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Decrease in *Methoprene tolerant* and *Taiman* expression reduces juvenile hormone effects and enhances the levels of juvenile hormone circulating in males of the linden bug *Pyrrhocoris apterus*

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ABSTRACT

Juvenile hormone (JH) produced by the corpus allatum (CA) stimulates vitellogenesis and reduces the synthesis of hexamerin proteins in adult females of *Pyrrhocoris apterus*. At present it is unknown whether the signaling pathway involving the JH receptor gene Methoprene tolerant (Met) and its binding partner Taiman (Tai), regulates the synthesis of accessory gland proteins (ACPs) and hexamerin proteins or effects male survival. Knockdown of genes by injecting *Met* dsRNA or *Tai* dsRNA, reduced the amount of ACPs whilst enhancing the amount of *hexamerin* mRNA in the fat body and the release of hexamerin proteins into haemolymph, as occurs after the ablation of CA. Lifespan was enhanced by injecting *Met* but not *Tai* dsRNA. Diapause associated with the natural absence of JH had a stronger effect on all these parameters than the ablation of CA or the knockdown of genes. This indicates there is an additional regulating agent. Both *Met* and *Tai* dsRNA induced a several fold increase in JH (JH III skiped bisepoxide) but a concurrent loss of Met or Tai disabled its function. This supports the view that the Met/Tai complex functions as a JH receptor in the regulation of ACPs and hexamerins.

1. Introduction

Juvenile hormone (JH) produced by the corpus allatum (CA) controls many events in an insect's life, including development and reproduction. JH keeps insects in the juvenile state until the final immature stage when a temporal drop in JH permits metamorphosis to the adult form (Wigglesworth, 1934, 1970; Nijhout, 1994; Gilbert et al., 2000; Jindra et al., 2013). In adult insects, JH has many other functions during reproduction, including the stimulation of oogenesis (Engelmann, 1970, 1990; Raikhel et al., 2005). In diapause the CA is deactivated, resulting in an inhibition in the synthesis and secretion of JH and cessation of reproduction (De Wilde and De Boer, 1969; Darjo, 1976; Hodkova, 1976).

Studies on the molecular basis of JH signaling has revealed that *Methoprene-tolerant* (*Met*), a member of the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) family of transcription factors (Ashok et al., 1998), is the JH receptor (Jindra et al., 2015b). This was discovered because certain mutants of *Drosophila melanogaster* are resistant to the toxicity of the JH mimic methoprene (Wilson and Fabian, 1986). RNA interference (RNAi)-mediated knockdown of a *Met* ortholog revealed that it is required if JH is to prevent precocious metamorphosis in both holometabolous and the hemimetabolous insects (Konopova and Jindra, 2007; Minakuchi et al., 2008, 2009; Konopova et al., 2011; Lozano and Belles, 2014; Smykal et al., 2014). In adult insects, the molecular basis of JH signaling has been studied mostly in females. The gene *Met* is implicated in oogenesis in different insects (Parthasarathy et al., 2010; Li et al., 2011; Sheng et al., 2011; Zou et al., 2013) including *Pyrrhocoris apterus* (Smykal et al., 2014). In response to JH, Met proteins form complexes with other bHLH-PAS proteins. There is genetic evidence that germ cell-expressed (Gce)/Met acts as a JH receptor during development of *Drosophila melanogaster* (Jindra et al., 2015b). Taiman (Tai) (also called FISC or SRC) is currently the best-known protein partner of Met (Charles et al., 2011; Li et al., 2011; Zhang et al., 2011; Lozano et al., 2014; Jindra et al.,

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2015a). Recently it has been shown that both *Tai* and *Met* are required for JH-induced vitellogenin synthesis in the fat body and ovarian maturation in *P. apterus* (Smykal et al., 2014). On the other hand, JH-induced circadian gene expression in the gut of *P. apterus* females (Bajgar et al., 2013a) requires *Met* and two circadian genes, *cycle* (*cyc*) and *Clock* (*Clk*), but not *Tai* (Bajgar et al., 2013b). Intriguingly, it is possible that Met interacts with many protein partners and so initiates the diverse functions of JH.

The role of JH in male reproduction is less well understood. The clear role of JH in the physiology of the male accessory gland (MAGs) was first suggested by Wigglesworth (1936). Later experiments indicate that JH is involved in the synthesis of male accessory gland proteins (ACPs) in many different insects (Chen, 1984; Gillot, 1988; Yamamoto et al., 1988; Gold and Davey, 1989; Ismail and Gillot, 1994; Herndon et al., 1997). Ablation of CA in males of *P. apterus* results in a decrease in the levels of ACPs, while application of the JH mimic methoprene results in an increase (Socha et al., 2004; Socha, 2006). Recent data indicate that Met and Tai are required for JH to stimulate the growth of MAGs (Urbanova et al., 2016), but their effect on ACPs is unknown. Mating activity in *P. apterus* is not affected by the ablation of CA (Zdarek, 1966, 1968; Blazkova et al., 2011), but diapause males do not mate (Hodkova et al., 1991; Hodkova, 1994). There are no studies on the effect of JH on fertility, but impaired fertility is reported in males of *D. melanogaster* with depleted accessory glands resulting from multiple mating (Lefevre and Johnson, 1962; Heifetz et al., 2001). However, in Met^{27} mutants of *D. melanogaster* the accumulation of protein in accessory glands is less but their fertility is not affected (Wilson et al., 2003). Furthermore, a deficiency in JH enhances the synthesis of hexameric proteins in the fat body and their release into the haemolymph (Bradfield et al., 1990; Jones and Sarkari, 1993). In diapausing males and females of *P. apterus* with a natural deficiency of JH, there is an increase in 70–80 kDa hexameric proteins in the haemolymph (Socha et al., 1991; Sula et al., 1995; Socha and Sula, 1992, 1996). In addition, in females of *P. apterus* from which the CA has been removed, there is an increase in hexameric mRNA in the fat body (Smykal et al., 2014). Finally, that JH affects ageing is indicated by the observation that diapause or the removal of the CA extends lifespan in acridid grasshoppers (Pener, 1972), the monarch butterfly *Danaus plexippus* (Herman and Tatar, 2001; Tatar and Yin, 2001) and *P. apterus* (Hodkova, 2008; Blazkova et al., 2011; Buricova and Hodkova, 2013).

The first aim of this paper is to determine whether RNAi-mediated knockdown of the genes *Met* and *Tai* affect male survival and reproduction (ACPs, hexamerins, fertility) in the same way as the removal of the CA of active males, or the natural absence of JH in diapause males of *P. apterus*.

Although it is known that both *Met* and *Tai* RNAi affect JH function (see above), their effect on the amount of JH is unknown. Therefore, the second aim of this study is to determine the effect of the RNAi-mediated knockdown of the genes *Met* and *Tai* on the level of $JHSB_3$ in the haemolymph of *P. apterus*. The chemical composition of heteropteran JH was only recently identified when JH III skiped bisepoxide ($JHSB_3$), methyl (2R, 3S, 10R)-2,3,10,11 bisepoxyfarnesoate, was identified as a novel JH in the stink bug, *Plautia stali* (Kotaki et al., 2009, 2011; Kaihara et al., 2012).

2. Material and methods

2.1. Experimental animals

Colonies of *P. apterus* (L.) (Heteroptera) were reared at 25 ± 2 °C under either a reproduction-promoting LD photoperiod of 18 h light or a diapause-promoting SD photoperiod of 12 h light/12 h darkness and supplied *ad libitum* with linden seeds and water. All experiments were done using adult males.

Males destined for allatectomy or injection with dsRNA were deprived of food for 24 h after adult ecdysis. Prior to each treatment animals were anesthetized by submerging them in water for 10 min. CA were removed through an incision in the neck membrane or they were injected with 4 μg/2 μl of concentrated dsRNA in Ringer solution. In the case of the control males the neck membrane was cut or they were injected with plain Ringer solution. Males were injected into the abdomen. The adults were provided with food immediately after treatment.

Fourteen day old virgin males were used for the dissection of the accessory glands, fat body and collection of haemolymph. Haemolymph was collected from cut antennae. Dissected tissues and haemolymph were immediately placed in Eppendorf tubes kept in liquid nitrogen, and stored at −85 °C until analyzed.

Males used for determining survival and fertility were kept individually or in pairs in Petri dishes. Each male was provided with a 4 day old virgin female once per week. In the fertility experiments, the previous female was then kept separately and the number of eggs and the percentage that hatched in each batch was recorded. Mortality was recorded three times per week.

2.2. Protein content of accessory glands (ACPs)

Accessory glands were dissected in Ringer solution. In each sample 4 accessory glands were homogenized in 50 μl of 50 mM phosphate buffer (pH 7.0) with 1 mM EDTA. Samples were treated with 5 μl streptomycin sulphate (10%) to remove nucleic acids (Reznick and Packer) and incubated for 15 min at room temperature (RT). The homogenate was then centrifuged at 3000*g* for 10 min at 4 °C. The resulting supernatant was used to determine the protein content using Pierce BCA Protein Assay Kit (ThermoScientific). Bovine serum albumin was used as a standard. A SpectraMax 384 (Molecular Devices) was used to measure absorbance at 562 am. The results are expressed in μg of protein per gland.

2.3. Haemolymph proteins

Polyacrylamide gel electrophoresis with sodium dodecyl sulphate (PAGE SDS) was carried out according to Laemmli (1970). Fresh gels of 10% were prepared by mixing TRIS buffer 8.8 pH with 0.1% SDS and polymerization solution with 30% acrylamide and 0.8% bisacrylamide dissolved in the same TRIS buffer. Gels, 0.7 mm thick, were used in a Bio-Rad electrophoresis apparatus Mini Protean III. Equivalents of 0.2 μl of haemolymph were used for each strip. Electrophoreses were run at 100 V for 2 h. After electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue R-250 in ethanol-acetic acid-water for 4 h. Gels were then washed several times with ethanol-acetic acid-water (2.5:1:6.5). Finally, the density of the blue bands corresponding to hexamerin proteins (70–90 kDa) was determined using a densitometer (GS 800, Bio-Rad).

2.4. cDNA cloning

Cloning of *P. apterus* cDNA encoding *Met* (Konopova et al., 2011) and *Tai* (Bajgar et al., 2013a,b) and hexamerin (Smykal et al., 2014) was carried using the methods of the cited authors.

2.5. RNA interference (RNAi)

Double-stranded (dsRNA) was prepared using the The MEGAscript® T7 Kit (Ambion) from plasmids containing the appropriate gene fragments as previously described (Konopova et al., 2011; Bajgar et al., 2013a,b) and 4 μg/2 μl of *Met* or *Tai* dsRNA was