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Importance of juvenile hormone signaling arises with competence of insect larvae to metamorphose

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ABSTRACT

Juvenile hormone (JH) postpones metamorphosis of insect larvae until they have attained an appropriate stage and size. Then, during the final larval instar, a drop in JH secretion permits a metamorphic molt that transforms larvae to adults either directly (hemimetaboly) or via a pupal stage (holometaboly). In both scenarios, JH precludes metamorphosis by activating the *Kr-h1* gene through a JH receptor, Methoprene-tolerant (Met). Removal of Met, Kr-h1, or JH itself triggers deleterious precocious metamorphosis. Although JH is thought to maintain the juvenile status throughout larval life, various methods of depleting JH failed to induce metamorphosis in early-instar larvae. To determine when does JH signaling become important for the prevention of precocious metamorphosis, we chose the hemimetabolous bug, *Pyrrhocoris apterus*, and the holometabolous silkworm, *Bombyx mori*. Both species undergo a fixed number of five larval instars. *Pyrrhocoris* larvae subjected to RNAi-mediated knockdown of *Met* or *Kr-h1* underwent precocious adult development when treated during the fourth (penultimate) instar, but younger larvae proved increasingly resistant to loss of either gene. The earliest instar developing minor signs of precocious metamorphosis was the third. Therefore, the JH-response genes may not be required to maintain the larval program during the first two larval instars. Next, we examined *Bombyx mod* mutants that cannot synthesize authentic, epoxidized forms of JH. Although *mod* larvae expressed *Kr-h1* mRNA at severely reduced levels since hatching, they only entered metamorphosis by pupating after four, rarely three instars. Based on findings in *Pyrrhocoris* and *Bombyx*, we propose that insect postembryonic development is initially independent of JH. Only later, when larvae gain competence to enter metamorphosis, JH signaling becomes necessary to prevent precocious metamorphosis and to optimize growth.

Introduction

Insect larvae grow through a number of successive instars, each terminated by a molt and ecdysis that replace the old larval cuticle with a new one. The molts are promoted by surges of ecdysteroids (Yamanaka et al., 2013). Secretion of the sesquiterpenoid juvenile hormone (JH) signals that a molt will produce another larva, thus enabling further growth (Hiruma and Kaneko, 2013; Nijhout et al., 2014). Only when larvae have attained an appropriate stage or size, the temporal absence of JH permits a metamorphic molt that transforms them into adults, either directly (hemimetaboly) or via an intermediate pupal stage (holometaboly). In either case, it is during the final larval instar that the animals commit to metamorphosis (Nijhout, 1994; Nijhout and Williams, 1974; Riddiford, 1994). The anti-metamorphic ("status quo") action of JH has been documented in a broad variety of insects by two types of experiments: First, untimely administration of JH early during the final larval or pupal stages blocks metamorphosis, causing a molt that repeats the previous juvenile stage. Second, premature removal of JH during pre-final larval instars leads to a precocious metamorphic molt, manifested by heterochronic development of adult characters such as wings and external genitals or, in holometabolans, formation of miniature pupae.

The effect of JH deprivation, achieved through decapitation or allatectomy (extirpation of the JH-producing corpora allata glands),

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was initially demonstrated for the hemimetabolous blood-sucking bug, *Rhodnius prolixus* (Wigglesworth, 1936, 1934) and the holometabolous silkworm, *Bombyx mori* (Bounhiol, 1938; Fukuda, 1944). Wigglesworth observed that when decapitated at the right time after feeding, even first-instar *Rhodnius* larvae could undergo a single molt to "precocious adults" with small genitals, partially grown wing pads, and an adult-specific cuticle pattern (Wigglesworth, 1934). He therefore argued that at least some insects were competent to metamorphose already as early larvae, and that any gradual development observed during the larval life occurred due to delayed or reduced JH secretion (Wigglesworth, 1954). Consequently, the notion that JH is required to maintain the larval character of all pre-metamorphic molts has been a paradigm of insect endocrinology to this day. However, the earliest time at which precocious *Bombyx* pupae could be obtained was after three, instead of the normal five larval instars (Bounhiol, 1938; Fukuda, 1944), even when allatectomy was performed on early second-instar larvae (K. Hiruma, personal communication). This record has not been broken even with modern techniques.

Indeed, various genetic methods of depleting JH have failed to induce precocious metamorphosis during early larval instars, suggesting that contrary to the paradigm, the juvenile character may not depend on JH until a later phase of larval development. Transgenic *Bombyx* silkworms expressing a JH-degrading esterase (JHE) throughout development pupated no earlier than after three larval instars (Tan et al., 2005). The recently characterized *dimolting* (*mod*) *Bombyx* mutants that lack a JH biosynthetic enzyme, JH epoxidase CYP15C1, metamorphosed to miniature pupae and adults only after completing three or four larval instars (Daimon et al., 2012). In *Drosophila melanogaster* that normally undergoes three larval instars, genetic ablation of the corpora allata cells did not reduce the instar number (Abdou et al., 2011; Liu et al., 2009; Riddiford et al., 2010) even though second-instar *Drosophila* larvae can be caused to pupate by other genetic manipulations (e.g., (Bialecki et al., 2002; Mirth et al., 2005; Zhou et al., 2004).

Interestingly, although instar numbers vary among and within insect species (Esperk et al., 2007), three larval instars are the lowest consensus. Exceptions include some parasitoid wasps with a single larval instar (Jarjees and Merritt, 2002) and histerid beetles that go through two larval instars (Achiano and Giliomee, 2005). Thus, it appears that in vast majority, juvenile insects must experience a minimum of three instars (or two larval molts) before gaining competence to metamorphose. The role of JH in the acquisition of this competence is unclear.

Recent progress in understanding of JH reception and molecular action (Jindra et al., 2013) now enables studies of the function of JH signaling genes throughout development. JH controls metamorphosis by activating a gene *Krüppel-homolog 1* (*Kr-h1*) that prevents precocious pupal and/or adult development in holometabolous (Kayukawa et al., 2014; Minakuchi et al., 2009, 2008) and hemimetabolous (Konopova et al., 2011; Lozano and Bellés, 2011) insects. The JH-dependent activation of *Kr-h1* requires the JH receptor, Methoprene-tolerant (Met) (Charles et al., 2011; Kayukawa et al., 2012; Konopova et al., 2011; Minakuchi et al., 2009; Parthasarathy et al., 2008). RNAi-mediated knockdown of *Met* or *Kr-h1* function triggers precocious pupation in larvae of the flour beetle, *Tribolium castaneum* (Konopova and Jindra, 2007; Minakuchi et al., 2009), and accelerates adult development in larvae of the linden bug, *Pyrrhocoris apterus* (Konopova et al., 2011).

In this study, we examine the role of the JH→Met→Kr-h1 anti-metamorphic pathway in preserving the juvenile status throughout larval development. We employ two complementary insect models with distinct development, the hemimetabolous *Pyrrhocoris* bugs and the holometabolous *Bombyx* silkworms. Systemic RNAi in *Pyrrhocoris* has previously revealed that silencing of *Met* and *Kr-h1* during the penultimate (fourth) larval instar induces precocious adult development (Konopova et al., 2011). Here, we find that *Pyrrhocoris* larvae are incapable of responding to disruption of JH signaling by developing adult-like features until in the third instar. Using the JH-deficient *Bombyx mod* mutants (Daimon et al., 2012), we show that while expression of the anti-metamorphic *Kr-h1* gene is drastically reduced in the absence of JH since hatching, this reduction is not sufficient for metamorphosis to take place. We conclude that insect larvae are initially incompetent to undergo metamorphosis, and do not rely on JH for maintenance of the juvenile status until late instars.

Materials and methods

Insect rearing and staging

Pyrrhocoris apterus bugs (short-winged form) were maintained at 25 °C under long-day photoperiod (18 h light/6 h dark) on dry linden seeds, and were supplemented with water. Eggs and hatchlings were collected daily. The five larval instars (hereafter referred to as L1 through L5; Fig. 1A) were distinguished based on body size, shape, and the degree of development of the wing pads. Staging within instars relied on measuring time after ecdysis.

The *Bombyx mori dimolting* (*mod*) mutants (Daimon et al., 2012) were maintained as a homozygous stock (*w-1*; *mod*) in the *white egg 1* (*w-1*) genetic background. The *w-1*; *pnd-1* (*pigmented and non-diapausing 1*) strain (hereafter referred to as "control") served for reference. Larvae of both strains were reared at 25 °C under 12 h light/12 h dark regime on mulberry leaves, and were synchronized just after ecdysing to each instar (day 0) and collected for mRNA expression analysis on the daily basis. During molts, animals were staged using the slippage of the old head capsule (HCS stage) as a morphological marker.

RNAi in Pyrrhocoris larvae

Double-stranded RNA (dsRNA) was synthesized using the T3 and T7 RNA polymerases with the MEGAscript kit (Ambion) from plasmids containing the appropriate gene fragments as described previously (Konopova et al., 2011). Using a glass capillary needle mounted in a micromanipulator, dsRNA was injected ventro-laterally into the abdomen of CO_2-anesthesized *Pyrrhocoris* larvae. Approximately 0.2 μg, 0.5 μg, 1 μg and 3 μg of dsRNA were delivered into larvae of L1, L2, L3 and L4 instars, respectively. Control animals were injected with equal amounts of heterologous dsRNA, derived from the *egfp* gene.

mRNA Quantification

In *Pyrrhocoris*, total RNA was extracted either from whole larvae (instars L1 and L2) or from their abdominal epidermis (instars L3-L5) using either TRIzol (Invitrogen) or the RNeasy Plus mini kit (Qiagen). After TURBO DNase treatment (Ambion), 150 ng of total RNA was used for cDNA synthesis using the SuperScript II reverse transcriptase (Invitrogen) and oligo$(dT)_{18}$ primers. Relative transcript levels were measured by quantitative PCR (qPCR) using the iQ SYBR Green Supermix kit and the C1000 Thermal Cycler (both Bio-Rad) and were normalized to mRNA levels of the *Pyrrhocoris* ribosomal protein (Rp49) as described previously (Konopova et al., 2011). Primers used for qPCR are listed in Table 1.

In *Bombyx*, either whole larvae were homogenized (instars L1 and L2), or the dorsal epidermis of the anterior four (L2), two (L3) or one (L4, L5) abdominal segments was dissected. RNA was then extracted using the RNeasy Plus mini kit (Qiagen), and 50 ng of total RNA was taken per cDNA synthesis reaction using the PrimeScript RT reagent kit (Takara Bio) with mixed oligo