ENDOCRINOLOGY OF FLEXIBLE DEVELOPMENT IN THE FLOUR BEETLE, *TRIBOLIUM FREEMANI*

by

KRISSANA RUANG-RIT

B.S., Chiang Mai University, Thailand, 1999
M.S., Chiang Mai University, Thailand, 2002

AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

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Abstract

Insect metamorphosis is driven by two major hormones, juvenile hormone (JH) and ecdysone (Ec). The presence of JH with an Ec peak in each stadium results in larval-larval molting whereas in the last larval instar a decline of JH to undetectable level combined with pulses of Ec leads to larval-pupal metamorphosis. Larval-pupal metamorphosis normally occurs after a certain number of larval instar and upon reaching a certain size (critical weight). However, in the flour beetle, *Tribolium freemani*, under crowded conditions larva continue larval-larval molting (LLC) without pupation for longer than 14 instars (6 months). Previous studies have implicated high JH titer as preventing the metamorphosis leading to supernumerary molts.

My investigation of JH roles in LLC started by asking whether suppression of JH would rescue the LLC phenotype and allow pupal metamorphosis. Using RNA interference (RNAi), I found that under crowded conditions RNAi of *T. freemani methyltransferase3* (*TfMT3*), which encodes a crucial enzyme for the final methylation step in the JH biosynthesis, or RNAi of *T. freemani Krüppel homolog1* (*TfKr-h1*), the JH downstream gene, did not rescue the larvae but resulted in prepupal lethality. Surprisingly, under crowded conditions prepupal lethality was rescued by RNAi of both *TfMT3* and *TfKr-h1* administered together, although developmental arrest occurred at the pharate adult stage; this is also the phenotype of *TfKr-h1* RNAi-treated larvae under isolated conditions.

In investigations of the role of Ec titer in LLC, lethality of the larvae with RNAi of *TfMT3* under crowded conditions was associated with the loss of the major ecdysteroid peak, while *TfKr-h1* RNAi-treated larvae under crowded conditions showed a delayed, but normal, Ec peak occurring at prepupal arrest. The pattern of Ec peak in RNAi of both *TfMT3* and *TfKr-h1*

together was similar to that with *TfKr-h1* RNAi alone. I suggest that a hormonal imbalance, high JH and high Ec in the prepupal arrest of *TfKr-h1* RNAi, was rescued by RNAi of both *TfMT3* and *TfKr-h1* for low JH and high Ec. These results demonstrate that the signaling pathways for LLC are through at least two independent pathways; JH biosynthesis and *TfKr-h1*-mediated JH response.

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Approved by:

Co-Major Professor
Yoonseong Park

Approved by:

Co-Major Professor
David Margolies

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Table of Contents

List of Figures

List of Tables

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Chapter 1 - Introduction

Insect metamorphosis and critical weight checkpoint

Within the Class Insecta, there are three developmental patterns from juvenile to adult stages; ametabolous, hemimetabolous, and holometabolous, depending on changes of the body form during development. The most derived form of development is holometabolous or complete metamorphosis. The holometabolous life cycle involves four life stages as egg, larva, pupa, and adult. It provides significant survival advantages by reducing competition for habitat or food resource between the different stages (i.e., larva vs. adult). During holometabolous development, larvae undergo metamorphosis to pupae when they reach a certain instar and weight. An unanswered question is how insects know when it is the time to metamorphose. Previous studies on tobacco hornworm, *M. sexta* (Davidowitz et al., 2003; Safranek and Williams, 1984), and fruit fly, *D. melanogaster* (De Moed et al., 1999) demonstrated the existence of checkpoints called "critical weight" for initiating the metamorphic process. The critical weight is the final weight for the onset of normal metamorphosis without being affected by starvation or external factors (De Moed et al., 1999; Safranek and Williams, 1984). The developmental checkpoints are determined by the number of larval instars and the size (or weight), which are separated from direct effects of nutritional status (Allegret, 1964; Nijhout, 1975). The threshold size corresponding to head-capsule width and weight may be the outcome and indicator of storage of sufficient amount of nutrient for metamorphosis. For pupal metamorphosis, the larva is required to reach the minimum viable weight (MVW), which means larvae store minimum nutrient that is sufficient to be able to survive thought metamorphosis (Mirth and Shingleton, 2012; Safranek and Williams, 1984). The next gate is "critical weight (CW)" that allows the onset of pupation.

Larvae stop feeding and start the metamorphic process when they attain the critical weight (Mirth and Shingleton, 2012; Safranek and Williams, 1984).

Hormonal regulation of insect development and metamorphosis

Metamorphosis is driven by two major insect hormones, juvenile hormone (JH) and ecdysone (Ec). JH is well-known for preventing metamorphosis while Ec is responsible for insect molting and for transforming larval tissues to pupal and adult structures (Nijhout and Williams, 1974; Riddiford, 1994; Suzuki et al., 2013). The periodic molting during the immature stage depends on the orchestration of both hormones. Because JH maintains cell division without differentiation (Truman et al., 2006), the larval cuticle is formed in the presence of JH with an Ec peak triggering the larval-larval molting (Nijhout and Williams, 1974; Riddiford, 1981, 2008). Inversely, a decline of JH to undetectable level and an increase in the Ec peaks leads to larval-pupal metamorphosis (Riddiford, 2008, 2012; Wigglesworth, 1954).

JH of insects are sesquiterpenoid molecules (Figure 1.1) (Williams, 1956) which are produced by the corpora allata (CA), located at the posterior part of the brain of insects, and secreted into the hemolymph. JH was first identified in *Rhodnius prolixus* as an inhibitory factor of metamorphosis (Wigglesworth, 1934, 1936, 1948). Commonly, the titer of JH in the final larval instar declines to very low levels or is absent, which allows immature insects to metamorphosis. In contrast, the application of the hemolymph containing JH from early immature insects or exogenous JH inhibits the metamorphosis (Fain and Riddiford, 1975; Wigglesworth, 1948). Biosynthesis of JH in the CA has been extensively studied, including the last steps for metabolic activation. Methylation and epoxidation of precursors (Figure 1.1), JH acid or farnesoic acid, is known to be the crucial JH regulatory step (Shinoda and Itoyama, 2003) in Lepidoptera (Sheng et al., 2008), Diptera (Niwa et al., 2008), and Coleoptera (Minakuchi et

al., 2008a). The *JH acid methyltransferase* (*JHAMT*) gene, encoding JHAMT enzyme in the late steps of JH biosynthesis, is highly expressed in the embryo and early larva and then gradually decreases to low level before pupation (Figure 1.2). Knockdown of *JHAMT* using RNA interference (RNAi) resulted in precocious metamorphosis in *T. castaneum,* which is the same as in allatectomized insects, thus showing that this enzyme is essential for JH biosynthesis (Minakuchi et al., 2008a; Pratt and Davey, 1972).

The receptor for JH was identified when JH gene expression was suppressed and insects showed resistant to the JH-analog Methoprene. Thus, the receptor gene was named as methoprene tolerance or Met (Konopova and Jindra, 2007; Wilson and Fabian, 1986). Like JH, Met is essential in preventing metamorphosis. After hormonal JH binds to Met, the JH/Met complex binds to a short DNA sequence motif named the JH-response element (JHRE) (Li et al., 2007), which activates JH-dependent gene transcription (Li et al., 2011; Zhang et al., 1996). One of the most well studied JH downstream genes is *Krüppel homolog1* (*Kr-h1*), which carries JHRE and mediates the repression of insect metamorphosis (Kayukawa et al., 2012). In *T. castaneum*, knockdown of JH biosynthesis or Met decreases *Kr-h1* transcript level, whereas *Kr-h1* level increases with exogenous JH treatment (Minakuchi et al., 2009; Minakuchi et al., 2008a). Therefore, it seems that the transcript levels of *Kr-h1* depends on the JH level.

In the last larval instar, after larvae attain critical weight, JH titer declines to undetectable levels at the onset of metamorphosis (Fain and Riddiford, 1975; Nijhout and Williams, 1974). After JH declines, the brain hormone, Prothoracicotropic hormone (PTTH), is released and stimulates ecdysteroid biosynthesis. In the last instar of lepidopterans and coleopterans, there are two peaks of ecdysteroids; a first, small peak prior to the wandering stage and a second, greater peak coinciding with pharate pupal development (Bollenbacher et al., 1975; Hirashima et al.,

1998). In dipterans and lepidopterans, alpha ecdysone (αEc) is known to be produced and released from the prothoracic glands (King et al., 1974; Yamanaka et al., 2013). However, 2-deoxyecdysone (2dE), which is the precursor of αEc, was found to be the third ecdysteroid component in the circulating system of the beetle, *Zophobas atratus* (Aribi et al., 1997a). Ecdysteroids in beetle may be in different forms from those in lepidopteran and dipteran insects; the site of biosynthesis (Delbecque et al., 1990), and differently hydroxylated ecdysteroids in the circulatory system and in the final active form. In lepidopteran and dipteran insects, the conversion of αEc to the most active form 20-hydroxyecdysone (20HE) (Figure 1.1) occurs at the target tissues through a specific hydroxylation by a cytochrome P-450. 20HE binds to the heterodimeric ecdysone receptor (EcR) and ultraspiracle (USP), and followed by binding to the ecdysone response element (EcRE) for regulation of the downstream genes (Bergman et al., 2004; Henrich, 2011; Riddiford et al., 2000; Sakurai et al., 1998; Yao et al., 1993).

As it was earlier described and hypothesized based on 20HE inducing polytene chromosome puffing in *D. melanogaster* (Ashburner et al., 1974), 20HE sequentially activates the expression of several ecdysone-response genes such as *Broad* (*Br*). The regulation of *Br* is complex and known to be controlled by both JH and ecdysone signaling pathways (Figure 1.3). The presence of JH during larval stages prevents *Br* expression, resulting in larval molt (Tracey, 1958; Zhou et al., 1998). However, when JH is absent, *Br* is induced by ecdysone. *Br* plays a critical role in the regulation of gene expression at the onset of metamorphosis (Karim et al., 1993); accomplishment of larval-pupal metamorphosis depends on *Br* expression during prepupal stage (Moeller et al., 2013; Parthasarathy et al., 2008).

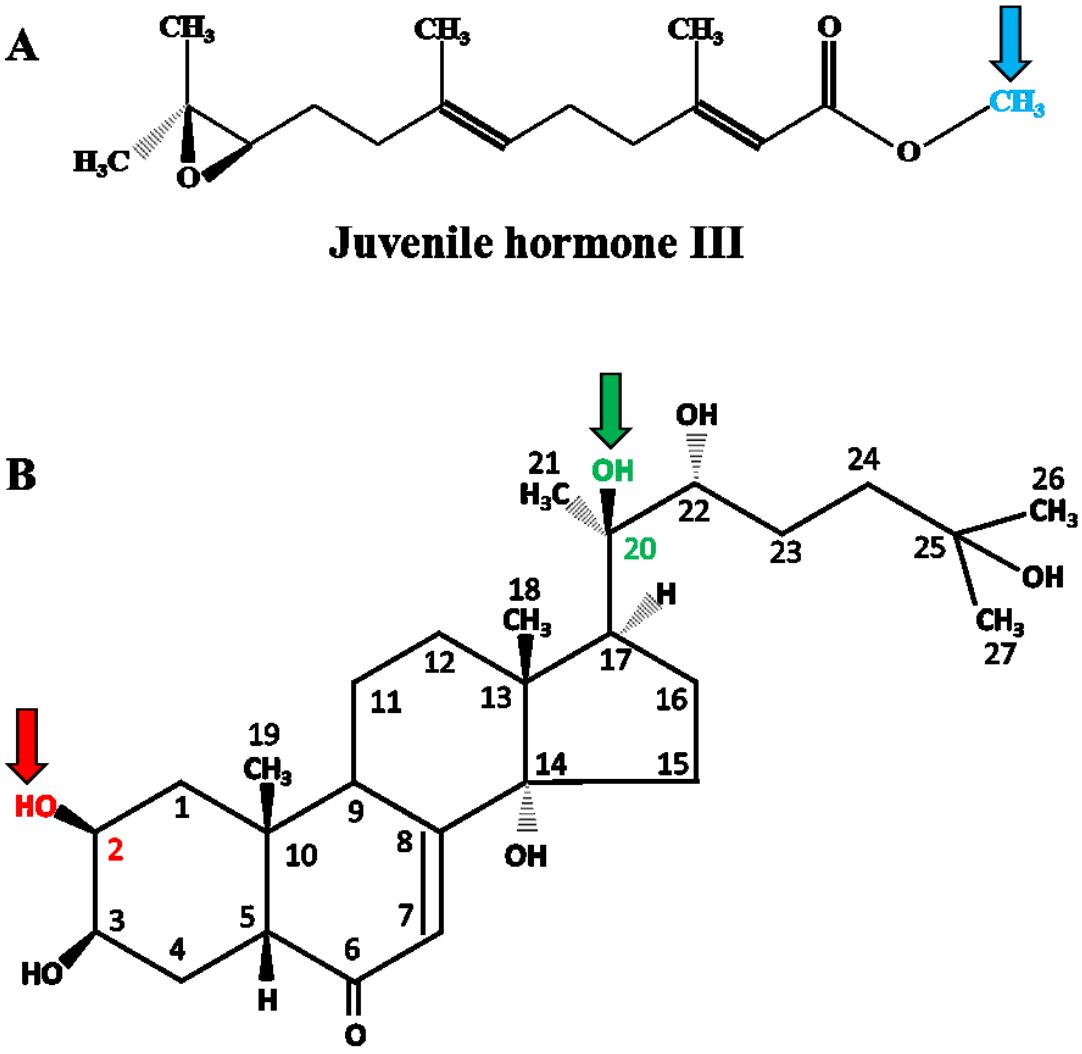

Figure 1.1 The structures of Juvenile hormone and 20-Hydroxyecdysone. (A) The structure of juvenile hormone III. The blue letter and blue arrow show the position of methylation (Devillers, 2013). (B) The structure of 20-hydroxyecdysone showing the number of carbon positions. The 2-deoxyecdysone is converted to alpha ecdysone by hydroxylation at position 2 (red number and red arrow). Then the hydroxyl substituent at position 20 (green number and green arrow) converts alpha ecdysone to 20-hydroxyecdysone (Smagghe, 2009).

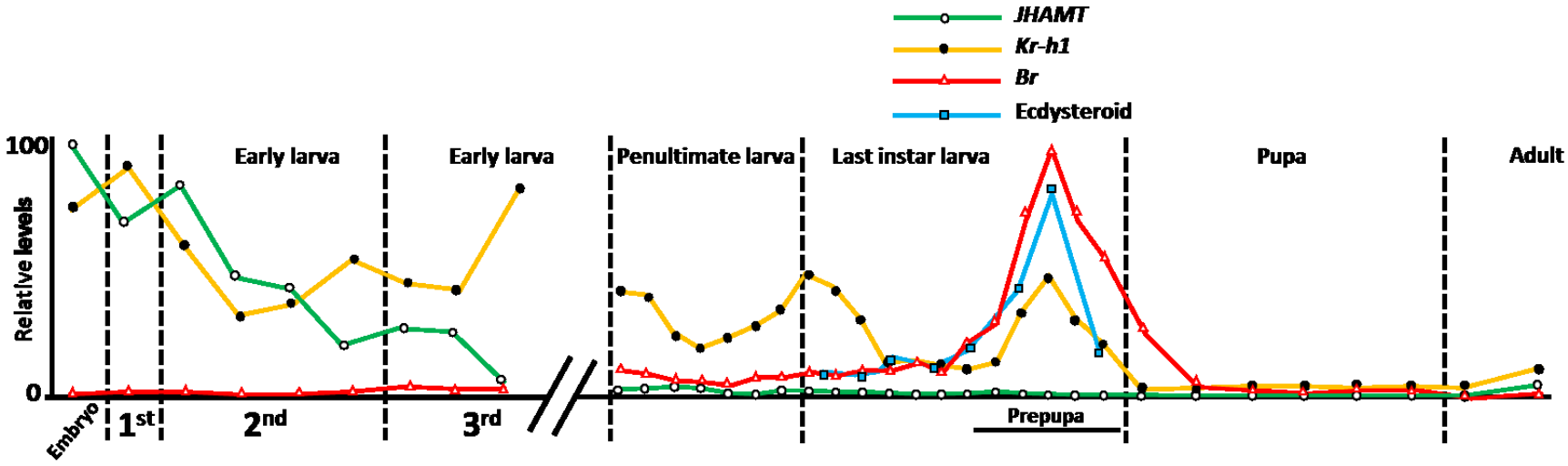

Figure 1.2 Developmental expression profiles of *JHAMT*, *Kr-h1*, and *Br* and the titers of Ec. Transcript levels of these genes are based on studies in *T. castaneum* for *JHAMT* by Minakuchi et al. (2008a) and for *Kr-h1, Br* by Minakuchi et al. (2009). The data of Ec titers is from *T. freemani* under isolated conditions by Ruang-Rit and Park (unpublished data). Abbreviations: *JHAMT*, *juvenile hormone acid methyltransferase*; *Kr-h1, Krùppel-homolog1*; *Br*, *Broad*; Ec, Ecdysteroids.

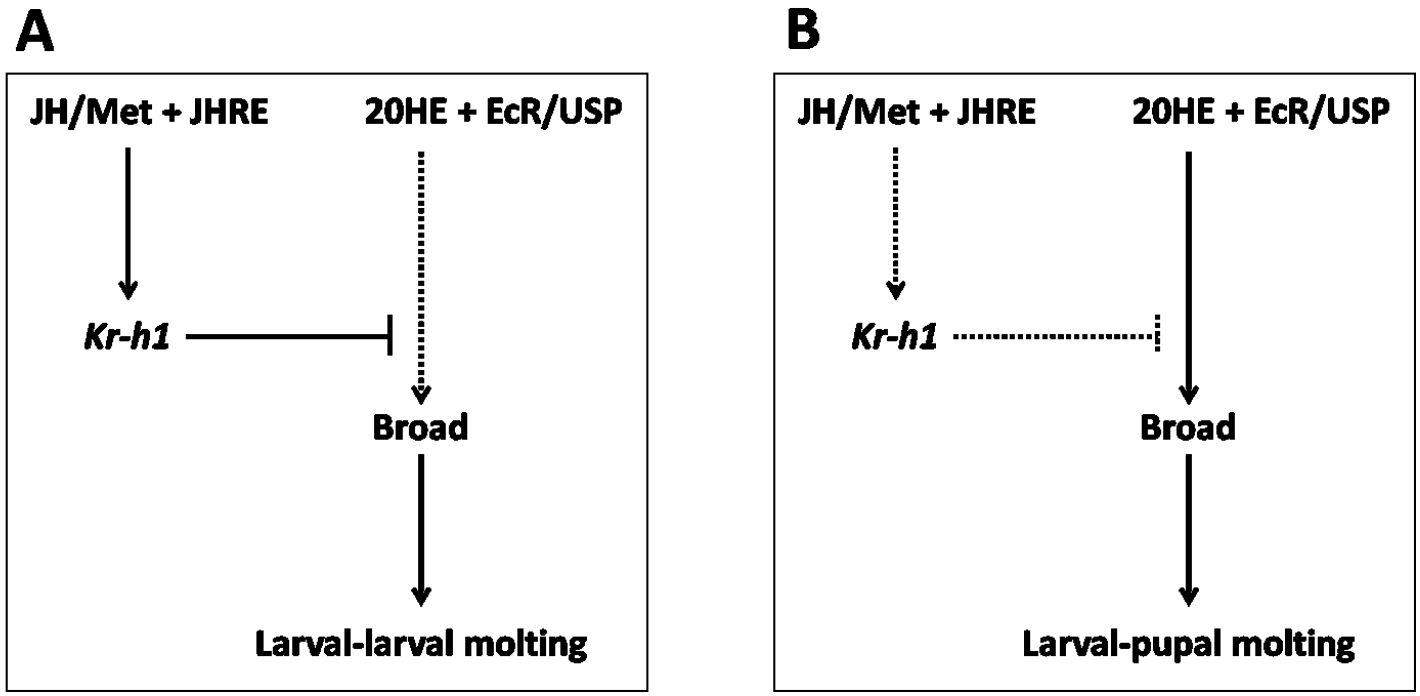

Figure 1.3 The model of larval-larval molting and larval-pupal molting. This model is modified from Riddiford (2012) and the studies by Minakuchi et al. (2009), Konopova and Jindra (2008), and Suzuki et al. (2008). (A) The high JH induces *Kr-h1* expression that suppresses *Br* expression resulted in larval-larval molting. (B) In the absent or low of JH, ecdysteroid peaks induce *Br* expression that causes larval-pupal metamorphosis. Abbreviations: JH, Juvenile hormone; Met, methoprene-tolerant; JHRE, Juvenile hormone response element; *Kr-h1*, *Krùppel homolog1*; 20HE, 20-hydroxyecsyone; EcR, Ecdysone receptor; USP, Ultraspiracle protein; *Br*, *Broad.*

Inhibition of metamorphosis and the endocrine hormones

Environmental factors resulting in the supernumerary molt in immature insects

The developmental period or number of larval instars up to metamorphosis may be flexible depending on environmental conditions. Delayed metamorphosis under unfavorable environmental conditions (Appendix table 1), including such variables as photoperiod, temperature, the quality and/or quantity of food, humidity, and density, has been shown in many different species of insects, although the physiological mechanism is not known yet in most cases. Delay in metamorphosis is often associated with supernumerary molting in the larval or nymphal stages, allowing flexible development time as an evolutionary outcome to survive in a fluctuating adverse environment (Esperk et al., 2007). Mostly, the number of larval instars increases after reaching MVW because larvae fail to attain the critical weight in adverse conditions (Jones et al., 1980; Safranek and Williams, 1984).

In the beetle *Tribolium freemani* (Kotaki et al., 1993; Nakakita, 1982), larva continues larval-larval molting under crowded conditions (LLC) without pupation until it dies. LLC in these beetles overrides the critical weight at 4.5 mg, while larva undergoes normal metamorphosis under isolated conditions (Figure 1.4) (Preuss, 2010). The supernumerary larval molts under crowded conditions may provide selective advantage by allowing these individuals to avoid being the victim of cannibalism that usually occurs on the immobile pupa. Evolution of this developmental pattern in *T. freemani* coincides with the occurrence of cannibalism in tenebrionid beetles (Appendix figure 1) (Alabi et al., 2008; Ichikawa and Kurauchi, 2009; Park et al., 1970; Tschinkel, 1981; Via, 1999).

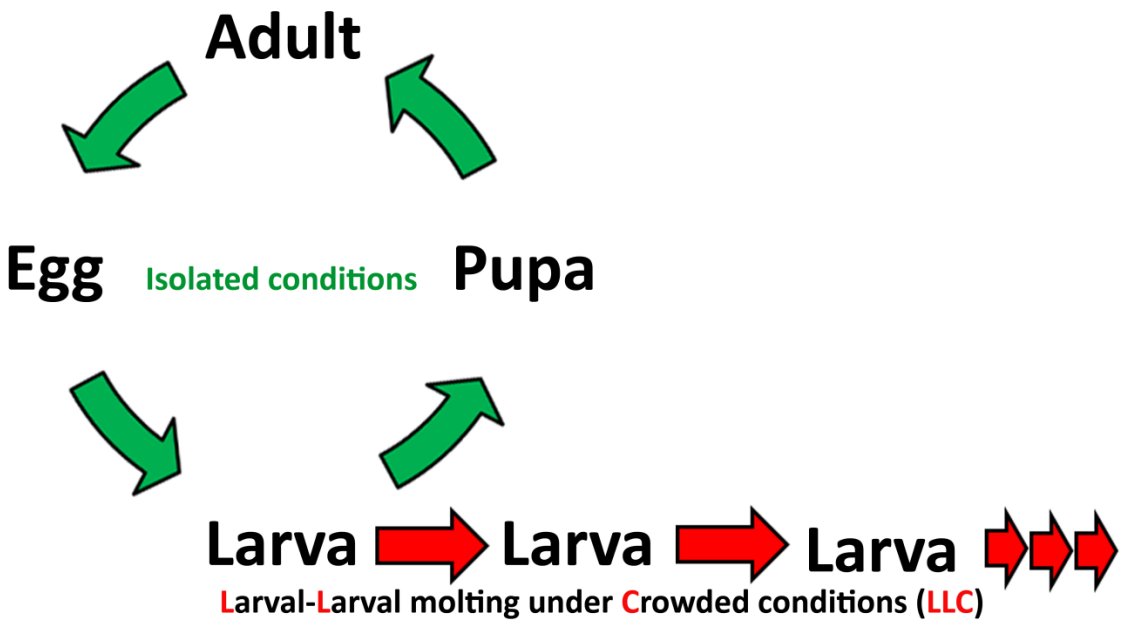

Figure 1.4 Development cycles of *T. freemani* under isolated and crowded conditions.

The endocrine hormones in regulation of LLC

Based on the studies in many holometabolous insects, JH has been implicated in supernumerary molting because experimentally applied exogenous JH mediated supernumerary molt (Kotaki et al., 1993; Quennedey et al., 1995; Riddiford, 1975), whereas the knockdown of JH biosynthesis induced precocious metamorphosis (Daimon et al., 2012; Minakuchi et al., 2008a). Because crowding induces supernumerary molts in *T. freemani*, it has been postulated that mechanical contact, which is more common in crowded conditions, stimulates the corpora allata (CA) to produce JH in mature larvae, resulting in larval-larval molting instead of larval-pupal molting (Kotaki et al., 1993). Additionally, it has been shown that, in *Rhodnius prolixus* if the CA, the endocrine organ that produces and secrets JH into hemolymph, is transplanted into last instar of immature stage larvae, it prevents metamorphosis (Riddiford and Ashburner, 1991; Wigglesworth, 1936, 1948). The necessity of JH for LLC was also suggested earlier in an

experiment with chemical allatectomy using precocene II (Nakakita, 1990). *T. freemani* reared in crowded conditions with diet containing precocene II showed 50% pupation within 30 days, while the larvae without precocene II showed 100% typical LLC.

Moreover, *Krüppel homolog1* (*Kr-h1*), downstream in the JH pathway, has shown an important role in metamorphosis. *Kr-h1* is a transcription factor that is upregulated by JH (Kayukawa et al., 2012; Minakuchi et al., 2008b). The expression of *Kr-h1* is simultaneous with JH and inducible by exogenous JH, whereas knockdown of JH biosynthesis results in reduction of *Kr-h1* (Minakuchi et al., 2009; Minakuchi et al., 2008b). The acceleration of larval-pupal transition also occurs in the suppression of *Kr-h1* (Minakuchi et al., 2009; Minakuchi et al., 2008b). Thus, *Kr-h1* also acts as insect metamorphic inhibition.

Thesis outline

In this thesis there are four chapters. **Chapter 1** provides a general review of hormonal regulation of insect metamorphosis. Two major hormones controlling insect metamorphosis, JH and ecdysteroids, were discussed in terms of production, changes in their titer in metamorphosis, and their downstream gene-regulations. Various environmental adverse effects that can delay metamorphosis and lead to supernumerary larva were listed for various insect species and reviewed. Specifically, studies in continuous supernumerary molts in *T. freemani* larvae under crowded conditions in the fully grown larva were reviewed.

In **Chapter 2**, I investigated the role of the JH pathway in supernumerary molting in *T. freemani* under crowded conditions. I tested whether LLC is induced by JH alone, which has been hypothesized in previous studies. I manipulated the JH signaling pathways using RNAi to investigate whether blocking the JH signaling pathway alone can rescue larvae under crowded conditions for pupation. Under crowded condition, suppression of JH biosynthesis by RNAi of

TfMT3, which encodes the critical metabolic enzyme JHAMT, resulted in arrest at pupation and, ultimately, death. The results suggest that there are at least two independent pathways blocking metamorphosis in crowded conditions. First, JH production is upregulated based on higher *JHAMT* expression in crowded conditions than in isolated conditions. Also, the application of exogenous JH can rescue larvae for at least 2 larval molts in the knockdown of JH biosynthesis under crowded conditions. Second, *TfKr-h1* transcription, the JH downstream responder, is independently induced by crowdedness although JH biosynthesis has been knocked down. Therefore, neither the suppression of JH biosynthesis nor *TfKr-h1* independently rescue the LLC. On the other hand, the combined knockdown of both *TfMT3* and *TfKr-h1* together could rescue the prepupal arrest by inducing further development to pharate adult, although the development was arrested at the eclosion under crowded conditions. However, the complete rescue of the metamorphosis in the crowded conditions appears to require additional factor(s).

In **Chapter 3,** I expanded the investigation into the cause of LLC to the role of ecdysteroids. I measured ecdysteroid titers in larvae treated with *dsTfMT3* and *dsTfKr-h1* to investigate whether crowded conditions affect the ecdysteroid production. Comparisons of ecdysone titers between crowded and isolated *T. freemani* treated with dsRNA revealed the lack of a major ecdysteroid peak in crowded conditions. The ecdysteroid titers in *dsTfMT3* treated larvae, which are lethal in crowded conditions, showed two small ecdysteroid peaks in the prepupal stage, but lacked the major peak. In case of *dsTfKr-h1*-treated larvae under crowded conditions, the level of ecdysteroids was suppressed until the delayed prepupal arrest, when an ecdysteroid peak coincide with the lethality. The results suggest that crowding of *dsTfMT3*-treated larvae suppresses ecdysteroid production indirectly or directly through the JH pathway. However, it was not possible to rescue the LLC or prepupal arrest to pupation by 20HE injection.

A complex endocrine remodeling in LLC is suggested as not only JH, but also synthesis of ecdysone and its downstream responses.

In the last chapter; **Chapter 4**, I provided a brief summary, discussion, conclusion and future direction to reveal more factors causing LLC.

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Chapter 2 - Juvenile hormone alone is insufficient to explain the development of supernumerary larva under crowded conditions in the flour beetle, *Tribolium freemani*.

Abstract

The flour beetle, *Tribolium freemani*, has a flexible development schedule that depends on the environmental conditions. Individuals developing in isolated conditions pupate at the end of the 8th or 9th instar. However, in crowded conditions, *T. freemani* and some other tenebrionid beetles, such as *T. castaneum, Zophobas atratus* and *Tenebrio molitor* show supernumerary larval molts and delayed pupation. Previous studies have shown that supernumerary larval molts can be induced by application of an endocrine factor, Juvenile Hormone (JH) or its analogue methoprene in isolated conditions. Therefore, delayed pupation in crowded conditions has been thought to be induced by JH. We tested this hypothesis by examining whether the opposite was true; that suppression of JH biosynthesis in crowded conditions allows normal metamorphosis to the pupal stage. We performed RNA interference (RNAi) to suppress expression of *T. freemani methyltransferase3* (*TfMT3*), which is a crucial enzyme for JH biosynthesis. As it is expected, the *TfMT3* RNAi induced precocious metamorphosis to pupal stage in *T. freemani* when larvae were reared in isolated conditions. However, in crowded conditions *TfMT3* RNAi resulted in prepupal arrest and death instead of pupal molting, although pupal characteristics were developed underneath the larval cuticle. Although JH biosynthesis had been knockdown, *Krüppel homolog1* (*Kr-h1*), which is normally induced by JH, is highly expressed in larvae treated with *dsTfMT3* under crowded conditions. However, the suppression of *TfKr-h1* alone also failed to induce the normal metamorphosis. Surprisingly, combination of *dsTfMT3* and *dsTfKr-*

h1 treatment could induce further development, but eventually arrested at the pupal-adult metamorphosis or pharate adult arrest. These results indicate that crowded conditions modulates multiple gene regulatory pathways independently, at least for upregulation of JH production and of *TfKr-h1* transcription independently. In addition, complete rescue of the metamorphosis in the crowded conditions appear to require additional factor(s).

Introduction

The programmed development of holometabolous insects is characterized by metamorphosis, which is defined by drastic changes in morphology during larva-pupa-adult transitions. This process is controlled mainly by combinations of two endocrine hormones, juvenile hormone (JH) and steroid molting hormone ecdysone (Ec) (Riddiford, 1994; Wigglesworth, 1970). The most well-known role of JH is preventing metamorphosis; if JH is present when Ec peaks in each instar, larval molting occurs, whereas Ec in the absence of JH induces larval-pupal and pupal-adult molting (Nijhout and Williams, 1974; Riddiford, 1994; Suzuki et al., 2013).

In addition to the programmed development of insects, environmental factors also influence the development schedule, while in most insects, the onset of metamorphosis normally occurs when fully grown larvae (FGL) attain some critical weight, without being affected by starvation or severe environmental factors (Nijhout, 1975; Safranek and Williams, 1984). In the flour beetle, *Tribolium freemani*, a crowded environment determines the developmental schedule even though larvae may have reached critical weight (Kotaki et al., 1993; Preuss, 2010). Under crowded conditions, the FGL fail to pupate but can undergo continuous larval-larval molting (LLC) for more than 6 months (Nakakita, 1982, 1983). This flexible developmental schedule under crowded conditions is because pupae, which are immobile, often become the victims of

cannibalism by mobile larvae and adults (Alabi et al., 2008; Tschinkel, 1981). Other closely related beetle species, including *Zophobas atratus* (Quennedey et al., 1995), *Tenebrio molitor* (Connat et al., 1991), and *T. castaneum* (Park, unpublished data) have also shown the similar developmental patterns under crowded conditions.

While the direct cause of LLC seems to be mechanical stimulation in crowded conditions (Tschinkel and Willson, 1971), an increased titer of juvenile hormone (JH) was proposed to be the causal factor of LLC. It was because the applications of JH analog in FGL of lepidopterans (Cymborowski et al., 1982; Dominick and Truman, 1985) and coleopterans (Quennedey et al., 1995) result in supernumerary larval molt. In *T. freemani*, studies on the hormonal control of LLC similarly implicated the involvement of JH (Kotaki et al., 1993). The study found that application of exogenous JH to the FGL of *T. freemani* under isolated conditions induced supernumerary larva. Conversely, larvae treated with an anti-JH agent, precocene II, pupated normally in crowded conditions (Nakakita, 1990), although the experiment using precocene II was not repeatable in my hand in this study.

Moreover, the suppression of JH by knocking down juvenile hormone acid methyltransferase (JHAMT), an essential enzyme for JH biosynthesis, resulted in precocious pupation in *T. castaneum* in isolated conditions (Minakuchi et al., 2008a). Involvement of JHAMT in JH biosynthesis has been confirmed by the activities of recombinant proteins of DmJHAMT of *Drosophila melanogaster* (Niwa et al., 2008), BmJHAMT of *Bombyx mori* (Shinoda and Itoyama, 2003), and methyltransferase3 (TcMT3) or TcJHAMT of *T. castaneum* (Minakuchi et al., 2008a).

Additionally, in the JH downstream pathways, *Krüppel homolog1* (*Kr-h1*) and *Broad* (*Br*), which are the most well studied JH downstream components, have shown important roles

in the metamorphosis. *Kr-h1* is a transcription factor that is upregulated by JH (Kayukawa et al., 2012; Minakuchi et al., 2008b). It acts as a metamorphic blocker because precocious larval-pupal transition was induced when *TcKr-h1* was suppressed in *T. castaneum* (Minakuchi et al., 2009). One of well-known *Kr-h1* actions further downstream in the JH pathway is suppression of *broad* (*Br*), an essential gene for larval-pupal metamorphosis (Zhou and Riddiford, 2001), although regulation of *Br* is complex and known to be controlled by both JH and ecdysone signaling pathways. *Br* is induced by ecdysone alone, but the presence of JH and *Kr-h1* prevents *Br* expression (Tracey, 1958; Zhou et al., 1998).

To investigate the roles of the components in JH signaling pathway in LLC, larval RNA interference (RNAi) was performed to suppress the gene expressions of *TfMT3* and *TfKr-h1*. Suppression of either *TfMT3* or *TfKr-h1* in crowded conditions resulted in early prepupal arrest and death, but RNAi of both *TfMT3* and *TfKr-h1* rescued the prepupal arrest, allowing further pupal development, though it eventually died in pharate adult. The results in this study suggest that crowded conditions likely activates both *TfMT3* for JH biosynthesis and *Kr-h1* independently.

Materials and Methods

Insects

The strain of *T. freemani* used in this study was provided by Dr. Richard W Beeman's laboratory. It was originally cultured from a population accidentally imported into Japan in stored product from Brazil. The beetle was transferred to Dr. Alexander Sokoloff and later maintained in Dr. Richard Beeman's laboratory for about 20 years. After I received *T. freemani* from Dr. Beeman, it was raised in our laboratory for more than 2 years in a bottle (7 cm diameter

x 12 cm height) with about 200 insects (mixed instar larvae and adults) in 80 grams wheat flour containing 5% yeast (by weight) Brewer's yeast (diet) at 30ºC and 30-40% relative humidity (RH). To collect eggs, adult beetles were placed in petri dishes (60 mm diameter x 15 mm height) with diet. Eggs (n=100) of *T. freemani* were collected and placed individually into wells of 96-well plates (7 mm diameter x 10 mm height/well, flat-bottom immune 96-well plates, Nunc^{Tm}, Denmark) containing diet for 25 mg per well. The plates were kept at 30 ºC under 18 h: 6 h, light: dark cycles and 30% RH. The development of *T. freemani* was observed daily in isolated and crowded conditions. The exuviae from ecdysis of each instar was used to determine the larval instar.

The crowded conditions were generated by adding a male adult of *T. castaneum* in the well containing one newly ecdysed 5th instar larva. The adult movements provided constant mechanical stimuli on the larva. Wheat flour was added into wells when diet reduces. Cleaning feces and old diet are necessary when feces and old flour are too much in the well, then adding new wheat flour. One hundred larvae were observed under isolated conditions, while thirty larvae were observed under crowded conditions.

Identification and cloning of *TfMT1*, *TfMT2*, *TfMT3*, *TfKr-h1*, and *TfBr*

The mRNA sequences of methyltransferase genes of *T. freemani* (*TfMT1, TfMT2,* and *TfMT3*), *Krüpel homolog1* (*TfKr-h1*) and *Broad* (*TfBr*) were identified in KSU Bioinformatics - *Tribolium* species BLAST search (http://129.130.115.231/blast) (unpublished) that contains a draft sequence of the *T. freemani* genome. The annotated sequences of *T. castaneum, TcMT1* (accession no. AB360761.1)*, TcMT2* (accession no. AB360762.1)*, TcJHAMT* (accession no. AB360763.1)*, TcKr-h1* (accession no. NM_001135763.1)*,* and common region of *TcBr* (accession no. XM_008194803.1, XM_008194805.1, and XM_008194807.1) were used as

query. mRNAs were isolated from the whole bodies of *T. freemani* pooled larvae. Total RNA was treated with RNase-free DNaseI and purified before cDNA synthesis. The template cDNA was synthesized with SuperScript® III reverse transcriptase (Invitrogen) using oligo dT primers. The concentration of mRNA for reverse transcription was approximately 500 ng per 10 µl reaction. Polymerase chain reaction (PCR) was performed to amplify *TfMT1, TfMT2, TfMT3, TfKr-h1,* and *TfBr* genes. The PCR conditions were 94ºC for 5s, 55 ºC for 30s, and 68 ºC for 30s, for 40 cycles. The PCR products were ligated to pGEM-T-Easy vector (Promega) for sequencing and for the template to sequence dsRNA.

Table 2.1 Primer sequences of *TfMT1, TfMT2, TfMT3, TfKr-h1,* and *TfBr* used for amplification, dsRNA synthesis, and quantitative PCR.

Primers	Sequences (5' – 3')	Product (bp)
Primers for qPCR		
qTfMT1-F	AAATTCGTTACCGATGCTGG	188
qTfMT1-R	GAGGTGAAGAAACACAAGTC	
qTfMT2-F	TCCCTGAACACAAACATCCT	184
qTfMT2-R	AAGTGATAGAGCGAAACCGT	
qTfMT3-F	AATGATGCGTCTTTTGTGAT	128
qTfMT3-R	TTGGGGATTTTCGGGAGTAA	
qTfKr-h1-F	CGGCAAATCATTCGGCTACA	185
qTfKr-h1-R	AGGATTCGTTCGAGGTGGAG	
qTfBr-F	CACAACATTTCTGTCTGCGGTG	200

Primers	Sequences (5' – 3')	Product (bp)
qTfBr-R	CACAGGGTGTTTGCAAGGAG	
Primers for dsRNA synthesis		
dsTfMT1-F	TAATACGACTCACTATAGGGCTTTATTCGGACTTCGGTGA	367
dsTfMT1-R	TAATACGACTCACTATAGGGTCAGCGTTTGAGGATAACTG	
dsTfMT2-F	TAATACGACTCACTATAGGGATGTGCTGGATGTTGGGTGT	274
dsTfMT2-R	TAATACGACTCACTATAGGGTTGAGTGCTTGAGGATGTTTG	
dsTfMT3-F	TAATACGACTCACTATAGGGGACCACATTTTCTCGTTTTATTG	376
dsTfMT3-R	TAATACGACTCACTATAGGGTTCGTTTTCAGGCAGTTTCTT	
dsTfKr-h1-F	TAATACGACTCACTATAGGGTTTGCAACGACACCTTCACC	510
dsTfKr-h1-R	TAATACGACTCACTATAGGGGTGAACTCATCGACACAGGC	

RNA interference

To synthesize double stranded RNA (dsRNA), the DNA templates for dsRNA synthesis were amplified by PCR with the T7 promoter sequence at the 5' end of each primer. To prevent cross reaction of *TfMT1*, *TfMT2*, and *TfMT3*, DNA sequences of these three genes were aligned (Appendix figure 2). The non-conserved regions of DNA sequences were used to amplify the DNA templates of dsRNA.

The dsRNAs of *TfMT1, TfMT2, TfMT3*, and *TfKr-h1* were synthesized by using MEGAscript T7 (Ambion) by templating the PCR product. The dsRNAs (*dsTfMT1, dsTfMT2, dsTfMT3*, *dsTfMT1+dsTfMT2, dsTfMT1+dsTfMT3*, *dsTfMT2+dsTfMT3, dsTfMT1+dsTfMT2+dsTfMT3,* or *dsTfKr-h1*) were dissolved in buffer (0.1 mM sodium phosphate, pH 7, containing 5 mM KCl). 200 ng of dsRNA (20 nL of 10 ng/nL) was injected

into the dorsal side of the second abdominal segment of 5th instar larvae within 24 hours after ecdysis. The 20 nL buffer was injected as control. After injection of dsRNAs or buffer, larvae were divided between isolated and crowded conditions. The number of insects and replications (total number/biological replication) were:

Under isolated conditions: buffer treatment, n = 22/2; *dsTfMT1* treatment, n = 15/2; *dsTfMT2* treatment, n = 15/2; *dsTfMT3* treatment, n = 23/2; *dsTfMT1+dsTfMT2*, n = 16/2; *dsTfMT1+dsTfMT3*, n = 15/2; *dsTfMT2+dsTfMT3*, n= 18/2; *dsTfMT1+dsTfMT2+dsTfMT3*, n = 15/2; and *dsTfKr-h1*, n = 22/2.

Under crowded conditions: buffer treatment, n = 15/2; *dsTfMT1* treatment, n = 15/2; *dsTfMT2* treatment, n = 15/2; *dsTfMT3* treatment, n = 19/2; *dsTfMT1+dsTfMT2*, n = 14/2; *dsTfMT1+dsTfMT3*, n = 17/2; *dsTfMT2+dsTfMT3*, n= 16/2; *dsTfMT1+dsTfMT2+dsTfMT3*, n = 14/2; and *dsTfKr-h1*, n = 24/2.

Analysis of gene expression by quantitative PCR (qPCR)

To examine the efficiency of RNAi in transcript suppression under isolated and crowded conditions, pooled RNA samples of *T. freemani* were isolated from three whole bodies of larvae in 6th and 7th instar larvae three days after molting with Trizol reagent solution (Ambion, Applied Biosystem, USA). RNAs were treated with RNase-free DNaseI. All RNA samples were converted to cDNA (500 ng per 10 µl reaction) with SuperScript® III reverse transcriptase (Invitrogen). The levels of transcript were quantified using a CFX connect Real-Time PCR Detection System (Bio-Rad, USA). The cDNAs were diluted for 4 times. Ribosomal protein 49 (Rp49) was used as internal standard for normalization. The qPCR was performed in 10 µl volume per reaction using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA) in three biological replications with two technical replications. The primers used for each gene are shown

in Table 2.1. The conditions of qPCR were 95 ºC for 5s and 60 ºC for 30s for 40 cycles, then analyzed melting curve with 65 ºC-95 ºC and 0.5 ºC increment for 5s per step.

Hormonal treatments

Methoprene (Sigma-Aldrich, USA) was dissolved in methanol and then diluted in acetone as described by Minakuchi et al. (2008). Methoprene (130 nL of 0.19 μg/μL) was topically applied to the dorsal side of 6th instar within 24 hours after molting of *T. freemani* larvae injected with dsRNAs or buffer. After treatment larvae were divided between isolated or crowded conditions.

Statistical analysis

Statistical differences were determined by Student's *t* test (p=0.05) and one-way or two-way analysis of variance (ANOVA) (p=0.05).

Results

The development of *T. freemani* under isolated and crowded conditions

The newly hatched larvae of *T. freemani* had white bodies to which egg shells were still attached. After hatching (1.8±0.6 days), larvae (n=100) molted to second instar and the body color became brown. Under isolated conditions there were 8 (n=52/100) or 9 (n=48/100) larval instars before pupation (Figure 2.1). The median duration of development time was 4.1±0.8 to 4.8±1.2 days in each instar from second to sixth. The duration of each stadium then increased to 5-6 days in 6th and 7th instars. The developmental period before pupation either in 8th or 9th instar was 13.9±4.6 or 14.5±3.2 days, respectively. The total development time from first instar to

pupal stage was 43.5±1.6 or 49.8±0.7 days for 8th or 9th larval instar, respectively. Pupal stage took 7.4±0.7 days to adult eclosion.

In simulation of crowded conditions, an adult male of *T. castaneum* was added to the newly ecdysed 5th-instar larva in each well of 96 well-plate. The durations of each instar under crowded conditions were similar to those of isolated larvae until the 8th or 9th instar. However, supernumerary instars (10th up to 14th instar) had longer durations in each stadium than the development time in earlier instars (1 to 9th instar) or in the instars of isolated larvae (Figure 2.2). Observations were stopped after larvae molted to 15th instar.

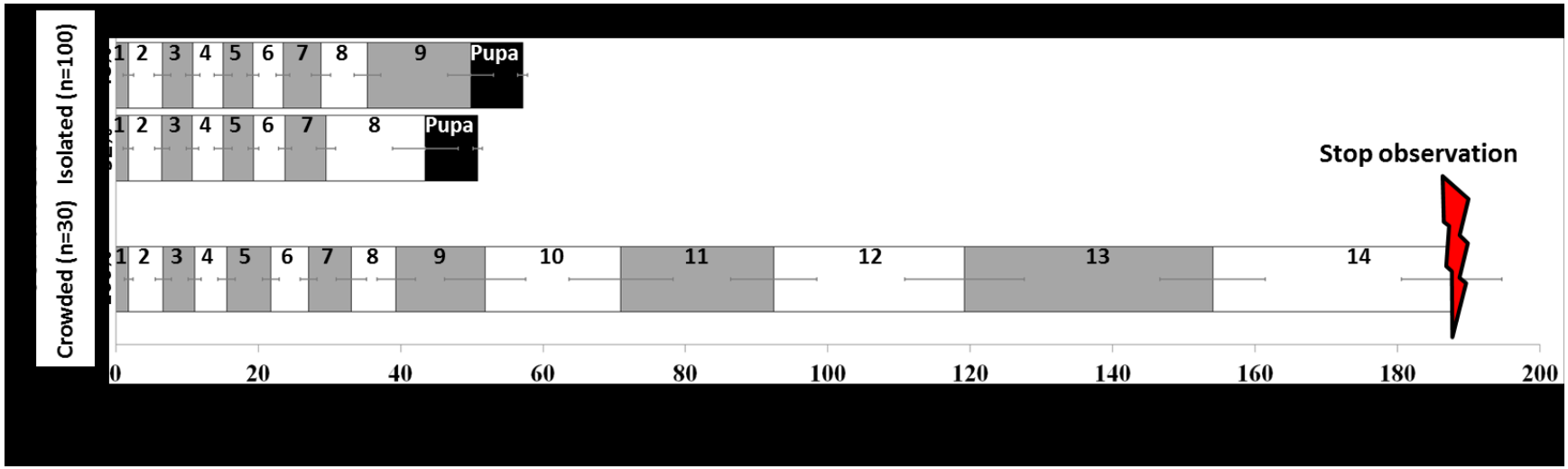


Figure 2.1 Development of *T. freemani* in isolated or crowded conditions. Numbers in boxes indicate different larval instars from 1st - 14th instar. This result is from 1-5 biological replications. Error bars show standard deviation of the development in each instar. For larvae under crowded conditions molt to the 15th instar, the observations were stopped.

Identification of three methyltransferase (*TfMT1, TfMT2,* and *TfMT3*), *krüppel homolog1* (*TfKr-h1*), and *Broad* (*TfBr*) genes

The mRNA sequences of three putative *methyltransferases* (*TfMT1*, *TfMT2*, and *TfMT3*), *Krùppel homolog1* (*TfKr-h1*), and the partial sequence for *Broad-complex* (*TfBr*) of *T. freemani* were obtained from BLAST searches in *T. freemani* genome database with query of *T. castaneum* sequences. After amplification and sequencing of the cDNA, the sequences of all putative genes from *T. freemani* were confirmed (Appendix figure 2). Comparison of methyltransferase amino acids with 4 different insects and three methyltransferases in the red flour beetle *T. castaneum* (*TcMT1*, *TcMT2*, and *TcJHAMT*) showed the *TfMT3* is orthologous to *TcJHAMT,* for which RNAi resulted in precocious metamorphosis in the *T. castaneum* (Minakuchi et al., 2008a), while *TfMT1* and *TfMT2* are orthologs of *TcMT1* and *TcMT2*, respectively, the functions of which are unknown (Figure 2.2 and 2.3A). The sequences for *TfKr-h1* (Figure 2.3B) and *TfBr* (Figure 2.3C) were also found to be the orthologs of *TcKr-h1* and *TcBr*, respectively, based on the blast result of the *T. freemani* genome.

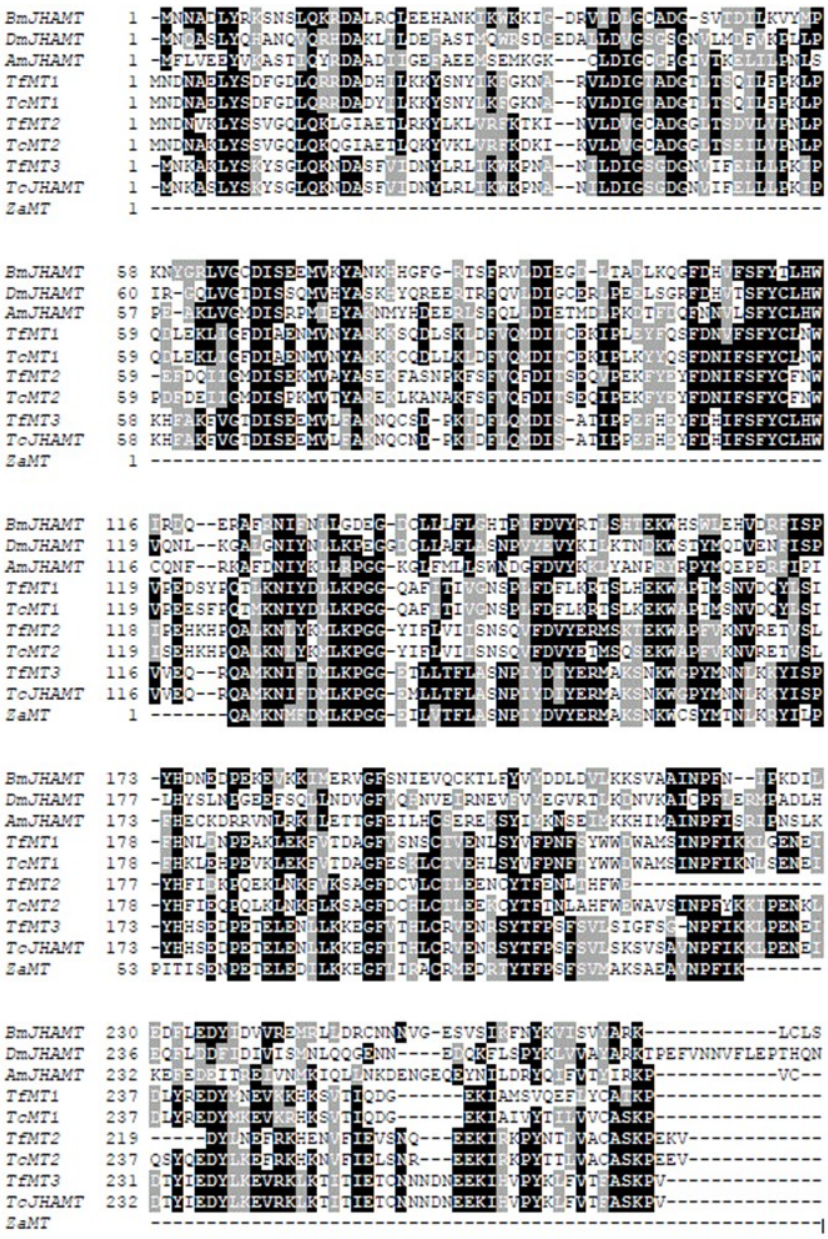


Figure 2.2 Alignment of the amino sequences of *T. freemani* methyltransferases with other insect JHAMTs. The alignment was done using ClustalW and the figure was generated in Boxshade 3.21. The number of the left side of the alignment indicated the position of residues in the sequence for each protein. Identical and conserve residue amino acids are indicated in black and grey backgrounds, respectively.

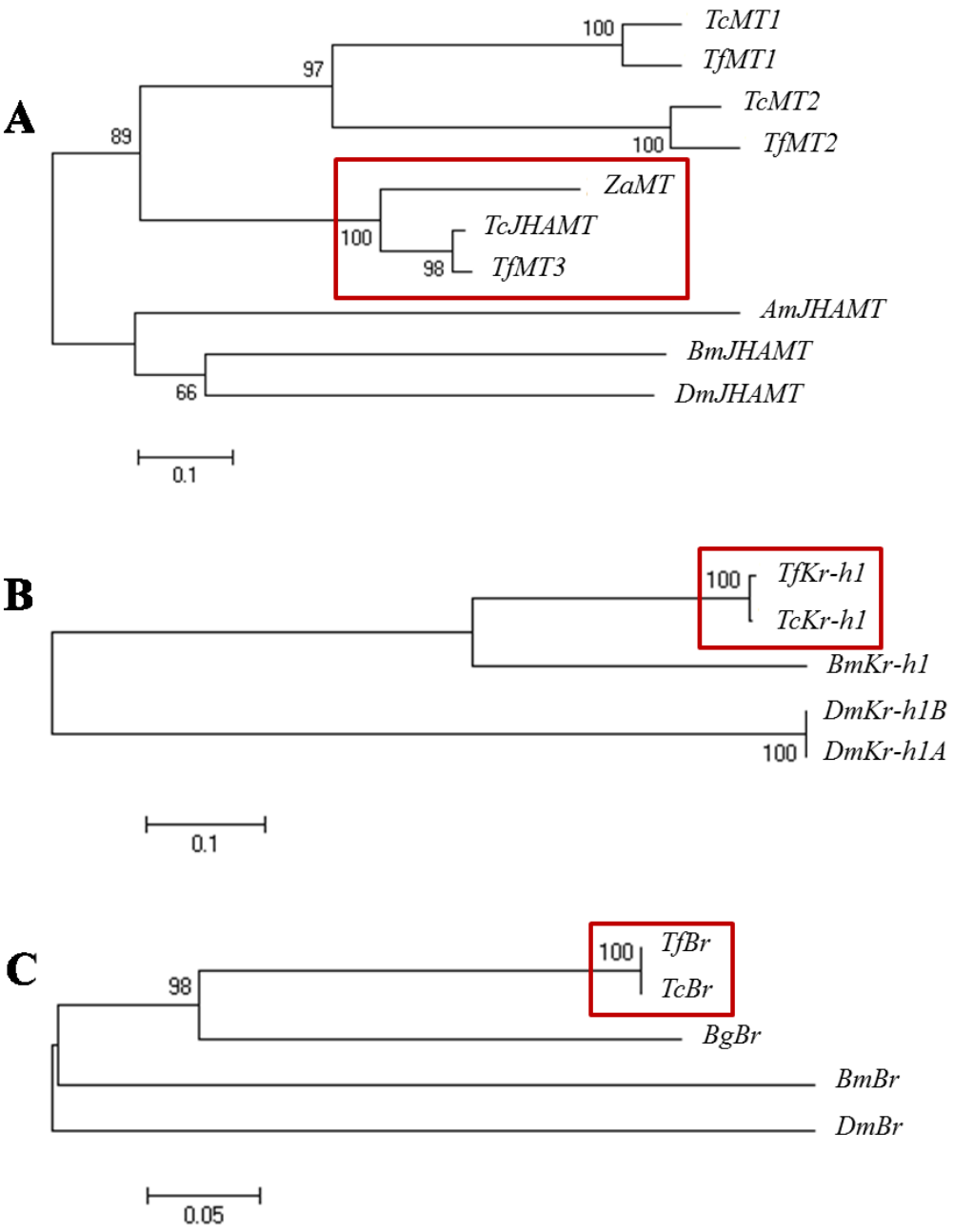


Figure 2.3 Phylogenetic relationship of *TfMT*s (1, 2, and 3) to other insect *methyltransferases* (A), *Krùppel-homolog1* (*Kr-h1*) (B), and *Broad* (*Br*) (C) was constructed by MEGA6 software with Neighbor-joining and shows bootstrapping values of the 1,000 trials at the nodes of the tree. The bar represents a distance unit of 0.1 or 0.05 when measuring the length of the branches.

Effects of *TfMT* RNAi on larval-pupal metamorphosis

The functions of *TfMTs* (1, 2, and 3) in the larval stage under isolated or crowded conditions were examined by injecting dsRNAs into newly molted fifth instar larvae. Control larvae were injected with 20 nL buffer only. Transcript levels from the larvae 3 days after molting to the 6th instar were used to quantify the transcript levels to confirm the efficiency of RNAi. Injections of *dsTfMT1*, *dsTfMT2*, or *dsTfMT3* for 200 ng (20 nL of 10 ng/nL) significantly suppressed the transcript levels of the counterparts compared to control larvae (5 – 30% expression levels of the control, Figure 2.4A-C).

Fifth instar larvae of *T. freemani* that were treated with dsRNAs of *TfMT1* or *TfMT2* showed slightly, but significantly, delayed development to pupal stage under isolated conditions (Figure 2.5). In contrast, larvae that were injected with *dsTfMT3* under isolated conditions had precocious pupation at the end of 6th or 7th instar resulting in small pupae (Figure 2.6). In addition, the injection of *dsTfMT3* with either *dsTfMT1* or *dsTfMT2,* or both, overrode the effects of delayed development in *dsTfMT1* and *dsTfMT2* treatments by accelerated pupations in *dsTfMT3* treatments (Figure 2.5).

The larvae kept in crowded conditions after the injections with *dsTfMT1* or *dsTfMT2* continued larval-larval molting without pupation, which is same as control larvae under crowded conditions. In contrast, injection of *dsTfMT3* in 5th instar larvae caused lethality (Figure 2.7A and C) in 6th instar (31.6%), 7th instar (63.2%), or 8th instar (5.3%). In addition, when the larval instar presumed to be older than 9th instar (about 2 months old; FGL) kept in crowded conditions was injected with *dsTfMT3,* the larva was arrested in prepupal stage and die without further molting. When the deaths of *dsTfMT3* treated larvae in crowded conditions were examined carefully, the

death occurred in the prepupal stage. Under the dried larval cuticle, presence of apparent pupal characteristics (eyes, wings, and gin traps) was obvious (Figure 2.7C).

The prepupal stage of the larvae treated with *dsTfMT3* under isolated conditions started after molting to 7th instar in about 10 days and pupate around 2 days later. On the other hand, the onset of prepupal stage of larvae treated with *dsTfMT3* under the crowded conditions was delayed to about 20-26 days after molting to 7th instar. The pupal characteristics underneath larval cuticle were observed after entering prepupal stage for 3-4 days. Under crowded conditions, the prepupae were flattened and died in about 5-6 days after entering the prepupal stage called “prepupal arrest or larval-pupal arrest” (Figure 2.7A, B).

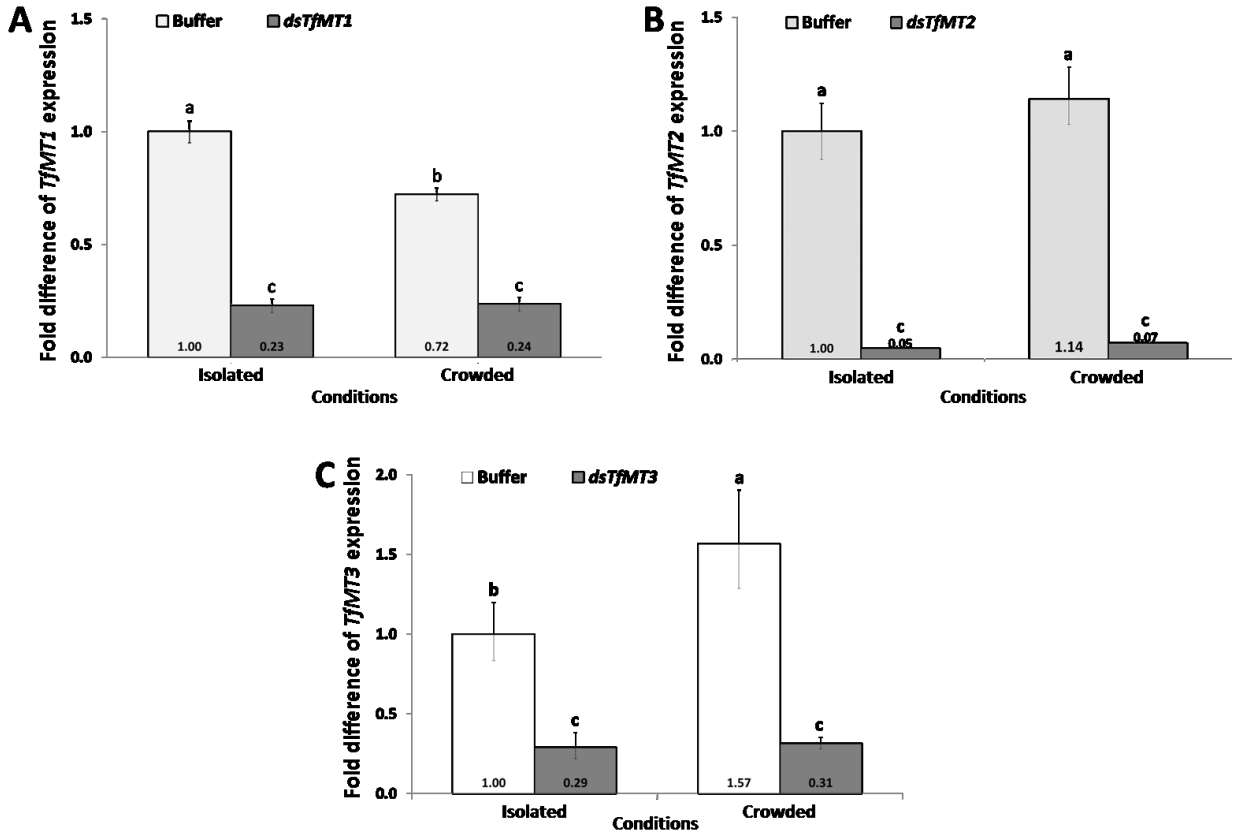


Figure 2.4 Transcript levels of *TfMT1* (A), *TfMT2* (B), and *TfMT3* (C) from dsRNA treated larvae 3 days after molting into 6th instar when kept under either isolated or crowded conditions (n=3 biological replications). One-way ANOVA with completely randomize design ($P<0.05$) was used for statistical analysis. Different letters indicated statistically significant differences. Error bars indicated standard deviation.

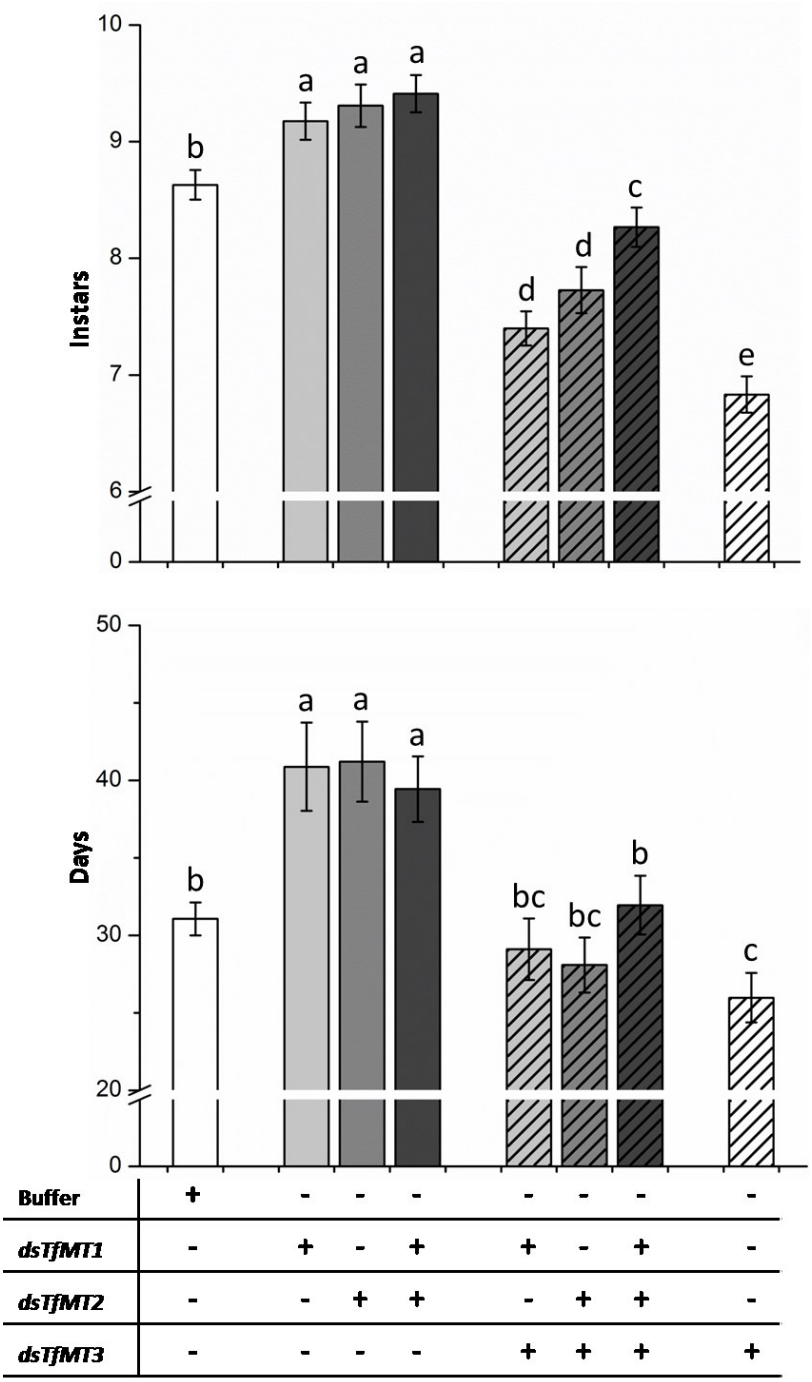
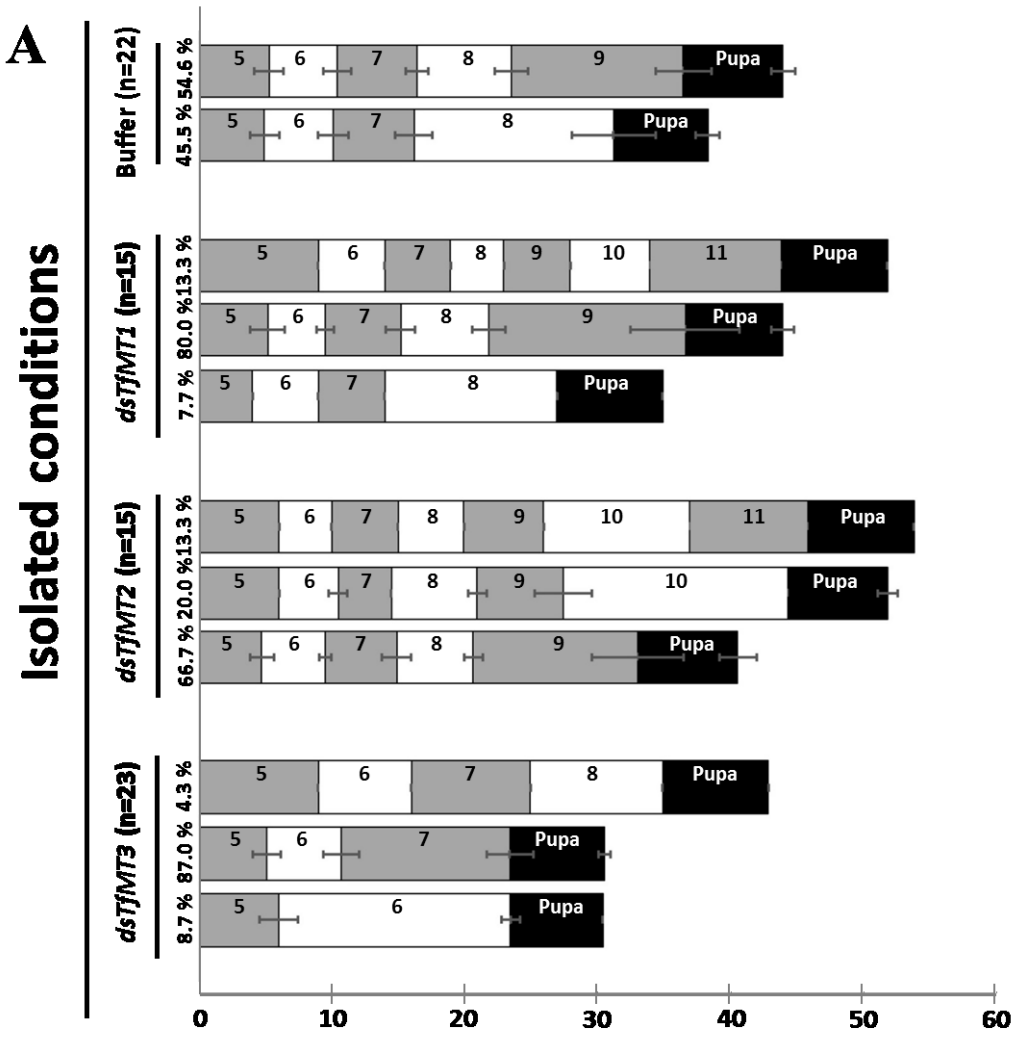


Figure 2.5 Number of instars and days to pupation with RNAi treatment under isolated conditions in *T. freemani*. Two-way factorial design of analysis of variance (ANOVA) with completely randomize design (P<0.05) was used for statistical analysis. Different letters indicated statistically significant difference. Error bars indicated standard deviation.


A
Isolated conditions
Buffer (n=22)
45.5 %
54.6 %
dsTfMT1 (n=15)
7.7 %
80.0 %
13.3 %
dsTfMT2 (n=15)
66.7 %
20.0 %
13.3 %
dsTfMT3 (n=23)
8.7 %
87.0 %
4.3 %
5
6
7
8
9
10
11
Pupa
0
10
20
30
40
50
60

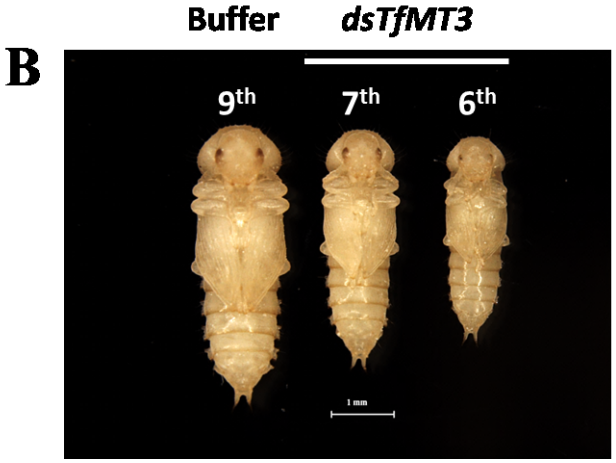

Buffer
dsTfMT3
B
9th
7th
6th
1 mm

Figure 2.6 The effects of RNAi of *TfMT1, TfMT2,* and *TfMT3* under isolated conditions. (A) The developmental period of larva after dsRNA injection in 5th instar. Numbers in the boxes indicate larval instars from 5th to 11th and pupal stage. Error bars indicate standard deviation of the development in each instar or stage. (B) Visual comparison of pupal size when 5th instar larvae were injected with *dsTfMT3* or buffer within 24 hours after ecdysis. Precocious pupation occurred in *dsTfMT3* injected larvae at the end of 6th or 7th instar, whereas buffer injected larvae pupate at the end of the 9th instar. Scale bar indicates 1 mm.

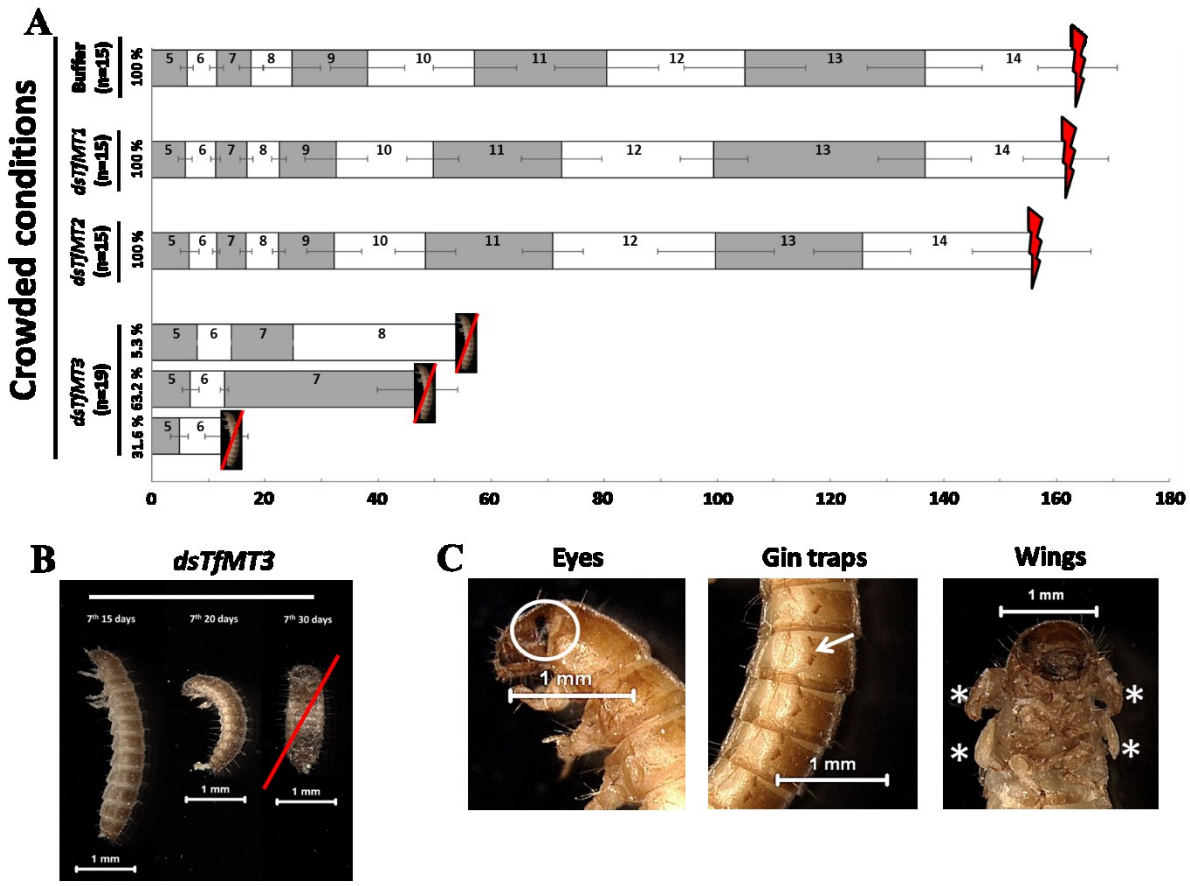


Figure 2.7 The effects of RNAi of *TfMT1*, *TfMT2*, and *TfMT3* under crowded conditions. (A) The developmental period of larvae injected with buffer or dsRNA. Numbers in boxes indicate larval instars from 5th to 14th. Error bars indicate standard deviation of the development in each instar. The red line indicates death in prepupal arrest. Observations were stopped after larvae reached the 15th instar. (B) The prepupal stage of RNAi of larvae after molting into 7th instar for 15, 20, and 30 days. (C) Pupal characteristics in *dsTfMT3*-treated larvae. Eyes (circle) and gin traps (white arrow) are shown underneath larval cuticle, while wings (asterisks) are shown after peeling larval cuticle out. Scale bar indicates 1 mm.

I tested whether application of the JH analog methoprene rescues the phenotype of the *dsTfMT3* treatment. In isolated conditions, the precocious pupation of *dsTfMT3* treatment was rescued by methoprene by additional larval molts. When methoprene was applied to *dsTfMT3* treated 6^{th} instar larvae within 24 hours after molting, larvae continued larval-larval molting and pupate at the end of 9^{th} or 10^{th} instar in isolated conditions. In crowded conditions, application of methoprene to the *dsTfMT3* treated larvae rescued larvae from immediate death, but death still occurred after 2 or 3 additional larval molts, whereas buffer injected larvae with methoprene treatment continued molting without death (Table 2.2). Therefore, the effect of *dsTfMT3* lasted longer than the effect of methoprene.

Table 2.2 Phenotype of *T. freemani* larvae injected with 200 ng *dsTfMT3* or buffer as control in 5th instar within 24 hours after molting and then treated with methoprene, JH analog, in 6th instar. Abbreviations: P = pupa, D = death, and L = larva. The developmental period of larvae in each treatment is shown in the figure 2.8-2.10.

dsRNA	Conditions	Treatment	n	Instars / phenotypes																	
				6th		7th		8th		9th		10th		11th		12th			13th		
				P	D	P	D	P	D	P	D	P	D	P	D	L	P	D	L	P	D
Buffer	Isolated	Solvent	31					14		15		2									
		Methoprene	10							5		5									
	Crowded	Solvent	15																15		
		Methoprene	14																14		
dsTfMT3	Isolated	Solvent	20	2		16		2													
		Methoprene	22					1		16		5									
	Crowded	Solvent	19		6		12		1												
		Methoprene	16						8		2		4		1			1			

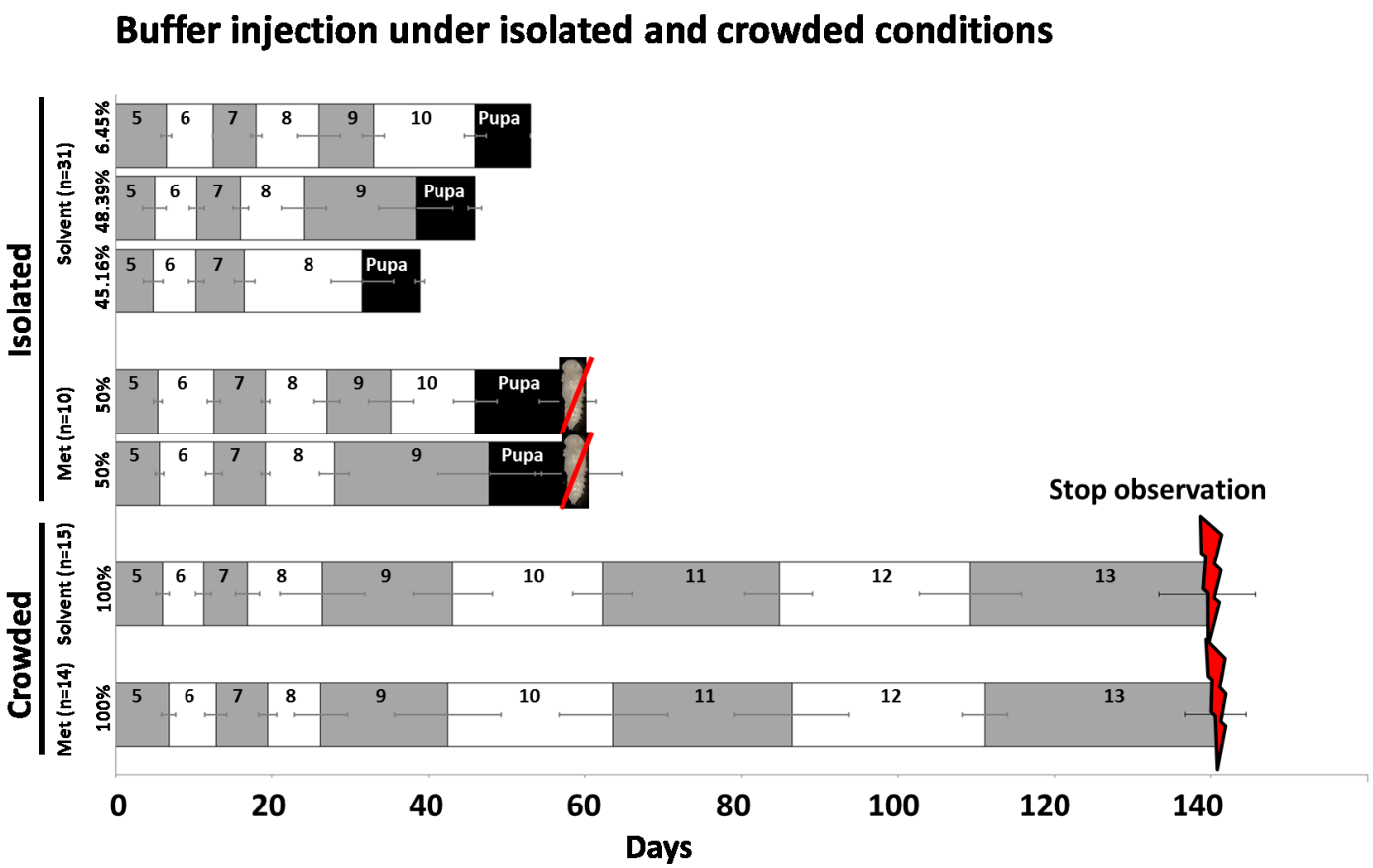


Figure 2.8 Larvae injected with buffer under isolated and crowded conditions in 5th instar and then treated with methoprene, JH analog, or solvent (acetone) in 6th instar of *T. freemani*. Numbers in boxes indicated larval instars from 5th- 13th instar and pupa. Error bars indicate standard deviation of the development in each instar. The red slash line on pupae indicates that pupae had died. Observations were stopped after larvae under crowded conditions reached the 14th instar.

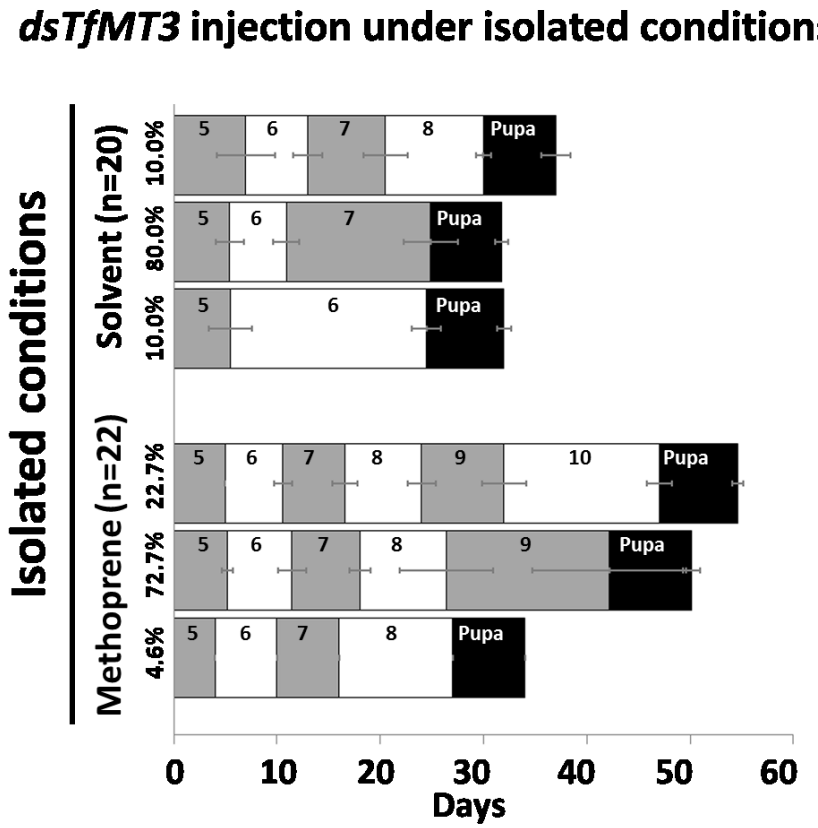


Figure 2.9 Larvae injected with buffer under isolated conditions in 5th instar and then treated with methoprene, JH analog, or solvent (acetone) in 6th instar of *T. freemani*. Numbers in boxes indicated larval instars from 5th- 10th instar and pupa. Error bars indicate standard deviation of the development in each instar.

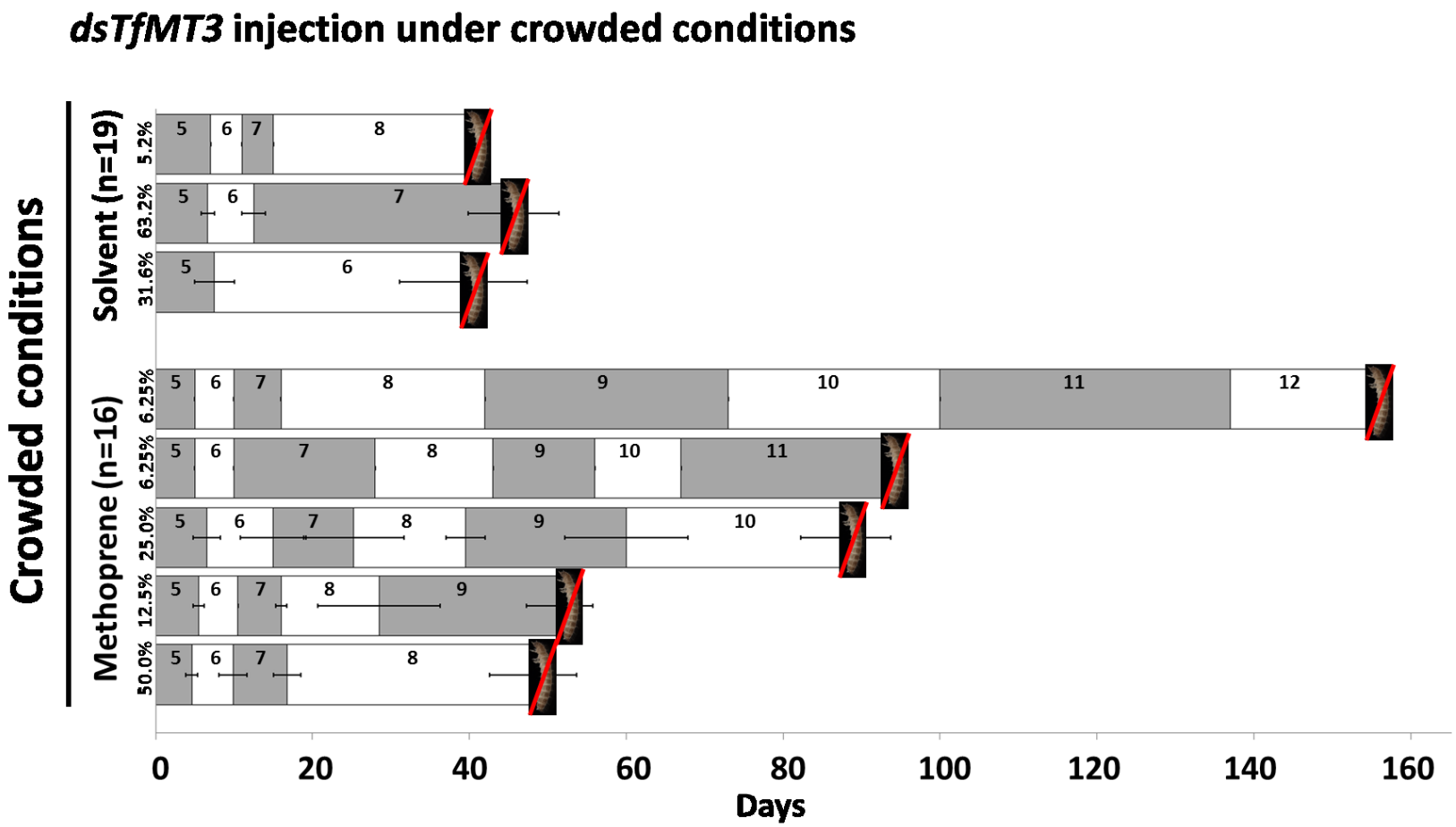


Figure 2.10 Larvae injected with buffer under crowded conditions in 5th instar and then treated with methoprene, JH analog, or solvent (acetone) in 6th instar of *T. freemani*. Numbers in boxes indicated larval instars from 5th- 12th instar. Error bars indicate standard deviation of the development in each instar. The red slash line indicates that prepupa had died.

The role of *TfKr-h1* in crowded conditions

Investigation of *TfKr-h1*, which is downstream in the JH signaling pathway, was conducted to test whether this gene is involved in the supernumerary molting under crowded conditions. *Kr-h1* is up-regulated by high JH and it suppresses expression of the further downstream gene *Broad* (*Br*) in *T. castaneum* (Minakuchi et al., 2009). In *T. freemani*, *TfKr-h1* transcript level was ~3-fold higher in larvae under crowded conditions than those in isolated conditions in 3-day old 7th larval instar (Figure 2.11A), consistent with the interpretation that high titer of JH in crowded conditions results in higher *TfKr-h1* transcript. However, *Br* transcript levels, which were negatively correlated with JH titer and *TfKr-h1* transcript level in the model insects *M. sexta* (Zhou and Riddiford, 2002), *D. melanogaster* and *T. castaneum* (Minakuchi et al., 2009; Minakuchi et al., 2008b), were not observed to be so in these samples. *TfBr* transcript level was 3-fold higher in *T. freemani* larvae kept under crowded condition than those in isolated conditions.

Treatment with *dsTfMT3,* which was expected to lower the JH level, significantly suppressed the *TfKr-h1* transcript in the same conditions (Figure 2.11A). However, despite *dsTfMT3* treatment, the *TfKr-h1* transcript level in larvae under crowded conditions increased by ~8-fold in larvae 15 days after molting to 7th instar. On the other hand, 1-day old prepupa from *dsTfMT3*-injected larvae kept under isolated conditions (about 11 days after molting to 7th instar) showed significantly lower level of *TfKr-h1* transcript than the 15-days old 7th instar larvae under crowded conditions (Figure 2.11A). A negative relationship between the levels of *TfKr-h1* and *TfBr* transcripts was obvious in the larvae treated with *dsTfMT3* (Figure 2.11B). Specifically, the high *TfKr-h1* transcript level in the larvae 15 days after molting to 7th instar under crowded

conditions coincided with the low *TfBr* transcript level and the lower *TfKr-h1* transcript level in isolated prepupal stage coincided with high *TfBr* transcript level.

In the case of *dsTfKr-h1* injection, the *TfKr-h1* transcript in either isolated or crowded larvae was significantly down-regulated compared to its transcript in buffer-injected larvae (Figure 2.12A). Surprisingly, *dsTfKr-h1* treatment not only suppressed the expression of *TfKr-h1* but also the expression of *TfBr* (Figure 2.12B), which was up-regulated in *Drosophila* (Tracey, 1958), since *Kr-h1* works upstream and suppresses the expression of *Br* (Minakuchi et al., 2009).

Most *dsTfKr-h1*- treated larvae precociously entered the prepupal stage in the 7th instar. A few *dsTfKr-h1*-treated larvae under isolated conditions continued development to the adult stage underneath the larval cuticle without molting to adult; these are called pupal-adult arrest or pharate adult arrest. The cuticle on head, thorax, short wings and ventral abdomen had become sclerotization. A few larvae (2/48) under isolated conditions showed larval cuticle split on the head and thoracic parts (Figure 2.14A-C). In contrast, arrested prepupae under crowded conditions of *dsTfKr-h1* treatment developed pupal characteristics such as eyes, gin traps, and wings underneath larval cuticle before they died, which was similar to *dsTfMT3* treated larvae under crowded conditions (Figure 2.7A). Therefore, the phenotype in the *dsTfKr-h1* treatment resulted in the same phenotype as in the *dsTfMT3* treatment under crowded conditions, suggesting that death at pupal molting may have been caused by same pathway.

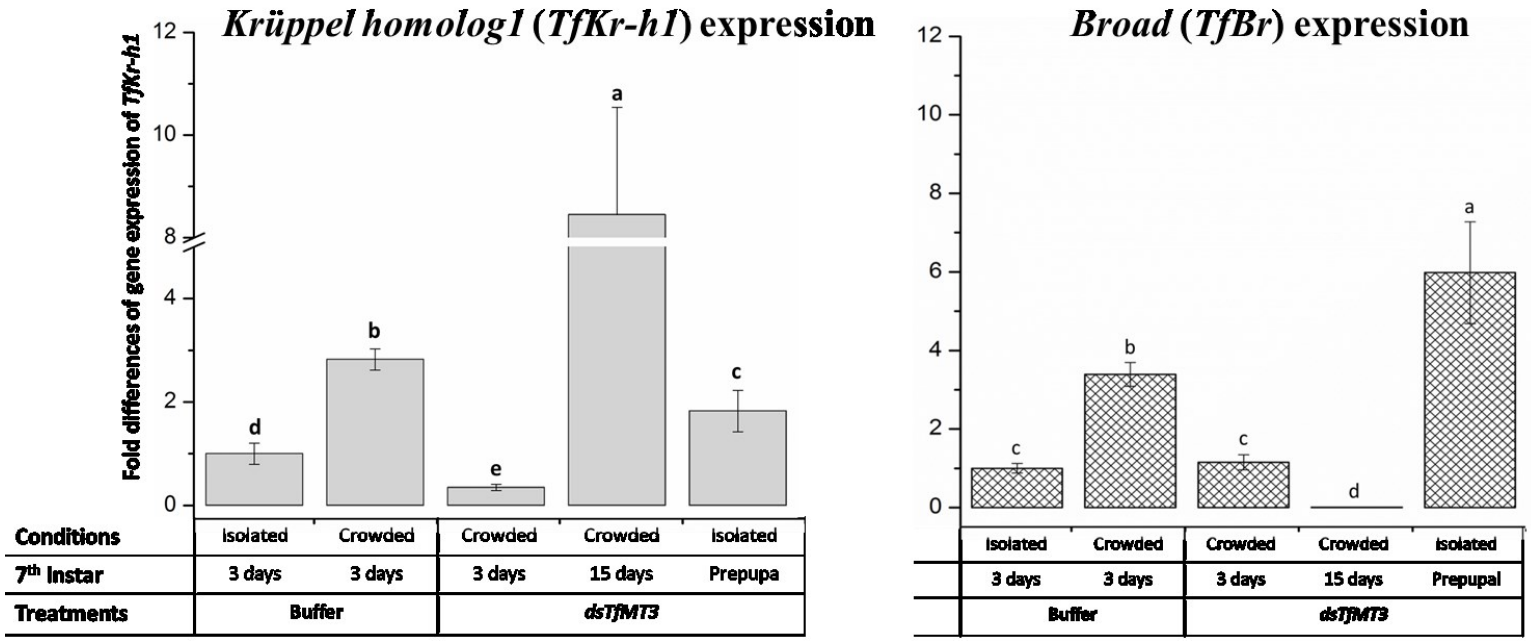


Figure 2.11 The *dsTfMT3* or buffer injection in 5th instar larvae and kept either under isolated and crowded conditions. Transcript levels of *TfKr-h1* (A) and *TfBr* (B) were then examined by quantitative PCR at two time points in crowded conditions (3 days or 15 days after molting to 7th instar, n = 3 biological replications) but only at one time point for larvae under isolated conditions (3 days). One-way analysis of variance (ANOVA) with completely randomized design (P<0.05) was used for statistical analysis. Different letters indicated statistically significant differences. Error bars indicated standard deviation.

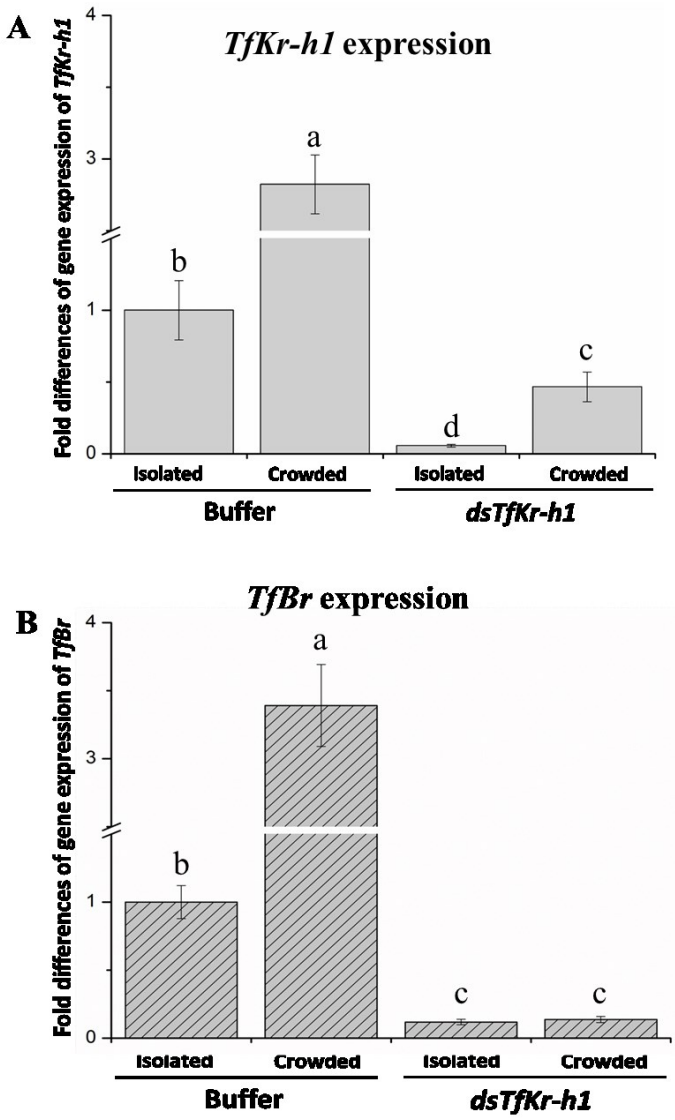


Figure 2.12 The *dsTfKr-h1* or buffer injection in 5th instar larvae and kept either under isolated and crowded conditions. Transcript levels of *TfKr-h1* (A) and *TfBr* (B) were then examined by quantitative PCR from RNA isolated three days after larvae had molted to the 7th instar (n = 3 biological replications). One-way analysis of variance (ANOVA) with completely randomized design (P<0.05) was used for statistical analysis. Different letters indicated statistically significant differences. Error bars indicated standard deviation.

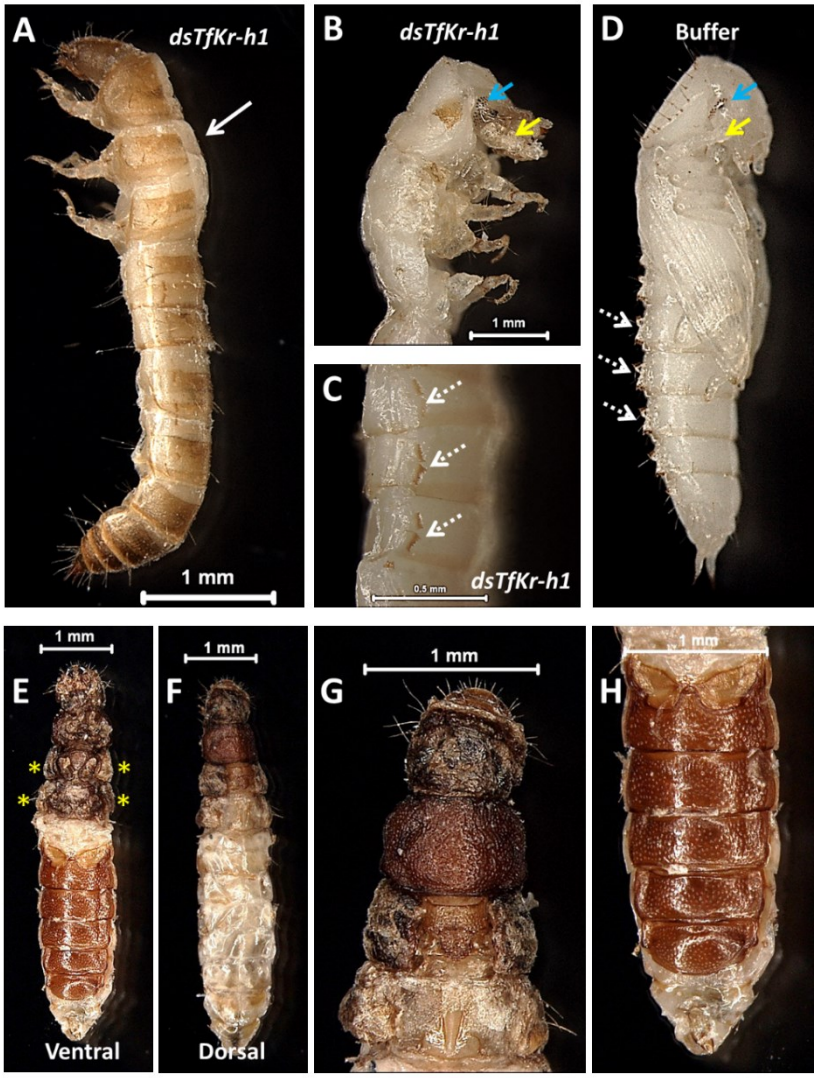


Figure 2.13 Phenotype of *TfKr-h1* RNAi under isolated conditions. (A) The *dsTfKr-h1* treated larva in isolated conditions arrested in prepupal stage had split of the cuticle (white arrow). (B-C) After removing the larval cuticle of *dsTfKr-h1* treated larva showing pupal eyes (blue arrows), antennae (yellow arrows), and gin traps (dot white arrows). (D) Control pupa from buffer injection. (E-H) The *dsTfKr-h1* treated larva arrested in the pharate adult stage in isolated conditions for 10 days showed adult-like cuticle on head, ventral abdomen, thoraxes and wings. Asterisks indicated wings.

The effect of double RNAi of *TfMT3* and *TfKr-h1*

Larvae treated with *dsTfMT3* under crowded conditions showed high expression of *TfKr-h1* and caused prepupal arrest, which was consistent with the occurrence of prepupal arrest in transgenic *B. mori* that overexpressed *Kr-h1* (Kayukawa et al., 2014). It confirmed that high *Kr-h1* with lack of JH was one of the cause of prepupal arrest (Figure 2.15A). However, the suppression of *TfKr-h1*, downstream of JH, under crowded conditions also caused prepupal arrest (Figure 2.15A). Because crowded conditions were proposed to induce high JH (Kotaki et al., 1993), the prepupal arrest in *dsTfKr-h1* treatment under crowded conditions might have been caused by high JH although *TfKr-h1* was suppressed. To reveal the role of JH and *Kr-h1* in repression of metamorphosis in crowded conditions, FGL were treated with both *dsTfMT3* and *dsTfKr-h1* for 200 ng of each gene. After dsRNA treatment, larvae were divided between isolated and crowded conditions. Larvae treated with both *dsTfMT3* and *dsTfKr-h1* together under isolated conditions were unable to shed larval cuticle but their development progressed to adult structures under larval cuticle. This was the same as with larvae treated with *dsTfKr-h1* alone under isolated conditions (Figure 2.14B). In contrast, larvae treated with *dsTfMT3* and *dsTfKr-h1* together under crowded conditions could be rescued from larval-pupal arrest to pharate adult arrest (Figure 2.15B). In pharate adult arrest, head and wings of those larvae under crowded conditions were sclerotized, whereas ventral abdomen maintained pupal characteristics under larval cuticle.

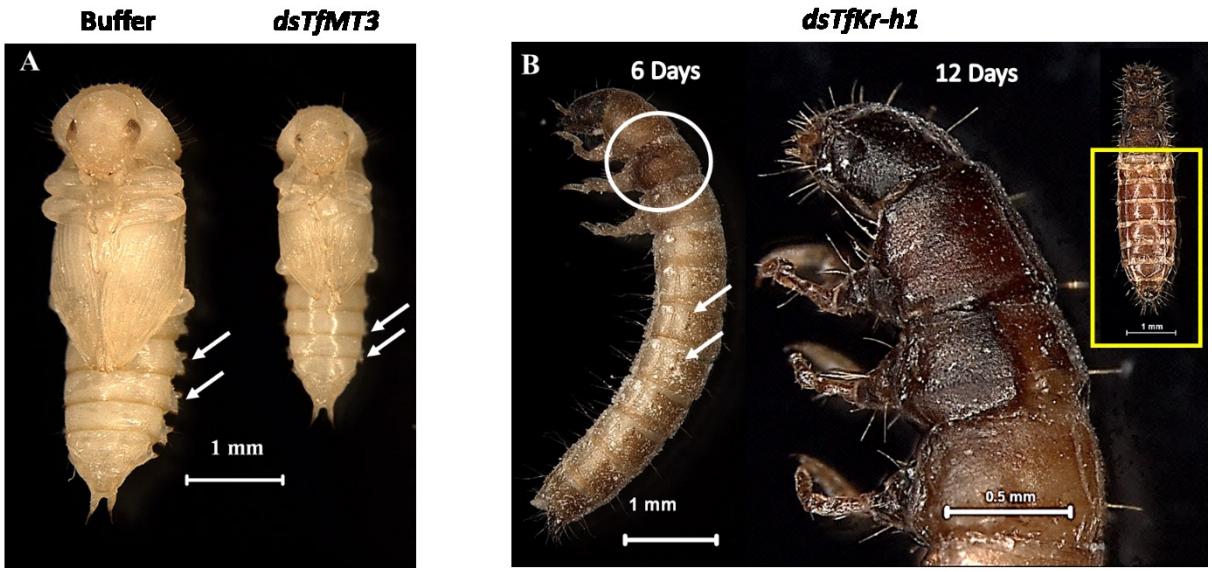


Figure 2.14 The phenotypes of dsRNA treatments under isolated conditions. (A) Pupae from buffer or *dsTfMT3* treatment in 5th instar larvae. Buffer treatment cause normal pupation at the end of 9th instar, while precocious pupation occurs at the end of 7th in *dsTfMT3* treated larvae. (B) Pharate adult arrest from fully grown larvae treated with *dsTfKr-h1* under isolated conditions. Wings were sclerotized (white circle) after entering prepupal stage for 6 days. Head, thorax, and ventral abdomen become adult-like cuticle after becoming prepupa for 12 days. White arrows indicate gin traps in pupae and in 6 days prepupal arrest. Yellow box indicates adult cuticle on ventral abdomen.

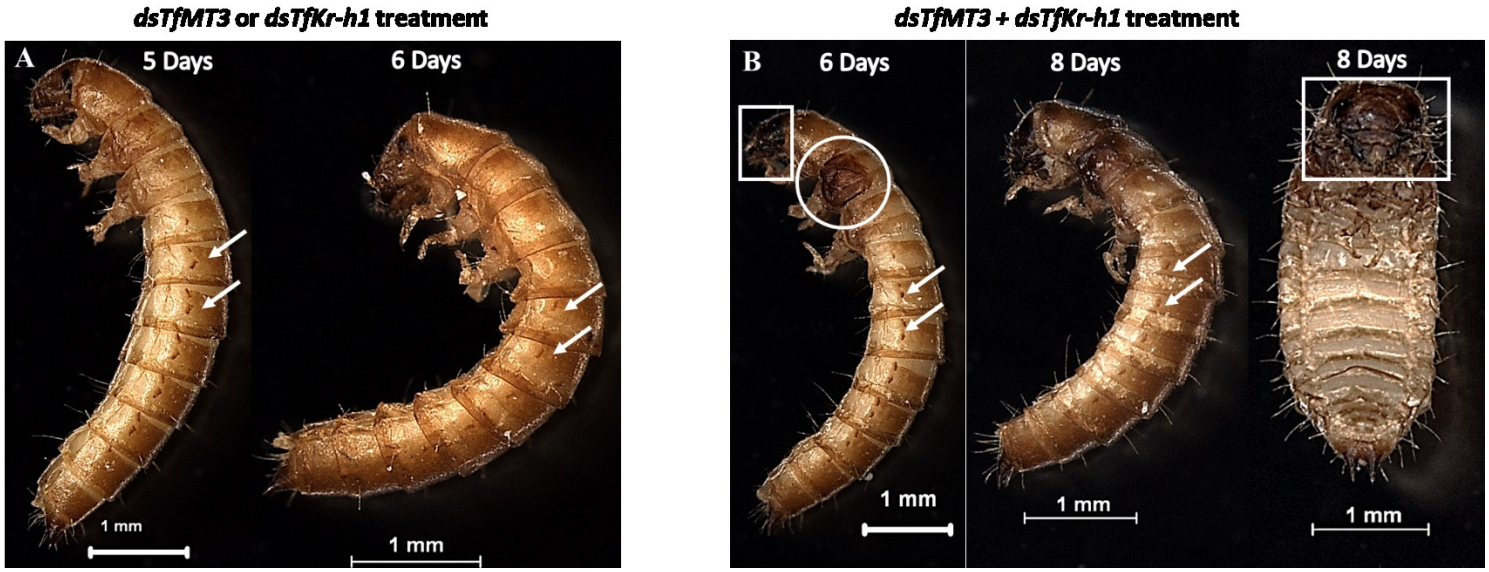


Figure 2.15 The phenotypes of *dsTfMT3* and *TfKr-h1* treatments under crowded conditions. (A) larval-pupal arrest in *dsTfMT3* or *dsTfKr-h1* treatment alone of fully grown larvae under crowded conditions. (B) The pharate adult arrest from the combination of both *dsTfMT3* and *dsTfKr-h1* treatment together in fully grown larvae under crowded conditions. There was sclerotization on head (white boxes) and wings (white circle) after entering prepupal stage for 6 days.

Discussion

In this study, I tested whether an endocrine factor, JH, is sufficient to explain the supernumerary larval molts in larval *T. freemani* when they are kept under crowded conditions. Although previous studies have shown JH or its analogue methoprene are sufficient to induce supernumerary larval molts in isolated larval stage (Kotaki et al., 1993), this study tested the opposite: that is, whether suppression of JH is sufficient to induce normal metamorphosis under crowded conditions

Development of *T. freemani* in isolated and in crowded conditions

There have been no reports of the exact life history of *T. freemani*, so we also described the number of larval instar and durations in isolated and crowded conditions. There were 8-9 larval instars under isolated conditions, which is different from the assumption by Imura et al. (1984) that *T. freemani* has 6 larval instars. They reported that larvae under isolated conditions pupate 21 days after hatching, whereas in our isolated conditions the shortest median duration of larval development was about 43 days. It is not clear whether the longer development time with increased larval instars in our experiment was caused by genetic changes after 30 years of laboratory history or by differences in other experimental conditions, such as temperature and humidity (Nakakita and Imura, 1981).

The larvae under crowded conditions continue to molt to at least a 15th larval instars. The inhibition of pupation under crowded conditions of *T. freemani* supports the previous studies by Nakakita (1982). In fact, the metamorphic inhibition may depend on degree of crowding. Nakakita (1982) demonstrated that high density (over 20 larvae per 2 gram diet in vial 55mm depth, 25 mm diameter) can inhibit pupation for more than 6 months, whereas low density

(below 5 larvae per 2 gram diet) pupate in one month. Larvae in my experimental set up of crowded conditions (1 larva with 1 adult in a well of 96 well-plates) also fail to pupate for more than 6 months (Figure 2.2). Our experimental set up is likely providing more severe mechanical stimulation in simulating the crowded conditions.

The roles of JHAMT orthologs in the development of *T. freemani*

TfMT1, TfMT2, and *TfMT3* are all grouped with *JHAMT* in other insects, suggesting these three copies are the product of recent gene expansions in the *Tribolium* species. RNAi-mediated knockdown of *TfMT1* and *TfMT2* under isolated conditions did not cause precocious larval-pupal metamorphosis, but delayed pupation by unknown reason, while larvae continued larval-larval molting under crowded conditions, which is same as the control. Only the injection of *dsTfMT3* in *T. freemani* under isolated conditions caused precocious larval-pupal metamorphosis. This was consistent with previous studied in *T. castaneum* by Minakuchi et al. (2008), in which methyltransferase activity toward JH acid occurred only in recombinant TcJHAMT (MT3 ortholog), but not with TcMT1 and TcMT2 proteins.

Precocious larval-pupal metamorphosis was caused by *dsTfMT3* injection of larvae in isolated conditions, whereas lethality occurred in prepupal stage of *dsTfMT3*-treated larvae under crowded conditions. The precocious pupation in isolated larvae is similar to the suppression of *TcJHAMT* in *T. castaneum* (Minakuchi et al., 2008a), as they are one-to-one ortholog. This suggests that *TfMT3* encodes JHAMT, the essential enzyme for JH biosynthesis as the biochemical activity was demonstrated in *T. castaneum* JHAMT (Minakuchi et al., 2008a).

Endocrine factors inhibiting the pupal molting in crowded conditions

The data in this study showed that endocrine mechanism leading to LLC is more complex than the simple explanation of high JH titer in crowded conditions. Blocking larval-pupal molting by exogenous JH in isolated conditions is logically insufficient as an explanation for the endocrine changes leading the LLC. Testing the inverse logic, whether lack of JH in crowded conditions permits the larval-pupal molting, was a main purpose of this study.

A previous study addressed this question by lowering JH using anti-JH, precocene II, under crowded conditions (Nakakita, 1990). That study concluded that the inverse logic was correct because precocene II treatments permitted the larval-pupal molting in crowded conditions. However, that is different from my observation; that is, larval death in the transition from larva to pupa in RNAi of *TfMT3* under crowded conditions. I also repeated the same experiment as Nakakita (1990) reported in experimental conditions that is as similar as possible to the report. In the previous report, 10 individuals in the 1 gram diet containing 1,000 ppm precocene II in a vial 25 x 55 mm (diameter x height) exhibited 50% pupation within 30 days while larvae without precocene II showed 100% metamorphic inhibition. However, I was unable to replicate the results; both control and precocene II treatments resulted ~50% pupation within 15 days. The different results of precocene II treatment might be attributed to different sensitivities of the beetles to crowdedness. Our *T. freemani* was a strain that has long been maintained in the lab rearing conditions and may have evolved to be less sensitive to crowded conditions, although our strain also originated from Japan presumably from the same stock.

Under crowded conditions, pupal characteristics developed underneath larval cuticle of *dsTfMT3*-treated larvae, but these larvae died without pupation. In general, larval-pupal molting occurs when JH disappears and ecdysone increases (Riddiford, 1976, 1981, 1994). The studies of

ecdysone titer in many insects, including *M. sexta* (Bollenbacher et al., 1975), *Z. atratus* (Aribi et al., 1997b), and *T. freemani* (Hirashima et al., 1998) showed that in the FGL before pupation, the small peak of ecdysone induces the onset of pupal commitment and start the wandering stage. Also, the pupal cuticle has been formed at this stage (Riddiford, 1978, 1981). Then, the second peak, which is greater than the first peak, induces pupal metamorphosis. Thus, it is likely that *dsTfMT3*-treated larvae of *T. freemani* could enter the wandering stage and develop pupal characteristics under crowded conditions with a small peak of ecdysone. However, inability of larval-pupal metamorphosis may be caused by lack of the second ecdysone peak that might be inhibited by crowded conditions. Therefore, the lethality in *dsTfMT3*-treated larvae suggests direct or indirect involvement of ecdysone pathway.

In addition, treatment of *dsTfMT3*-injected larvae with methoprene, a JH analog, delayed but did not rescue the lethality under crowded conditions. Although dsRNA-treated larvae under crowded conditions continued molting at least 2-3 times after methoprene application, they eventually died in the subsequent molting. This observation also supports that the crowded conditions suppress a JH-independent pathway for pupal molting, likely ecdysone signaling pathway. Alternatively, the inability of methoprene-rescue may be caused by the effect of crowded conditions on the downstream of JH pathway.

Effects of crowded conditions on the JH downstream, *Kr-h1* and *Br*

There are two unexpected findings in the study of JH downstream pathways framed on the studies in *Drosophila, T. castaneum* and *B. mori*, model insects (Kayukawa et al., 2014; Minakuchi et al., 2008b; Pecasse et al., 2000). First, based on experiments using the application of exogenous JH and suppression of JH biosynthesis, the aforementioned studies suggest that JH upregulates *Kr-h1* and in turn *Kr-h1* expression downregulates *Br* in this pathway. However, in

my study, the *TfKr-h1* mRNA level increased in crowded conditions, even in the *dsTfMT3*-injected larvae in which JH biosynthesis was suppressed. This suggests the presence of a JH-independent pathway that increased expression of *TfKr-h1* under crowded conditions. Furthermore, the prepupal arrest in *dsTfMT3*-treated larvae under crowded conditions, in which *TfKr-h1* was highly expressed, was same as allatectomized larvae of transgenic *B. mori* that overexpressed *BmKr-h1α* (Kayukawa et al., 2014). Thus, prepupal arrest and lethality in *dsTfMT3*-treated larvae under crowded conditions are likely caused by lack of JH and overexpression of *Kr-h1* from JH-independent pathway.

Secondly, suppression of *TfKr-h1* by dsRNA treatment resulted in lower expression of *TfBr,* which is contradictory to results from mutant *D. melanogaster* (Pecasse et al., 2000; Tracey, 1958) and previous suggestion (Riddiford, 2012). In *T. castanuam* and *B. mori,* the transcriptional profiles of *Kr-h1* showed that *Kr-h1* reappears during the prepupal stage while *Br* is also expressed, suggesting that *Kr-h1* is required for *Br* expression. Moreover, *dsTfKr-h1* larvae were arrested in prepupal stage after molting into 7th instar. It supports previous studies in *T. castaneum*, *Blattella germanica*, and *Pyrrhocoris apterus* that *Kr-h1* plays critical role for preventing precocious metamorphosis (Konopova et al., 2011; Lozano and Belles, 2011; Minakuchi et al., 2009).

Effect of suppression of genes in JH signaling pathway

Isolated larvae treated with both *dsTfMT3* and *dsTfKr-h1* together were arrested in pupal-adult metamorphosis, which is the same as was observed in the *dsTfKr-h1* treatment alone. This suggests that downstream of *Kr-h1* is the cause of pharate adult arrest in isolated conditions. Previous study in *T. castaneum* (Minakuchi et al., 2009) and our data in *T. freemani* showed that *Br*, ecdysone-response gene and downstream of *Kr-h1*, was downregulated in *dsTfKr-h1*-treated

larvae. Therefore, the inability of treated larvae to shed larval cuticle may be affected by losing *Br* expression.

Interestingly, although prepupal arrest under crowded conditions was caused by the knockdown of JH or *Kr-h1* alone, it could be rescued to pharate adult arrest by the treatment with both *dsTfMT3* and *TfKr-h1* together. Previous study in *B. mori* showed that prepupal arrest and lethality were caused by low JH with high *Kr-h1* expression (Kayukawa et al., 2014). These results indicate that imbalance of JH and *Kr-h1*, which is independently induced by crowded conditions in the suppression of either *JH* or *Kr-h1*, is the cause of prepupal arrest and lethality later. Therefore, it is likely that the balance of both JH and *Kr-h1* in crowded conditions is required for either larval-larval molting with high of both or larval-pupal-adult development with low of both.

In conclusion, based on our results and previous studies, larval-larval molts require the presence of JH that induces *Kr-h1* but represses *Br*, while in the process of larval-pupal metamorphosis, JH and *Kr-h1* disappear on the onset of pupal commitment. Then, *Br* is expressed in prepupal stage initiated by surge of Ec and re-expression of *Kr-h1,* JH-independent induction, to complete larval-pupal metamorphosis (Figure 1.2).

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Chapter 3 - The roles of ecdysteroids in supernumerary molts of the flour beetle, *Tribolium freemani,* in the crowded conditions

Abstract

Pupal metamorphosis in the development of *Tribolium freemani* is density-dependent. The larvae undergo supernumerary molts without pupation when they are under crowded conditions. In the previous chapter, I have shown that this developmental pattern is not fully explained by high titer of juvenile hormone (JH) under the crowded conditions, but some additional factor is required. Knockdown of JH biosynthesis by RNA interference (RNAi) of *JH-methyltransferase3* gene (*TfMT3*) and RNAi of *Krüppel homolog1* (*TfKr-h1*) downstream in the JH pathway, causes lethality in crowded conditions instead of larval-pupal metamorphosis. In this study, I investigated possible involvement of ecdysteroids in this lethal phenotype. *dsTfMT3*-treated larvae undergo normal pupation under isolated conditions, in which there are two ecdysteroid peaks, a small rise of ecdysteroid (about 20 pg/larva) followed by greater peak (about 700 pg/larva). On the other hand, *dsTfMT3*-treated larvae in crowded conditions showed two small ecdysteroid peaks (about 15 and 40 pg/larva) before they died. In the case of *dsTfKr-h1* treatment, ecdysteroid peaks in isolated larvae were similar to buffer treated larvae during prepupal stage, but the decline in ecdysteroids did not occur at the time of pupal-adult eclosion, resulting in pupal-adult arrest. The levels of ecdysteroids in *dsTfKr-h1*-treated larvae under crowded conditions did not increase during larval stage but dramatically increased in prepupal arrest, leading to lethality. However, the injection of 20HE into prepupae of *dsTfMT3*- or *dsTfKr-h1*-treated larvae under crowded conditions did not rescue larval development to pupation. Interestingly, the injection of ecdysteroids into *dsTfMT3*-treated larvae before the prepupal stage under crowded conditions caused larval-larval molting, with short wings developing on the

thorax. The results suggest that crowded conditions not only suppress ecdysteroid production, but also block the process downstream of ecdysteroid, resulting in larval-larval molting.

Introduction

The transition from larva to pupa in holometabolous insects is controlled by two major hormones, ecdysteroid and juvenile hormone (JH). Ecdysteroid in hemolymph includes structurally similar forms of α-ecdysone (αEc or also called ecdysone) such as 2-deoxyecdysone (2dE) and 20-hydroxyecdysone (20HE). Although αEc is the form synthesized and released from prothoracic gland in many insects (Bollenbacher et al., 1975; Chino, 2012; Gilbert et al., 1997; Marchal et al., 2010), 2-deoxyecdysone is known to be the third ecdysteroid released in the circulating system in coleopteran insect (Aribi et al., 1997a). 2-deoxyecdysone is likely metabolized sequentially to αEc and 20HE as the major active form of the hormone (Petryk et al., 2003; Yamanaka et al., 2013). During the larval stage, a single peak of ecdysteroid (Aribi et al., 1997b; Connat et al., 1991) in the presence of JH correlates with larval-larval molts. On the other hand, if JH is low or absent in the last instar larva, ecdysteroid is the major hormone responsible for transforming larval tissues into pupal tissues and the molt to pupal stage (Nijhout and Williams, 1974; Riddiford, 1994; Suzuki et al., 2013). Previous studies in lepidopterans and coleopterans on the larval-pupal metamorphosis showed that there were two peaks of ecdysteroid; a small peak at the onset of the prepupal stage and greater peak during the prepupal stage (Aribi et al., 1997b; Bollenbacher et al., 1975; Delbecque and Sláma, 1980). These pulses of ecdysteroid regulate expressions of downstream genes (i.e. E74, E75, and *Broad* complex) that are essential for larval-pupal metamorphosis (Thummel, 2002).

Depending on the environmental and nutritional status, a flexible developmental schedule may occur in some species of insects, which is likely mediated by the ecdysteroid hormones and

JH, and the factors in control of the hormone biosyntheses and in responses to the hormones. For pupal metamorphosis, the larval weight reaching to the minimum viable weight (MVW) is required for survival of the last larval instar. The next gate is "critical weight" of the larva that allows the onset of pupation (Mirth and Shingleton, 2012; Safranek and Williams, 1984). Larva that fail to reach critical weight, but with weight over the MVW, remains a feeding larva until it reaches the critical weight. The required threshold of critical weight is lowered over time on low quality diets; therefore, a larva that reaches minimal viable weigh but before the critical weight will eventually pupate because of lowered critical weight threshold (Davidowitz et al., 2003; De Moed et al., 1999). The hormonal system controlling this process is not fully understood, but the involvement of ecdysone, insulin, and their downstream signaling has been suggested (Nijhout et al., 2014; Rewitz et al., 2013; Wu and Brown, 2006).

The flour beetle, *Tribolium freemani*, shows a flexible pupal metamorphosis schedule. Larvae fail to pupate and continue larval-larval molting under crowded conditions (LLC) although larval size has reached critical weight. The LLC can continue at least 6 months (Kotaki et al., 1993). The advantage of remaining in the larval stage by supernumerary molts may be to avoid cannibalism on the immobile pupa (Alabi et al., 2008; Ichikawa and Kurauchi, 2009; Park et al., 1970; Tschinkel, 1981; Via, 1999). Previous studies proposed that high titer of JH is the cause of the LLC (Kotaki et al., 1993; Minakuchi et al., 2008a). This hypothesis was based on induction of supernumerary molting of isolated last larval instar instead of pupation by exogenous JH treatment. However, in an inversely structured experiment, the knockdown of JH biosynthesis in *T. freemani* larvae under crowded conditions was unable to induce larval-pupal metamorphosis but larval lethality in prepupal arrest (Chapter 2). Therefore, I concluded that the development of supernumerary larvae of *T. freemani* under crowded conditions could not be

explained by high titer of JH alone. Other factor(s) in addition to the JH are likely involved in the LLC. The lethality by the suppression of JH biosynthesis in the crowded conditions is likely due to misalignment of the complex system consisted of more than one factor.

In this study, I investigated the role of ecdysteroids in LLC of *T. freemani* by measuring the 20HE levels from larvae treated with suppression of the genes in the JH signaling pathways under isolated and crowded conditions. The lethality caused by suppression of JH by *dsMT3* treatment under crowded conditions is associated with lack of large ecdysteroid peak. However, injections of 20HE could not rescue the phenotype, suggesting that there are complex additional signaling pathways involved in the LLC.

Materials and Methods

Ecdysteroid titers in the larvae injected with dsRNAs (*dsTfMT3* and *dsTfKr-h1)*

Sample preparation for enzyme immunoassay

Normally, larvae reared under isolated conditions from first instar pupated at the end of 8^{th} or 9^{th} instar. The total developmental time was 43.5±1.6 or 49.8±0.7 days. The developmental period before pupation either in 8^{th} or 9^{th} instar was 13.9±4.6 or 14.5±3.2 days, respectively. Since the developmental time is not simultaneous, fully grown larvae (FGL) which were more than 2 months in mass rearing after hatching were used to determine ecdysteroid (Ec) titers without dsRNA treatment. FGL were isolated from mass rearing. After isolation for 4 days, most of FGL become prepupal stage, stop feeding and have crooked immobile posture. The ecdysteroid was collected after isolation in every day: larval 1, 2, 3 days, prepupal stage 0, 1, 2, pupal stage 0 to 7 days. The larvae under crowded conditions were collected after the larvae

were individually placed in 96-well plate with one adult *T. castaneum* in every day until 14th day that coincides with adult eclosion in isolated conditions.

Under isolated conditions a majority of larvae injected with *dsTfMT3* precociously pupated at the end of 7th instar. Therefore, we concluded that the Ec pulse was in the 7th instar in both isolated and crowded conditions. Larvae injected with *dsTfMT3* were collected in 7th instar kept in isolated conditions after molting for 0, 1, 2, 3, 4, 6, 8 days and prepupal stage. Larvae similarly injected but kept in crowded conditions were sampled after molting for 0, 1, 2, 3, 4, 5, 6, and additionally sampled every other day up to 30 days.

Injection of *dsTfKr-h1* causes pharate adult arrest under isolated conditions whether injected in 5th instar or in FGL. Therefore, *dsTfKr-h1*treated FGL were used to determine Ec titer. In isolated conditions, larvae were collected after *dsTfKr-h1* injection for 1, 2, 3 days and every day after entering prepupal stage until day 10 which coincides to adult eclosion in buffer treated larvae under isolated conditions. Crowded larvae were collected every day until 13th day.

Those larvae were sampled in the afternoon between 2-6 pm. The ecdysteroids were isolated from whole body of larvae by homogenizing a single larva in 200 µl absolute methanol using mechanical homogenization followed by ~10 second pulses of 500 amplitude ultrasonic processor (model GE5020PB, Midsci, USA). Samples were centrifuge at 12,000 g for 15 minutes. Supernatants were transferred to new tube and kept at -80 °C until use.

Enzyme immunoassay (EIA)

The assay of 20HE was performed in 96 well-plates (NUNC, Roskide, Denmark). Plate was coated overnight at room temperature with 0.5 µg goat anti-rabbit antibody (Sigma-Aldrich, USA) in 90 µl phosphate buffer saline (PBS; 0.01 M Na2HPO4, 0.01 M NaH2PO4.2H2O, 0.15 M NaCl, pH 7.4) for each well. The wells were blocked by blocker buffer (0.025 M Na2HPO4,

0.025 M NaH2PO4.2H2O, 0.15 M NaCl, 1mM Na2EDTA, 0.1% BSA, 0.002% sodium azide) for overnight. The plate was washed with PBS containing 0.05% Tween 20 (0.05% PBST). The samples, dried and resuspended in the EIA buffer, and series concentration of 20HE standard were loaded into wells, each in 50 µl EIA buffer. Rabbit anti-20HE antibody, a generous gift from Timothy Kingan (Kingan, 1989), was diluted 1:100,000 in 50 µl and was added to all wells except blank wells. Peroxidase-conjugated 20-hydroxyecdysone (20HE-HRP) was diluted 1:10,000 in 50 µl was added to all wells including blank wells. The plate was shaken in an orbital shaker at room temperature for 5 minutes and stored at 4 °C for overnight. The contents were discarded and the wells were washed with 0.05% PBST three times. 100 µl of substrate solution, 3, 3', 5, 5' – tetramethylbenzidine (TMB), was added to each well to estimate enzyme activity for 10 minutes. The enzyme reaction was terminated by adding 1M phosphoric acid. Plate was read at 450 nm using a microplate reader.

Ecdysteroid injections

To elucidate the role of 20HE in larvae under crowded conditions, 20HE (Santa Cruz Biotechnology, USA) was dissolved in absolute methanol to 1 M. Then it was diluted to 1 mM in injection buffer (0.1 mM sodium phosphate, pH 7, containing 5 mM KCl). The 1mM 20HE in injection buffer was injected for 50 ng (104 nL) into larvae under crowded conditions in the afternoon between 2-6 pm. The injections were made for last instar larvae treated with buffer; prepupal stage of *dsTfMT3* and *dsTfKr-h1,* and feeding stage of *dsTfMT3* treated larvae. The prepupal stage in crowded conditions is indicated by nonfeeding, inactive larva which is in crooked posture.

Results

Ecdysteroid titers in the FGL treated with buffer

When FGL were isolated from mass rearing, they started entering the prepupal stage about 4 days after isolation. Pupation occurred after 2 days in the prepupal stage. In contrast, FGL under crowded conditions were in feeding stage at the time of adult eclosion of individuals kept in isolated conditions. The LLC was characterized by the supernumerary moltings in FGL and the duration of each instar increases up to about 30 days.

In our EIA, the antibody showed reactivity mainly toward 20-hydroxyecdysone, while it also cross-reacted to α-ecdysone (αEc) with one-tenth (1/10) sensitivity (Appendix figure 3). Therefore, the results of EIA-based quantification are expressed by 20-HE immunoreactivity equivalent, while we conservatively name it as ecdysteroid titer in this study.

In the FGL treated with buffer, ecdysteroid titers significantly increased in prepupal stage and decrease when larval-pupal molting (Figure 3.1A). There were two peaks of ecdysteroids: a small peak of 80±9 pg/larva (Figure 3.1B) immediately before the prepupal stage and the greater peak of 1,504±278 pg/larva before the pupation. In the pupal stage, the levels gradually increase and peak on day 4 of 3,022±421 pg/pupa, and decrease at the time of adult eclosion.

On the other hand, the FGL reared in crowded conditions had no increase of ecdysteroids from day 1 to days 14. The levels of ecdysteroids were in the range of 5-25 pg/larva. Although this study measuring the 20HE found no ecdysteroids increased up to 14 days in this study, based on previous study in *Z. atratus* (Aribi et al., 1997b), an ecdysteroid peak is expected for the supernumerary molt which usually occurs after 30 days in the case of *T. freemani*.

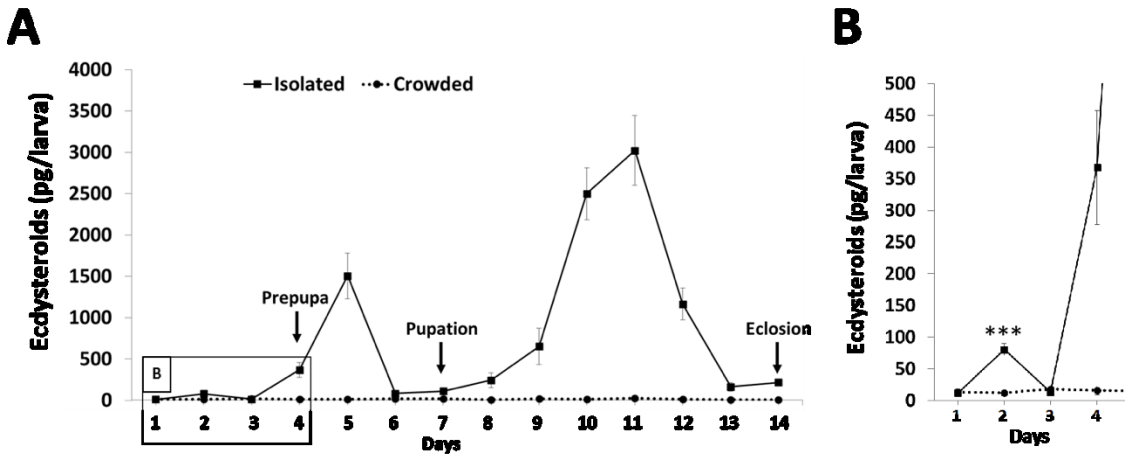

Figure 3.1 Ecdysteroid titers in fully grown larvae under isolated and crowded conditions. Enzyme immunoassay (EIA) results are shown by picograms ecdysteroids per larva. Black squares and solid lines indicate ecdysteroid titers from isolated larvae. Black circles and dotted lines indicate ecdysteroid titers from crowded larvae. (A) Prepupal stage begin after isolate for 4 days. (B) Magnificatioin of the same data between days 1 and 4 after isolation, showing small peak at day 2. Each data point is average of 3 individual with the standard deviation. Student's *t*-test (P<0.001) was used for statistical analysis at day 2.

Ecdysteroid titers in the larvae treated with *dsTfMT3*

Larvae treated with *dsTfMT3* as 5th instar larvae under isolated conditions underwent precocious metamorphosis in the 7th instar. The prepupal stage lasted about 10 days after molting into 7th instar larvae and they pupated 12 days after. On the other hand, entry into the prepupal stage by larvae treated with *dsTfMT3* under the crowded conditions was delayed to about 24 days after molting into the 7th instar, and they died shortly after entering the prepupal stage.

Isolated larvae treated with *dsTfMT3* showed two peaks of ecdysteroids (Figure 3.2A) in the 7th instar, which are same as the FLG treated with buffer in isolated conditions. The first

small peak is at day 8 (about 20 pg/larva) which is ~ 2 days before the onset of prepupal stage. The second large peak occurred at day 11 or 1 day after entering prepupal stage (about 700 pg/larva), and followed by dramatic declining of the titer before pupation.

In contrast, ecdysteroid levels in *dsTfMT3*-treated larvae under crowded conditions were very low for the first 20 days, at which time they were in feeding stage after molting into the 7^{th} instar. Small peaks of ecdysteroids in crowded larvae were delayed to 22^{nd} - 26^{th} day, which is about the time just before the prepupal arrest occurred. The ecdysteroid titers slightly increase to about 15 pg/larva for the first peak and to about 40 pg/larvae for second peak coinciding for the time entering prepupal stage under crowded conditions (Figure 3.2C).

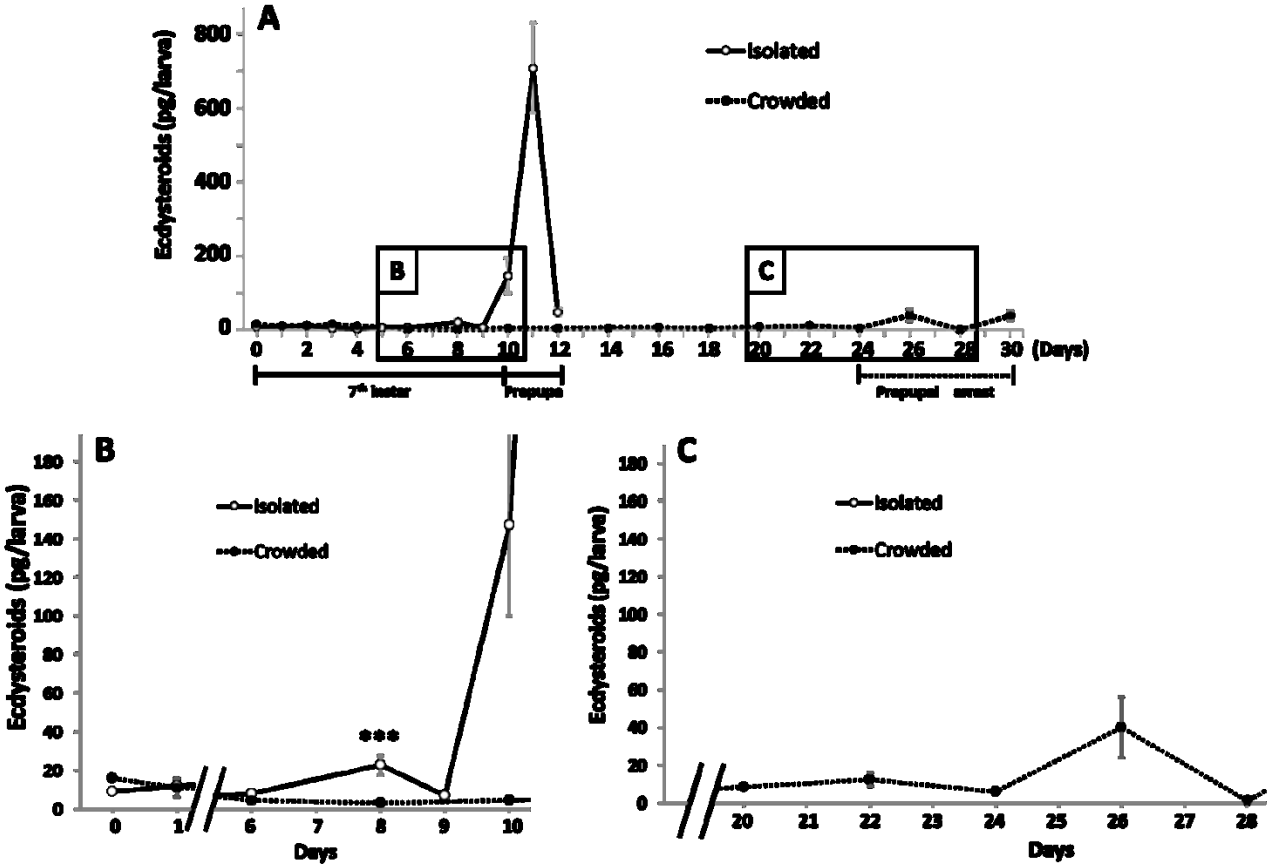

Figure 3.2 Ecydsteroid titers in 7th instar larvae treated with *dsTfMT3* under isolated and crowded conditions. Open circles and solid lines indicate ecdysteroid titers from isolated larvae. Black circles and dotted lines indicate those from crowded larvae. (A) Whole 7th larval instar, showing precocious prepupal stage in isolated larvae and prepupal arrest in crowded larvae. (B and C) Magnification of the same date between days 6 to 10 and days 20 to 28, showing small peaks in isolated and crowded larvae, respectively. Each data point is average of 3 individual with the standard deviation. Student's *t*-test (P<0.001) was used for statistical analysis at day 8.

Ecdysteroid titers in the larvae treated with *dsTfKr-h1*

Krüppel homolog1 (*Kr-h1*) is an early JH-induced gene that works downstream of JH (Kayukawa et al., 2012; Minakuchi et al., 2008b). The suppression of *Kr-h1* in *T. castaneum* induced precocious larval-pupal transition, suggesting that expression of *Kr-h1* prevents metamorphosis (Minakuchi et al., 2009). In *T. freemani*, more than 50% of isolated FGL treated with *dsTfKr-h1* enter prepupal stage at day 4 after dsRNA injection. Prepupae showed pupal eyes 3-4 days after becoming prepupae, while other pupal characteristics (wings, gin traps, and legs) were reveal after peeling larval cuticle. Ten days after entering prepupal stage they were arrested in the pharate adult or pupal-adult stage. In arrested pharate adults, the ventral abdomen, wings, thorax, legs, and head were sclerotized to be adult-like cuticle. However, although pupal and adult characteristics had developed underneath the larval cuticle, they were unable to molt or shedding old cuticles.

Increasing ecdysteroids in *dsTfKr-h1*-treated FGL coincided with buffer-treated FGL during prepupal stage (Figure 3.3). Ecdysteroid titers increased at day 4 and peaked at day 5, or 1 day after entering the prepupal stage. While the size of the ecdysteroid peak in prepupae of buffer-treated FGL and *dsTfKr-h1*-treated FGL were the same, the pupal ecdysteroid peak of *dsTfKr-h1* treated FGL was about 1,500 pg/pupa, which was significantly lower than in the buffer-treated FGL in normal pupa. Interestingly, the titer of ecdysteroid was maintained at a high level, whereas buffer-treated insect showed significant reduction of ecdysteroid at the end of papal stage.

The FGL treated with *dsTfKr-h1* under crowded conditions were still in the feeding stage until ~day 14. Then *dsTfKr-h1*-treated FGL enter the prepupal stage by stopping feeding and becoming inactive with a crooked posture. Prepupae were unable to pupate and died in 3-4 days,

but the pupal characteristics developed underneath larval cuticle. The levels of ecdysteroids in the larvae under crowded conditions were very low until the onset of prepupal stage. The peak of ecdysteroids at day 1 of prepupal arrest was about 1,200 pg/larva, which was not significantly difference than the ecdysteroid peak in day 1 of prepupae in isolated conditions (Figure 3.4).

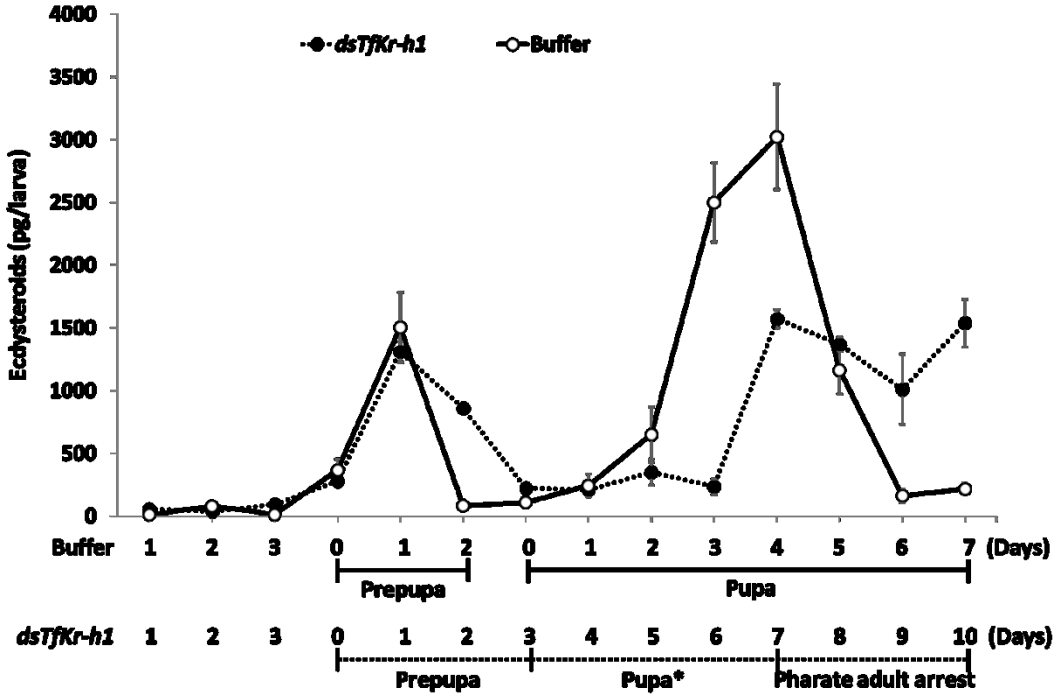

Figure 3.3 Ecdysteroid titers in fully grown larvae treated with buffer or *dsTfKr-h1* under isolated conditions. Open circles and solid line indicate ecdysteroid titers from buffer treated larvae. Black circles and dotted line indicate ecdysteroid titers from *dsTfKr-h1* treated larvae. The prepupal and pupal stages of buffer treated larvae is indicated by horizontal solid line under number of days in each stage, while horizontal dotted line under number of days is indicated prepupal, pupal stage, and pharate adult arrest of *dsTfKr-h1* treated larvae. Pupa with asterisk (pupa*) in *dsTfKr-h1* treatment indicates the appearance of pupal characteristics (eyes and gin traps) under larval cuticle. Each data point is average of 3 individual with the standard deviation.

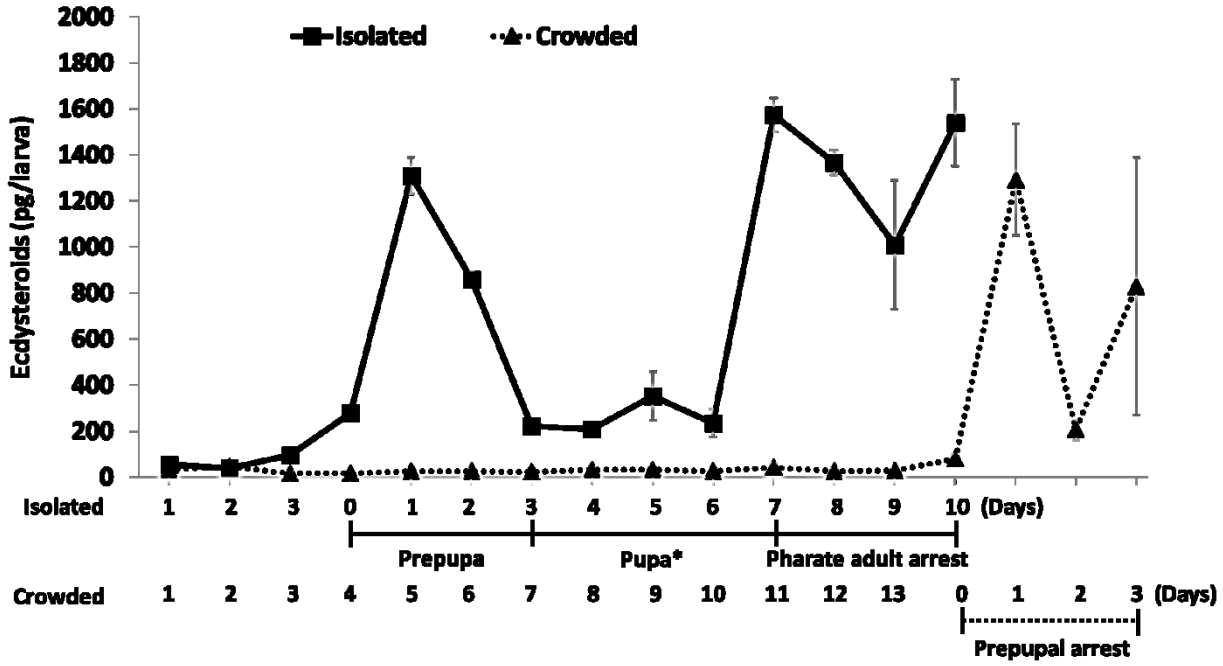

Figure 3.4 Ecdysteroid titers in fully grown larvae treated with *dsTfKr-h1* under isolated and crowded conditions. Black squares and solid line indicate ecdysteroid titers from isolated larvae, while black triancles and dotted line indicate those from crowded larvae. Horizontal solid line under number of days indicates prepupal stage in isolated larvae; dotted line under number of days indicates prepupal in crowded larvae. Pupa with asterisk (pupa*) indicates the appearance of pupal characteristics (eyes and gin traps) under larval cuticle. Each data point is average of 3 individual with the standard deviation.

Injection of 20HE into buffer, *dsTfMT3* or *dsTfKr-h1* treated larvae under crowded conditions

Injection of 20HE into FGL treated with buffer under crowded conditions did not induce larval-pupal metamorphosis; those larvae continued supernumerary molts. In dsRNA treated larvae, injection of 20HE in the prepupal stages of either *dsTfMT3* or *TfKr-h1* treatment did not rescue the larvae for pupation; those larvae died.

In contrast, injection of 20HE into feeding stages of 7th instar *dsTfMT3*-treated larvae under crowded conditions resulted in successful molt to next larval instar (10/11). Interestingly, after molting most of these larvae (8/10) showed short wings on both meso- and meta-thorax (Figure 3.5), whereas other organs such as eyes, antennae, legs, and abdomen were still in the larval form. These larvae died a few days later.

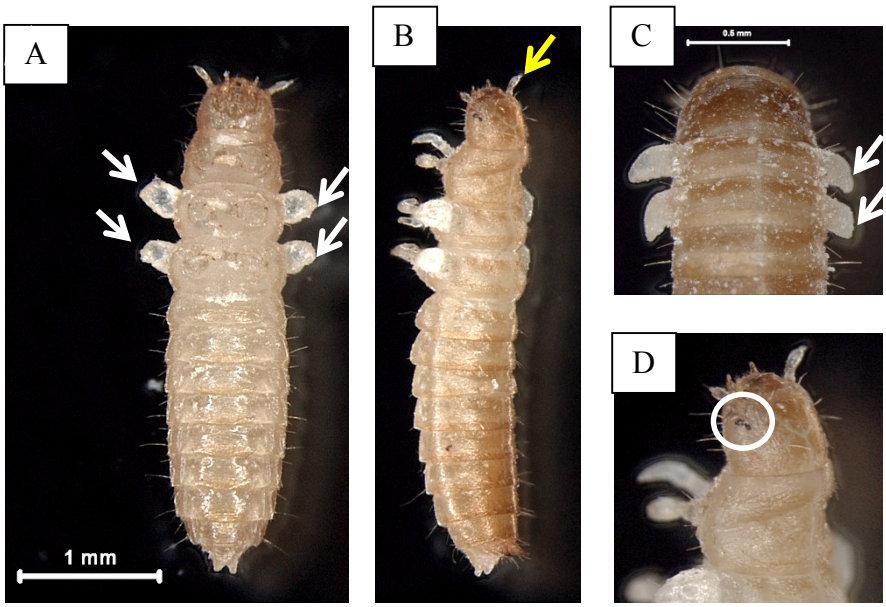

Figure 3.5 The *TfMT3* RNAi larvae injected with 20HE in 7th instar feeding period under crowded conditions. (A, C) White arrows indicate short wings. (B) Yellow arrow indicates larval antennae. (D) White circle indicates eye in larval form. Scale bar =1 mm.

Discussion

In isolated conditions, FGL treated with buffer showed two sequential peaks of ecdysteroids, one small and another larger peak, which is similar to previous reports of peaks in *Z. atratus* (Aribi et al., 1997b), *Dermestes maculatus* (Delbecque and Sláma, 1980) and *M. sexta* (Bollenbacher et al., 1981; Bollenbacher et al., 1975). In contrast, there is no increase of ecdysteroids in FGL under crowded conditions in days 1-14, which is about the time needed for adult eclosion in isolated conditions. Although the peak did not occur in the first 14 days of observation, the duration for a stadium of FGL under crowded conditions in *T. freemani* is about 30 days until the next supernumerary molt (Ruang-Rit et al., unpublished data), and a peak of ecdysteroids likely occurs toward the end of the stadium. Likewise, in supernumerary molts of *Z. atratus* and normal larval-larval molt of *Tenebrio molitor*, a single peak of ecdysteroids occurs at the beginning of apolysis, which is about 4-6 days before ecdysis (Aribi et al., 1997b; Connat et al., 1991).

The effect of crowding also delays the peak of ecdysteroids in larvae treated with dsRNA (*dsTfMT3* or *dsTfKr-h1*). Previous studies of supernumerary moltings in *T. molitor*, *Z. atratus,* and *Calliphora erythrocephala* stated that external stimulation was the cause of delayed ecdysteroid release (Aribi et al., 1997b; Berreur et al., 1979; Hirashima et al., 1995). Mechanical stimulation has been proposed to induce JH which may directly suppressed ecdysone secretion, causing a delay of the ecdysteroid peak (Kotaki et al., 1993; Sakurai et al., 1989; Tschinkel and Willson, 1971). However, in this study JH suppression by *dsTfMT3* treatment did not induce an increase in ecdysteroid, but in crowded conditions it delayed small ecdysteroid peak. Therefore, high JH alone in crowded conditions is not the cause of suppression and delay of the small

ecdysteroid surge; there are other factor(s) that suppress ecdysteroid other than high JH under crowded conditions.

The ecdysteroid peaks of *dsTfMT3* treated larvae under isolated conditions which underwent precocious metamorphosis in 7th instar were similar to isolated FGL in having two ecdysteroid peaks, one small followed by another larger peak (Figure 3.2 A, B). In the case of *dsTfMT3*-treated larvae under crowded conditions, a small peak which is lower (~17 folds) than that in prepupae in isolated *dsTfMT3*-treated larvae coincided with prepupal arrest at day 26 (Figure 3.2 C). These results suggest that crowding of *dsTfMT3*-treated larvae suppressed ecdysteroid production (Figure 3.6 C). Even though the peak of ecdysteroids in larvae under crowded conditions in prepupal arrest is much lower, the pupal characteristics had developed underneath the cuticle in larvae in prepupal arrest. In *M. sexta*, pupal cuticle could be induced by low level of 20HE (~25-100 ng/ml) in the absence of JH (Riddiford, 1978, 1981; Wolfgang and Riddiford, 1986). Thus, it appears that pupal cuticle formation in crowded *dsTfMT3*-treated larvae in prepupal arrest was induced by the small peak of ecdysteroid in the absence of JH.

Although the titers of ecdysteroids in *dsTfKr-h1*-treated FGL under isolated conditions were not different from buffer-treated FGL, prepupae of the former could not shed their larval cuticle to become pupae. However, pupal characteristics developed underneath the larval cuticle, which is similar to the effect of *TcKr-h1* suppression in *T. castaneum* (Minakuchi et al., 2009). Furthermore, there was some development to the adult underneath larval cuticle of larvae in isolated conditions, but pupal-adult eclosion of *dsTfKr-h1*-treated FGL of *T. freemani* was blocked and arrested in the pharate adult stage, showing that titers of ecdysteroid did not decline at the time of adult emergence. Normally, after the peak of ecdysteroids in the pupal stage levels of ecdysone declined, allowing *ftz transcription factor 1* (*βFTZ-F1*) to initiate ecdysis-triggering

hormone (ETH) release from Inka cells (Kingan et al., 1997; Parvy et al., 2005; Žitňan et al., 1999). Thus, high titers of ecdysteroid that did not decline in isolated *dsTfKr-h1*-treated larvae may have blocked the expression of *βFTZ-F1* and blocked the release of ETH in *T. freemani*. This suggests that *TfKr-h1* is required for regulating the decline of ecdysteroid during pupal-adult eclosion. Like *dsTfKr-h1*-treated larvae under isolated conditions, prepupae of *dsTfKr-h1*-treated larvae were unable to pupate under crowded conditions, although pupal characteristic developed. However, under crowded conditions they died 3-4 days after entering the prepupal stage without developing adult characteristics, which took about 7-8 days after pupation in *dsTfKr-h1*-treated larvae under isolated conditions. It seems that factors (genes or hormones) which are essential for molting and pupal survival were affected by the crowded conditions.

Exogenous 20HE injected at the prepupal stage of *dsTfMT3* or *dsTfKr-h1* treated larvae under crowded conditions were still unable to pupate. Moreover, isolated *dsTfKr-h1*-treated FGL were also unable to shed larval cuticle, although the ecdysteroid peak in prepupal stage was increased same as prepupae from buffer-treated larvae under isolated conditions. Interestingly, the injection of 20HE into feeding 7th instar *dsTfMT3*-treated larvae under crowded conditions induced larval-larval molting with the formation of short wings on larval thorax. This phenotype of larvae with wings was similar to *T. castaneum* larvae treated with RNAi of an isoform of *Broad* (*Br*) gene *Br-Z3* (Suzuki et al., 2008). *Br* is known to be an early ecdysone-response gene which is essential for larval-pupal metamorphosis. *Br* plays a critical role as a central regulator of gene expressions at the onset of metamorphosis in *Drosophila* (Karim et al., 1993; Uhlirova et al., 2003).

On the other hand, exogenous 20HE injected into buffer-treated FGL under crowded conditions did not induce larval-pupal metamorphosis but rather induced larval-larval molting.

Previous studies in lepidopteran and dipteran insects demonstrated that the presence of JH prevents transformation of larval tissues into pupal tissues, causing larval-larval molting in the pulse of ecdysone (Nijhout and Williams, 1974; Riddiford, 2012; Tsutsumiuchi et al., 1989). In *M. sexta*, JH also prevents the expression of *Br* in larval epidermis that exposes to 20HE (Figure 3.6A) resulted in suppression of pupal cuticle formation (Zhou et al., 1998). Assuming that suppression of *TfMT3* blocks the synthesis of JH, this result suggests that crowded conditions induce JH (Figure 3.6 C) that in turn suppresses *Br* expression-mediated LLC. Konopova and Jindra (2008) and Minakuchi et al. (2009) showed that *Br* is not only downstream of ecdysone but also downstream of JH and *Kr-h1* in JH signaling pathway. However, knockdown of JH biosynthesis or *TfKr-h1* could not induce larval-pupal metamorphosis. In Chapter 2, I have shown that *Br* expression was suppressed in *dsTfMT3*-treated larvae under crowded conditions and in *dsTfKr-h1*-treated larvae in both isolated and crowded conditions. Moreover, in *T. castaneum*, loss of *Br* results in failure of normal larval-pupal metamorphosis (Konopova and Jindra, 2008; Parthasarathy et al., 2008). Therefore, *Br* is likely responsible for the independent suppression that occurred in crowded conditions.

In conclusion, the effects of crowded conditions on larvae of *T. freeman,* either treated with buffer or dsRNA, appear to have at least four independent pathways:

- **JH induction**: Previous studies in *T. freeman* proposed high JH titer in crowded condition as the causal factor for supernumerary larvae (Hirashima et al., 1998; Hirashima et al., 1995; Kotaki et al., 1993; Nakakita, 1990). In this study, although high JH (from application of a JH analog) is likely associated with crowded conditions, suppression of JH biosynthesis by RNAi of *TfMT3* alone failed to recapitulate the pupal

molting in crowded conditions. This result strongly suggests that JH alone is not the only factor for LLC, but additional factor(s) need to be coordinated with JH.

- ***Kr-h1* induction**: Based on the study in Chapter 2, *Kr-h1* is highly expressed in the larvae under crowded conditions even when JH production is suppressed, whereas JH is generally known as the factor for induction of *Kr-h1* expression. Our experiment is similar to the recent report that overexpression of *Kr-h1* in *B. mori* with removal of the corpora allata, which is the organ for JH synthesis, resulted in prepupal arrest (Kayukawa et al., 2014). Therefore, the prepupal lethality of *dsTfMT3* treatment under crowded conditions in *T. freemani* is same as the experimental manipulation in *B. mori;* low JH and high *Kr-h1*. I also provided another line of support for the cause of prepupal arrest in *dsTfMT3* treated *T. freemani* in crowded condition as low JH titer with high *Kr-h1*. Suppression of both *TfMT3* and *TfKr-h1* together under crowded conditions could rescue the early prepupal lethality to further development and die in late pupa under the larval cuticle. Therefore, larval-pupal metamorphosis requires suppression of both JH and *Kr-h1*, and these two factors are independently controlled in the crowded condition for high JH and high level of *TfKr-h1*.
- **Suppression of ecdysteroid**: I found several lines of evidence that ecdysteroid titer is affected by crowded conditions. First, delayed larval molting in LLC is associated with a delayed ecdysteroid peak. Second, prepupal arrest of *TfMT3* is also associated with lack of large ecdysteroid peak. Although antagonistic control of ecdysteroid by JH was previously proposed (Sakurai et al., 1989), it does not fit in this specific case where the prepupal arrest of *TfMT3* RNAi exhibited very low ecdysteroid. Therefore, suppression

of ecdysone under crowded conditions is likely through a mechanism independent from JH titer.

- **Suppression of *Br* expression**: The current model for JH and ecdysteroid pathways placed *Br* downstream on the ecdysteroid pathway, but suppressed by high JH titer through *Kr-h1* as shown in Figure. 3.6A (Riddiford, 2012). Pupal metamorphosis coincides with suppression of the JH pathway and with activation of the ecdysone pathway through *Br*. In this study, suppression of *Br* expression in larvae treated with *dsTfMT3* or *dsTfKr-h1* was observed under crowded conditions (Chapter 2). Injection of ecdysone into animals treated with *dsTfMT3* could rescue them from lethality, but they molted to larva with large wing pads, which is the same phenotype shown by the RNAi of *Br* in *T. castaneum* pupal arrest. Therefore, the larvae in crowded conditions manipulated for suppression of JH signaling with high ecdysone showed low *Br* expression and *Br* RNAi phenotype. This result suggests that the suppression of *Br* expression is independent from JH and *Kr-h1,* and ecdysteroid in crowded conditions.

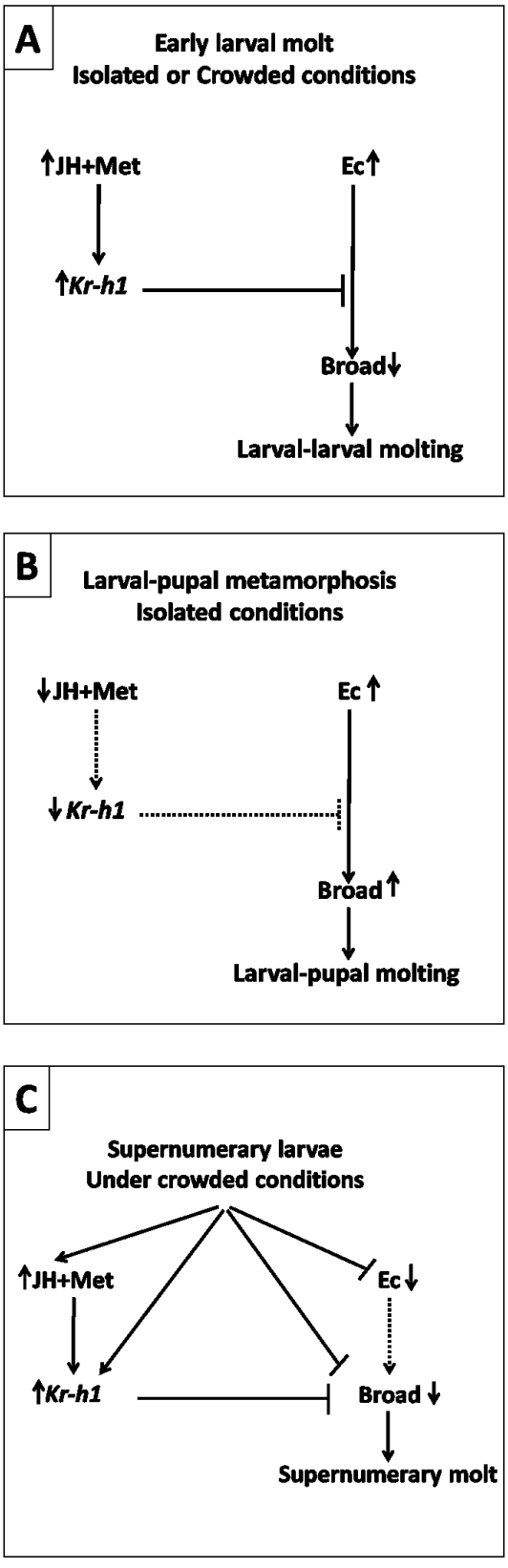

Figure 3.6 The model of larval molting and metamorphosis in *T. freemani*. This model is modified from Riddiford (2012) and based on our results and the studies by Minakuchi et al. (2009), Konopova and Jindra (2008), and Suzuki et al. (2008). (A) Larval-larval molting in early larval stage. High JH induces *Kr-h1* expression that suppresses *Br* expression, resulting in larval-larval molting. (B) Larval-pupal metamorphosis under isolated conditions. If JH is absent or low at the onset of the prepupal stage, ecdysteroid induces *Br* expression that causes larval-pupal metamorphosis. (C) Supernumerary molt under crowded conditions. In crowded conditions, JH and *Kr-h1* are induced while ecdysteroid and *Br* are suppressed, causing LLC. Abbreviations: JH, Juvenile hormone; Ec, ecdysone; *dsTfMT3*, double strand RNA of *methyltransferase3*; Met, methoprene-tolerant; *Kr-h1*, *Krüppel homolog1*. *Br*, *Broad.*

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Chapter 4 - Summary and Discussions

Juvenile hormone (JH) and ecdysteroids (Ec) are the major hormones in insect development and metamorphosis. The orchestrative actions of these hormones lead to major developmental events: ecdysis and metamorphosis. The metamorphosis process from larva to pupa requires reaching a critical weight, resulting in declining JH and increasing Ec to induce metamorphosis. (Davidowitz et al., 2003; Safranek and Williams, 1984). However, in the flour beetle, *T. freemani* even after larvae reach critical weight, metamorphosis is inhibited under crowded conditions. In crowded conditions, this insect shows continued larval-larval molting (LLC) that can continue for at least 6 months. Previous studies speculated that development under crowded conditions may stimulate the corpora allata (CA), the site of JH biosynthesis and secretion, to produce high JH (Kotaki et al., 1993; Nakakita, 1982, 1990). Classical experiment have shown transplanting the CA induces extra molting in immature *Rhodnius prolixus* (Wigglesworth, 1936, 1948). Moreover, application of exogenous JH in the fully grown larvae (FGL) resulted in supernumerary molt in *Z. atratus* and *T. freemani* under isolated conditions (Kotaki et al., 1993; Quennedey et al., 1995). Inversely, the knockdown of JH acid methyl transferase *JHAMT,* a crucial enzyme in the final steps of JH biosynthesis, can cause precocious metamorphosis in coleopteran insects (Minakuchi et al., 2008a) in isolated conditions. In addition, chemical allatectomy using precocene II has shown mild, but not full, effects in rescuing LLC for pupal molting (Nakakita, 1990). I tested the hypothesis that the effect of crowded conditions on LLC is due to high JH by using RNA interference (RNAi) mediated suppression of enzymes that are essential for JH biosynthesis (**Chapter 2**).

I performed RNAi on three paralogous *JHAMT* genes, *TfMT1*, *TfMT2*, and *TfMT3*. Only RNAi-mediated knockdown of *TfMT3* under isolated conditions caused precocious

metamorphosis, which is similar to results of a previous study in *T. castaneum* (Minakuchi et al., 2008a). The phenocopy of *TfMT3* RNAi phenotype suggest that the *TfMT3* is the orthology of *TcJHAMT* in *T. castaneum*. On the other hand, the suppression of *TfMT3* in larvae under crowded conditions led to larval-pupal arrest and lethality, in which the larvae could not shed the cuticle or complete metamorphosis, although pupal characters such as pupal eyes, wings, and gin traps, developed underneath the larval cuticle in the prepupal stage (called larval-pupal arrest or prepupal arrest). The failure in the larval-pupal molting in *dsTfMT3* injection under crowded conditions suggests the presence of another endocrine system in addition to JH as a gate for the metamorphic program.

Quantitative PCR (qPCR) showed that *Krüppel homolog1* (*TfKr-h1*), downstream in the JH pathway, was highly expressed in *dsTfMT3* treatment under crowded conditions. The same prepupal arrest in low JH and high *Kr-h1* was also shown in a synthetic condition in a transgenic silkworm, allatectomy of transgenic *Bombyx mori* overexpressing *BmKr-h1α* (Kayukawa et al., 2014). This strongly suggests that prepupal arrest in *dsTfMT3*-treated larvae under crowded conditions was caused by overexpression of *Kr-h1*, which is likely a JH-independent pathway activated by the crowded conditions.

Suppression of *TfKr-h1* alone showed the same phenotype as *dsTfMT3* injection, that being prepupal arrest in larvae under crowded conditions. However, under isolated conditions, the developmental arrest occurred at the pupal-adult stage called pharate adult arrest. In contrast, the knockdown of both *TfMT3* and *TfKr-h1* together could rescue prepupal arrest and resulted in further development to pharate adult arrest under crowded conditions. This result indicates that high in both JH and *Kr-h1* is normal under crowded conditions, but imbalance of this condition by RNAi for suppression of either JH or *Kr-h1* resulted in prepupal arrest, which was also shown

in *B. mori* (Kayukawa et al., 2014). Although suppression of both JH and *Kr-h1* rescued the prepupal arrest, these pupae eventually arrested at the pharate adult stage in which different body part showed different degrees of maturation for adult tissue (Table 4.1.). The complex phenotype indicates involvement of another endocrine hormone, ecdysone, which has been well known for its functions in insect development.

In **Chapter 3**, the role of 20-hydroxyecdysone (20HE) was tested as another possible controller of the LLC, which may be essential with the role of JH. The 20HE levels were measured in ds*TfMT3*- and *dsTfKr-h1*- treated larvae under isolated and crowded conditions. Our enzyme immunoassay (EIA) showed that 20HE antibody not only reacts to 20HE but also cross-reacted to α-ecdysone (αEc) with 10-fold lower detection sensitivity. Thus, levels of ecdysone from the immunoreactivity were called ecdysteroids. There were two peaks of ecdysteroids, a small peak followed by a large surge, during larval-pupal transformation in isolated conditions of larvae treated with *dsTfMT3*, which is similar to ecdysteroid titers in buffer treated larvae and in other tenebrionid beetles (Aribi et al., 1997). On the other hand, *dsTfMT3*-treated larva under crowded conditions showed only the small peak of ecdysteroids, but lacked the subsequent large ecdysteroid peak. Moreover, the small peak of ecdysteroids in crowded larvae treated with *dsTfMT3* was delayed to about 26 days while in isolated conditions the first small peak of ecdysteroids was about 8 days after molting to 7th instar. This results suggests that crowded conditions suppress ecdysteroid production. However, the small peak of ecdysteroids in the suppression of JH biosynthesis was sufficient to induce pupal characteristics, which is consistent with the study in *M. sexta* that has pupal cuticle formation in low level of 20HE (~25-100 ng/ml) in the absence of JH (Riddiford, 1978, 1981; Wolfgang and Riddiford, 1986).

In the case of *dsTfKr-h1* treatment, titers of ecdysteroid in larvae under isolated conditions were not different from buffer-treated larvae in the prepupal stage. However, ecdysteroids in *dsTfKr-h1*-treated larvae did not show the decline of the ecdysteroid that normally coincide with adult emergence. The decline of ecdysteroids is required to release ecdysis-triggering hormone (ETH) (Kingan et al., 1997; Parvy et al., 2005; Žitňan et al., 1999). Ecdysteroids at high titer inhibit ETH release resulting in pupal-adult arrest. Thus, my results suggest that *Kr-h1* is essential for the decline of ecdysteroids during pupal-adult metamorphosis. In contrast, the peak of ecdysteroids in larvae treated with *dsTfKr-h1* under crowded conditions was delayed to about 14 days, in which prepupal arrest occurred in 5-6 days after the titers of ecdysteroid was raised.

Because larvae in prepupal arrest could not molt to pupal stage, I attempted to rescue the lethality of *dsTfMT3*- and *dsTfKr-h1*- treated larva under crowded conditions by injections of 20HE. The injection of 20HE at the prepupal stage, which coincided with the first small natural peak of 20HE, did not induce larval-pupal metamorphosis. In contrast, the injection of 20HE into *dsTfMT3*-treated larva before the prepupal stage resulted in larval-larval molting with the development of a pair of short wings. This suggests that RNAi-treated larvae under crowded condition not only suppressed 20HE, but it might have also suppressed processes downstream of Ec that regulate cuticle shedding.

Broad (*Br*) gene is downstream of *Kr-h1* and the ecdysone-response gene, merging the JH and ecdysone pathways. The expression of *Br* is induced by 20HE but suppressed by JH (Reza et al., 2004). However, in contrast to the expectation, *Br*- was suppressed in the larvae treated with *dsTfMT3* (low JH) under crowded conditions, and in the larvae treated with *dsTfKr-h1* in both isolated and crowded conditions. *Br* has been shown to have a critical role during

metamorphosis; when *Br* expression was disrupted, larvae were lethal in *Drosophila* while larval-pupal transition failed in *B. mori* (Kiss et al., 1988; Uhlirova et al., 2003). In addition, suppression of *Tcbr* in *T. castaneum* resulted in failure of larval-pupal metamorphosis and showed larval-adult intermediate (Parthasarathy et al., 2008; Suzuki et al., 2008). Moreover, larvae treated with *dsTfMT3* and injected with 20HE under crowded condition showed short wings on the larval thorax, which was similar to *T. castaneum* larvae treated with RNAi of an isoform of *Broad* (*Br*) gene *Br-Z3* (Suzuki et al., 2008). Thus, our results support the conclusion that *Br* is responsible for larval-pupal arrest under crowded conditions.

Table 4.1 The consolidated summary of results from the knockdown of *TfMT3* and *TfKr-h1* under isolated and crowded conditions, and the application of JH analog (methoprene) and ecdysone.

Treatment	Isolated conditions					Crowded conditions					
						dsTfMT3					
	dsTfMT3	*dsTfMT3* + Methoprene	*dsTfKr-h1*	*dsTfKr-h1* + *dsTfMT3*		-	Ec 1[2]	Ec 2[3]	*dsTfMT3* + Methoprene	*dsTfKr-h1*	*dsTfKr-h1* + *dsTfMT3*
Change in endocrine system predicted	JH ↓ *Kr-h1* ↓	JH ↑ *Kr-h1* ↑	JH ↓ *Kr-h1* ↓	JH ↓ *Kr-h1* ↓		JH ↓ *Kr-h1* ↑	JH ↓ *Kr-h1* ↑	JH ↓ *Kr-h1* ↑	JH ↑ *Kr-h1* ↑	JH ↑ *Kr-h1* ↓	JH ↓ *Kr-h1* ↓
	Ec ↑	Ec ↓	Ec ↑	Ec ↑		Ec ↓	Ec ↑	Ec ↑	Ec ↓	Ec ↓	Ec ↓
Observed results	Precocious pupation	Larval [1] molting	Pupal arrest	Pupal arrest		Prepupal arrest	Larval molting	Prepupal arrest	Larval [4] molting	Prepupal arrest	Pupal arrest
Eyes	Pupal eyes	Larval eyes	Pupal eyes	Pupal eyes		Pupal eyes	Larval eyes	Pupal eyes	Larval eyes	Pupal eyes	Pupal eyes
Wings	Normal wings	-	Short Wings	Short Wings		Short Wings	Short Wings	Short Wings	-	Short Wings	Short Wings
Gin traps	Gin traps	-	Gin traps	Gin traps		Gin traps	-	Gin traps	-	Gin traps	Gin traps
Lethality	-	-	Lethal	Lethal		Lethal	Lethal	Lethal	-	Lethal	Lethal

[1] Pupation after molting 2-3 times and died in pupal stage

[2] Ec1 indicates the injection of Ec in feeding stage

[3] Ec2 indicates the injection of Ec in prepupal stage.

[4] Eventually arrested in prepupal stage and died after molting 2-3 times

Conclusion and Future Directions

Even though knockdown of *TfMT3* can cause precocious pupation in *T. freemani* under isolated conditions, my study demonstrated that suppression of JH is not sufficient to rescue larval-pupal molting under crowded conditions. Normally, down regulation of JH induces a high peak of Ec leading to metamorphosis, as it is in *T. freemani* under isolated conditions. In contrast, *T. freemani* treated with *dsTfMT3* under crowded conditions had low Ec titers. It is still unknown how crowded conditions suppress the ecdysteroid induction. Low titers of ecdysone may be due to low ecdysone biosynthesis. It is possible that crowded conditions inhibit ecdysteroid biosynthesis by inhibiting PTTH or other signaling factors upstream from Ec. Control of Ec biosynthesis under crowded condition in beetles remains a question to be investigated in the future.

The suppression of *Kr-h1*, downstream of JH, in larvae under isolated conditions caused pharate adult arrest while, in crowded conditions, it caused prepupal arrest. Although, the treatment of *TfMT3* or *TfKr-h1* alone under crowded conditions resulted in prepupal arrest, suppression of both JH and *Kr-h1* rescued the prepupal arrest but ultimately led to pharate adult arrest. Ec measurement in pharate adult arrest of *dsTfKr-h1* treatment alone under isolated conditions showed that Ec did not decline at the time of adult emergence. The regulation of *Kr-h1* in declining Ec during pupal-adult eclosion should be further studied.

The injection of Ec into the prepupal stage of larvae treated with *dsTfMT3* or *dsTfKr-h1* under crowded conditions did not rescue the treatments and cause larval-pupal molt. My interpretation of this failure is that crowded conditions may suppress not only the ecdysteroid

induction but also inhibit downstream of ecdysone such as *broad* or ecdysone receptor (EcR). The effects of crowded conditions on the downstream factors also need to be elucidated.

In contrast, the injection of Ec into *dsTfMT3*-treated larvae before the prepupal stage causes larval-larval molt with the resulting larvae having short wings. This may be due to downregulation of *Br*, an interpretation supported by the *Br* suppression in *T. castaneum* (Suzuki et al., 2008). However, the regulation of *Br* in insect development is still poorly understood. The study of *Br* in development and metamorphosis needs to be investigated.

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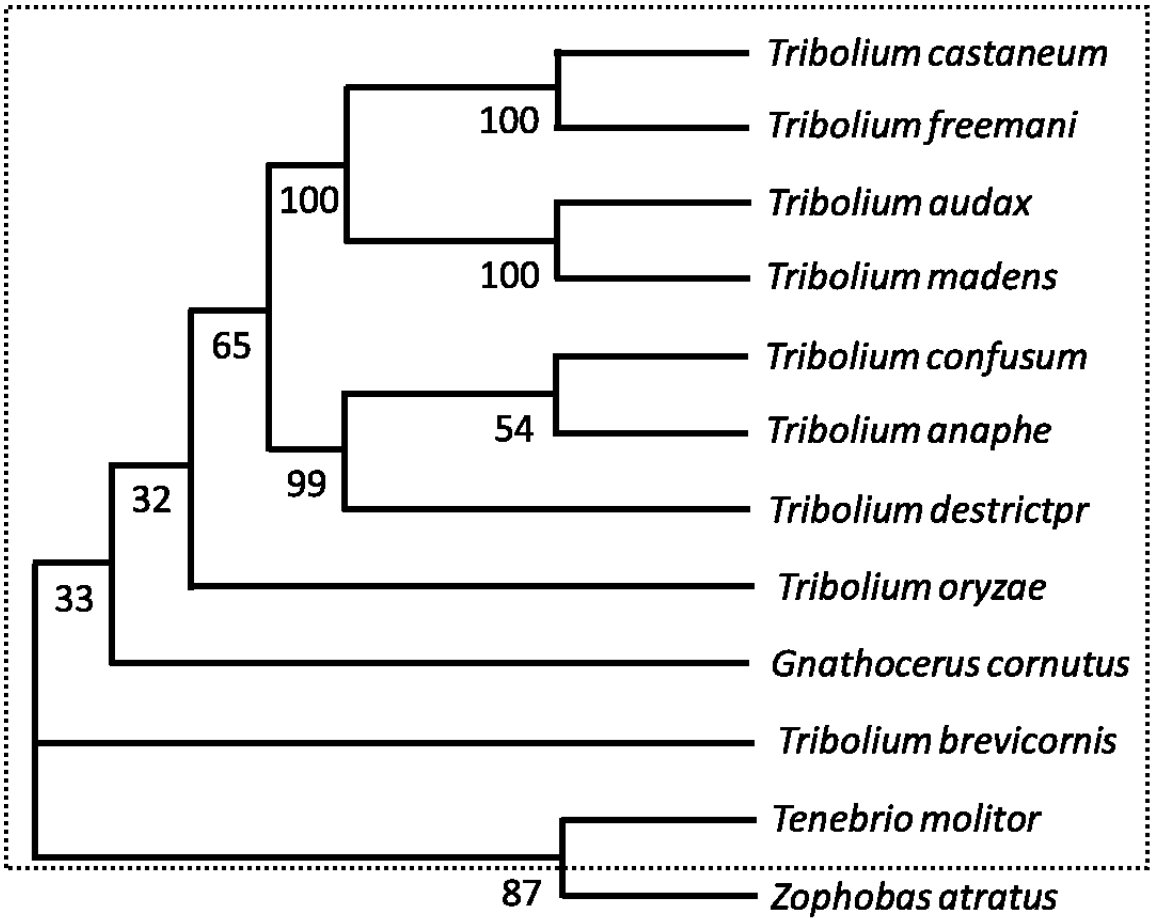
Appendix chapter - 1

In coleopterans, the crowdedness does not only affect *T. freemani* and *Z. atratus* but also other beetles in Family Tenebrionidae. Evolutionary origin of the larval-larval molting under crowded (LLC) might be rooted from the common ancestor of Tenebrionid beetles. While clear cases of LLC have been reported in *T. freemani* and *Z. atratus*, other Tenebrionidae species, *T. castaneum* and *T. molitor* also have shown conditions LLC-like development pattern. In *T. castaneum*, the LLC up to 9th instar with a delay of 2 months to reach to pupal stage occurs in crowded conditions (Park, unpublished data). Similarly, at the weight that larvae are able to pupate, larvae of *T. molitor* reared in crowded conditions continue larval molt about 50% and then pupate eventually (Connat et al., 1991).

Based on the phylogeny based on the comparisons of mitochondrial and nuclear markers (*cytochrome oxidase I, 16S ribosomal DNA, wingless, 28S ribosomal DNA, and histone H3*), the LLC has likely evolved from at the root of the tree shown in appendix figure 1. However, divergence of this phenotype within this lineage is also apparent, such as loss or weakens responses to crowded condition depending on the lineage. Although the divergence of *T. freemani* and *T. castaneum* is estimated to be a relatively recent event about 11.6 to 47 million years ago (Angelini and Jockusch, 2008), they show different degrees of LLC. Many other species in this group is not known for the presence or absence of LLC.



Appendix figure 1 Cladogram showing the relationships among Tenebrionid species. The tree is based on a combined analysis of *cox1, 16S rDNA, wg, 28S rDNA, and histone H3* (Angelini and Jockush, 2008) indicated by dotted line and added one additional taxa *Z. atratus* because larvae of *T. freemani* and *Z. atratus* show same developmental pattern under crowded conditions. The beetle, *Z. atratus,* was added on the analysis by using amino acids of *cox1* with Neigbor-joining/maximum likelihood for 1,000 bootstrap replications.

Appendix table 1 The environmental factors involved in the supernumerary molt of insects modified from Esperk et al. (2007)

Environmental factors	Insect order	Insect species
Photoperiod		
	Dictyoptera	*Opisthoplatia orientalis* (Zhu and Tanaka, 2004)
	Orthoptera	*Allonemobius fasciatus* (Tanaka and Brookes, 1983)
	Lepidoptera	*Pyrrharctia isabella* (Goettel and Philogène, 1978)
		Sesamia nonagrioides (Fantinou et al., 1996)
		Sasakia charonda (Kato, 1989)
		Platynota idaeusalis (Rock and Shaffer, 1983)
	Coleoptera	*Psacothea hilaris* (Shintani and Ishikawa, 1998)
Low Temperature		
	Odonata	*Brachythemis contaminate* (Mathavan, 1990)
	Lepidoptera	*Harrisina brillians* (Roltsch et al., 1990)
		Agrotis ipsilon (Santos and Shields, 1998)
		Prionoxystus robiniae (Solomon, 1973)
		Diatraea grandiosella (Jacob and Chippendale, 1971)
		Lycaena hippothoe (Fischer and Fiedler, 2001)
		Porthetria dispar (Leonard, 1966)
		Copitarsia decolora (Gould et al., 2005)
		Spodoptera frugiperda (Ali et al., 1990)
		Pararge aegeria (Shreeve, 1986)
		Galleria mellonella (Cymborowski and Boguś, 1976)
		Herpetogramma licarsisalis (Jensen and Cameron, 2004)
		Adoxophyes orana (Milonas and Savopoulou-Soultani, 2000)

Environmental factors	Insect order	Insect species
		P. idaeusalis (Rock and Shaffer, 1983)
High Temperature		
	Odonata	*Orthetrum sabina* (Mathavan, 1990)
	Orthoptera	*Chorthipus brunneus* (Hassall and Grayson, 1987),
		Gryllus bimaculatus (Behrens et al., 1983)
		Melanoplus differentialis (Bellinger and Pienkowski, 1987, 1989)
		Melanoplus atlanis (Shotwell, 1930)
	Lepidoptera	*Syntypistis punctatella* (Kamata and Igarashi, 1995)
	Hymenoptera	*Nematus oligospilus* (Charles and Allan, 2000)
	Coleoptera	*Monochamus carolinensis* (Pershing and Linit, 1988)
		Tenebrio molitor (Ludwig, 1956).
Both higher and lower than favorable temperature		
	Dictyoptera	*O. orientalis* (Zhu and Tanaka, 2004)
	Hemiptera	*Nasanobia ribisnigri* (Diaz and Fereres, 2005)
	Lepidoptera	*D. lineolate* (Rodriguez-del-Bosque et al., 1989)
		A. ipsilon (Archer et al., 1980; Santos and Shields, 1998)
		Acleris minuta (Weatherby and Hart, 1986)
		P. stultana (Zenner-Polania and Helgesen, 1973)
		S. exigua (Ali and Gaylor, 1992)
	Coleoptera	*Anoplophora malasiaca* (Adachi, 1994)
		P. hilaris (Shintani and Ishikawa, 1997)
		Anthrenus sarnicus (Armes, 1990)
		Dermestes lardarius (Fleming and Jacob, 1986)
		Trogoderma glabrum (Beck, 1971a, b)
		Oryzaephilus surinamensis (Beckett and Evans, 1994)
		T. obscurus (Fiore, 1960)

Environmental factors	Insect order	Insect species
Humidity		
	Lepidoptera	*Corcyra cephalonica* (Russell et al., 1980)
		A. ipsilon (Archer et al., 1980; Santos and Shields, 1998)
	Coleoptera	*Sitophilus oryzae* (Pittendrigh et al., 1997)
		A. sarnicus (Armes, 1990)
		D. lardarius (Fleming and Jacob, 1986)
		T. glabrum (Archer and Strong, 1975; Beck, 1971a)
		T. variabile (Archer and Strong, 1975; Beck, 1971a)
		O. surinamensis (Collins et al., 1989)
		T. molitor (Urs and Hopkins, 1973)
Quality and quantity of food (low nutrient content)		
	Lepidoptera	*Malacosoma disstria* (Jones and Despland, 2006)
		Streblote panda (Calvo and Molina, 2004)
		Orgyia antiqua (Esperk and Tammaru, 2006)
		A. ipsilon (Santos and Shields, 1998)
		Anticarsia gemmatalis (Conti, 1982; Waters and Barfield, 1989) *C. decolora* (Gould et al., 2005)
		Heliothis armigera (Casimero et al., 2000)
		Pseudopulsia includes (Kidd and Orr, 2001; Strand, 1990) *S. frugiperda* (Pencoe and Martin, 1981, 1982)
		Galleria mellonella (Allegret, 1964)
		Samea multiplicalis (Taylor, 1984)
		Tineola bisselliella (Titschack, 1930)

Environmental factors	Insect order	Insect species
Quality and quantity of food (low nutrient content)		
		A. minuta (Weatherby and Hart, 1986)
		Adoxophyes orana (Milonas and Savopoulou-Soultani, 2000)
		Argyrotaenia sphaleropa (Bentancourt et al., 2003)
		Bactra verutana (Frick and Wilson, 1978)
		Bonagota cranaodes (Bentancourt et al., 2004)
		C. occidentalis (Schmidt and Lauer, 1977)
	Coleoptera	*Tribolium castaneum* (Mukerji and Sinha, 1953)
		T. confusum (Schwardt, 1927)
Quality and quantity of food (Starvation)		
	Lepidoptera	*Bombyx mori* (Kato and Sumimoto, 1968)
		Chilo partellus (Ochieng'-Odero et al., 1994)
		P. dispar (Leonard, 1970a, b)
		A. orthogonia (McGinnis and Kasting, 1959)
		S. litura (Morita and Tojo, 1985)
		S. punctatella (Kamata and Igarashi, 1994)
		Manduca sexta (Jones et al., 1980; Nijhout, 1975)
	Coleoptera	*T. glabrum* (Archer and Strong, 1975; Beck, 1971a)
Density (Solitary or Isolated conditions)		
	Orthoptera	*M. differentialis* (Dingle and Haskell, 1967)
		Nomadacris septemfasciata (Albrecht, 1957)
		Ornithacris turbida (Antoniou, 1973)
		S. gregaria (Albrecht, 1957)

Environmental factors	Insect order	Insect species
Density (Crowded conditions)		
	Lepidoptera	*S. litura* (Morita and Tojo, 1985)
		P. dispar (Leonard, 1968)
	Hymenoptera	*Arge nigrinodosa* (Adachi, 1981)
		A. nipponensis (Adachi, 1981)
	Coleoptera	*T. molitor* (Connat et al., 1991)
		Zophobas atratus (Quennedey et al., 1995)
		T. freemani (Kotaki et al., 1993; Nakakita, 1982) *T. castaneum* (Park, unpublished data)

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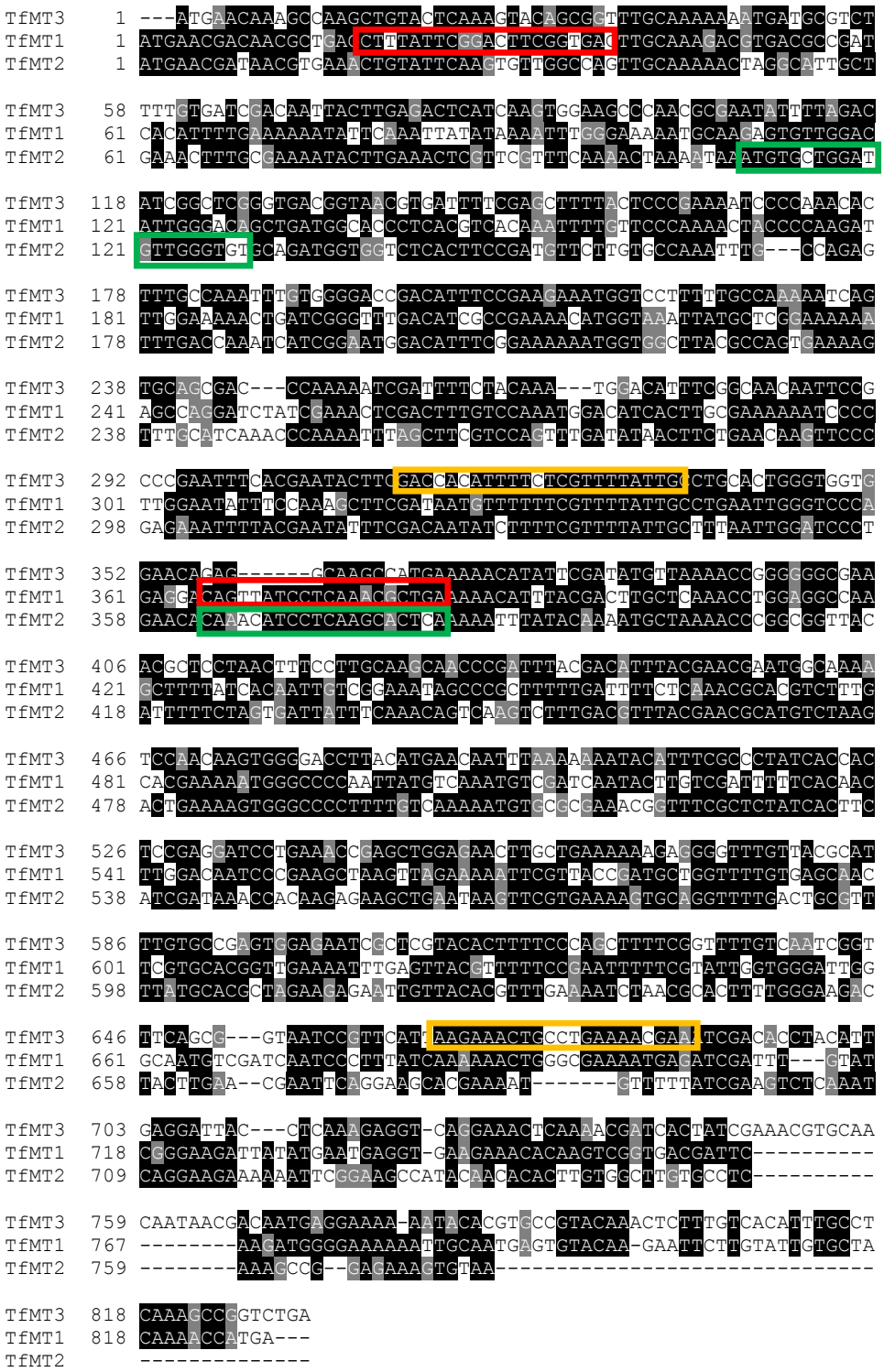
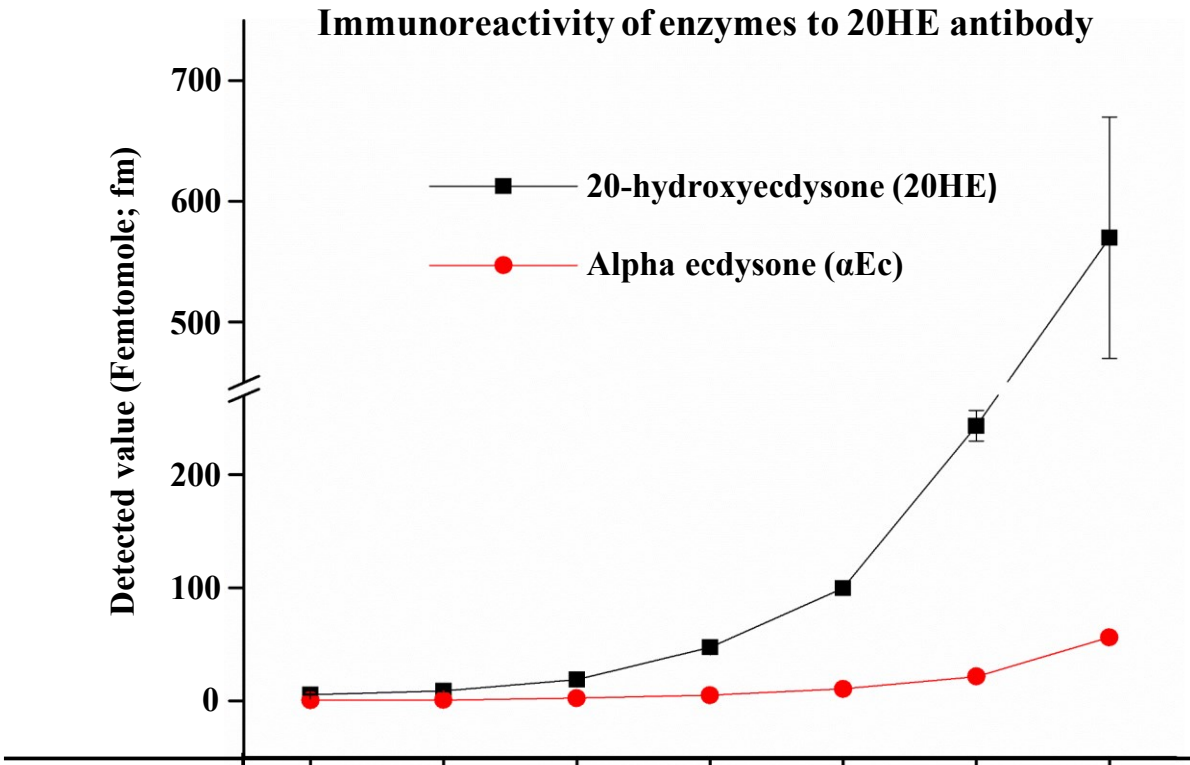
Appendix chapter - 2



Appendix figure 2 Multiple sequence alignment of methyltransferase cDNA in *Tribolium freemani* (*TfMT1*, *TfMT2*, and *TfMT3*). Red, green, and yellow boxes indicate forward and reverse primers for amplifying DNA templates for double strand RNA synthesis of *TfMT1*, *TfMT2*, and *TfMT3*, respectively. The alignment was done using ClustalW and the figure was generated in Boxshade 3.21. The numbers of the left side of the alignment indicate the position of nucleic acids of each gene. The black and grey background indicate identical and conserved nucleic acids, respectively.

Appendix chapter - 3



Standard value	5 fm	10 fm	20 fm	50 fm	100 fm	200 fm	500 fm
20HE	5.6 fm	8.9 fm	18.7 fm	47.3 fm	99.7 fm	242.9 fm	570.0 fm
αEc	0.5 fm	0.7 fm	2.5 fm	5.0 fm	10.5 fm	21.4 fm	56.1 fm

Appendix figure 3 The immunoreactivity of alpha ecdysone and 20-hydroxyecdysone to 20-hydroxyecdysone antibody. The red line with red circle is the alpha ecdysone that cross reacted to anti-20-hydroxyecdysone with one-tenth (1/10) the sensitivity.