Studies on the mechanisms underlying the acquisition of competence for metamorphosis in the silkworm, *Bombyx mori*

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List of publications

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Abbreviations

20E	20-hydroxyecdysone
Br–C	*Broad-Complex*
BTB	Broad-Tramtrack-Bric-a-brac
CA	Corpora allata
CC	Corpora cardiaca
cDNA	Complementary DNA
chinmo	*Chronologically inappropriate morphogenesis*
CNS	Central nervous system
dib	*Disembodied*
DNA	Deoxyribonucleic acid
E75	*Ecdysone inducible gene E75*
E93	*Ecdysteroid induced protein 93F*
EcR	*Ecdysone receptor*
EGFP	Enhanced Green Fluorescent Protein
HCS	Head capsule slippage
HMGS	3-hydroxy-3-methylglutaryl-CoA synthase
IGF	Insulin/insulin-like growth factor
JH	Juvenile hormone
JHAMT	Juvenile hormone acid O-methyltransferase
JHEH	JH epoxide hydrolase
K × S	Kinshu × Showa
Kr-h1	*Krüppel-homolog 1*
MEKRE93	Methoprene tolerant-Krüppel homolog 1-E93
Met	*Methoprene-tolerant*
MH	Molting hormone
MIF	Metamorphosis-initiating factor
miRNA	MicroRNA
mRNA	Messenger RNA
ncRNA	Non-cording RNA
nm-g	*Non-molting glossy*
nvd	*Neverland*

O/E	Over-expressed
PCR	Polymerase chain reaction
PG	Prothoracic gland
phm	*Phantom*
pnd	*Pigmented and nondiapausing egg*
PTTH	Prothoracicotropic hormone
qRT-PCR	Quantitative reverse-transcription PCR
RNA	Ribonucleic acid
RNAi	RNA interference
rp49	*Ribosomal protain 49*
rRNA	Ribosomal RNA
sad	*Shadow*
SEM	Scanning electron microscope
spo	*Spook*
SRC	Steroid receptor co-activator
Tai	Taiman
TALEN	Transcription activator-like effector nuclease
tRNA	Transfer RNA
USP	*Ultraspiracle*
w-1	*White egg 1*
βFtz-F1	*Fushi-Tarazu F1*

1. General Introduction

1.1. Type of metamorphosis in insects

Insects are pancrustacean hexapod invertebrates of the class Insecta, derived from crustaceans about 450 million years ago. They form the largest group within the arthropod phylum, which has evolved extensively and adapted to every habitat on Earth. A key feature of the unprecedented prosperity of insects was metamorphosis (Belles, 2020). Metamorphosis, together with drastic physiological and morphological changes such as the acquisition of wings, must have contributed to the remarkable diversity of insects by enabling them to exploit different habitats and resources (Nicholson et al., 2014; Rainford et al., 2014; Truman and Riddiford, 1999).

Insects are divided into three major evolutionary lineages, characterised by the degree of morphological change that accompanies their metamorphic molt (Fig. 1.1). The Ametabola is the most basal group of insects without significant metamorphosis, in which the body form of nymphs and adults is largely identical except for the development of external genitalia and internal reproductive organs. The innovation of wings enabled insects to fly and led to further modifications of post-embryonic development. The hemimetabola is an incomplete metamorphosis in which adults differ from nymphs in having functional wings, in addition to the previous features. Wings and other adult appendages gradually appear as the nymphs grow. The holometabola is a complete metamorphosis. The development of adult segmental appendages, wings and eyes is suppressed during the larval stage. During metamorphosis, these adult structures undergo rapid growth and differentiation and are externalised to form the adult appendages.

Metamorphosis in the Holometabola requires two molting cycles, the first to externalise the undifferentiated adult structures and the second to allow differentiation of the adult morphology.

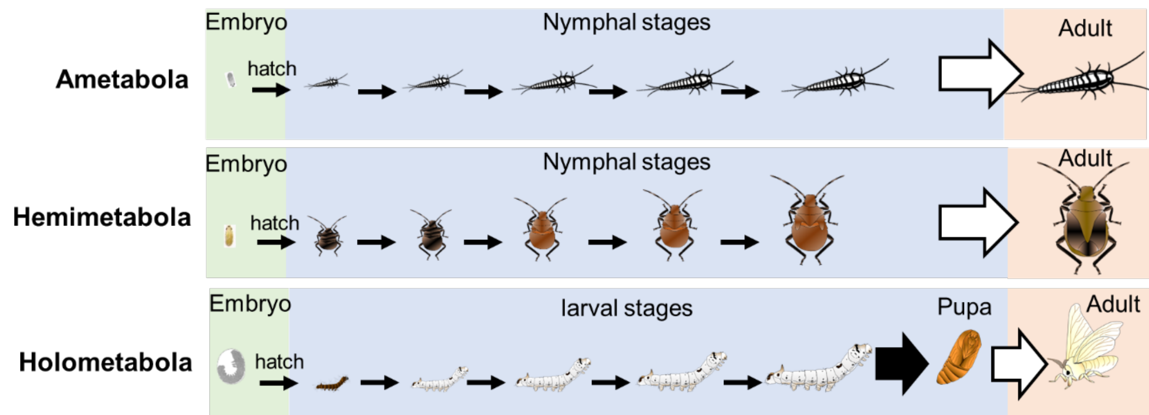


Fig. 1.1. Types of postembryonic development in insects. White arrows indicate imaginal molt. The black thick arrow indicates metamorphic (pupal) molt.

1.2. Endocrine control of molting and metamorphosis

The invertebrate endocrine system is cell-cell communication involving a complex network of hormone signalling that affects target cells. These endocrine processes use a variety of hormones to regulate growth, development, metabolism, morphogenesis and other physiological parameters (Hartenstein, 2006). The insect endocrine system is an important link between the environment and various physiological and developmental events and is the best known and most studied hormone system due to the economic and ecological importance of these invertebrates (deFur, 2004). Development and metamorphosis from embryo to adult in holometabolous insects are events regulated mainly by two types of hormones: ecdysteroids and juvenile hormones (JHs) (Riddiford, 1993).

Ecdysteroid and JH signaling pathways with their respective hormones are the most well-studied routes in insects. In general, for all the insects studied to date, it has been reported that these two signaling pathways work synergically to regulate development and metamorphosis (Noriega, 2014; Riddiford, 2012). Holometabolous insects undergo multiple rounds of larval-larval molts before reaching the pupal stage, where drastic changes in physiology and morphology occur, leading to the sexual maturation of the individual and the emergence of the active adult stage. Molts and metamorphosis are induced by peaks in ecdysteroids release. Additionally, the timing of ecdysteroids pulses, as well as the presence or absence of JHs, will condition molting or metamorphosis, due to the anti-metamorphic action of JHs (Berger *et al*., 1992; Riddiford, 1978; Talbot *et al*., 1993).

A vast variety of changes in gene expression are involved in the control of insect developmental transitions. These changes are under the control of several key hormones and neuropeptides, upon the regulation of steroid hormone, ecdysone (Yamanaka *et al*., 2013). All these processes (Fig. 1.2.) are initiated in response to external stimuli through the secretion of prothoracicotropic hormone (PTTH), which is produced by two pairs of lateral neurosecretory brain cells. PTTH secretion stimulates the prothoracic gland (PG) in the hemolymph, where ecdysone is primarily synthesized from cholesterols. This hormone is initially inactive and is converted into its active form 20-hydroxyecdysone (20E) in the peripheral tissues and fat body by a pool of ecdysteroidogenic genes (Miyakawa *et al*., 2018; Riddiford, 2012; Rewitz *et al*., 2006). The classical knowledge around the control of ecdysone pointed out that PTTH-producing neurons integrate and evaluate environmental and developmental stimulations to determine when the progress to the next developmental stage should take place. Subsequent study revealed that basal levels of ecdysteroids can negatively alter systemic growth prior to maturation (Colombani *et al*., 2005; Delanoue *et al*., 2010; Boulan *et al*., 2013, 2015) and the Corazonin-PTTH neuronal axis is the coordinator of ecdysone biosynthesis (Imura *et al*., 2020). Together, new evidence has shown that, in addition to PTTH, act on the PG to control ecdysteroidogenesis, suggesting that PG is the "decision making center" of developmental transitions (Yamanaka *et al*., 2013). Along with ecdysone, JH has primary roles in regulation of development and reproductive maturation.

JH is a hormone which is unique to insects, synthesized in the corpora allata (CA) in the brain, dispersed into hemolymph and acts at peripheral

tissues. It is converted into active form by a series of enzymes (Goodman and Cusson, 2012; Miyakawa *et al*., 2018; Riddiford, 2012; Shinoda and Itoyama, 2003). JH biosynthesis is regulated by a wide variety of factors, including 20E and JH itself (Goodman and Cusson, 2012). Moreover, the JH epoxide hydrolase (JHEH) is involved in JH catabolism by reducing JH titers in critical developmental periods (Goodman and Cusson, 2012; Seino *et al*., 2010).

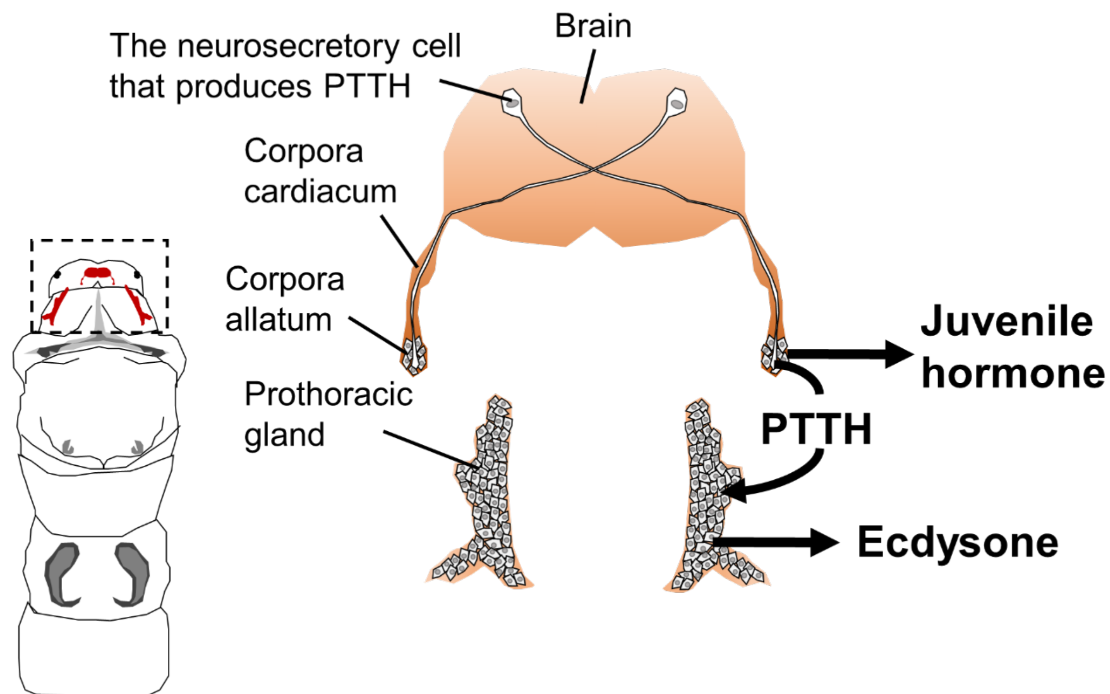


Fig. 1.2. Key organs and hormones in the control of insect development. A schematic illustration of the location of endocrine organs in a larva of *Bombyx mori* (left). Organs are indicated in red. Neurosecretory cells that secrete PTTH have cell bodies in the brain and axon terminals in the corpora allata. Nonneural endocrine cells in the prothoracic glands secrete ecdysone. The corpora allata also contain nonneural endocrine cells that secrete juvenile hormone. Hormones are in bold face.

1.3. Receptors and transcription factors

In peripheral target cells, ecdysone and JH bind to specific receptors. 20E binds to a nuclear receptor superfamily member, ecdysone receptor (EcR). This receptor forms a heterodimer with the ultraspiracle (USP) protein, an orphan nuclear receptor for which no endogenous ligand has been unambiguously stabilized (Goodman and Cusson, 2012). The hormone-activated EcR/USP complex acts as a transcription factor by binding to hormone response elements of downstream genes, thus triggering a cascade of changes in the expression of numerous downstream genes responsible for the major insect developmental transitions (Yao *et al*., 1992, 1993). Although USP does not contribute to the intrinsic function of EcR, it is an obligatory heterodimeric partner of the receptor required for both high-affinity ligand and DNA binding, as it acts as an allosteric effector (Browning *et al*., 2007). Moreover, USP has been described as the JH receptor, as well as the methoprene-tolerant (Met)receptor (Riddiford, 2012). The nuclear receptor USP, an orthologue of the vertebrate retinoid X receptor (RXR), binds JHs and it has been suggested that this interaction may be necessary for the dimerization of EcR/USP (Iwema *et al*., 2009).

The JH-receptor complex induces the expression of the *Krüppel-homolog 1* (*Kr-h1*), a C2H2 type zinc finger transcription factor. During larval stages, *Kr-h1* transduces the anti-metamorphic action of JH in holometabolous and hemimetabolous species (Kayukawa *et al*., 2014; Konopova *et al*., 2011; Lozano and Belles, 2011; Minakuchi *et al*., 2008a, 2008b). *Kr-h1* prevents larvae from undergoing precocious larval-pupal transition by suppressing the pupal specifier gene *Broad-Complex* (*Br–C*), a Broad-Tramtrack-Bric-a-brac (BTB) domain-C2H2-zinc finger transcription factor (Jindra *et al*., 2013;

Kayukawa *et al*., 2014). Further, *Kr-h1* inhibits the precocious adult transition by repressing the transcription of the metamorphic gene *Ecdysteroid induced protein 93F* (*E93*), which encodes a Pipsqueak-family transcription factor (Belles and Santos, 2014; Kayukawa *et al*., 2017). The JH, Met, Kr-h1 and E93 pathway (the MEKRE93 pathway: Belles and Santos, 2014, Fig. 1.3.) is found as a principal route of insect metamorphosis. However, several questions are left to be addressed to fully understand the insect life cycle.

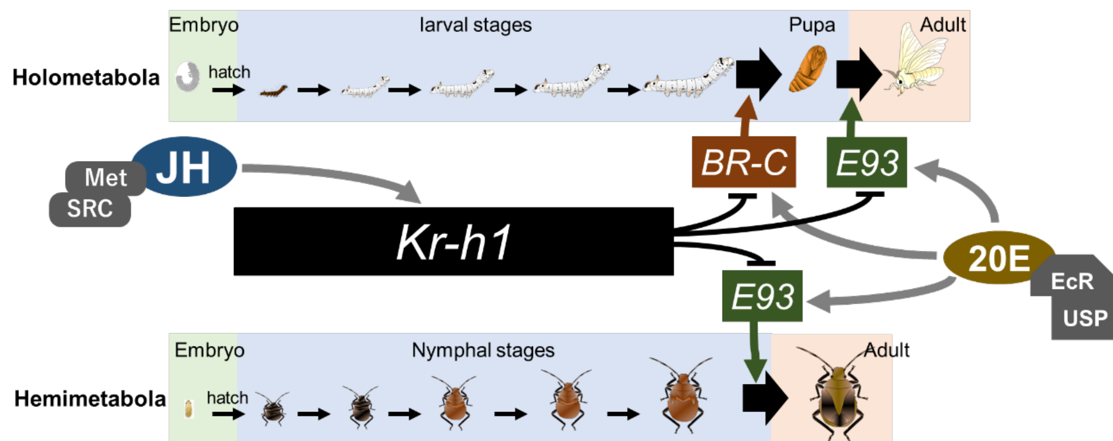


Fig. 1.3. A model JH pathway representing regulation of metamorphosis. Grey lines represent the induction of genes by hormones. Black lines represent interactions in a given pathway. Brown and green lines represent induction of pupal and adult metamorphosis, respectively.

1.4. MicroRNAs and insect metamorphosis

Cells contain a variety of non-coding RNAs (ncRNAs), including components of the gene expression machinery such as tRNAs, rRNAs and regulatory RNAs, which influence the expression of other genes. However, it has long been thought that almost all genes encode proteins, so ncRNA genes were effectively invisible..

Recently, it has become clear that ncRNAs are diverse and that a significant proportion of genes in all organisms do not encode proteins. The miRNAs are a class of small ncRNAs that have been recognised to be numerous and phylogenetically extensive (Lau et al., 2001; Lee and Ambros, 2001). miRNA genes produce short transcripts of about 21 nucleotides that function as antisense regulators of other RNAs. miRNAs were first described in 1993 by Lee (Lee et al., 1993) and later named miRNAs by Ruvkun (2001). To date, thousands of miRNAs have been identified in various organisms by random cloning and sequencing or in silico prediction.

Numerous studies have demonstrated the relationship between miRNAs and the metamorphosis process in insects. Some of them suggested that miRNA expression is induced or reduced by insect hormones (Chawla and Sokol, 2012; Sempere et al., 2002; Sempere et al., 2003), others suggested that miRNA targets genes involved in insect development (Biryukova et al., 2009; Caygill and Johnston, 2008; Ronshaugen et al., 2005; Sokol et al., 2008). Sokol et al. (2008) showed that the *D. melanogaster let-7*-complex locus (*let-7-C*; which includes *let-7*, *miR-100* and *miR-125*) is expressed mainly in the pupal and adult neuromusculature. Let-7-C knockout flies appeared

morphologically normal but displayed defects in several adult behaviours (i.e. flight, motility and fertility).

In insects, it is also known that the expression of some miRNAs are influenced by metamorphic hormones, and *vice versa*. Among holometabolan species, a study using the ecdysteroid deficient *D. melanogaster* mutants revealed that *let-7-C* levels were significantly reduced when ecdysteroid synthesis was impaired (Sempre *et al*., 2003). The role of *Br-C* was also studied using *npr* (a *Br-C* complementary group) mutant files that lack *Br-C*. The work showed *let-7-C* had much lower levels in homozygous *npr* mutant flies, suggesting that ecdysteroid induces *let-7-C* expression via *Br-C* (Sempre *et al*., 2003). Similar relationships between let-7-C, ecdysteroid and *Br-C* was tested in the cockroach, *Blattella germanica*. In *D. melanogaster*, ecdysteroid signaling through *EcR* involves a positive feedback loop that increases *EcR* levels (Karim and Thumel, 1992). *miR-14* modulates this loop by limiting the expression of *EcR* (Varghese and Cohen, 2007). In *B. germanica*, *dicer-1* depletion resulted in an increase of *Kr-h1* level. Further work concluded that miR-2 scavenges *Kr-h1* transcripts when the transition from nymph to adult stage should proceed (Lozano *et al*., 2015).

Multiple miRNAs have been reported in regulation of ecdysone biosynthesis in *Chilo supperessalis* (He *et al*., 2017). In *D. melanogaster*, studies showed that *bantam* and *miR-8* are involved in metamorphosis by controlling ecdysone biosynthesis (Moeller *et al*., 2017; Lim *et al*., 2020). miR-14 is involved in the shutdown of ecdysteroid biosynthesis after ecdysis in *B. mori* (He *et al*., 2019). Meanwhile, there are only few data available on

the possible action of miRNAs on JH synthesis or reception. Recently, *miR-8* has been identified as a positive endogenous regulator of JH biosynthesis, and *bantam* miRNA inhibits *JHMAT* transcription (Zhang *et al*., 2021). Computational analysis predicted the involvement of some miRNAs, such as *let-7, miR-14* and *miR-278*, in regulation of JH biosynthesis (Qu *et al*., 2017). However, functions of these miRNA have not been tested *in vivo*.

1.5. Aim of the thesis

In this study, I addressed the molecular mechanisms underlying the pupal metamorphosis in holometabolous insects. The identity of a larva is determined by the action of Kr-h1 and loss of Kr-h1 results in precocious metamorphosis so does the removal of JH (Jindra *et al*., 2015; Konopova *et al*, 2011; Truman and Riddiford, 2019). However, at least two exceptions to this rule are rising.

First, in a higher Dipteran, *Drosophila melanogaster*, the removal of Kr-h1 does not induce precocious metamorphosis or affect the numbers of larval-larval molt (Pecasse *et al*., 2000). Truman and Riddiford (2022) demonstrated that *chronologically inappropriate morphogenesis* (*chinmo*) mediates maintenance of the larval state through chromatin modification as well as by repression of both *Br-C* and *E93* in *D. melanogaster*. However, the function of *chinmo* or the mechanisms underlying the induction of it have not been tested.

Second, knockout study of JH biosynthetic genes or receptor gene in the silkworm, *Bombyx mori*, resulted in production of normal hatchilings and these larvae can progress through the next two larval instars in the absence of JH and Kr-h1 (Daimon *et al*., 2015). Similar results were reported in a hemimetabolous *Pyrrhocoris* bug upon RNAi depletion of the JH receptor *Met* or its target *Kr-h1* (Smykal *et al*., 2014). Therefore, the absence of JHs or JH signaling alone is not sufficient to induce precocious metamorphosis in very young larvae (Daimon *et al*., 2015, Smykal *et al*., 2014).

If not JH and Kr-h1, then what is the determinant that specifies the larval status in very young phase? Here I set out to test two of my hypotheses, 1) Unidentified factor(s) which enables larvae to express *Br-C* present in late larval stages. 2) Heterochronic microRNA is involved in the control of larval to pupal transition.

2. Implantation assays using the integument of early stage *Bombyx* larvae

Abstract

It is widely accepted that the anti-metamorphic action of insect juvenile hormones (JHs) is required to inhibit larval-pupal metamorphosis. However, recent studies using RNAi or knockout techniques reveal that larval status may be maintained independently of JHs during the early larval stages. To investigate why larvae of very early instars do not have competence to metamorphose and how they acquire this competence through larval development, I revisited the classic experiments of Piepho (ca. 1930s) and performed implantation assays using the integument of very young larvae of the silkworm, *Bombyx mori*.

Here, I demonstrate that when the integuments of neonate larvae or newly molted second instar larvae are implanted into last instar host larvae, they are able to directly produce pupal cuticle at the time of pupal metamorphosis of the host. To investigate whether the pupal commitment of implants from the neonate first instar larvae is repressed by JHs, the integuments of *Met1* knockout larvae lacking a functional JH receptor were implanted into penultimate instar larvae. I found that the implants of *Met1* knockout neonate larvae produced patched pupal cuticles after the host larval molt, whereas those of the wild-type strain produced only larval cuticle without any trace of pupal cuticle.

Taken together, results suggest that the epidermis of very early instar larvae can be pupally committed when provided with unidentified blood-borne factor(s) present in final-instar larvae, and that JHs can block the action of that factor(s) to prolong the feeding period until larvae attain a size appropriate for metamorphosis.

2.1. Introduction

Insect juvenile hormones (JHs) prevent precocious metamorphosis until larvae attain an appropriate size for metamorphosis (Jindra *et al*., 2015, Jindra *et al*., 2013, Nijhout, 1998, Riddiford, 2012). Although it is widely accepted that this "*status quo*" action of JHs is required to maintain the larval status throughout the larval stage, several attempts to deplete JHs using classic and modern techniques have failed to induce precocious metamorphosis in very early larval instars (Abdou *et al*., 2011, Aboulafia-Baginsky *et al*., 1984, Bounhiol, 1938, Daimon *et al*., 2012, Daimon *et al*., 2015, Feyereisen and Jindra, 2012, Fukuda, 1944, Furuta *et al*., 2007, Riddiford *et al*., 2010, Smykal *et al*., 2014, Tan *et al*., 2005). For example, in the silkworm *Bombyx mori*, a classic model in insect physiology, the depletion of JH by neck ligation or allatectomy (surgical removal of the JH-producing endocrine glands, the corpora allata) failed to induce precocious metamorphosis in first (L1) or second (L2) instar larvae (Bounhiol, 1938, Fukuda, 1944). Similarly, recent genetic studies have shown that L1 or L2 larvae cannot undergo pupal metamorphosis even though they carry null mutations in *CYP15C1* and *JHAMT* genes that encode JH biosynthetic enzymes (Daimon *et al*., 2012, Daimon *et al*., 2015). In addition, null mutants of the JH receptors, *Met1* and *Met2*, do not exhibit any pupal characteristics during the L1 and L2 larval stages (Daimon *et al*., 2015). Knockout larvae of *Met1* can reach L3 with the overall appearance of larvae, but have small patches of pupal cuticles in specific regions in the thoracic

and abdominal segments, and eventually die without eating. Furthermore, many of the *Met1* mosaic L3 larvae can reach L4 and become larval–pupal mosaics. In severe cases, approximately a half of the bodies of the mosaic L4 larvae was covered with pupal cuticle, yielding a half-larval, half-pupal mosaic (Daimon *et al*., 2015). Dramatic metamorphosis is first observed at larval molt from L3 to L4, but never in larvae molting from L2 to L3. Therefore, it appears that a metamorphic molt cannot be induced until molting from L3 to L4, except in very limited regions of the epidermis. Similar results were reported in a hemimetabolous *Pyrrhocoris* bug upon RNAi depletion of the JH receptor *Met* or its target *Kr-h1* (Smykal *et al*., 2014) that represses metamorphosis in response to JH (Kayukawa *et al*., 2012, Kayukawa *et al*., 2014, Konopova *et al*., 2011, Minakuchi *et al*., 2009, Smykal *et al*., 2014). Taken together, the results of both classic and modern experiments consistently suggest that larval status is maintained independently of JH signaling during very early nymphal/larval stages, in both hemimetabolous and holometabolous insects alike. Therefore, the long-held paradigm that JHs prevent premature metamorphosis throughout the larval stages should be modified, as the absence of JHs or JH signaling alone is not sufficient to induce precocious metamorphosis in very young larvae (Daimon *et al*., 2015, Smykal *et al*., 2014).

In contrast to these observations, classic experiments by Piepho, (Piepho,1938a; Piepho, 1938b) showed that the epidermis of L1 larvae of the greater wax moth, *Galleria mellonella*, could produce pupal cuticles. When implanted into the body cavity of last instar larvae, the epidermis of the L1

larvae produced pupal cuticles at the time of pupal metamorphosis in the hosts (Piepho, 1938a, Piepho, 1938b). These experiments suggest the possibility that L1 tissues are able to be pupally committed (determination to show pupal characteristics at the following molt) and directly metamorphose to pupal tissues when placed in the physiological milieu of the last instar larvae, albeit that as a whole organism, L1 larvae seem to be incompetent to metamorphose (Daimon *et al*., 2015, Smykal *et al*., 2014). Considering the stark differences between the results of experiments at the tissue and organismal levels, Daimon *et al*. (2015) postulated a humoral factor that confers competence for metamorphosis in larval tissues.

To my knowledge, implantation experiments using the epidermis of very young larvae have not been reported since the original reports by Piepho (Piepho,1938a; Piepho, 1938b) in any insects including the tobacco hornworm *Manduca sexta* or *Bombyx mori*. This is likely because the roles of JHs in early larvae have not received much attention and that it is technically difficult to handle the very small tissues of young larvae. Therefore, it remains unclear if the results of Piepho(Piepho,1938a; Piepho, 1938b) are specific to Galleria or if they can be extended to other insects.

In order to understand why very early instar larvae are incompetent to metamorphose, and how they acquire the competence necessary for metamorphosis through larval growth and development, I first performed implantation experiments using the integument of very young larvae of *Bombyx*. I demonstrated that the epidermis of L1 and L2 larvae was able to

produce pupal cuticle when implanted into last instar larvae (L5), as shown in *Galleria* by Piepho (Piepho,1938a; Piepho, 1938b). Surprisingly, even the epidermis of neonate L1 larvae successively produced pupal and adult cuticles at the time of pupal and adult metamorphosis in the host, respectively, when implanted into the last instar *Bombyx* larvae. In addition, to observe the effects of JHs on the process of pupal commitment, I used *Met1* knockout larvae ($Met1^{-/-}$) as a donor of the epidermis to determine the role of JHs in this process. Our results suggest that L1 or L2 epidermis can be pupally committed when provided with some blood-borne factor(s) present in late larval stages, whereas JHs can block the action of that factor(s) to prolong the larval feeding period until larvae attain a size appropriate for metamorphosis.

2.2. Materials and methods

2.2.1. Insects

Silkworms were reared on an artificial diet at 24–27 °C under standard conditions as described previously (Daimon *et al*., 2003). The F1 hybrid strain Kinshu × Showa (K × S; control strain) was purchased from Ueda Sanshu Ltd. (Ueda, Japan). A knockout silkworm strain with a null mutation in the JH receptor gene *Met1* ($Met1^{\Delta 67/+}$; *w-1/w-1*) (Daimon *et al*., 2015) was obtained from the National Agriculture and Food Research Organization, Japan. To obtain $Met1^{-/-}$ animals for implantation experiments, sibling crosses of $Met1^{+/-}$ adults were performed in each generation as previously described (Daimon *et al*., 2015).

2.2.2. Implantation of larval integument

To determine whether precocious metamorphosis could be induced in the tissues of very early (L1 and L2) larvae, I performed implantation assays of the integument according to previously published methods with some modifications (Fukamoto *et al*., 2006, Muramatsu *et al*., 2008, Riddiford, 1976, Riddiford, 1978). When L3–L5 larvae were used as donors, pieces of the dorsal abdominal integuments (~3 mm × 3 mm) from the second abdominal segment were dissected from newly molted donor larvae. When L1 and L2 larvae were used as donors, dorsal thoracic and abdominal

integuments above the spiracles were dissected. Muscle and fat body tissues were carefully removed from the dissected integuments prior to implantation. Integuments were rinsed once with PBS (137 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) prior to implantation. Each of the pieces was implanted into day 0 5th (last) or 4th (penultimate) instar larvae at the intersegmental membrane between the 3rd and 4th abdominal segments of the control strain K × S. The recipient larvae (i.e., hosts) were anesthetized by chilling on ice prior to implantation. To facilitate the formation of the cyst, the pieces of the integument were folded in half so that the epidermal cell layer was on the outside.

2.2.3. Genotyping

As *Met1* homozygous knockouts are larval lethal and the mutant allele is maintained as a heterozygous stock (Daimon *et al*., 2015), the three segregating genotypes (i.e., $Met1^{+/+}$, $Met1^{+/-}$, and $Met1^{-/-}$) were determined using PCR on the genomic DNA template. Each individual larva from the *Met1* line was genotyped when used as a donor of integument for the implantation assay. To achieve this, I individually collected the carcasses of each larva after dissection of the integument, and subjected them to extraction of genomic DNA and subsequent PCR genotyping as described previously (Daimon *et al*., 2015).

2.2.4. Observation of recovered integuments

The implanted host larvae were reared in a group (when the donor was the control strain) or individually (when the donor was the *Met1* strain) until they became L5 larvae or metamorphosed into pupae or adults. The recovered cysts were fixed in 70% (v/v) ethanol, and investigated for the formation of larval, pupal, or adult cuticles using a stereomicroscope (Leica, EZ4W, Wetzlar, Germany) and a scanning electron microscope (SEM; VE-8800, Keyence, Japan).

2.3. Results

2.3.1. Capacity of early-instar epidermis to produce pupal cuticle

I first addressed the question of whether the epidermis of very young *Bombyx* larvae are capable of being pupally committed when implanted into a last instar host, as demonstrated by Piepho (Piepho,1938a; Piepho, 1938b) for the wax moth, *Galleria*. To accomplish this, I used the standard silkworm strain (K × S), dissected small pieces of the integument from the larvae (K × S) at selected developmental stages (neonate L1 or newly molted L2–L5 larvae), and implanted them into newly molted last instar (L5) larvae (Fig. 2.1 and Table 2.1). Both donors and hosts were denied access to food during the molting period, and newly molted individuals were collected and subjected to implantation.

I implanted the integument of donors (L1–L5) into L5 hosts and recovered the implants 3–5 days after pupation. As I experienced difficulty in recovering the implants from pupae, the overall recovery rate of the implants from host pupae was relatively low (34%, 79/232) (Table 2.1). However, the overall successful rate of cyst formation by the implanted integument was high enough (87%, 66/79) (Table 2.1) to carry out the implantation assays. After recovery, the formed cysts were opened and the structure of the newly produced cuticle was examined under a stereomicroscope and SEM (Fig. 2.1). In *Bombyx*, the same epidermal cells sequentially produce larval, pupal, and adult cuticles, which are easily

distinguished based on color and surface structures. As shown in Fig. 2.1, I found that the cysts produced pupal cuticles at the time of pupal metamorphosis of the hosts. As shown in *Galleria* (Piepho, 1938a, Piepho, 1938b), the production of a pupal cuticle was induced irrespective of the developmental stage of the donor animals (Table 2.1). Notably, even in the case of neonate L1 larvae, the cysts produced pupal cuticle when hosts metamorphosed to pupae (Fig. 2.1A and A′). I did not find any trace of new larval cuticle (e.g., patches of larval cuticle) in the pupal cuticle newly produced by the cysts.

Next, I examined whether implants from L1 and L2 donors could produce pupal and subsequently adult cuticles (Fig. 2.2 and Table 2.2). To this end, I implanted the epidermises of L1 or L2 larvae into L5 larvae, and dissected the implants after eclosion of the adult hosts. The recovery of implants from adults was much easier in comparison to recovering implants from pupae, and I was able to recover the implants from all host animals (100%, 40/40) (Table 2.2). Most of the recovered cysts (82%, 31/38) formed two layers of cuticles (Fig. 2.2A). SEM analyses showed that the outermost cuticle of the cysts possessed characteristics of adult cuticle, such as smooth surface structure and differentiated scale and socket cells (Fig. 2.2D), whereas the inner cuticle had a pupal character (Fig. 2.2C). This suggests that the cysts produced a new cuticle twice (i.e., first pupal cuticles and then adult cuticles) during the pupal and adult phases of metamorphosis of the host, respectively. Seven of 38 cysts (18%) did not produce the adult cuticle (Table 2.2), likely because these cysts died or failed to develop during the

pupal period.

Taken together, these results clearly demonstrate that the epidermis of very young *Bombyx* larvae can directly commit to the metamorphic program when exposed to the physiological milieu of last instar larvae and pupae. This contrasts with the observations that, as a whole organism, L1 or L2 larvae are incompetent to metamorphose (Daimon *et al*., 2012, Daimon *et al*., 2015, Fukuda, 1944, Furuta *et al*., 2007, Tan *et al*., 2005).

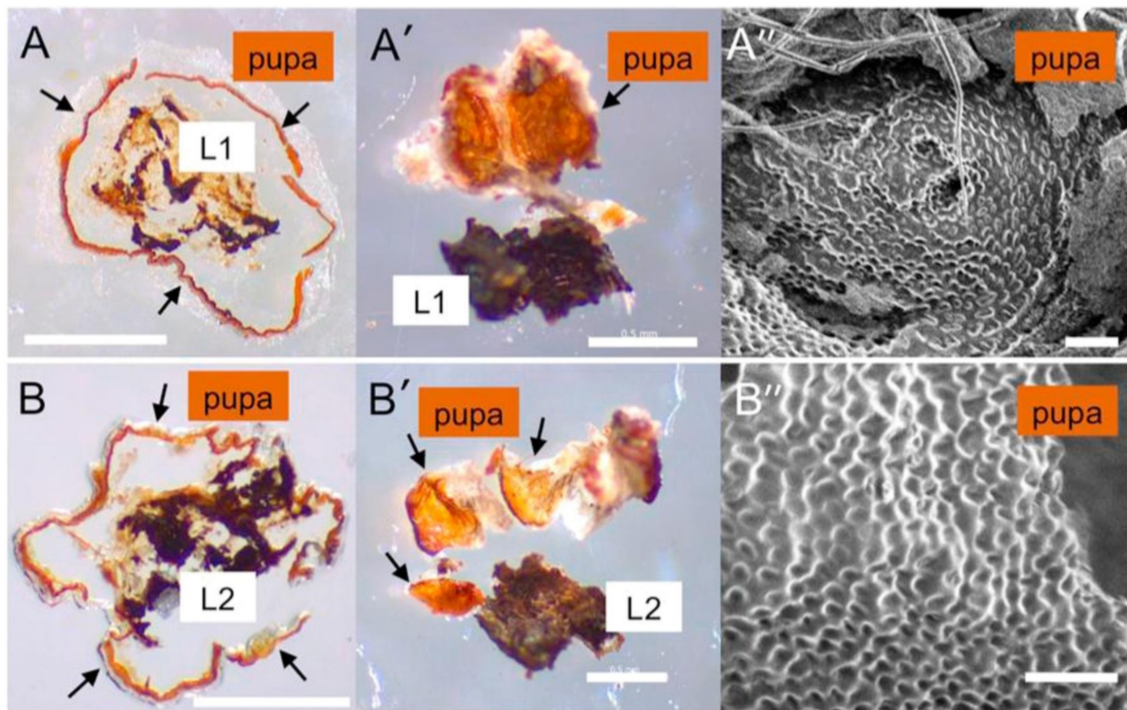


Fig. 2.1. Implantation assays of the integument of the control strain. Pieces of integument from neonate L1 larvae (A, A′, and A″) or newly molted L2 larvae (B, B′, and B″) (strain K × S) were implanted into last instar (L5) larvae. Implants were dissected out 3–5 days after pupation of the host. Frozen sections of the cysts were prepared (A and B) or opened (A′ and B′) to observe the newly formed cuticles. The cysts produced new, brown pupal cuticle at the time of pupal metamorphosis of the host. The SEM analysis showed that the newly produced cuticle possessed the pupal characteristic structures (A″ and B″). Arrows indicate the newly produced pupal cuticle that enclosed the old L1 (A and A′) or L2 (B and B′) cuticles. Scale bars: 0.5 mm in A, A′, B, and B′; 20 μm in A″ and B″.

Table 2.1. Pupal metamorphosis of the epidermis implanted into last instar host larvae.[a]

Stage(strain)		Total operation	Recovered implants	Newly formed cuticles during pupal metamorphosis [b]			Cyst formation failed
Donor (K × S)	Host (K × S)			Larval	Pupal	Adult	
L1 day 0	L5 day 0	78	24	0	19	0	5
L2 day 0	L5 day 0	36	16	0	14	0	2
L3 day 0	L5 day 0	24	5	0	4	0	1
L4 day 0	L5 day 0	47	19	0	18	0	1
L5 day0	L5 day 0	47	15	0	11	0	4

[a] Pooled results from three independent experiments are shown.

[b] Cysts were recovered from host pupae 3–5 days after pupation.

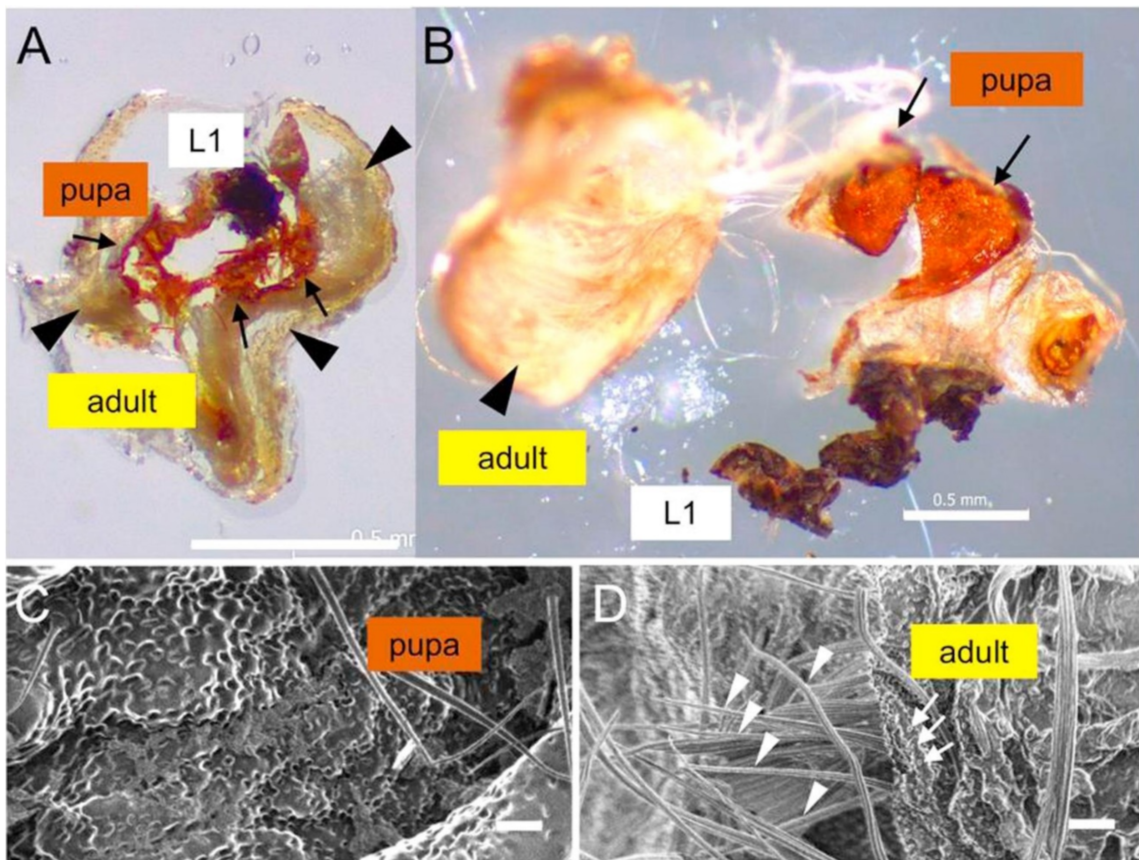


Fig. 2.2. Pupal and adult metamorphosis of L1 implants. (A and B) Pieces of integument from neonate L1 larvae (strain K × S) were implanted into last instar larvae. Cysts were recovered after adult emergence. Frozen sections of the cysts were prepared (A) or opened (B) to observe newly formed cuticles. Cysts sequentially produced pupal (arrows) and adult (arrowheads) cuticles during the pupal and adult metamorphosis of the hosts. Scale bar: 0.5 mm. (C and D) SEM micrographs of pupal (C) and adult (D) cuticles produced by the cysts. Arrows indicate socket cells and arrowheads indicate scales, which are specific to the adult cuticle. Scale bar: 20 μm.

2.3.2. Roles of JHs in the pupal commitment of epidermal implants

As described above, the epidermis of L1 or L2 larvae can be pupally committed when implanted into last instar host larvae. Thus, the next aim was to determine whether the pupal commitment of the implants from L1 larvae is repressed by JHs. As moderately high concentrations of JHs are present in the hemolymph of penultimate instar larvae (L4) (Furuta *et al.*, 2013, Sakurai and Niimi, 1997), I implanted the integuments of neonate larvae into L4 larvae and recovered them from L5 larvae. As shown in Fig. 2.3 and Table 2.3, the implanted L1 epidermis of the control strain produced only larval cuticle without any trace of pupal cuticle. This suggests that the implanted L1 epidermis was sensitive to JHs and thus pupal commitment was inhibited by JHs.

To further investigate the roles of JHs, I performed similar experiments using the integument of *Met1* knockout larvae, which carry a null mutation in the JH receptor *Met1* (Daimon *et al.*, 2015). If JHs prevent pupal commitment of the implanted epidermis, then the epidermis from $Met1^{-/-}$ larvae should be pupally committed even when implanted into L4 hosts. To test this prediction, I implanted the epidermis from individually genotyped $Met1^{+/+}$, $Met1^{+/-}$, and $Met1^{-/-}$ neonate larvae into L4 hosts, and recovered the implants from L5 larvae. As shown in Fig. 2.4 and Table 2.3, cysts of the $Met1^{+/+}$ and $Met1^{+/-}$ genotypes produced larval cuticles after the hosts molted to L5. In contrast, I found small patches of pupal cuticle in the larval cuticles produced by $Met1^{-/-}$ cysts (Fig. 2.4C). I did not observe any

pupal patches in the case of *Met1*$^{+/+}$ or *Met*$^{+/-}$ cysts (Fig. 2.4A and B). This suggests that the pupal commitment of L1 epidermis is prevented by JHs, and that pupal commitment can occur, albeit partially, even in the presence of JHs if the L1 epidermis is deprived of the JH receptor.

Table 2.2. Adult metamorphosis of the epidermis implanted into last instar host larvae.[a]

Stage(strain)		Total operation	Recovered implants	Newly formed cuticles during pupal metamorphosis [b]			Cyst formation failed
Donor (K × S)	Host (K × S)			Larval	Pupal	Adult	
L1 day 0	L5 day 0	19	19	0	3[c]	16[d]	0
L2 day 0	L5 day 0	21	21	0	4[c]	15[d]	2

[a] Pooled results from two independent experiments are shown.

[b] Cysts were recovered from host adults.

[c] Cysts appeared to fail to produce adult cuticles during adult metamorphosis. Instead, they produced new, second pupal cuticles.

[d] Pupal cuticles were formed inside the newly formed adult cuticles.

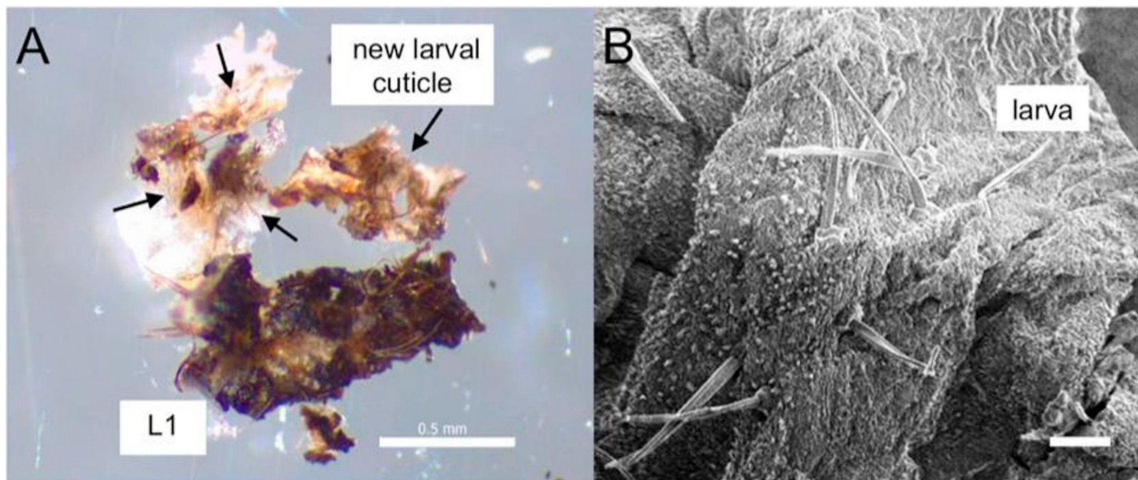


Fig. 2.3. Implantation assay of L1 integument into penultimate instar larvae. (A) Pieces of integument from neonate L1 larvae (strain K × S) were implanted into penultimate instar (L4) larvae, and recovered after the molt to L5. Arrows indicate newly formed larval cuticle. Scale bar: 0.5 mm. (B) SEM micrographs of newly formed larval cuticles. Scale bar: 20 μm.

Table 2.3. Molting and metamorphosis of the epidermis implanted into penultimate instar host larvae.

Stage (strain or genotype)		Total operation	Recovered implants	Newly formed cuticles during the molt from L4 to L5 [b]			Cyst formation failed
Donor [a]	Host (K × S)			Larval	Pupal	Adult	
L1 day 0 (K × S)	L5 day 0	15	15	11	0	0	4
L1 day 0 (*Met1* $^{+/+}$)	L5 day 0	22	22	11	0	0	11
L1 day 0 (*Met1* $^{+/-}$)	L5 day 0	23	23	18	0	0	5
L1 day 0 (*Met1* $^{-/-}$)	L5 day 0	11	11	3	4[c]	0	4

[a] Genetypes of donor larvae from the *Met1* knockout line were individually detemined by PCR.

[b] Cysts were recovered from last instar larvae.

[c] Patched pupal cuticles were formed.

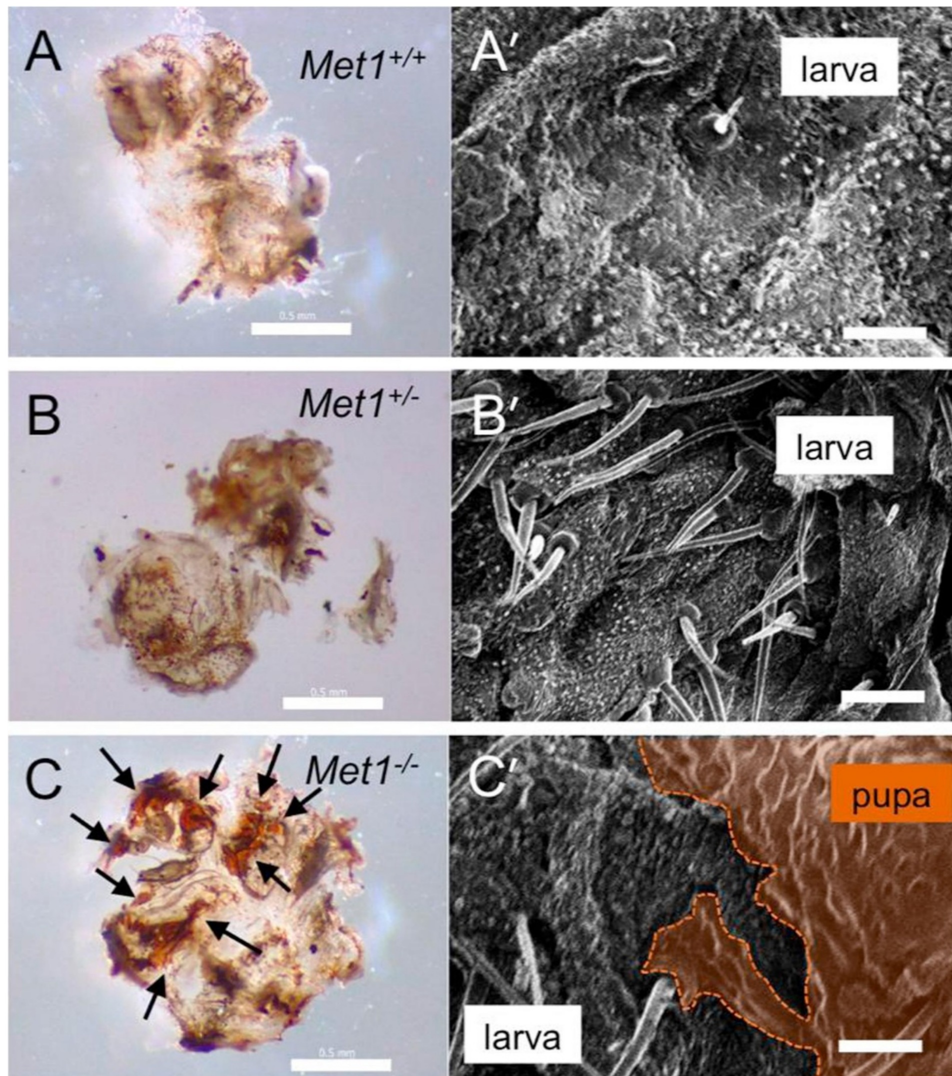


Fig. 2.4. Implantation assay using *Met1* knockout larvae. Pieces of integument from neonate L1 larvae of the *Met1* knockout line were implanted into L4 larvae, and implants were dissected out 3–5 days after the molts to L5. Experiments were performed individually to determine the *Met1* genotypes of donor insects (see Materials and Methods). The epidermis of *Met1*$^{+/+}$ (A, A′) or *Met1*$^{+/–}$ (B, B′) produced larval cuticle, whereas that of *Met1*$^{–/–}$ (C) produced patches of pupal cuticle (indicated by arrows). SEM micrographs (A′–C′) showed that *Met1*$^{–/–}$ cysts produced mosaics of larval and pupal (indicated by brown dotted line) cuticles. Scale bars: 0.5 mm in A–C, 20 μm in A′ and C′, 40 μm in B′.

When integuments from L1 larvae of the Met1 line were implanted into L5 hosts, pupal cuticle formed in nearly the entire implant regardless of whether the genotype was *Met1*$^{+/+}$, *Met1*$^{+/-}$, or *Met1*$^{-/-}$ (Fig. 2.5 and Table 2.4). This suggests a physiological difference between the internal environments of L4 and L5 larvae, where L5 hemolymph would have a higher activity in inducing pupal commitment of the implanted epidermis.

Taken together, I have demonstrated that the epidermis of L1 and L2 larvae can be pupally committed in the endocrine milieu of last instar larvae, although L1 or L2 *Bombyx* larvae are incompetent to metamorphose as a whole organism (Daimon *et al*., 2012, Daimon *et al*., 2015, Fukuda, 1944, Furuta *et al*., 2007, Tan *et al*., 2005).

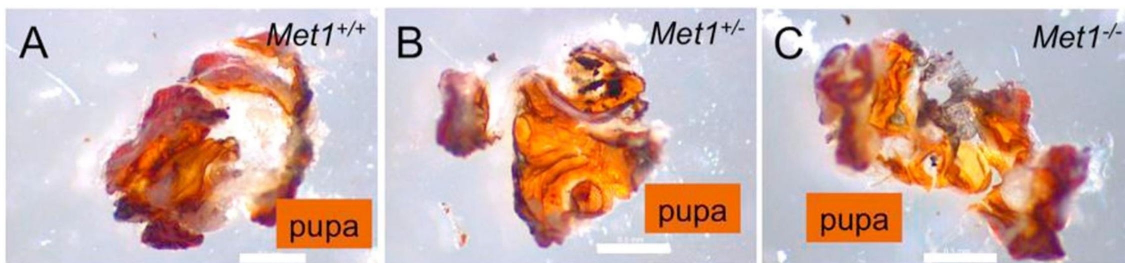


Fig. 2.5. Pupal metamorphosis of *Met1* knockout implants in L5 hosts. Pieces of integument from neonate L1 larvae of the *Met1* knockout line were implanted into L5 larvae, and implants were recovered 3–5 days after pupal metamorphosis of the hosts. The three genotypes (A, *Met1*$^{+/+}$; B, *Met1*$^{+/-}$; C, *Met1*$^{-/-}$) all produced pupal cuticles that covered the nearly entire region of the implant. Scale bars: 0.5 mm.

Table 2.4. Pupal metamorphosis of the Met1 knockout epidermis implanted into last instar host larvae.

Stage (strain or genotype)		Total operation	Recovered implants	Newly formed cuticles during the molt from L4 to L5 [b]			Cyst formation failed
Donor [a]	Host (K × S)			Larval	Pupal	Adult	
L1 day 0 (*Met1* $^{+/+}$)	L5 day 0	22	13	0	7	0	6
L1 day 0 (*Met1* $^{+/-}$)	L5 day 0	24	13	0	4	0	9
L1 day 0 (*Met1* $^{-/-}$)	L5 day 0	10	4	0	3[c]	0	1

[a] Genetypes of donor larvae from the Met1 knockout line were individually detemined by PCR.

[b] Cysts were recovered from host pupae 3–5 days after pupation.

2.4. Discussion

A growing body of evidence has shown that the absence of JHs or JH signaling alone is insufficient for larvae to undergo pupal metamorphosis, as RNAi or knockout of JH biosynthetic or JH receptor genes, and overexpressing a JH-degrading enzyme, as well as the surgical removal of the corpora allata, have failed in inducing precocious metamorphosis in very young larvae (Daimon *et al*., 2012, Daimon *et al*., 2015, Fukuda, 1944, Furuta *et al*., 2007, Tan *et al*., 2005). Conversely, the classic experiments of Piepho, 1938a, Piepho, 1938b have demonstrated that the epidermis of neonate (L1) *Galleria* larvae could directly produce pupal cuticle when implanted into last instar larvae. However, to our knowledge, there has been no further research based on implantation experiments using the epidermis of very young larvae since Piepho (1938a, b).

In this study, I revisited Piepho's experiments using *Bombyx*. I have demonstrated that the epidermis of very early instar *Bombyx* larvae (L1 and L2) are able to directly commit to the pupal program when implanted into last instar larvae (Fig. 2.1 and Table 2.1). As pupal cuticle was not formed upon implantation into penultimate (L4) instar larvae (Fig. 2.3 and Table 2.3), it is likely that the L1 epidermis is sensitive to JHs which prevent its pupal commitment. However, the epidermis of L1 larvae lacking the JH receptor (*Met1*$^{-/-}$) formed patched pupal cuticle after the L4 to L5 molt of the host (Fig. 2.4C), indicating that pupal commitment of the implanted epidermis is indeed prevented by JHs present in the host.

Recent studies have demonstrated that pupal and adult development of holometabolous insects are controlled by *broad* and *E93*, respectively, and stage-specific interactions among broad, *E93*, and *Kr-h1* are critical for pupal and adult metamorphosis (Belles and Santos, 2014, Urena *et al.*, 2016, Urena *et al.*, 2014). In a previous study, Daimon *et al.* (2015) proposed that the inability of very young larvae to undergo pupal metamorphosis could be attributed to the absence of an unidentified humoral factor that is required for the metamorphic induction of broad, a pupal specifier gene (Jindra *et al.*, 2015, Jindra *et al.*, 2013, Konopova and Jindra, 2008, Muramatsu *et al.*, 2008, Reza *et al.*, 2004, Riddiford, 2012, Suzuki *et al.*, 2008, Uhlirova *et al.*, 2003, Zhou *et al.*, 1998, Zhou and Riddiford, 2002). The results presented here are in line with this hypothesis, as they suggest that this humoral factor circulates in the hemolymph of L5 hosts and confers the competence for metamorphosis to the implanted L1 epidermis. The action of this humoral "competence factor" would be blocked by JHs as shown in our implantation assays. If this hypothesis is true, several issues need to be addressed, such as when and where this factor is produced, how it confers competence for metamorphosis or promotes pupal commitment, and whether a similar system is conserved in other insects. I do not know the answers to these questions at this point, and further study is required to provide more clues to better understand the hormonal control of insect metamorphosis. Notably, in *Manduca*, it was proposed that a blood-borne factor overrides the suppression of imaginal disc formation by JHs (Allee *et al.*, 2006, MacWhinnie *et al.*, 2005, Truman *et al.*, 2006). This factor, termed

metamorphosis-initiating factor (MIF), was presumed to be released in response to nutritional cues. A subsequent study demonstrated that the MIF corresponds to insulin-like peptides (Koyama *et al*., 2008). It is thus of interest to investigate whether insulin-like peptides are involved in the process of pupal commitment in the epidermis.

It is also not clear at this point why pupal commitment occurred partially in the epidermis of *Met1*$^{-/-}$ L1 larvae when implanted into L4 hosts (Fig. 2.4C), whereas almost all the cells appeared to be pupally committed when implanted into L5 (Fig. 2.5C). One possible reason is that the time required for the completion of pupal commitment in the epidermis is unusually long in *Bombyx* (~3 days) (Muramatsu *et al*., 2008) in comparison to *Manduca* (~24 h) (Riddiford, 1978). During the L5 stage of the control strain used in this study, pupal commitment of the epidermis gradually occurs between days 3 and 6 (Muramatsu *et al*., 2008), which is followed by a large peak of ecdysone on day 8 (Sakurai *et al*., 1998). In contrast, the ecdysone titer begins to increase from day 2 and peaks on day 3 during L4 (Koyama *et al*., 2004). Therefore, the relatively short time period between implantation (on day 0) and the commencement of the rise of ecdysone (from day 2) in the L4 hosts may have caused a "partial" commitment of the implants. It is therefore of interest to perform similar experiments in other lepidopteran insects such as *Manduca*, in which the time required for the completion of pupal commitment of the epidermis is much shorter than in *Bombyx*. Alternatively, abundance of the "competence factor" may gradually increase

throughout larval development, where the quantity of the factor during the L4 larval instar may not be sufficient to induce pupal commitment in all the cells of the implanted *Met1*$^{-/-}$ L1 epidermis.

It is known that the minimum number of larval instars in holometabolous insects is generally three and rarely two (Esperk *et al.*, 2007), but there are few exceptions. An example of this extremely rare phenomenon are some cave beetles, known to pupate after only a single larval instar (Cieslak *et al.*, 2014a, Cieslak *et al.*, 2014b). Together with these observations, the results of our implantation assays may suggest that the occurrence of at least one larval-larval molt is not a prerequisite for epidermal cells to become competent to metamorphose.

One novel aspect of our present study is the use of genome-edited animals in the context of transplantation experiments. Implantation assays of the integument are one of the classic techniques in insect physiology and have been extensively used to investigate the hormonal control of molting and metamorphosis (Riddiford, 1978). As demonstrated here, I am now in the position to perform classic experiments using genome-edited insects. Recent rapid advances in genome-editing tools have enabled the sophisticated modification of genes in a wide variety of insects and arthropods (Daimon *et al.*, 2014, Hwang *et al.*, 2013, Joung and Sander, 2013). Therefore, I am hopeful that the present study will encourage the coupling of the classic experimental biology with genome-editing technologies to overturn or confirm fundamental ideas, as well as develop

new paradigms in insect physiology.

3. Roles of MicroRNA *let-7* in metamorphic transition

Abstract

The heterochronic microRNA *let-7*, which was first identified in *Caenorhabditis elegans*, controls the timing of developmental programs, and *let-7* triggers the onset of the juvenile-adult transition in bilaterians. The expression of *let-7* is strongly induced during the last larval stage of *C. elegans* and is highly expressed in the late last instar larvae/nymphs of the fly *Drosophila melanogaster* and the cockroach *Blattella germanica*. In the silkworm, *Bombyx mori*, the expression of *let-7* remarkably increases in the corpus cardiacum-corpus allatum complex (CC-CA) at the beginning of the last larval instar and is maintained at high levels during this instar.

To determine the biological function of *let-7* in *B. mori*, I generated a *let-7* knockout line and a transgenic UAS-*let-7* line. The *let-7* knockout larvae were developmentally arrested in the prepupal stage and became pupal-adult intermediates after apolysis. When *let-7* was ubiquitously overexpressed under the transcriptional control of an Actin3-GAL4 driver, developmental timing and growth of larvae were severely impaired in the penultimate (L4) instar, and these larvae underwent precocious metamorphosis from L4.

Furthermore, results showed that reception and signaling of ecdysteroids and juvenile hormones (JHs) normally occurred in the absence of *let-7*, whereas the biosynthesis of ecdysone and JHs were affected by disruption and overexpression of *let-7*. Together, the present study demonstrates that *let-7* is required for the coordination of the biosynthesis of ecdysone and JH to ensure the developmental transition during the metamorphosis of *B. mori*.

3.1. Introduction

MicroRNAs (miRNAs) comprise a large family of short noncoding RNAs that regulate transcript abundance through binding to target mRNAs and facilitate degradation of mRNAs via the RNA-induced silencing complex (Carthew and Sontheimer, 2009; Djuranovic *et al*., 2011, 2012). Hundreds of distinct eukaryotic miRNA genes are involved in development, growth, differentiation, and metabolism (Ambros, 2004; Bartel and Chen, 2004; Kidner and Martienssen, 2004; Lim *et al*., 2003; Plasterk, 2006).

The heterochronic miRNA *let-7*, which was first identified in the nematode *Caenorhabditis elegans*, controls the timing of developmental programs. For example, loss-of-function mutations in *let-7* cause repetition of larval cell fates during adult stages. Conversely, overexpression of *let-7* causes larval cell fates to be retarded and are replaced by precocious adult fate during the larval stage (Reinhart *et al*., 2000; Hutvágner *et al*., 2001; Ketting *et al*., 2001). Remarkably, *let-7*, which is not restricted to *C. elegans*, is evolutionarily highly conserved among diverse animals, including annelids, mollusks, vertebrates, and the fly *D. melanogaster* (Pasquinelli *et al*., 2000). Moreover, *let-7* expression correlates with the onset of adult differentiation in all bilaterians tested, suggesting that role of *let-7* as a key determinator of adult differentiation, indicating that its function has been conserved throughout evolution (Pasquinelli *et al*., 2000).

The stage specificity of the ecdysozoan clade, which includes

nematodes and insects, is defined by the numbers and types of molts. For example, in insects, developmental transitions in the life cycle such as molting and metamorphosis are primally regulated by the actions of ecdysteroids and juvenile hormones (JHs). Pulses of 20-hydroxyecdysone (20E) induce the larval-larval molt in the presence of JHs. When the JHs titer declines to a trace level in the final instar, larval-pupal and pupal-adult metamorphoses are induced by 20E (Belles, 2020; Nijhout, 1994). Topical application of JHs or JH analogs during larval development of numerous insects inhibits normal metamorphosis, leading to supernumerary larval molts or prepupal arrest (Kadono-okuda *et al*., 1986; Kamimura and Kiuchi, 2002). Conversely, the deprivation of JH by the surgical removal of the corpora allata (CA) from the penultimate larvae induces precocious metamorphosis (Dominick and Truman, 1985). Thus, the major function of JH is to prevent larvae from precociously turning into the next stage. The molecular mechanisms of JH-mediated repression of insect metamorphosis are characterized by the events as follows (Jindra *et al*., 2013): The JH-receptor complex induces the expression of the Krüppel-homolog 1 (Kr-h1) transcription factor. During larval stages, Kr-h1 transduces the anti-metamorphic action of JH in holometabolous and hemimetabolous species (Kayukawa *et al*., 2014; Konopova *et al*., 2011; Lozano and Belles, 2011; Minakuchi *et al*., 2008a, 2008b). Kr-h1 prevents larvae from undergoing precocious larval-pupal transition by suppressing the pupal specifier gene *Broad-Complex* (*Br–C*) (Jindra *et al*., 2013; Kayukawa *et al*., 2014). Further, Kr-h1 inhibits the precocious adult transition by repressing the transcription

of the metamorphic gene Ecdysteroid induced protein 93F (E93) (Belles and Santos, 2014; Kayukawa *et al*., 2017).

Numerous studies show that *let-7* interacts with the components of the 20E pathway. For example, in *D. melanogaster*, expression of the *let-7* cluster (*let-7*, *miR-100* and *miR-125*) is indirectly induced by 20E during the prepupal stage (Bashirullah *et al*., 2003; Chawla and Sokol, 2012; Garbuzov and Tatar, 2010), and the 20E-inducible transcription factor Br–C mediates the 20E response (Sempere *et al*., 2002). Moreover, *let-7* contributes to developmental timing (Pasquinelli *et al*., 2000; Sempere *et al*., 2002) and regulates diverse processes during pupal-adult metamorphosis, such as wing development, neurogenesis, innate immunity, and circadian rhythm (Caygill and Johnston, 2008; Garbuzov and Tatar, 2010; Wu *et al*., 2012; Chen *et al*., 2014; Chawla *et al*., 2016). Similarly, in the cockroach Blattella germanica, expression of the *let-7* cluster significantly correlates with a 20E surge during nymphal-adult metamorphosis. Further, the functions of *let-7* clusters are associated with wing development during metamorphosis (Rubio and Belles, 2013). In the oriental fruit fly *Bactrocera dorsalis*, *let-7* is co-expressed with the ecdysteroid-inducible gene *E75* during the prepupal stage to regulate molting and metamorphosis by targeting *E75* (Peng *et al*., 2019).

Although *let-7* regulates 20E-induced insect metamorphosis, insufficient data are available to indicate how *let-7* interacts with JHs or the components of the JHs signaling pathway to regulate developmental transitions in insects. *In vitro*, the JH analog methoprene reduces the

stimulatory effect of 20E on the *let-7* cluster in *Drosophila* S2 cells (Sempere *et al*., 2003). A similar inhibitory effect of a JH analog occurs in *B. germanica* (Rubio *et al*., 2012). The opposing effects of JH and 20E support the conclusion that Br–C mediates the regulation of the *let-7* cluster. To our knowledge, the study of Song *et al*. (2018) provides the only evidence that *let-7* interacts with JH signaling pathway. In the migratory locust *Locusta migratoria*, *let-7* and *miR-278* bind to the *Kr-h1* coding sequence and downregulate its expression. Application of *let-7* and *miR-278*-mimics reduces the level of *Kr-h1* transcripts, leading to the precocious appearance of adult-specific patterns in the nymphal pronotum. Further, these molecules markedly decrease the accumulation of yolk protein precursors, arrest ovarian development, and block oocyte maturation in adults. However, further studies have apparently not been conducted to identify the mechanisms through which *let-7* contributes to JH-dependent developmental transitions in insects.

Here I asked whether *let-7* and insect hormones, ecdysteroid and JH, coordinately regulate the juvenile-adult transition in the silkworm Bombyx mori. Liu *et al*. (2007) reported that the expression of *let-7* significantly increases during the early larval instar (L2), which occurs much earlier than pupal metamorphosis (after L5), during which it is expressed at its maximal levels in the prepupal and pupal stages. Our present detailed spatial and temporal analyses of *let-7* showed that expression of *let-7* is largely restricted to the corpus cardiacum-corpus allatum complex (CC-CA), which produces JH, and is constitutively maintained throughout the last larval instar

independently of 20E in the hemolymph. I next analyzed the functions of *let-7* during the larval-pupal transition using *let-7* knockout silkworms as well as transgenic silkworms overexpressing *let-7*. Our phenotypic analyses of these lines demonstrate the roles of *let-7* during larval-pupal metamorphosis.

3.2. Materials and Methods

3.2.1. Experimental animals and dissection

Silkworms were reared on an artificial diet (Kuwanohana) at 24 °C–27 °C under previously described standard conditions (Daimon *et al*., 2003). For the implantation assays, the F1 hybrid strain Kinshu × Showa (K × S; control strain) was purchased from Ueda Sanshu Ltd. (Ueda, Japan). Tissues were dissected at various developmental stages in phosphate-buffered saline (PBS, 137 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4).

3.2.2. TALEN-mediated knockout silkworm

Knockout silkworms were generated using TALENs as previously described (Daimon *et al*., 2015; Takasu *et al*., 2013). Briefly, 400 ng of TALEN mRNAs (200 ng per pair) were coinjected into preblastoderm embryos of a *pnd* (*pigmented and nondiapausing egg*) *w-1* (*white egg 1*) strain, which is a standard strain for performing transgenesis (Tamura *et al*., 2000). An established *let-7* knockout line was not outcrossed to other standard strains to enable comparisons of phenotypes and gene expression levels in the same genetic background. The parental *pnd w-1* strain was used as a control for the *let-7* knockout strain.

3.2.3. *piggyBac* vectors and transgenic silkworms

I anchored a sequence of the *let-7* stem-loop (Fig. 2A), miRBase accession number MI0004968), with BlnI sites at the termini and used it to amplify the cognate sequence from genomic DNA of the last instar of the standard p50 strain using the primers Bmlet7_BlnI_F1 and Bmlet7_BlnI_R1 (Table S1). The *let-7* amplicon was digested with BlnI and inserted into the BlnI site of the plasmid pBacMCS[UAS-3 × P3-EGFP] (Sakudoh *et al*., 2007), which encodes the eye-specific 3 × P3-EGFP marker, to generate the *piggyBac* vector pBac[UAS-let-7; 3 × P3-EGFP]. Transformants were isolated using a standard protocol (Tamura *et al*., 2000), and G1 embryos were screened using a fluorescence stereomicroscope equipped with an EGFP filter (Leica) to obtain the UAS-let-7 line. The ubiquitous GAL4 expressing strain BmActin3-GAL4 (strain name: 193-2) (Uchino *et al*., 2006) was crossed with the established UAS-let-7 strain to produce silkworms expressing the gene of interest.

3.2.4. Phenotypic analysis

Approximately 25%–50% of the eggs from 4 batches produced by the *let-7* knockout line were used to determine hatchability and day of hatching. Hatched larvae were collected each day in individual 96-well plates, and unhatched eggs were collected on day 20 after oviposition (AO). I analyzed unhatched embryos only at stage 25 (pigmentation of trachea)

according to Daimon *et al*. (2015). In the present study, unhatched embryo (stages 1–24) comprised <10% of the eggs. To investigate the development of *let-7* knockout and *let-7* overexpressing larvae, larvae were individually reared in a Petri dish (90 mm diameter, 20-mm deep). Their development was recorded daily until they reached pupal metamorphosis or died. The body weights of larvae were measured after every molt and pupation. To examine the detailed morphological phenotypes, arrested animals and wild-type animals (larvae, pupae, or adults) were fixed in 70% (v/v) ethanol and observed using a stereomicroscope (Leica) and a scanning electron microscope (SEM; VE-8800, Keyence).

3.2.5. Implantation assay

To test whether pupal metamorphosis was induced in the tissues of the *let-7* mutant final instar larvae, I performed implantation assays of the integument according to a published method (Inui and Daimon, 2017). Briefly, integuments (approximately 9 mm^2) from the second abdominal segment were dissected from newly molted donor larvae. Muscles and fat tissues dissected from the integuments were carefully removed. Integuments were rinsed once with PBS before implantation. Each integument was implanted into the body cavity of L5 day 0 instar larvae of K × S. The implanted host larvae were individually reared until metamorphosis to pupae. To determine the presence of pupal cuticles, the recovered cysts were fixed in 70% (v/v) ethanol and observed using a stereomicroscope (Leica) and a

scanning electron microscope (SEM; VE-8800, Keyence).

3.2.6. Quantitative reverse-transcription PCR (qRT-PCR) analysis

Total RNA extracted using TRIzol reagent (Invitrogen) or the RNeasy Plus mini kit (Qiagen) was used to synthesize the cDNA or the first-strand cDNA using ReverTra Ace qPCR RT Master Mix with a gDNA Remover kit (Qiagen). *rp49* served as the internal reference. qRT-PCR was performed in a 10-μl reaction volume containing 1 μl of template cDNA (equivalent to 0.5 ng of total RNA), THUNDERBIRD SYBR qPCR Mix (TaKaRa), and 10 pM each primer. PCR conditions were as follows: 95 °C for 30 s, 1 cycle; followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. Reactions were performed using a Thermal Cycler Dice Real Time System Single (TaKaRa). The absence of byproducts was confirmed using melting curve analysis (95 °C for 15 s, 60 °C for 30 s, 95 °C for 15 s; 1- cycle each) in the same instrument.

To determine the expression patterns of *let-7*, I used the Mir-X miRNA First-Strand Synthesis Kit (Clontech) to convert miRNAs into cDNAs. To quantify the miRNAs, I performed qRT-PCR using the same conditions described above. *U6* served as internal reference. The qPCR primers are listed in Table 3.1.

3.2.7. Genotyping

The *let-7* homozygous knockouts are larval-lethal, and the mutant allele must therefore be maintained as a heterozygous stock. The three segregating genotypes *let-7*$^{+/+}$, *let-7*$^{+/-}$ and *let-7* $^{-/-}$ were determined using PCR analysis of the genomic DNA template. I genotyped the larvae from the *let-7* line used for qRT-PCR and as donors of integument for the implantation assay. For this purpose, I collected the carcass of each larva after dissection of tissues and extracted genomic DNA for subsequent PCR genotyping as previously described (Daimon *et al*., 2015).

3.3. Results

3.3.1. Spatial and temporal expression of *let-7* during larval development

I first examined the spatial expression pattern of the mature *let-7* sequence (let-7-5p) using 13 tissues representing four developmental stages (L4 day 3, L4 HCS, L5 day 3 and L5 Spinning). I found that *let-7* was highly expressed in the CC-CA on day3 L5 (Fig. 3.1A) when the corpora allata (CA) ceases the production of JH (Fig. 3.1B) (Kinjoh *et al*., 2007). I detected relatively small amounts of *let-7* in tissues such as the silk gland, fat body, muscle, testicle, and ovary (Fig. 3.1A). Notably, the expression of *let-7* was mainly limited to L5.

I next analyzed the temporal expression pattern of *let-7* in the CC-CA, I detected drastic changes during larval development as follows: The expression of *let-7* significantly correlated with the molting peak of ecdysteroid during the L4 stage. In contrast, *let-7* was constitutively expressed throughout the L5 stage, although I detected a slight decrease in the timing of gut purge (Fig. 3.1B). The expression of *let-7* was undetectable on pupal day 0. These results show that the expression pattern of *let-7* significantly changed, particularly after the final larval-larval (L4/L5) molt. I therefore hypothesized that *let-7* was temporally involved in the metamorphic processes of the CC-CA, such as the temporal suspension of the biosynthesis of JHs.

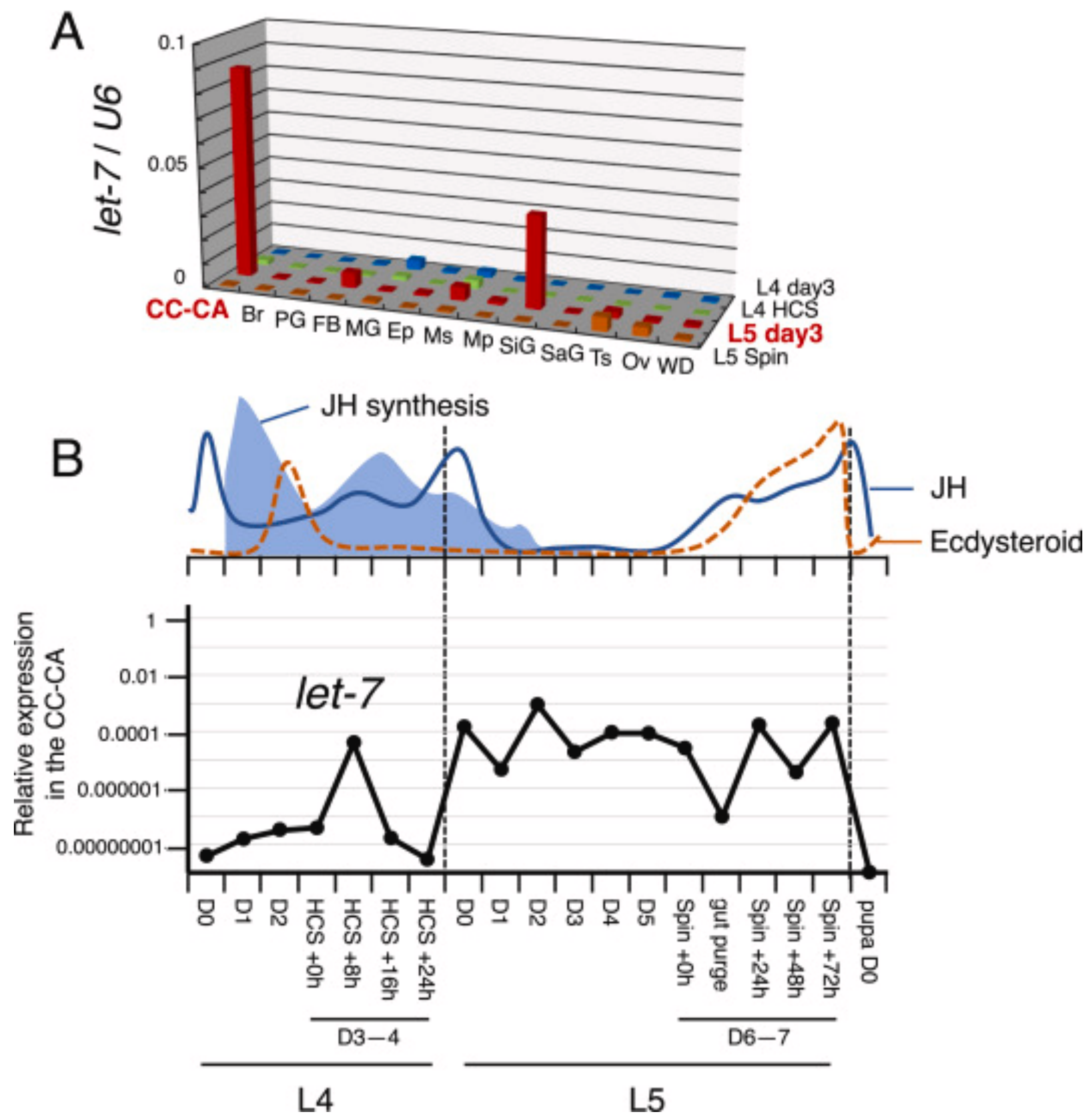

A
let-7 / U6
0.1
0.05
0
CC-CA
Br
PG
FB
MG
Ep
Ms
Mp
SiG
SaG
Ts
Ov
WD
L4 day3
L4 HCS
L5 day3
L5 Spin
B
JH synthesis
JH
Ecdysteroid
Relative expression in the CC-CA
1
0.01
0.0001
0.000001
0.00000001
let-7
D0
D1
D2
HCS +0h
HCS +8h
HCS +16h
HCS +24h
D0
D1
D2
D3
D4
D5
Spin +0h
gut purge
Spin +24h
Spin +48h
Spin +72h
pupa D0
D3−4
D6−7
L4
L5

Fig. 3.1. Spatial and temporal expression of *let-7*. (A) qRT-PCR analysis of the spatial expression of *let-7* in the silkworm strain *pnd w-1*. The level of *let-7* was determined by qRT-PCR and was normalized to that of the internal U6 control. RNA samples were collected from larvae on day 3 of the 4th instar (L4 day 3), 4th instar larvae showing head capsule slippage (L4 HCS), larvae on day 3 of the 5th instar (L5 day 3), and larvae immediately after exhibiting spinning behavior (Spin). CC-CA, corpus cardiacum-corpus allatum complex; Br, brain; PG, prothoracic gland; FB, fat body; MG, midgut; Ep, epidermis; Ms, muscle; Mp, Malpighian tubule; SiG, silk gland; SaG, salivary gland; Ts, testis; Ov, ovary; and WD, wing disc. Tissues were collected from three individuals, and pooled samples were analyzed. (B) Temporal expression pattens of *let-7* (black line with closed circle). The levels of genes normalized to that of internal U6 control. The expression levels of genes were determined using qPCR. Developmental stages are defined as h/days subsequent to developmental events such as molting, head capsule slippage, and spinning. The JH titer is from Niimi and Sakurai (1997). Developmental changes in the rate of JH biosynthesis by *B. mori* CA in vitro was based on Kinjoh *et al*. (2007). The ecdysteroid titer is from Koyama *et al*. (2004; 4th instar), Sakurai *et al*. (1998; 5th instar) and Kaneko *et al*. (2006; 5th instar). Tissues were collected from three individuals, and pooled samples were analyzed.

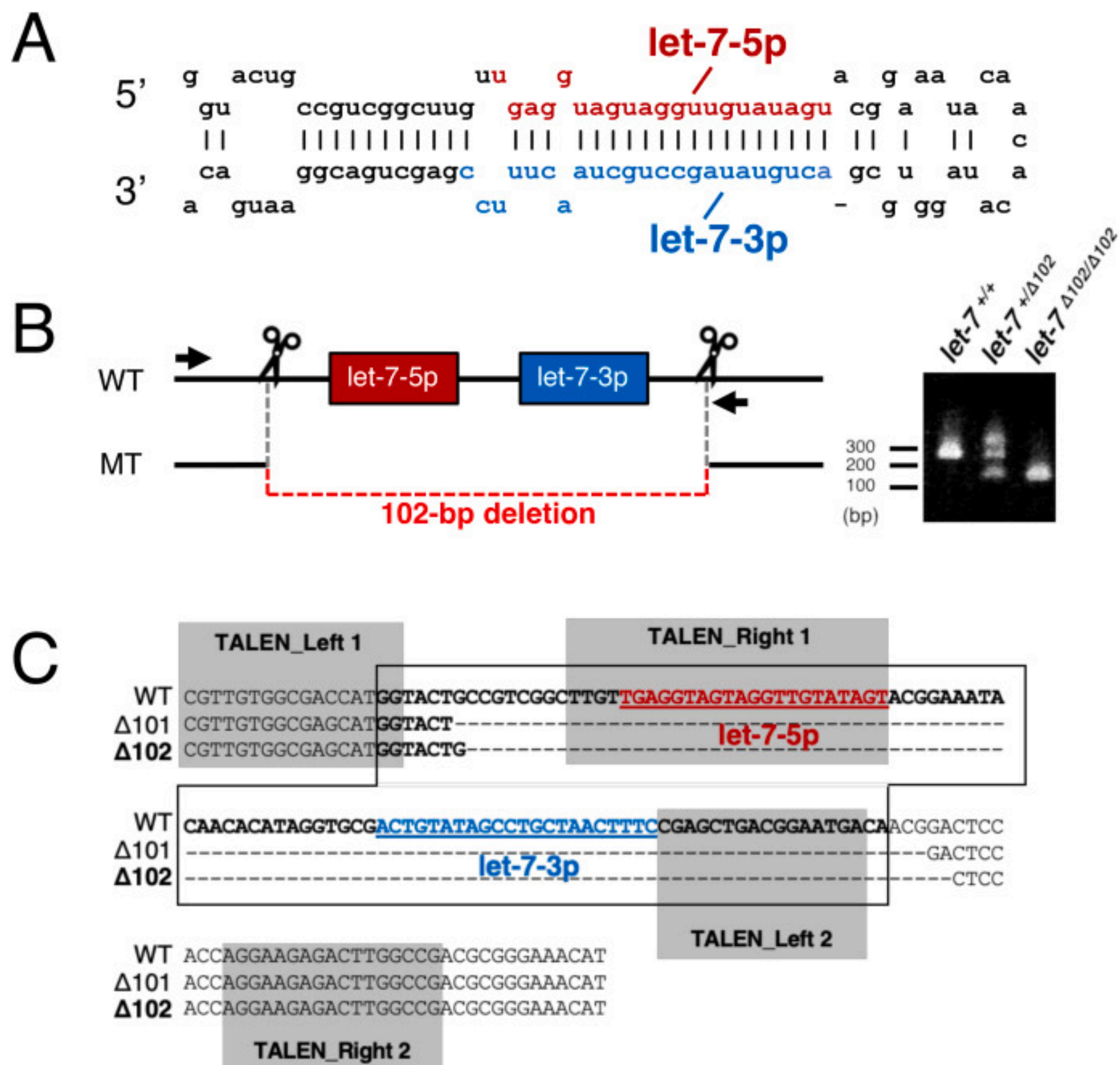


Fig. 3.2. *let-7* knockout alleles. (A) The stem-loop structure of *B. mori let-7*. (B) Schematic representations of mutant alleles used in this study (left). A 102-bp deletion was identified in *let-7*, and this deletion caused a null mutation in *let-7* mature sequences (let-7-5p and let-7-3p). genomic PCR showing the presence of the 102-bp deletion (right). Genotyping primers (Table S1) that flank the deletion are indicated by arrows. (C) TALEN binding sites are indicated by gray boxes. TALEN mRNAs were injected into early embryos, and the two mutant alleles with 101- and 102-bp deletions that induce large null mutation were recovered.

3.3.2. Establishment of a *let-7* knockout strain using TALENs

To determine the biological function of *let-7 in vivo*, I generated a *let-7* knockout line. For this purpose, I employed a TALENs-mediated gene targeting approach, because TALENs enable efficient targeted gene disruption in *B. mori* (Daimon *et al*., 2015; Takasu *et al*., 2013). To induce large gene disruptions, I designed two target sites at the junctions of the *let-7* sequence (Fig.3. 2A and B). TALEN mRNA was injected into early-stage embryos of the *pnd w-1* strain, a standard strain for transgenesis (Tamura *et al*., 2000). The hatched larvae (G0) were crossed to the parental strain (*pnd w-1*) to obtain the G1 generation.

I employed PCR using the let-7_F2 and let-7_R1 primers to identify the induced mutations in G1 adults (Table 3.1). I was able to identify two adults harboring mutant *let-7* alleles. These adults were crossed to the parental strain, and mutant alleles were genetically fixed in the next generation (G2). Nucleotide sequence analysis revealed that deletions of 101-bp and 102-bp deletion in each allele, both of which introduced a mutation that resulted in a null phenotype (Fig. 3.2C). I further confirmed that these two knockout lines exhibited the same developmental phenotype (data not shown). I analyzed the line with the 102-bp deletion allele in the experiments described below.

3.3.3. Disruption of *let-7* causes defects in metamorphosis

To investigate the development of the *B. mori let-7* knockout, I used the G4 embryos and larvae generated from the heterozygous G3 sibling cross (*let-7*$^{+/-}$ × *let-7*$^{+/-}$). When I first examined the effects of the *let-7* knockout on embryonic development, I found that the hatchability of embryos was unaffected by the absence of *let-7* (Fig. 3.3A). Further, the day of hatching was not affected compared with that of the control strain (Fig. 3.3B). These results provide compelling evidence that *let-7* is not involved in developmental processes during embryogenesis, consistent with the absence of detectable *let-7* expression throughout embryonic stages (Liu *et al*., 2007).

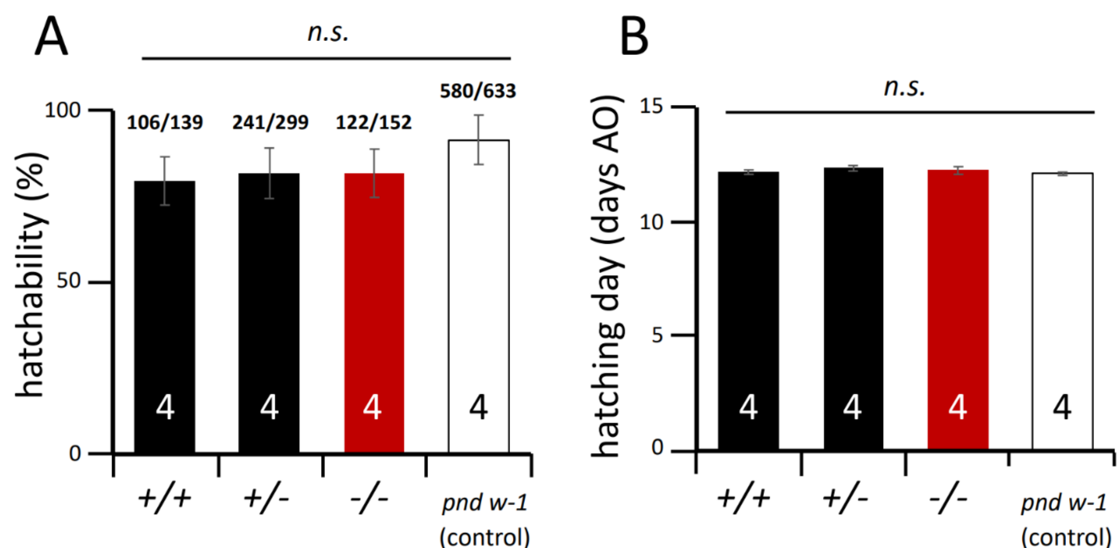


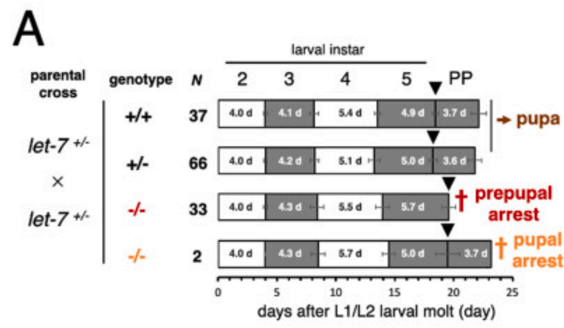
Fig. 3.3. Effects of *let-7* knockout on embryogenesis. (A) Hatchability and (B) day of hatching of mutant strains and the control strain (pnd w-1). Hatched larvae or unhatched embryos were counted, and their genotypes were individually determined using PCR. Genotypes of *let-7* knockout line (+/+, +/- and -/-) are shown below the bars. Bars indicate the mean± SD for (A) hatchability or (B) the hatching day after oviposition (days AO) of each genotype. The numbers above the bars indicate the number of hatched/total, and the number of batches used for this experiment are indicated inside the bars. n.s. indicates no significant differences compared with the control strain ($P \geq 0.05$, ANOVA).

I next analyzed the effects of the null *let-7* mutation on postembryonic development. The *let-7*$^{+/-}$ and *let-7*$^{-/-}$ larvae suffered relatively high rates of lethality during larval development compared with the control *let-7*$^{+/+}$ strain (Fig. 3.4D). In *let-7*$^{-/-}$ larvae, the phenotypes segregated after L5. The majority of *let-7*$^{-/-}$ larvae (25/35; 74%) were arrested in the prepupal stage, and 6% of *let-7*$^{-/-}$ larvae (2/35) metamorphosed to pupae subsequent to L5. Further, the growth of the *let-7*$^{-/-}$ larvae was significantly affected, particularly after the molt to L5 (Fig. 3.4A). The *let-7*$^{-/-}$ larvae exhibited a prolonged feeding period during L5 and became giant L5 larvae when feeding ceased (Fig. 3.4B and C). These *let-7*$^{-/-}$ larvae subsequently exhibited wandering behavior and formed cocoons. Approximately 5 days after wandering, they eventually died in their cocoons after apolysis (Fig. 3.4C').

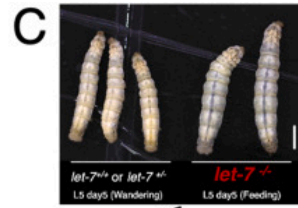
When I used forceps to remove the L5 cuticles of the *let-7*$^{-/-}$

prepupae, I found that the metamorphic growth of imaginal discs and primordia (i.e., wing discs, antennae, and eyes), as well as the degeneration of larval abdominal legs, was induced after apolysis (Fig. 3.4C''). Our observation revealed that *let-7*$^{-/-}$ larvae produced pupal cuticles at the time of apolysis (Fig. 3.5A). The compound eyes developed rows of ommatidia, although they were not fully shaped as in normal adults (Fig. 3.5B). Other structures, such as antennae and legs, appeared to be less affected by the loss of *let-7*, and continued pupal program in arrested animals (Fig. 3.5C and D). Although the antennae had overall pupal appearance, they developed rudimentary adult sensillae, showing the partial formation of adult structures (Fig. 3.5C). Finally, the legs did not seem to have any trace of structures typical for adults (Fig. 3.5D). Taken together, these morphological phenotypes indicate that *let-7* is required for proper differentiation of pupal characters by suppressing precocious adult development during pupal metamorphosis.

A

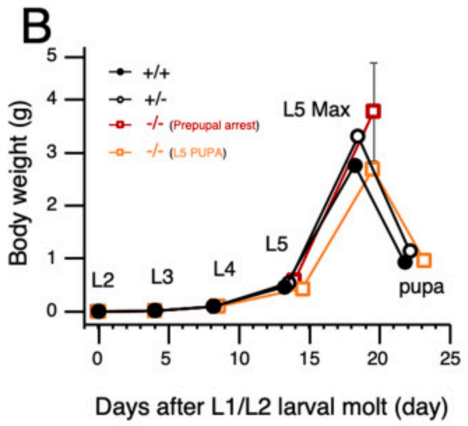


C



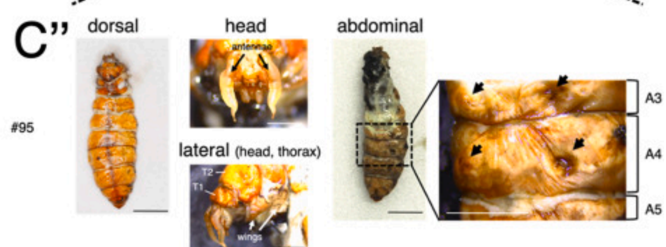
C'

B



C''

#95



D

Genotype	*N*	larval lethal stage L1	L2	L3	L4	L5	pupation
let-7 +/+	37				3	1	33
let-7 +/-	66	3	3	2	1	9	48
let-7 -/-	35		2	4	2	25	2

Fig. 3.4. Phenotypes of *let-7* knockout *B. mori*. (A) Duration of larval periods. Larvae were individually reared until pupation. Each bar indicates a period (mean ± SD) of each developmental stage, i.e. larval (L2–5) and prepupal (PP). *let-7*$^{-/-}$ larvae (G4) were generated from heterozygous G3 sibling crosses (*let-7*$^{+/-}$ × *let-7*$^{+/-}$). Genotypes of larvae and the numbers of individuals are indicated on the left. Numbers along the horizontal axis indicate days after the L1/L2 larval molt. (B) Analysis of body weight during development of *let-7*$^{-/-}$ larvae. L2–5, body weight of newly molted larvae; L5 Max, maximal larval body weight of 5th instar larvae, and pupa, pupal body weight. Each point indicates the mean ± SD. (C, C′ and C″) The pupal-adult intermediate phenotype of L5 *let-7*$^{-/-}$ larvae. Giant *let-7*$^{-/-}$ larvae found in G4 (C, right). (C′) *let-7*$^{-/-}$ larvae that eventually died after apolysis. (C″) Metamorphic growth of imaginal discs in L5 *let-7*$^{-/-}$ larvae. The old L5 cuticles of the arrested *let-7*$^{-/-}$ prepupae were removed using forceps. Evagination of wing discs and antennae (middle left, indicated by arrows) was observed. In addition, the degeneration of larval abdominal legs was observed (middle right, indicated by arrows). Scale bars = 1 cm in C and C” (dorsal and abdominal) and 5 mm in C'and C” (head and lateral), respectively. (D) Development of *let-7* knockouts.

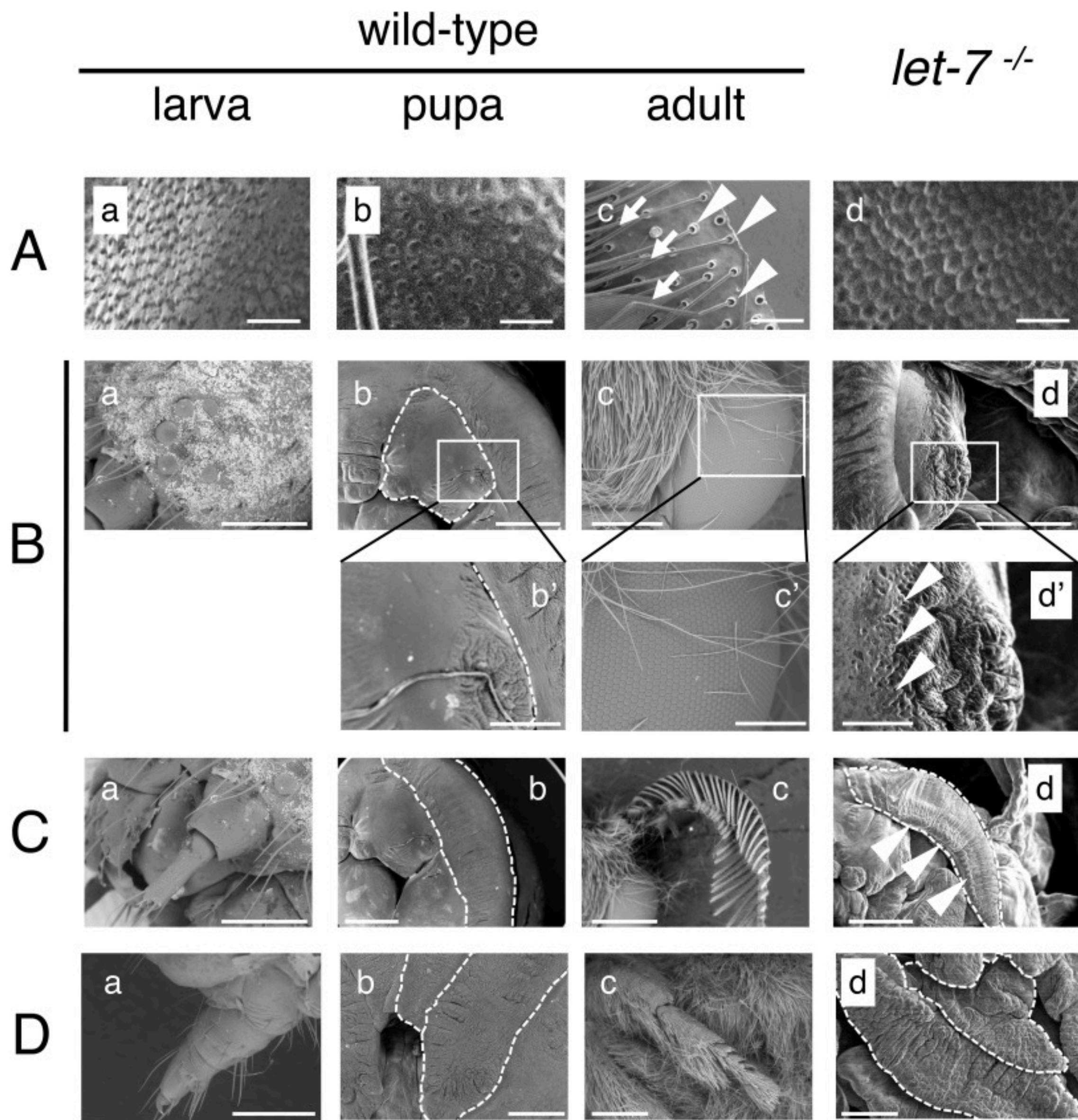

wild-type
larva
pupa
adult
let-7 -/-
A
B
C
D
a
b
c
d
b'
c'
d'

Fig. 3.5. *let-7* is required for proper differentiation of pupal characters. (A) Cuticle surface on the dorsolateral region of A1 segment is shown. Arrowheads indicate socket cells and arrows indicate scales (c). (B) Larval stemmata (a) differentiate into pupal compound eyes (b, b') during pupal metamorphosis. Rows of ommatidia appear in *let-7* knockout animals (d', arrowheads), although they were not fully shaped as in normal adults (c'). (C) Larval antennae (a) are replaced by pupal antennae (b) during pupal metamorphosis. In *let-7* knockout animals, antennae lose larval characters but, unlike those of pupae, they rudimentary develop sensillae (d, arrowheads). (D) Separations of leg segments seen in adult legs (c) were not observed in legs of *let-7* knockout animals (d). Scale bars = 20 µm (Aa–d), 100 µm (Bd'), 250 µm (Bb', Bc), 500 µm (Ba, Bc, Bd, Ca, Db, Dd), 1 mm (Bb, Cb, Cc, Cd, Da, Dc).

3.3.4. Effects of *let-7* disruption on the hormonal regulation of pupal metamorphosis

To investigate the effect of *let-7* knockout on hormonal regulation of pupal metamorphosis, I determined expression profiles of the JH-inducible genes and ecdysteroid-inducible genes in the epidermis during L5. I selected *Kr-h1* isoforms α and β, which are classified as JH-early inducible genes responsible for the repression of metamorphosis (Kayukawa *et al*., 2014; Konopova *et al*., 2011; Lozano and Belles, 2011; Minakuchi *et al*., 2008a, 2008b). I observed decreased levels of *Kr-h1α/β* transcripts in the *let-7*$^{+/+}$ larvae during the L5 feeding period as well as a transient peak of expression during the prepupal stage (Fig. 3.6A). These gene expression profiles were consistent with those previously reported (Kayukawa *et al*., 2014). In contrast, prepupal peaks of *Kr-h1α/β* were delayed and reached higher levels in *let-7*$^{-/-}$ larvae compared with those of the control strain.

I next determined the levels of expression of ecdysteroid-inducible genes in tissues that included the transcription factors *Br–C, E75A, E75B, βFtz-F1, E93A*, and *E93B*), which are involved in the ecdysteroid signaling pathway (Hiruma and Riddiford, 2010). In the epidermis of *let7*$^{+/+}$ larvae, these genes were highly expressed before the onset of pupation (Fig. 3.6B), corresponding to the elevation of ecdysteroid levels in hemolymph. In the tissues of *let-7*$^{-/-}$ larvae, the pattern of expression of these six genes were similar to those of the *let-7*$^{+/+}$ larvae. However, the peak levels of these transcripts were delayed, reflecting the extended feeding period (Fig. 3.6B).

Importantly, expression of the pupal specifier gene *Br–C*, which is upregulated at the late stage of last instar in holometabolan insects, including *B. mori* (Konopova and Jindra, 2008; Belles and Santos, 2014; Ureña *et al.*, 2014, 2016; Daimon *et al.*, 2015), was detected in the absence of *let-7* expression.

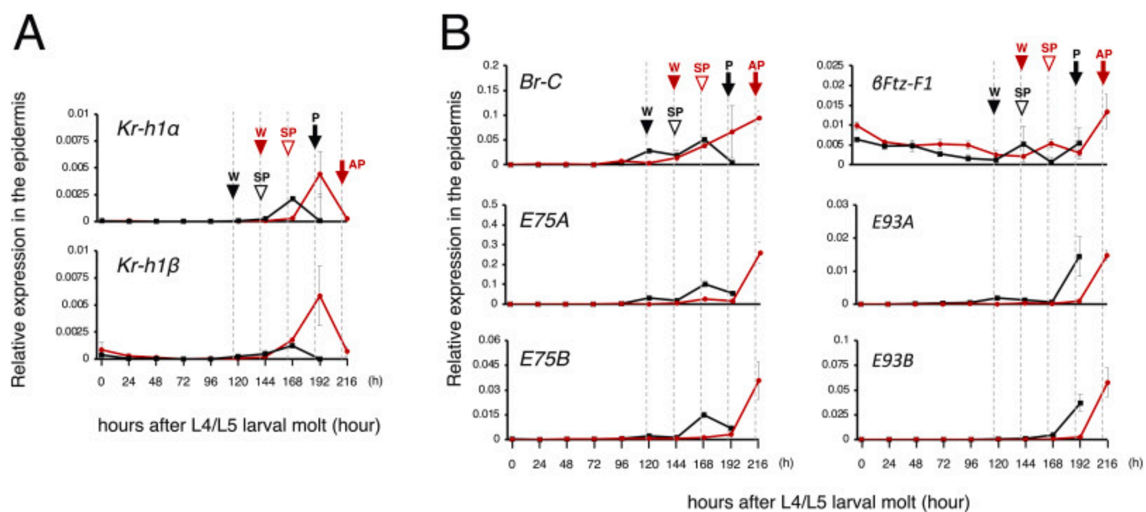


Fig. 3.6. mRNA levels of genes involved in ecdysteroid or JH signaling pathways in *let-7* knockouts. qRT-PCR analysis of genes involved in JH (A) and ecdysteroid (B) signaling pathways in the epidermis of the control (*let-7*$^{+/+}$ larvae, indicated by black lines and black squares) and *let-7*$^{-/-}$ larvae (indicated by red lines and red circles). mRNA levels were normalized to those of *rp49*. Numbers below indicate developmental stages, which are defined as hours after L4/L5 ecdysis. W, wandering (filled arrowhead); SP, spinning (blank arrowhead); P, pupation (black arrow); and AP, apolysis (red arrow). Vertical dotted lines indicate the timing of metamorphic events. The results represent mean ± SD (N = 3 biological replicates).

To determine whether the delay in expression of ecdysteroid-inducible genes in *let-7*$^{-/-}$ larvae was caused by the delay of ecdysteroidogenic activity in the PG, I analyzed the Halloween genes *nvd, nm-g, spo, phm, dib*, and *sad* (Fig. 3.7). In control PGs, expression levels of these genes peaked before pupal metamorphosis (Fig. 3.7). In the PG of *let-7*$^{-/-}$ larvae, expression of these genes peaked before apolysis, although the timing was delayed (Fig. 3.7). Thus, these results indicate that delayed ecdysteroidogenic gene expression in the PG delayed expression of the ecdysteroid-inducible genes in *let-7*$^{-/-}$ larvae. Moreover, expression levels of phm and sad were significantly affected in *let-7* knockouts, suggesting that *let-7* regulates the expression of *phm* and *sad*.

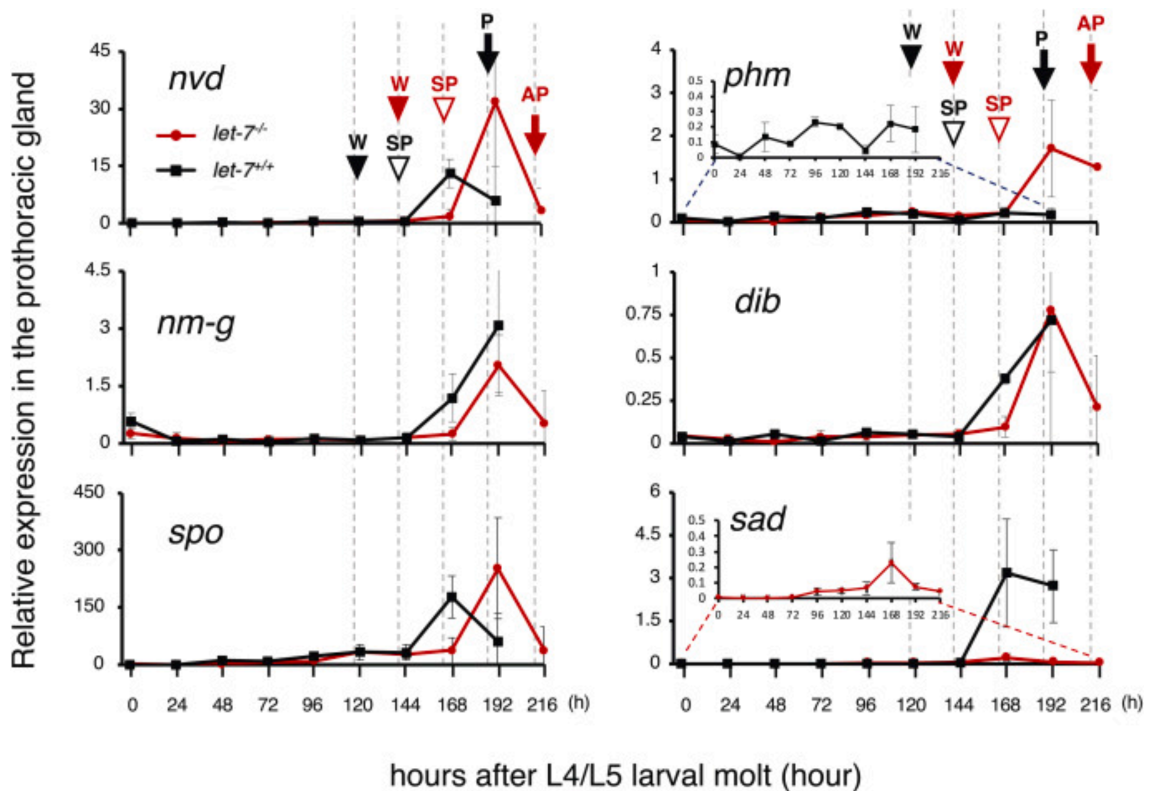
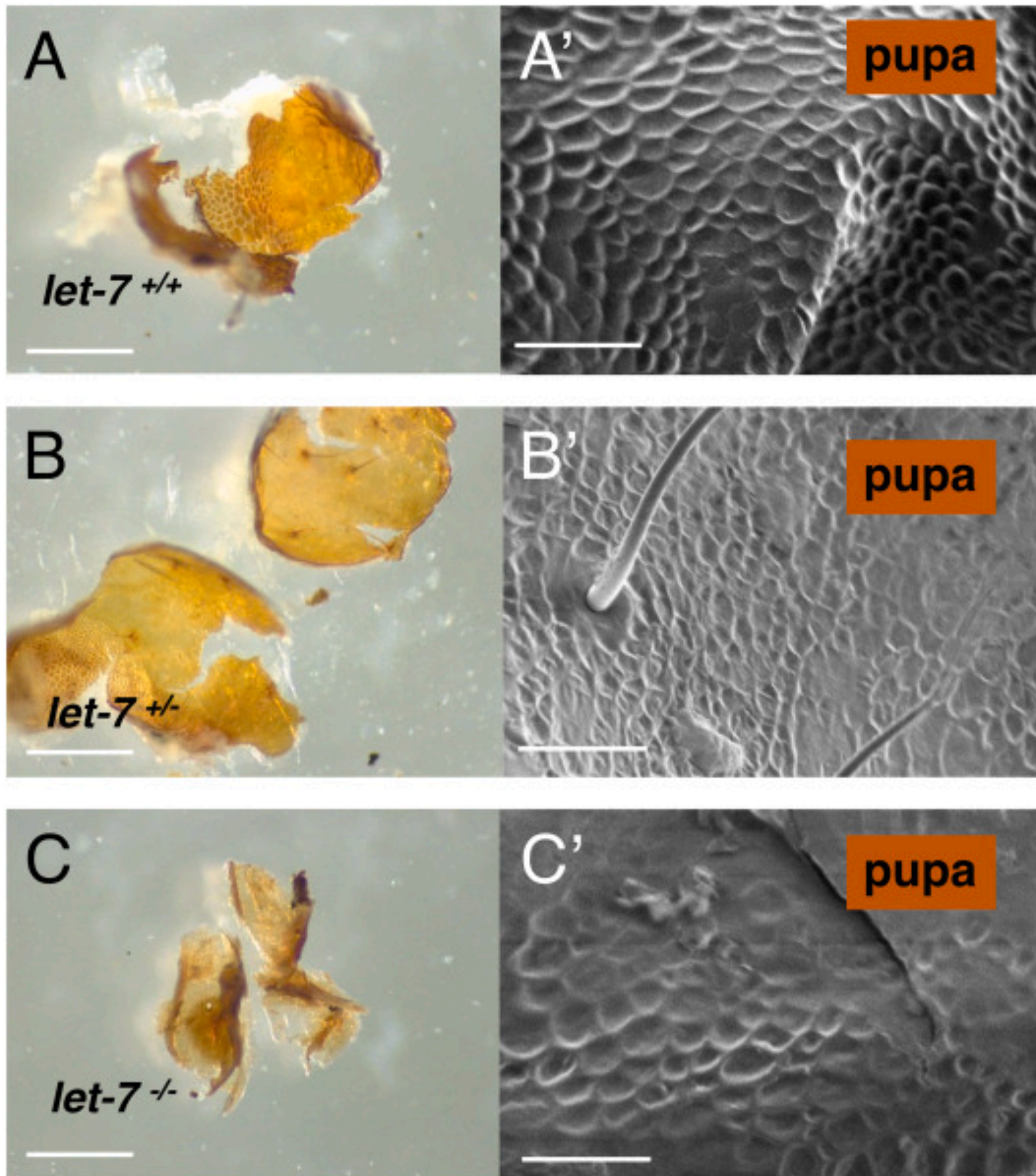


Fig. 3.7. Expression of Halloween genes in the prothoracic gland (PG) of *let-7* knockouts. qRT-PCR analysis of the expression of ecdysteroid biosynthetic genes in the PG of the control (*let-7*$^{+/+}$ larvae, black lines and black squares) and *let-7*$^{-/-}$ larvae (red lines and red circle). mRNA levels were normalized to those of *rp49*. Numbers below indicate developmental stages defined as hours after 5th instar ecdysis. W, wandering (filled arrowhead); SP, spinning (blank arrowhead); P, pupation (black arrow); and AP, apolysis (red arrow). Vertical dotted lines indicate the timing of metamorphic events. The results represent the mean ± SD (N = 3 biological replicates).

I next conducted implantation assays using *let-7*$^{-/-}$ integuments to determine whether the epidermis was committed to pupal metamorphosis in the absence of *let-7*. For this purpose, I dissected small pieces of the integument from last instar (L5) *let-7*$^{-/-}$ larvae and implanted them into larvae of the last instar standard strain (K × S) (Fig. 3.8D). I implanted the integument of donors into L5 hosts and recovered the implants 3 days after pupation. I examined the microscopic structure of the newly produced cuticle. I found that the implants produced pupal cuticles at the time of pupal metamorphosis regardless of genotype (*let-7* $^{+/+}$, *let-7* $^{+/-}$ or *let-7*$^{-/-}$) (Fig. 3.8). Thus, the epidermis can be pupally committed in the absence of let-7 when exposed to the hormonal milieu of wild-type larva. Together, these results suggest that *let-7* is not required for reception and signaling of JH and ecdysteroids and further suggest that *let-7* is not involved in metamorphic processes in the epidermis.



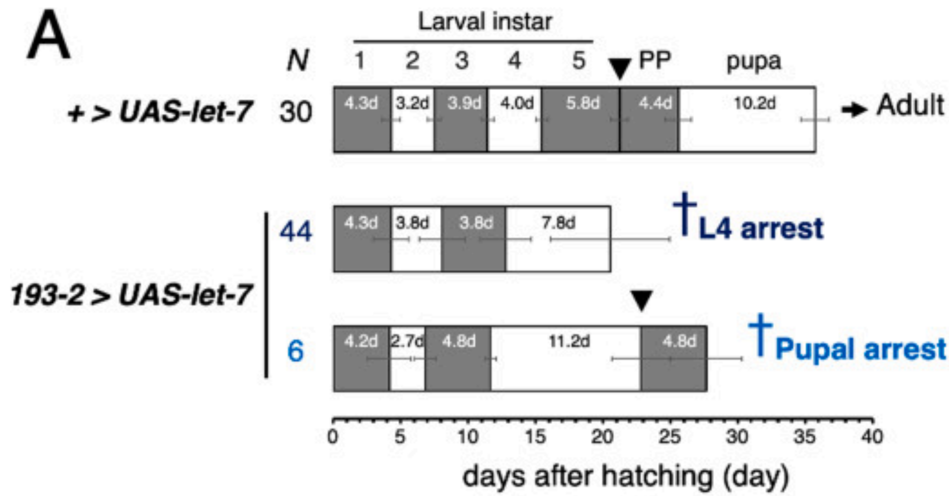
D

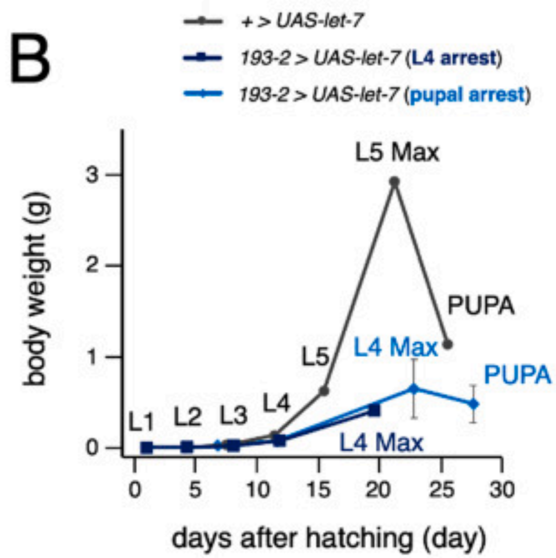
genotype of donor	total operation	recovered implants	Newly formed cuticles during pupal metamorphosis			Cyst formation failed
			Larval	Pupal	Adult	
let-7 $^{+/+}$	7	4		4		
let-7 $^{+/-}$	9	5		4		1
let-7 $^{-/-}$	5	5		3		2

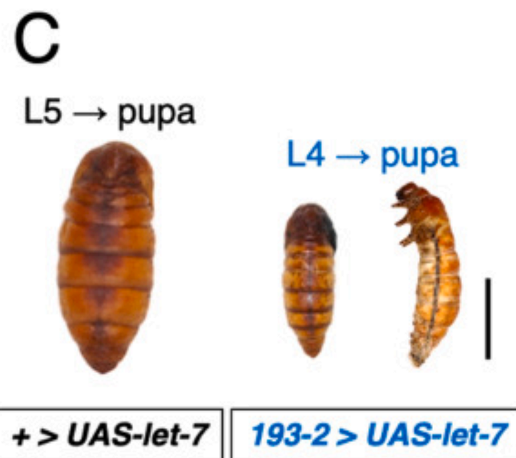
Fig. 3.8. Implantation assay using *let-7* knockout larvae. (A–C) Pieces of the integument of just molted L5 larvae of the *let-7* knockout line were implanted into L5 K × S larvae, and the implants were dissected 3–5 days after pupation of the hosts. Experiments were individually performed to determine the *let-7* genotypes of donor insects. Stereomicroscopic observations (A–C) and SEM analysis (A′–C′) showing the donor epidermis-produced pupal cuticle independent of genotype. Scale bars = 0.5 mm and 20 μm in A–C and A′–C′, respectively. (D) Pupal metamorphosis of the epidermis implanted into last instar host larva. Pooled results from three independent experiments are shown. Genotypes of donor larvae from the *let-7* knockout line were individually determined by PCR. Integuments were recovered from 3 to 5 days after pupation.

3.3.5. Overexpression of *let-7* causes precocious pupal metamorphosis from the penultimate larval instar

To further investigate the biological functions of *let-7*, I generated transgenic silkworms overexpressing *let-7* (Actin3-GAL4>UAS-let-7; abbreviated let-7O/E). The development and survival of *let-7*O/E larvae were recorded at the beginning of each larval instar and when they metamorphosed into pupae. Most *let-7*O/E larvae reached L4, although the developmental timing and growth was severely impaired during L4 (Fig. 3.9A). Interestingly, 35/50 (70%) were developmentally arrested during this instar, whereas 6/50 (12%) *let-7*O/E larvae underwent precocious metamorphosis from the penultimate (L4) instar to form small pupae (Fig. 3.9C and D). However, there was no significant difference between the growth trajectories of the two groups of *let-7*O/E larvae during L4 (Fig. 3.9B).







D

	N	larval lethal stage					metamorphosis				
		L1	L2	L3	L4	L5	L1	L2	L3	L4	L5
+ > UAS-let-7	30	1			1						28
193-2 > UAS-let-7	50	5	2	2	35	-				6	

Fig. 3.9. Phenotypes of transgenic *B. mori* overexpressing *let-7*. (A) Duration of larval periods. Larvae were individually reared until pupation. Each bar indicates the period (mean ± SD) of each developmental stage, i.e. larval (L1–5) and prepupal (PP). + > UAS-let-7 larvae served as controls. The numbers of individuals are indicated on the left and the days after hatching are on the horizontal axis. (B) Analysis of body weight during the larval development of *B. mori* overexpressing *let-7*. L1, body weight on day 1 L1; L2–5, body weight of newly molted larvae; L5 Max, maximum larval body weight of 5th instar larvae, L4 Max, maximum larval body weight of 4th instar larvae; and PUPA, pupal body weight. Each point indicates the mean ± SD. (C) Images of pupae of GAL4/UAS transgenic lines. Larvae overexpressing *let-7* underwent precocious pupal metamorphosis from L4 (right). Control *B. mori* with the UAS-*let-7* construct underwent pupal metamorphosis from L5 (left). Scale bar = 1 cm. (D) Development of *B. mori* larvae after overexpressing *let-7*.

3.3.6. Effects of *let-7* overexpression on the hormonal regulation of pupal metamorphosis

In *B. mori*, depletion of JH induces precocious pupal metamorphosis (Daimon *et al*., 2012, 2015; Feyereisen and Jindra, 2012; Furuta *et al*., 2007; Tan *et al*., 2005). Therefore, I investigated expression of *Kr-h1α*. I found that the expression levels of *Kr-h1α* during the L4 stage of *let-7O/E* larvae remained low compared to the control +>UAS-let-7 strain, although a transient expression peak was observed before pupation (Fig. 3.10). The overall low expression of *Kr-h1* in *let-7O/E* larvae may account for their precocious pupal metamorphosis. I further found that the expression of *Br–C* precociously arose during the L4 stage in *let-7O/E* larvae (Fig. 3.10).

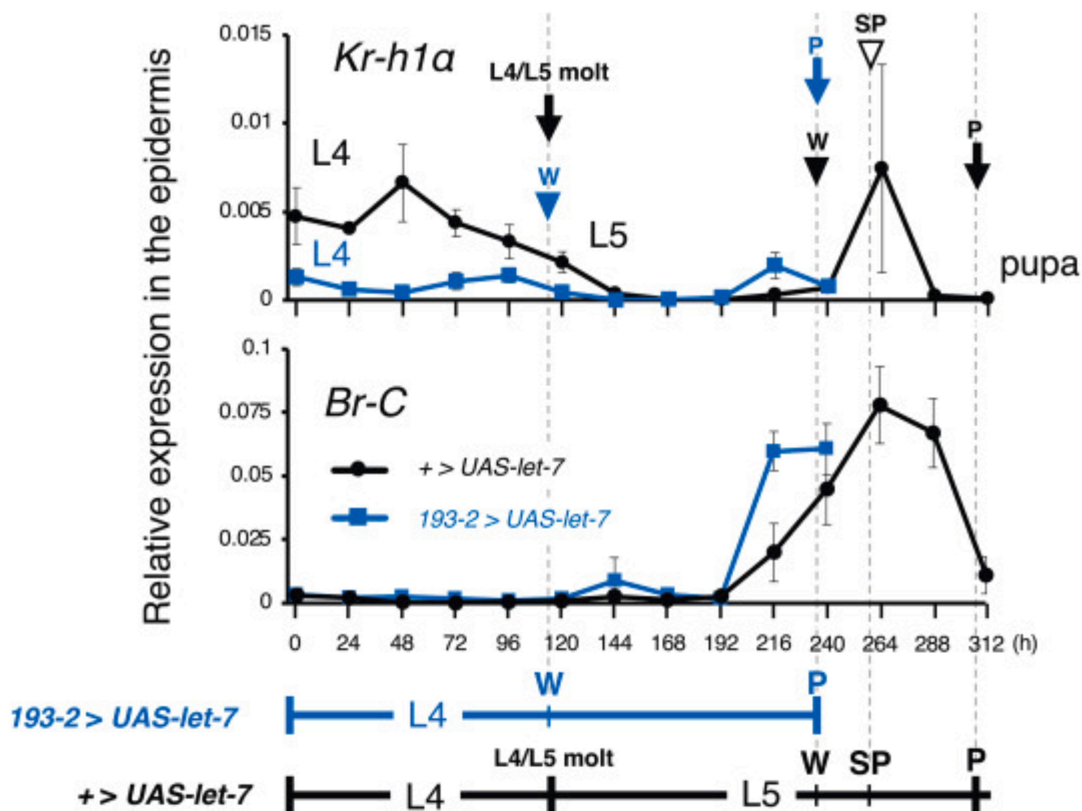


Fig. 3.10. mRNA levels of genes involved in ecdysteroid or JH signaling pathways in *B. mori* overexpressing *let-7*. qRT-PCR analysis of genes involved in JH signaling pathways (*Kr-h1α*, upper) and ecdysteroid signaling pathways (*Br–C*, lower) in the epidermis of the control (+>UAS-*let-7*, indicated by black lines and black circles) and *193-2*>UAS-*let-7* larvae (indicated by blue lines and blue squares). mRNA levels were normalized to those of *rp49*. Numbers below indicate developmental stages, which are defined as hours after 4th instar ecdysis. Marks on the graph and colored lines below the horizontal axis indicate larval duration and timing of metamorphic events. L4/L5 molt, larval molt of control strain; W, wandering; SP, spinning; and P, pupation. Vertical dotted lines indicate the timing of metamorphic events. The results represent mean ± SD (N = 3 biological replicates).

3.4. Discussion

The timing of *let-7* induction, which plays a central role in the heterochronic pathway of *C. elegans*, controls the switch from larval to adult cell fates (Reinhart *et al*., 2000). The gene encoding *let-7* is widely conserved among bilaterians (Pasquinelli *et al*., 2000) and is expressed in an adult-specific manner in all bilaterians tested, suggesting that it may play a conserved role in controlling late temporal transformation across animal phylogeny. In *D. melanogaster* and *B. germanica*, the expression of *let-7* is induced in late last instar larvae/nymphs (Bashirullah *et al*., 2003; Sempere *et al*., 2002; Rubio and Belles, 2013). Here I show that the expression of *let-7* is most restricted to the last larval instar (L5) of *B. mori.* I detected high levels of *let-7* in the CC-CA and much lower levels in the fat body, muscles, and silk gland of L5 day 3 larvae (Fig. 3.1A). Liu *et al*. (2007) previously reported that *let-7* is abundantly expressed in the head of L5, day 3 *B. mori* larva, suggesting that the expression of *let-7* in the head may reflect that in the CC-CA. Interestingly, the timing of *let-7* induction in the CC-CA coincided with the decline of the JH synthetic activity in the organ. Therefore, I investigated in detail the expression pattern of *let-7* in the CC-CA. After the last larval-larval ecdysis, JH synthesis gradually declines and ceases 3 days after ecdysis (Kinjoh *et al*., 2007). Overall, JH biosynthetic activity is modulated by the expression of JH biosynthetic enzymes in the CA, primarily *JHAMT* (Shinoda and Itoyama, 2003). Here I found that *let-7* was constitutively expressed during the declining phase of expression of *JHAMT*

mRNA (L5 D0–D4) (Daimon *et al*., 2012; Shinoda and Itoyama, 2003) and was expressed at its highest level on L5 D2 (Fig. 3.1B).

Here, the levels of *let-7* decreased upon the onset of the gut purge, increased again 24 h after the onset of spinning, remained high during spinning, and became undetectable at the time of pupation, when titers of hemolymph JH and ecdysteroid rise again (Fig. 3.1B). These results suggest that the signal of 20E alone is insufficient to induce *let-7* during the last instar stage of *B. mori.* Several studies show that the expression of *let-7* is enhanced by 20E (Chawla and Sokol, 2012; Garbuzov and Tatar, 2010; Rubio *et al*., 2012) and that the 20E response is mediated by *Br–C* transcription factors (Sempere *et al*., 2002) in *D. melanogaster*. Moreover, *let-7* is induced by the molting peak of 20E in the wing buds of *B. germanica* (Rubio and Belles, 2013). Here I detected the peak of *let-7* expression around the molting peak of 20E during the penultimate (L4) stage, suggesting that *let-7* expression is temporally regulated by 20E during this stage. However, *let-7* was induced in the absence of 20E during early L5. I further found that the expression levels of *Br–C* during this period were very low (Fig. 3.11).

The biological functions of *let-7* have been studied in insects (reviewed in Belles, 2017). For example, numerous studies in *D. melanogaster* show that *let-7* and *miR-125* (an ortholog of *C. elegans lin-4*) mediate diverse morphogenetic processes during pupal to adult metamorphosis (Caygill and Johnston, 2008; Sokol *et al*., 2008; Wu *et al*., 2012). Further, *let-7* is associated with innate immunity (Garbuzov and Tatar,

2010). In *B. germanica*, the functions of *let-7* cluster are associated with wing development during nymphal to adult metamorphosis (Rubio and Belles, 2013). In contrast, our present study of *B. mori* reveals that the most severe phenotype of *let-7* knockouts was developmental arrest during the prepupal stage. Further, the effects of *let-7* disruption were limited to the last larval instar. In contrast to our observation, Ling *et al*. (2014) found that the majority of *B. mori* larvae died during L3 without apparent signs of precocious metamorphosis when *let-7* was depleted by a microRNA sponge. The reason for the distinct phenotypes between our study and that of Ling *et al*. (2014) remains to be elucidated, but one explanation would be the technical difference between the two studies (i.e., knockout vs. microRNA sponge).

Recent genetic studies reveal that *Br–C* and *E93* regulate pupal and adult metamorphosis, respectively, of holometabolous insects and that stage-specific interactions among *Br–C, E93*, and *Kr-h1* are critical for pupal and adult metamorphosis (Belles and Santos, 2014; Ureña *et al*., 2014, 2016). Moreover, depletion of *Br–C* leads to incomplete pupation (Konopova and Jindra, 2008; Uhlirova *et al*., 2003). However, particularly in *B. mori,* somatic mosaic analysis of *Br–C* shows that epidermal cells carrying homozygous mutations in *Br–C* are unable to produce pupal cuticle at pupal metamorphosis (Daimon *et al*., 2015). Our present gene expression analysis demonstrates that the induction of ecdysone-inducible genes, including *Br–C*, normally occurred at the time of apolysis (Fig. 3.6B), suggesting that the phenotype of *let-7*$^{-/-}$ larvae was independent of *Br–C* action. Further, our

implantation assays using *let-7*$^{-/-}$ integuments consistently demonstrated that *let-7* was not required for reception and signaling of JH and ecdysteroids.

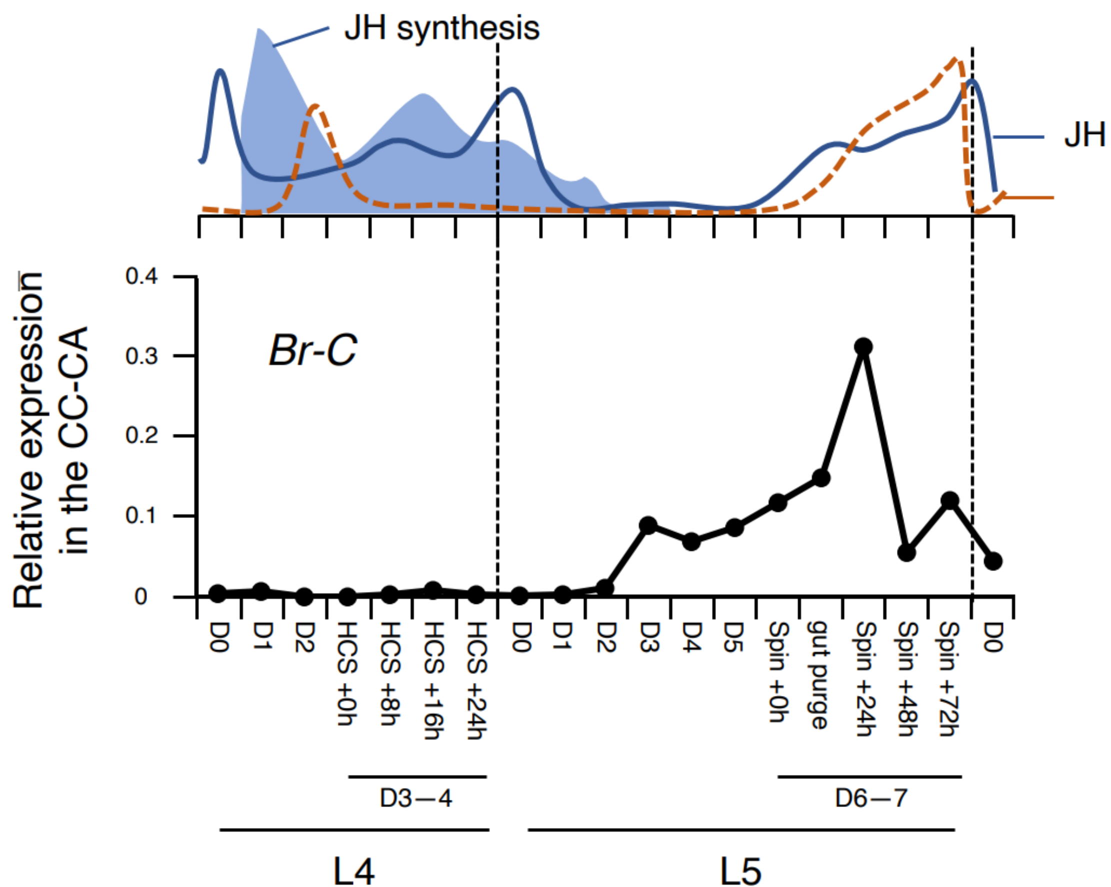


Fig. 3.11. Temporal expression pattens of *Broad-Complex* in *B. mori* CC-CA. mRNA levels were determined using qRT-PCR. The levels of gene expression normalized to that of the internal *rp49* control. Developmental stages are defined as h/days after molting, head capsule slippage, and spinning. Vertical dotted lines indicate the timing of molts. The JH titer is from Niimi and Sakurai (1997). The ecdysteroid titer is from Koyama *et al*. (2004; 4th instar), Sakurai *et al*. (1998; 5th instar) and Kaneko *et al*. (2006; 5th instar). Tissues were collected from 3 individuals, and pooled samples were analyzed.

Homozygous mutants of genes encoding key JH biosynthetic enzymes, *JHAMT* and *CYP15C1*, and a JH receptor designated *Met1*, undergo precocious pupal metamorphosis or exhibit signs of precocious metamorphosis subsequent to the L3 stage (Daimon *et al*., 2012, 2015). Further, low mRNA levels of *Kr-h1α/β* are maintained in these mutants (Daimon *et al*., 2015; Smykal *et al*., 2014). Similarly, ubiquitous overexpression of *let-7* in *B. mori* larvae was associated with low levels of *Kr-h1* mRNA during the L4 stage and precocious pupal metamorphosis during this stage (Fig. 3.9, Fig. 3.10). The *let-7O/E* larvae exhibited these JH-deficient characteristics. However, the anti-metamorphic actions of JH alone cannot account for the phenotype of the homozygous *let-7* mutant, because *Kr-h1* mRNA levels in the *let-7* knockouts remained low before the onset of spinning (Fig. 3.6A).

A possible explanation of the phenotype of the *let-7* knockouts is that *let-7* targets transcripts of some key enzyme(s) of JH biosynthesis, such as *JHAMT*, leading to a reduction of JH biosynthetic activity in the CC-CA of last instar larvae, and consequently leads to a reduction of *Kr-h1* expression. Our expression analysis of *Kr-h1* transcripts in the epidermis of *let-7* overexpressing larvae supports this model (Fig. 3.10). However, our expression analysis showed that *Kr-h1* mRNA levels in the epidermis of *let-7* knockouts were low during the feeding period (0–96 h in Fig. 3.6A). Moreover, I showed that in the *let-7* homozygous mutants, expression of the ecdysteroid-inducible genes was delayed (Fig. 3.6B). The delay was shown to be due to a late expression of the ecdysteroid biosynthetic enzymes in the

PG (Fig. 3.7). In the PG of *let-7* knockouts, I observed a delay in the expression of most of the Halloween genes (i.e., *nvd, nm-g, spo,* and *dib*). Expression levels of *phm* and sad were significantly affected by the absence of *let-7*; while the expression of *phm* highly increased relative to the normal level after spinning, sad mRNA remained nearly undetectable throughout the last instar (Fig. 3.6). Although I do not know the mechanisms by which *let-7* regulates *phm* and *sad, let-7* may directly or indirectly regulate the Halloween genes in the PG. Further studies are necessary to understand the roles and significance of *let-7* (and other miRNAs) in the regulation of PG activity.

Given that *let-7* regulates JH biosynthetic activity by targeting some of the enzymes critical for JH biosynthesis, it can be assumed that *let-7* coordinates the role of JH in the timing of the release of the prothoracicotropic hormone (PTTH). In *B. mori*, the effect of JH clearly depends on its concentration and the timing of its administration. When a low dose (0.01 μg) of methoprene, a JH analog, is administered before the release of PTTH on day 4 of the last instar, the feeding period is extended, whereas there is no detectable effect when injected after the release of PTTH (Sakurai, 1984). According to the observations of Sakurai (1984), I speculate that loss of *let-7* causes JH to be produced at higher concentrations at the beginning of last instar stage, and the JH concentration, in turn, extends the feeding period and delays the expression of Halloween genes in the PG.

In the present study, two important findings on the role of *let-7* in *B.*

mori were made: First, the expression of *let-7* was found to be localized to the CC-CA and upregulated during the early last instar stage but not by 20E signaling. Second, *let-7* regulated pupal metamorphosis via controlling the biosynthesis of ecdysteroid and JH. Identification of the pathway through which *let-7* controls hormone synthetic activity will contribute to a better understanding of the molecular mechanism underlying the control of metamorphosis in insects.

Table 3.1. List of primers used in this study.

Gene	Primer name	Nucleotide sequence (5' to 3')	purpose
Pre-let-7	Pre-let-7_BlnI_F1	gatcctaggCGCCTAAAGAAGTTACAGCTTA	Vector construction
	Pre-let-7_BlnI_R1	gcacctaggGTGGTCTTGTGAGGAATGTTTC	
let-7	let-7-F1	GTGGTCTTGTGAGGAATGTTTC	Genotyping
	let-7-R1	GTACGTACCCTCAAATCGGTAAT	
let-7	let-7-5p_qPCR-F1	TGAGGTAGTAGGTTGTATAGT	qRT-PCR
Kr-h1α	BmKrh1α_qRT-PCR_FW	CACAACCTACGCCAACATTAGAAACG	qRT-PCR (isoform-specific)
	BmKrh1α_qRT-PCR_RV	ACTGATGAACTCGCTCCTCGTCAC	
Kr-h1β	BmKrh1β_qRT-PCR_FW	GAAACAATTTCGTTCTTCAGGTGACG	qRT-PCR (isoform-specific)
	BmKrh1β_qRT-PCR_RV	TCGTGCGTGTGCTGTAAGCG	
Broad-C	BmBRC_qPCR_FW	CGCAACACTTCTGTCTCCGATGG	qRT-PCR (common region)
	BmBRC_qPCR_RV	TTGAGGCTTTTCCCGTCGCA	
E75A	BmE75A_qPCR	AGAAGCCCTTGCAGCCCTCG	qRT-PCR (isoform-

	_FW		specific)
	BmE75A_qPCR_RV	CGCTACGATGTGCCTACGTCCG	
E75B	BmE75B_qPCR_FW	GGTGCGAACCATGTCGTGTGG	qRT-PCR (isoform-specific)
	BmE75B_qPCR_RV	TCGAGCAGCCCGAGTCACTG	
βFTZ-F1	BmFTZ-F1_qPCR-F1	TCTCAAGTGGATTGGGCAAG	qRT-PCR
	BmFTZ-F1_qPCR-R1	ATCCAAAACCAGCATAACAGACC	
E93A	BmE93A_qPCR_FW	GCCCGTACAAAAAAAAGAACTCGAAG	qRT-PCR (isoform-specific)
	BmE93A_qPCR_RV	TCACGACGTGCTCCATTCCG	
E93B	BmE93B_qPCR_FW	TGCACAATCGGCAGAGAGTCG	qRT-PCR (isoform-specific)
	BmE93B_qPCR_RV	GCAAAAGAGATGTTTGTAGCGGTTTG	
nvd	Bmnvd-qPCR-F1	TCCCGTACTGCACGAAATCA	qRT-PCR
	Bmnvd-qPCR-R1	CGTGATGTGCATTAGCGATG	
nm-g	Bmnmg-qPCR-	TGTGATAGTGGACTCGGTGGG	qRT-PCR

	F1		
	Bmnmg-qPCR-R1	GGCTTTCGCTGCTTCGGTTTC	
spo	Bmspo-qPCR-F1	CCATCTTGGAATGTCTGCGG	qRT-PCR
	Bmspo-qPCR-R1	GGAGTTGCGTCTCGTCCTGA	
phm	Bmphm-qPCR-F1	GACCCAACGGCACTGTATATGAGAG	qRT-PCR
	Bmphm-qPCR-R1	GCCCACTGCAATGGGATCAC	
dib	Bmdib-qPCR-F1	CGATACAACAGCCTACACGACAAGCTTCG	qRT-PCR
	Bmdib-qPCR-R1	CCTTCTGCAGCCATCTACCTATTCCAATCG	
sad	Bmsad-qPCR-F1	TAGAGCTCAAAGTGG	qRT-PCR
	Bmsad-qPCR-R1	TGTCGTATCTCCAGCGGCG	
rp49	BmRp49_qPCR_FW	CAGGCGGTTCAAGGGTCAATAC	qRT-PCR
	BmRp49_qPCR_RV	TGCTGGGCTCTTTCCACGA	

4. General Discussion

4.1 What is the "Competence factor"?

4.1.1. Competence factor as a "positive factor"

The results of implantation study (Chapter 2) presented the hypothesis, as they suggest that this humoral factor circulates int the hemolymph of L4 and L5 hosts and confers the competence for metamorphosis to the implanted L1 epidermis. The action of this humoral "competence factor" would be blocked by JHs as shown in the implantation assays. If this hypothesis is true, several issues need to be addressed, such as when and where this factor is produced, how it confers competence for metamorphosis or promotes pupal commitment, and whether a similar system is conserved in other insects. I do not know the answers to these questions at this point, and further study is required to provide more clues to better understand the hormonal control of insect metamorphosis.

Notably, in the eye and leg imaginal primordia of last instar larvae of *M. sexta*, JH failed to inhibit disc formations in these primordia when the larvae that were feeding. This failure of JH to suppress disc formation in feeding last instar larvae cannot be explained by the enhanced clearance of the applied hormone, because topical treatment of the leg primordia with JH was also ineffective in feeding larvae. Therefore, nutritional cues apparently result in the release of a metamorphosis-initiating factor (MIF) that overrides the suppression of disc formation by JH (Allee *et al.*,2006; MacWhinnie *et al.*, 2005; Truman *et al.*, 2006). Further, Truman *et al.* (2006) confirmed

suggested that MIF overrides supresseion of *Br-C* expression by JH, thus MIF would be a candidate for “competence factor”.

MIF corresponds to insulin-like peptides. In *D. melanogaster*, two out of seven insuline-like peptides are induced nutrition-depended manner (Brogiolo *et al*., 2001; Ikeya *et al*., 2002). Both loss and gain of function analysis of *Drosophila* insulin /insulin-like growth factor (IGF) have shown that its signaling regulates growth, size and longevity at the cellular and organismal levels (Saucedo *et al*., 2003; Mirth and Riddiford, 2007). In lepidopteran larvae, an insulin-like peptide, bombyxin, stimulates cell proliferation in the wing discs (Nijhout and Grunert, 2002; Nijhout *et al*., 2007). Intriguingly, in *B. mori*, bombyxin is secreted from neurosecretory cells of the brain in response to sugar intake at the beginning of the fifth instar (Masumura *et al*., 2000).

More direct evidence for the correspondence of IGR and MIF was obtained by Koyama *et al*. (2008). Koyama and his colleagues showed that insulin can overcome the suppressive effects of JH on the induction of *Br-C* and pupal commitment in the wing discs in starved final instar *M. sexta* larvae, even when exogenous JH is applied topically during the molt to the final instar. Moreover, this effect of insulin appears to be directly on the discs since their *in vitro* studies using cultured wing discs showed that insulin suppresses the ability of JH to inhibit the increase in *Br-C* mRNA that occurred when early fifth instar discs were incubated in hormone-free conditions. Therefore, further studies using IGR signaling mutant silkworms

will shed light upon the elucidation of "positive competence factor" (Fig. 4.1.a).

4.1.2. Competence factor as a "negative factor"

Although my implantation assays suggested the existence of humoral "competence factor" which induces *Br-C* expression, existence of unknown factor(s) which suppresses metamorphosis in very young instar larvae in *B. mori* cannot be ruled out.

Recently, Truman and Riddiford (2022) demonstrated that *chronologically inappropriate morphogenesis* (*chinmo*) mediates maintenance of the larval state through chromatin modification as well as by repression of both *Br-C* and *E93* in *D. melanogaster*. In *D. melanogaster,* the timing of *chinmo* expression first appears late embryonic stage, during germband retraction and dorsal closure, continues at high levels until "critical weight", the growth-related checkpoint that allows the preparation for pupal metamorphosis. Down-regulation of *chinmo* after critical weight checkpoint is accompanied by the up-regulation of *Br-C*, which is consistent with that expected for a larval control gene. Surprisingly, Truman and Riddiford (2022) found that requirement for Chinmo to suppress *Br-C* expression extends back to the L1 stage when the imaginal discs begin their proliferation. The function of *chinmo* in suppressing *E93* expression occurs earlier in the mid-embryogenesis. I also examined the expression pattern of

chinmo in several non-Drosophid holometabolans including *B. mori* (data not shown) and found *chinmo* expression levels are high in neonate L1, gradually decrease through larval development and reach to the ground levels at middle of the last instar, when the expression of *Br-C* rises. The expression patterns in holometabolous insects I tested was concordant with that of *D. melanogaster*, leading me to suggest the existence of non-JH "negative factor" that induces *chinmo* (Fig. 4.1.b). Further studies such as depletion or over-expression of *chinmo* is required for the better understanding of the mechanisms underlying the maintenance of larval feature in the very young instar.

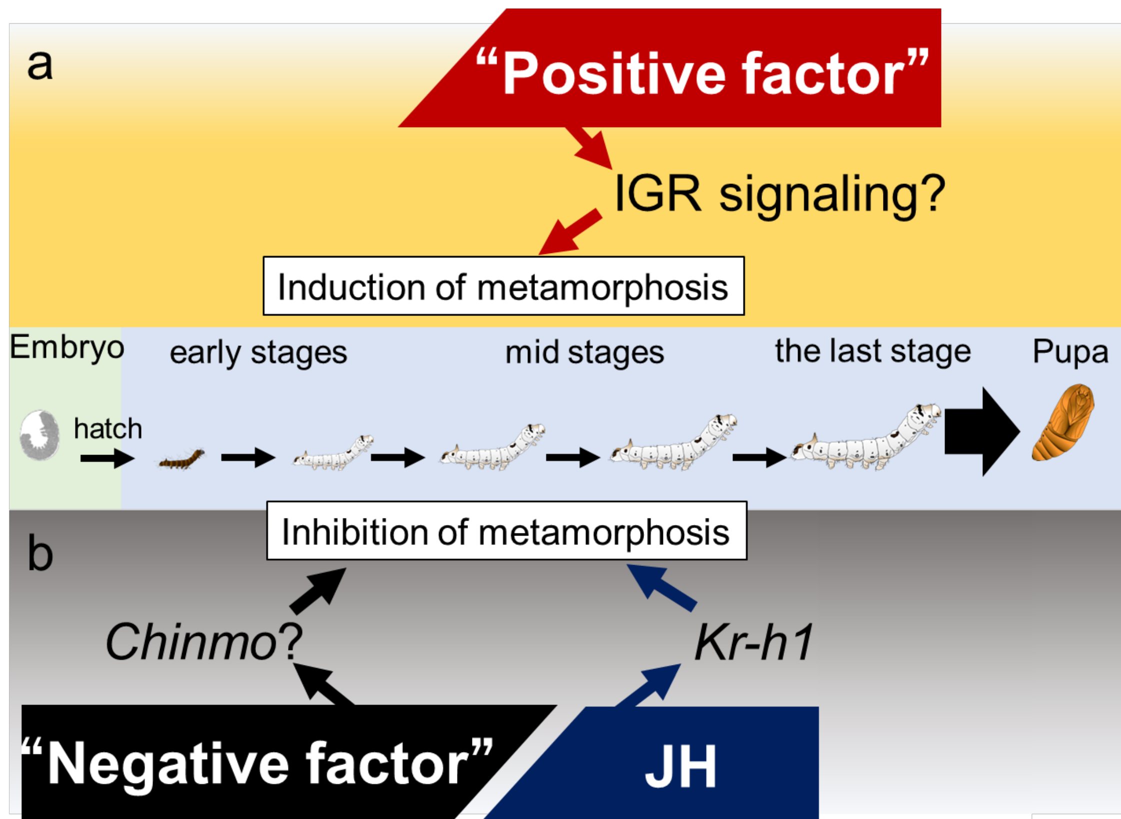


Figure 4.1. Summary of the contrast hypothesis of "competence factor". a) competence factor as a positive factor. b) competence factor as a negative factor. Each bars in a and b represents putative effective period of factors.

4.2. Putative target genes of *let-7* in regards to control of metamorphosis

In Chapter 3, two important findings on the role of *let-7* in *B. mori* were made: First, the expression of *let-7* was found to be localized to the CC-CA and upregulated during the early last instar stage but not by 20E signaling. Second, *let-7* regulated pupal metamorphosis via controlling the biosynthesis of ecdysteroid and JH. However, I have not identified the pathway through which *let-7* controls hormone synthetic activity. Here I remark *let-7*'s potential target genes involved in the JH biosynthesis.

JHAMT, which encodes a rate-determining JH biosynthetic enzyme, has already been listed as candidates. Qu *et al*. (2017) determined interactions of *let-7* and 3'UTR of *JHAMT* mRNA cloned from several arthropods, using *Renilla* firefly luciferase assays in *Drosophila* S2 cells. Their *in vitro* assays revealed that *let-7* can interact *JHAMT* in wide variety of arthropods for example, in insects; such as *Anopheles gambiae, Tribolium castaneum* and *D. melanogaster,* and in crustaceans; *Daphnia pulex, Neocaridina denticulate* and *Strigamia maritima*, in the chelicerate *Tachypleus tridentatus*. All these results suggest that *let-7* could directly modulate *JHAMT* expression in arthropods.

Several exogenous factors such as 20E, biogenic amines (octopamine, glutamate, and dopamine), peptides (allatotoropin, allatostatin, ecdysis-triggering hormone, sex peptide and short neuropeptide F act together in a

stage-specific manner to guarantee the precise production of JH in each larval stage (Hiruma and Kaneko, 2013; Zhang *et al.*, 2022). In addition, the insulin/insulin-like growth factor (IIS)/ target of rapamycin (TOR) signaling pathway mediates the transduction of nutritional signals into JH biosynthesis by regulating JH biosynthetic enzymes such as 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) (Belgacem and Martin, 2007; Koyama *et al.*, 2013; Maestro *et al.*, 2009; Zhu *et al.*, 2020). These factors are secreted from central nervous system (CNS) and affect JH biosynthesis in the CA. As I found that the expression of *let-7* is highly localized in the CC-CA, it is possible that *let-7* is involved in given signaling pathways.

Recently, the molecular mechanisms underlying the regulation of JH biosynthesis in the CA is revealed step by step. TGF-β signaling has been reported to regulate JH biosynthesis via the expression of *JHAMT* in the CA of *D. melanogaster* and *Gryllus bimaculatus* (Huang *et al.*, 2011; Ishimaru *et al.*, 2016). In *B. germanica*, Seven-up and FTZ-F1 drive the expression of HMGS in the CA to control JH production in adulthood (Borras-Castells et al., 2017). Ventral veins lacking (Vvl)/Drifter, a POU domain transcription factor, regulate JH biosynthesis via upregulating the *JHAMT* transcript in *T. castaneum* (Cheng *et al.*, 2014).

Further studies are required to investigate the involvement of *let-7* in those signaling pathways mentioned above in order to understand the role of miRNAs in development of insects.

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