larvae need to experience at least one (e.g., L2 pupae in D. melanogaster [43]) or two (e.g., B. mori) larval-larval molts and/or require a certain length of time of postembryonic development in order to acquire competence for metamorphosis.

The silkworm is a classic model organism that has been used for pioneering studies in genetics, physiology, and biochemistry [5]. The availability of whole genome data [8], post-genomic tools [10], and unique mutant resources [6], together with the classic “status quo” responses to JHs in this insect [14,15,17], makes the silkworm well-suited for study of hormonal control of growth and development. Indeed, these advantages have greatly contributed to the identification of essential components in the biosynthesis of ecdysteroids, the insect molting hormones [44]. Moreover, recent success in targeted gene disruption using a zinc-finger nuclease [45] increases the utility of this model organism. We are hopeful that our present study will encourage further studies on other “moltinism” strains in the silkworm, and consequently pave the way for a greater understanding of physiological control, developmental plasticity, and evolutionary history of the number of larval molting in insects, which may reflect adaptive strategies of insects to diverse environmental conditions. It is also noteworthy that the late step of the JH biosynthetic pathway is insect-specific and is therefore a potential target for biorational insecticides [46].

Materials and Methods

Insects and cell lines

Silkworms were reared on an artificial diet or mulberry leaves at 25–27°C under standard conditions as described previously [47]. The silkworm strain t011 (mod/mod) was obtained from Kyushu University [6]. The Spodoptera frugiperda S9 and Drosophila melanogaster S2 cells were maintained as described previously [48]. To determine the developmental profile of mod larvae, larvae from two batches of t011 were individually reared in plastic dishes, and their developmental stages were recorded at ~8-h intervals.

Hormonal treatments

The JH analog, methoprene (a kind gift from S. Sakurai) was applied to newly molted third or fourth instar larvae (~8–12 h after molting). Methoprene was diluted with acetone and the selected doses (0.01–10 mg/larva) were topically applied to the dorsum using a 10-μl Hamilton microsyringe. The same volume of acetone was applied as a control.

Positional cloning of the mod locus

Positional cloning of the mod locus was performed as described previously [49]. Codominant PCR markers and p50T-specific PCR markers were generated for each position of the scaffold Bm_scaf16 (chromosome 11) [9], and used for genetic analysis (Figure 2A and Figure S1). Homozygotes of the mod locus were collected from the BG1 population [t011×p50×x011] based on the phenotype of precocious pupation.

Cloning of CYP15C1

Total RNAs were collected from CA of day 0 fifth instar larvae of p50T and Kinshu × Showa strains and used for 5’- and 3’-rapid amplification of cDNA ends (RACE) using the GeneRacer Kit (Invitrogen). PCR was performed using the primers listed in Table S1. The PCR products were subcloned and sequenced as described previously [47]. The obtained cDNA sequence was deposited in the GeneBank (accession number: AB124839).

Quantitative RT–PCR (qRT–PCR) analysis

qRT-PCR was performed essentially as described previously [21]. The primers used for the quantification of the CYP15C1 transcript are listed in Table S1.
In situ hybridization

*In situ* hybridization was performed as described previously [50]. A *CYP15C1* cDNA fragment (~1.1 kb) was amplified by PCR listed in Table S1 and subcloned into a pDrive plasmid vector (Qiagen).

Chemicals

(2E,6E)-farnesoic acid (FA) and (2E,6E)-methyl farnesoate (MF) were purchased from Echelon Research Laboratories (Salt Lake City) and racemic JH III from Sigma. JH III acid was prepared from the racemic JH III as described previously [21]. (R)-JH III was a kind gift from W.G. Goodman.

Enzyme assays of CYP15C1 in S2 cells

*CYP15C1* overexpression in S2 cells was achieved using a GAL4/UAS system [51]. To generate a vector for expressing CYP15C1 under the control of the UAS promoter (UAS-CYP15C1-HA), a cDNA fragment coding the entire CYP15C1 ORF was ligated into the pUAST vector. UAS-GFP-RN3 [52] was used as a negative control. UAS-CYP15C1-HA or UAS-GFP-RN3 was transfected with the Actin5C-GAL4 construct (a gift from Yasushi Hiromi, National Institute of Genetics, Japan). Forty-eight hours after transfection of S2 cells in a 60-mm dish, the old medium was replaced with 2 ml of fresh medium. S2 cells were detached from the bottom of the dish by pipetting, and 1 ml of the cell suspension was transferred to a siliconized glass test tube. FA or MF (100 μM at final concentration) was then added to the tube. After incubation at 25°C for 16 h, 500 μl of medium was collected and mixed with 500 μl of acetonitrile. Samples were centrifuged for 10 min at 15,000 rpm, followed by incubation at 25°C for 10 min. After filtration using a 0.2 μm filter, 10–20 μl of each sample was subjected to HPLC analysis as described below.

Establishment of SF9 cells stably expressing CYP15C1 and enzyme assay

A cDNA with the full ORF of *CYP15C1* cDNA was subcloned into the pIZT/V5-His vector (Invitrogen). The plasmid was transfected into SF9 cells with Cellfectin reagent (Invitrogen), then transfected into Sf9 cells with Cellfectin reagent (Invitrogen). The plasmid was amplified by PCR listed in Table S1 and subcloned into a pDrive plasmid vector (Qiagen).

Analysis of the stereospecificity of JH III acid generated by CYP15C1

The stereospecificity of the epoxide group of JH III acid formed by CYP15C1 was analyzed as follows under semi-dark conditions. SF9/CYP15 cells were cultured in medium containing 10 μg/ml FA for 48 hrs. An equal volume of CH3CN was added to the medium (2 ml), vortexed vigorously and centrifuged at 4,800 rpm for 10 min. One ml of 1 M CH3COONH4 (pH 5.5) was added to the supernatant and extracted with 5 ml of CH3CH2Cl; this step was performed 5 times. The extract was dehydrated with anhydrous Na2SO4 and evaporated to dryness in vacuo at 40°C, then the residue was dissolved in 200 μl of CH3CN, 50 μl of MeOH and 100 μl of TMS-diazomethane were then added and the solution was incubated at room temperature for 30 min. The reaction was dried with an N2 gas stream, the residue dissolved in 100 μl of hexane, and subjected to a normal-phase HPLC (column, Shinseido Sg80, 250 × 4.6 mm ID; solvent, hexane-EtOH, 99:1; flow rate, 0.5 ml/min; detection, UV 211 nm). The peak corresponding to JH III (r.t. = 9.8 min) was collected. The stereospecificity of the epoxide group of the JH III was analyzed by a chiral-HPLC (column, Chiralpack IA, 250 × 4.6 mm ID, DAICEL; solvent, hexane-EtOH, 99:1; flow rate, 0.5 ml/min; detection UV 219 nm) as described previously [31].

Purification of JHs from hemolymphs and preparation for LC-MS analysis

Ten microliters of deuterium-substituted JH III (d3-JH III) [53] in toluene (67.1 μg/ml) was transferred to a clean glass tube to which 0.5 ml of methanol was added. The hemolymph sample (100 μl) was then added and mixed vigorously, and 1.5 ml of 20% NaCl was added to the JH sample. JH was extracted by partition with 0.5 ml hexane; this step was performed three times. The combined solvent containing JH (1.5 ml) was evaporated under a stream of nitrogen. One hundred microliters of methanol and 2 μl trifluoroacetic acid were added to the crude JH extract and mixture heated at 60°C for 30 min. After removal of the methanol, methylhydroxyn derivates of JH (JH-MHs) were purified using a Pasteur pipette packed with 1.0 g of aluminum oxide (activity grade III, ICN Ecocomd) prewashed with hexane. After loading the extract and washing with 2 ml of 30% ether in hexane, JH-MHs were eluted with 2 ml of 50% ethyl acetate in hexane and then dried under a stream of nitrogen. The residue was dissolved in 25 ml of 80% acetonitrile containing 5 μM sodium acetate.

Analytical condition for LC-MS

The HP1100 MSD system (Agilent) was equipped with a 150 × 3 mm C18 reversed phase column (UG80, Shinseido) protected by a guard column with 70% acetonitrile containing 5 μM sodium acetate at a flow rate of 0.4 μl/min. For MS analysis, electrospray ionization in the positive mode was used under the conditions of drying gas temperature at 320°C with 10 l/min flow rate, ionization voltage of 70 V. Under these conditions, selected ion masses for each JH-MH were monitored as [M+Na]+, i.e., m/z 321, 324, 335, and 349 for JH III, d3-JH III, JH II, and JH I, respectively.

Transgenic rescue experiments

Overexpression of *CYP15C1* was performed in transgenic silkworms using the GAL4/UAS system as described previously [25,27,54]. A coding sequence of CYP15C1 was introduced into a silkworm UAS vector carrying the marker gene 3xP3-EGFP. B. mori transformants were established using standard protocols [10].
To overexpress CYP15C1 on the mod/mod background, established UAS lines and an enhancer trap line ET14 [27] were crossed with the t011 strain, and the resultant F1 animals were sib mated to obtain the F2 generation. In the F2 generation, we collected animals showing premature pupation with white eyes (i.e., mod/ mod; w*/w*) and confirmed the presence of the fluorescent marker gene using a fluorescent microscope (SZX12, Olympus). The established w*/l; mod lines carrying UAS-CYP15C1 or ET14 were crossed, and their offspring were examined to determine whether precocious metamorphosis was blocked by CYP15C1 overexpression.

Supporting Information

Figure S1 Detailed procedure for positional cloning of the mod locus. (A) Mating scheme for mapping the mod locus. A single-pair cross between a female p50T (wt) and a male t011 (mod/mod) [6] produced the F1 offspring. Then, the male informative cross (t011 cross between a female p50T (wt) and a male t011 (mod/mod)) produced the F1 generation. In the F2 generation, we collected animals showing premature pupation with white eyes (i.e., mod/ mod; w*/w*) and confirmed the presence of the fluorescent marker gene using a fluorescent microscope (SZX12, Olympus). The established w*/l; mod lines carrying UAS-CYP15C1 or ET14 were crossed, and their offspring were examined to determine whether precocious metamorphosis was blocked by CYP15C1 overexpression.

Table S1 PCR primers used in this study.

Table S2 Substrate specificity of CYP15C1 to FA and MF. Sf9 or Sf9/CYP15C1 cells (~1.2×10^6) were cultured with 200 ml of medium containing 2 μg of FA or MF at 26°C for 2 h (Exp. 1) or 6 h (Exp. 2), and the production of JHA III or JH III in the medium was quantified by HPLC. Mean ± SD (N=3); ND, not detected.

Acknowledgments

We thank S. Sakurai (Kanazawa Univ.) for providing methoprene, W. G. Goodwin (Univ. of Wisconsin-Madison) for (R);JH III, Y. Hiromi (Natl. Inst. Genet.) for the Actin5C-GAL4 plasmid, M. Yoshiyama for technical assistance, and M. Kawamoto for clerical assistance.

Author Contributions

Conceived and designed the experiments: T Daimon, T Kozaki, R Niwa, S Katsuma, H Sezuttsu, T Shimada, T Shinoda. Performed the experiments: T Daimon, T Kozaki, R Niwa, I Kobayashi, K Kuruta, T Namiki, K Uchino, M Nakayaka, K Itoyama, T Shinoda. Analyzed the data: T Daimon, T Kozaki, R Niwa, T Namiki, T Shinoda. Contributed reagents/materials/analysis tools: I Kobayashi, K Uchino, Y Banuo, T Tamura, K Mita, H Sezuttsu. Wrote the paper: T Daimon, T Kozaki, R Niwa, K Kuruta, T Shinoda.

References

1. Sehnal F (1985) Growth and lifecycles. In: Kerkut GA, Gilbert LI, eds. Comprehensive insect physiology, biochemistry and pharmacology. Oxford: Pergamon Press. pp 1–81.
2. Esperk T, Tammaru T, Nylin S (2007) Intraspecific variability in number of larval instars in insects. J Econ Entomol 100: 627–645.
3. Nijhout HF (1998) Insect hormones. Princeton: Princeton University Press.
4. Nijhout HF (1981) Physiological control of molting in insects. Amer Zool 21: 631–640.
5. Goldsmith MR, Shimada T, Abe H (2004) The genetics and genomics of the silkworm, Bombyx mori. Annu Rev Entomol 50: 71–100.
6. Banno Y, Fujii H, Kawaguchi Y, Yamamoto K, Nishikawa K, et al. (2005) A derived vector. Nat Biotechnol 18: 81–84.
7. Shimomura M, Minami H, Saaetsugu Y, Ohnagay H, Satoh G, et al. (2009) KARIObase: an integrated silkworm genome database and data mining tool. BMC Genomics 10: 486.
8. Tamura T, Tahura C, Royer C, Kanda T, Abraham E, et al. (2000) Germ line transformation of the silkworm Bombyx mori L. using a <p>transposon</p> derived vector. Nat Biotechnol 18: 81–84.
9. Oota S, Watanabe A, Tokunaga H (1956) Genetical study on a spontaneous mutant in the brain-CA complex. Whole-mount in situ hybridization of CYP15C1 and JHAMT in the brain-CC-CA complex on day 2 of the fourth instar and day 4 of the fifth instar. Magnified images of CAs indicated by arrows are shown below each panel. Signals were not detected when sense probes were used for analysis.
10. Hervig C, Koener JF, Unnithan GC, Feyereisen R (2004) CYP15A1, the cytochrome P450 that catalyzes epidioxylation of methyl farnesolate to juvenile hormone III in cockroach corpora allata. Proc Natl Acad Sci U S A 101: 4024–4029.
11. Imamura M, Nakai J, Inoue S, Quan GX, Kanda T, et al. (2003) Targeted gene Goodman (Univ. of Wisconsin-Madison) for (R);JH III, Y. Hiromi (Nat. Inst. Genet.) for the Actin5C-GAL4 plasmid, M. Yoshiyama for technical assistance, and M. Kawamoto for clerical assistance.
12. Ninaki O, Doira H, Chikushi H (1980) Genetical studies of the mod locus. J Insect Biotec Seric (In Japanese) 26: 77–81.
13. Tan A, Tanaka H, Tamura T, Shiotoku T (2005) Precocious metamorphosis in transgenic silkworms overexpressing juvenile hormone esterase. Proc Natl Acad Sci U S A 102: 11751–11756.
14. Werck-Reichhart D, Feyereisen R (2000) Cytochromes P450: a success story. Genes Drugs 1173–1179.
15. Andersen JF, Walding JK, Evans PH, Bowers WS, Feyereisen R (1997) Cytochrome P450 that catalyzes epoxidation of methyl farnesoate to juvenile hormone III in cockroach corpora allata. Proc Natl Acad Sci U S A 104: 2977–2982.
16. Imamura M, Nakai J, Inoue S, Quan GX, Kanda T, et al. (2003) Targeted gene Goodman (Univ. of Wisconsin-Madison) for (R);JH III, Y. Hiromi (Nat. Inst. Genet.) for the Actin5C-GAL4 plasmid, M. Yoshiyama for technical assistance, and M. Kawamoto for clerical assistance.
17. Fukuoka S (1944) The hormonal mechanism of larval molting and metamorphosis in the silkworm. J Fac Sci Tokyo Univ Sect IV 6: 477–532.
18. Helvig C, Koener JF, Unnithan GC, Feyereisen R (2004) CYP15A1, the cytochrome P450 that catalyzes epidioxylation of methyl farnesolate to juvenile hormone III in cockroach corpora allata. Proc Natl Acad Sci U S A 101: 4024–4029.
19. Imamura M, Nakai J, Inoue S, Quan GX, Kanda T, et al. (2003) Targeted gene Goodman (Univ. of Wisconsin-Madison) for (R);JH III, Y. Hiromi (Nat. Inst. Genet.) for the Actin5C-GAL4 plasmid, M. Yoshiyama for technical assistance, and M. Kawamoto for clerical assistance.
29. Kaneko Y, Shinoeda T, Hiruma K (2011) Remodeling of the corpora cardiaca and the corpora allata during adult metamorphosis in Bombyx mori: identification of invisible corpora cardiaca by the expression of adipokinetic hormone. Appl Entomol Zool 46: 67–93.

30. Minakuchi C, Namiki T, Yoshiyama M, Shinoeda T (2008) RNAi-mediated knockdown of juvenile hormone acid O-methyltransferase gene causes precocious metamorphosis in the red flour beetle Tribolium castaneum. FEBS J 275: 2919–2931.

31. Niwa R, Namiki T, Honda N, Yoshiyama M, Itoyama K, et al. (2008) Juvenile hormone acid O-methyltransferase in Drosophila melanogaster. Insect Biochem Mol Biol 38: 714–720.

32. Sheng ZT, Ma L, Cao MX, Jiang RJ, Li S (2008) Juvenile hormone acid methyl transferase is a key regulatory enzyme for juvenile hormone synthesis in the Eri silkworm, Samia cyrtoidea. Arch Insect Biochem Physiol 69: 143–154.

33. Noriega FG, Reis JM, Koerner JF, Valenzuela JG, Hernandez-Martinez S, et al. (2006) Comparative genomes of insect juvenile hormone biosynthesis. Insect Biochem Mol Biol 36: 366–374.

34. Maestro JL, Pascual N, Treiblmayr K, Lozano J, Belles X (2010) Juvenile hormone acid O-methyltransferase in juvenile hormone biosynthesis in the desert locust, Schistocerca gregaria. Insect Biochem Mol Biol 41: 219–227.

35. Marchal E, Zhang J, Badisco L, Verlinden H, Hult EF, et al. (2011) Final steps of invisible corpora cardiaca by the expression of adipokinetic hormone. Appl Entomol Zool 46: 87–93.

36. Nouzova M, Edwards MJ, Mayoral JG, Noriega FG (2011) A coordinated expression of biosynthetic enzymes controls the flux of juvenile hormone precursors in the corpora allata of mosquitoes. Insect Biochem Mol Biol 41: 660–669.

37. Chung H, Satul T, Pasricha S, Srithar M, Batterham P, et al. (2009) Characterization of Drosophila melanogaster cytochrome P450 genes. Proc Natl Acad Sci U S A 106: 5731–5736.

38. Laufer H, Borst D, Baker FC, Reuter CC, Tsai LW, et al. (1987) Identification of a juvenile hormone-like compound in a crustacean. Science 235: 202–205.

39. Harshman LG, Song KD, Casas J, Schuurmans A, Kuwano E, et al. (2010) Bioassays of compounds with potential juvenoid activity on the 'Black Box' of the ecdysteroid biosynthesis pathway. Development 137: 1991–1999.

40. Kuwano E, Takeya R, Eto M (1985) Synthesis and anti-juvenile hormone activity of 1-substituted-5-[(E)-2, 6-dimethyl-1, 5-heptadienyl]imidazoles. Agric Biol Chem 49: 483–486.

41. Furuta K, Ashibe K, Shirahashi H, Fujita N, Yamabita H, et al. (2007) Synthesis and anti-juvenile hormone activity of ethyl 4-(2-benzylalkoxy)benzoate and their enantiomers. J Pestic Sci 32: 99–105.

42. Kaneko Y, Furuta K, Kuwano E, Hiruma K (2011) An anti-juvenile hormone agent, ethyl 4-(2-benzylalkoxy)benzoate, inhibits juvenile hormone synthesis through the suppression of the transcription of juvenile hormone biosynthetic enzymes in the corpora allata in Bombyx mori. Insect Biochem Mol Biol 41: 786–794.

43. Zhou X, Zhou B, Truman JW, Riddiford LM (2004) Overexpression of brnt1: a new insight into its role in the Drosophila prothoracic gland cells. J Exp Biol 207: 1151–1161.