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Research Article

Juvenile hormone regulation of HMG-R gene expression in the bark beetle *Ips paraconfusus* (Coleoptera: Scolytidae): implications for male aggregation pheromone biosynthesis

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Abstract. Juvenile hormone III (JH III) induces acyclic isoprenoid pheromone production in male *Ips paraconfusus*. A likely regulatory enzyme in this process is 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-R). To begin molecular studies on pheromone production, a 1.16-kb complementary DNA representing approximately one-third of *I. paraconfusus HMG-R* was isolated by polymerase chain reaction and sequenced. The predicted translation product is 59% and 75% identical to the corresponding portion of HMG-R from the fruit fly, *Drosophila melanogaster*, and the German cockroach, *Blattella germanica*, respectively. Northern blots show that topical application of JH III increases *HMG-R* transcript levels in male thoraces in an apparent dose- and time-dependent manner. These data support the model that JH III raises *HMG-R* transcript levels, resulting in increased activity of the isoprenoid pathway and de novo pheromone production.

Key words. Juvenile hormone; HMG-R; gene regulation; insect; Coleoptera: Scolytidae; bark beetle; isoprenoid; pheromone.

3-Hydroxy-3-methylglutaryl CoA reductase (HMG-R) is a major regulatory enzyme of the isoprenoid pathway in animals [1]. The activity of this enzyme may be controlled at transcriptional, translational and/or post-translational levels, although an increase in HMG-R activity usually correlates with an increase in the corresponding gene transcript [2]. This enzyme has been studied in insects primarily because of its implied role in regulating juvenile hormone (JH, a sesquiterpenoid) production [3]. Also, since insects lack squalene synthase, HMG-Rs from these animals may not be regulated by sterols as observed in mammals [4].

In certain bark beetles (Coleoptera: Scolytidae), mevalonate-based, acyclic isoprenoids play a critical role in reproductive biology as aggregation pheromones [5–7]. The aggregation pheromone produced by the male California fivespined ips, *Ips paraconfusus* Lanier, contains a 10:1 mixture of the acyclic monoterpenoids (4*S*)-(−)-ipsenol (2-methyl-6-methylene-7-octen-4-ol) and (4*S*)-(+)-ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) [8, 9]. A synergistic bicyclic monoterpenoid, (1*S*,2*S*)-*cis*-verbenol, is also necessary to elicit complete aggregation behavior. Pheromone production begins after the insect has tunneled through the bark of pines (*Pinus* spp.) and

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has begun feeding on the phloem [10]. Although it was initially thought that myrcene, present in pine oleoresin [11], was directly hydroxylated for pheromone production [12–14], recent studies show that synthesis is also de novo [6, 7, 15, 16].

Pheromone synthesis is induced in unfed male *I. paraconfusus* by topical application of JH III or the JH analogue, fenoxycarb [17, 18]. In the congeneric *I. pini* (Say), topical application of JH III to males increases the incorporation of labeled acetate, but not mevalonate, into ipsdienol in a dose-dependent manner [7]. With male *I. pini*, feeding on host phloem stimulates both de novo ipsdienol production in vivo and JH III synthesis from isolated corpora allata [7]. Treatment of male *I. duplicatus* Sahlberg with the HMG-R inhibitor, compactin, decreases the amount of ipsdienol produced in both fed and JH analogue-treated insects [5, 19]. Topical JH III application also causes flight muscle degradation in both sexes of *I. paraconfusus* [20]. Together, these data support the model that feeding induces JH synthesis, which in turn increases the flux of carbon through the isoprenoid pathway. It is likely that the source of energy and carbon for pheromone production comes from feeding and possibly through recycling of flight muscle tissue [21].

Based on biochemical data from *I. pini* [7], we hypothesized that HMG-R is important for controlling isoprenoid pheromone production in male *I. paraconfusus*. Since de novo isoprenoid pheromone synthesis is induced by JH III, an extension of this hypothesis is that HMG-R transcript levels should also increase in response to JH III. As an initial step in investigating the role of HMG-R in bark beetle pheromone production, and also to begin to address the question of JH effects on adult male tissues, we isolated *I. paraconfusus* HMG-R complementary DNA (cDNA) (*HMG-R*) and studied the effects of dose and incubation time following topical JH III treatment on HMG-R transcript levels.

Materials and methods

Insects. Immature insects were collected from standing, infested *Pinus radiata* D. Don (Contra Costa County, California) between December 1996 and March 1997, reared to adults and used as described [6, 7]. JH III (Sigma; 5.33 μg in 0.5 μl of acetone) was applied topically to each ventral abdomen [7] 20 h before RNA isolation. The 20-h time point was chosen as this is approximately when maximal pheromone synthesis occurs (J. A. Tillman et al., unpublished observations). Control insects were similarly treated with 0.5 μl of acetone. For the dose-response experiment, JH III was applied in 53 ng, 0.53 μg, 4.31 μg, 5.30 μg, 8.6 μg and 53 μg doses to each of 10 males per treatment. For the time course experiment, thoraces from 25–30 males treated with 5.3 μg of JH III or acetone were isolated and pooled for each time point (0, 8 and 20 h following treatment). Otherwise, each sample for the other experiments contained 10 intact adults.

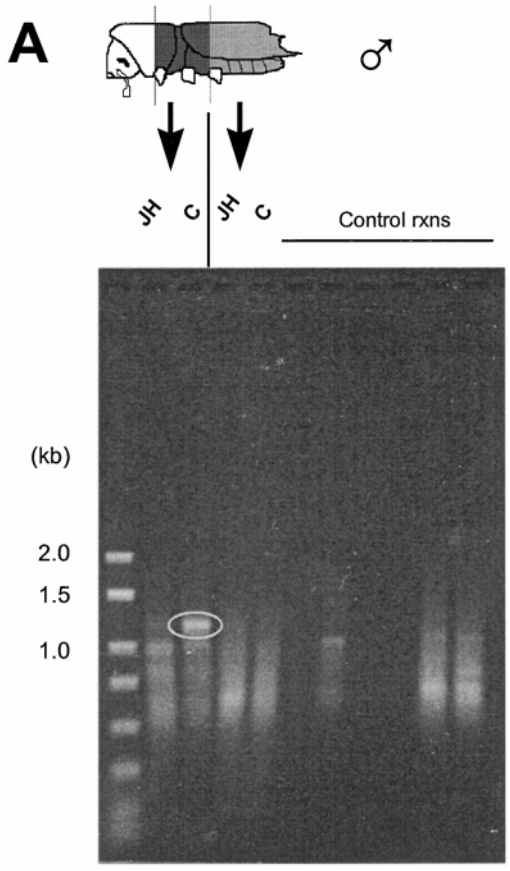
PCR, cloning and sequencing. Polyadenylated [poly(A)$^+$] RNA was prepared using QuickPrep Micro spin columns according to the manufacturer's protocol (Pharmacia). First strand cDNA templates for 3′ rapid amplification of cDNA ends polymerase chain reaction (RACE PCR) reactions were prepared using 1 μg of poly(A)$^+$RNA isolated from males treated with JH III or acetone (controls) using Superscript II RNase H$^-$ reverse transcriptase (Life Technologies) and the 'T_{17}CSX' primer [22]. A portion of *HMG-R* cDNA was amplified by 3′ RACE using a 32-fold degenerate forward primer ('Parahmd', GAICNATGGGNATGAAYATG, where I = inosine, N = A,C,G or T, and Y = C or T) which corresponds to part of the highly conserved catalytic domain of the protein, and CSX. The cycling profile was 1 min at 95 °C; 40 cycles of 40 s at 94 °C, 1 min at 62 °C, 2.5 min at 72 °C; and a final 8-min extension at 72 °C. PCR products were purified on low-gelling-temperature agarose (FMC), isolated using Gelase enzyme (Epicenter Technologies), and ligated into pT7Blue plasmid (Novagen). Recombinant clones were sequenced with an ABI Prism 310 automated sequencer using dye terminators.

All primers for PCR and sequencing were purchased from Life Technologies (Bethesda, MD, USA).

Northern hybridization. Poly(A)$^+$ RNA (1.2–2.1 μg/lane) was separated on glyoxal agarose gels [23], transferred to Hybond N nylon membrane (Amersham), and immobilized by ultraviolet cross-linking. The blots were hybridized with a portion of *I. para-confusus* HMG-R ('ParapT7'; see 'Results') or, to control for loading in each lane, mouse β-actin (Stratagene). Hybridization probes were labeled with ^{32}P-deoxycylidine triphosphate (dCTP) by PCR [24] and purified by Sephadex G-50 spin columns. The blots were washed [23] under stringent (HMG-R; twice for 10 min at room temperature with 2 × SSC, 0.1% SDS and once for 30 min at 70 °C with 0.2 × SSC, 0.1% SDS) or moderate (β-actin; twice for 10 min at room temperature with 2 × SSC, 0.1% SDS and once for 10 min at 42 °C with 0.2 × SSC, 0.1 % SDS) conditions and exposed using a BioRad Molecular Imager. Densitometry was done using Molecular Analyst software (BioRad).

Results

Previous in vitro experiments have demonstrated that the pheromone precursor ipsenone is synthesized in



B

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D. mel  TGDRMGMNMVSKALRWPFAEFTLHFPDMQIISLSGNFCCDKKPAAINWIK    50
B. ger  TGDAMGMNMLSKGTEVALAYVQQVYPDMEILSLSGNFCTDKKPAAVNWIE    50
I. para -----GMNMLSKGTEYSLKLCQRAFEDMEILSLSGNFCTDKKPAAVNWIE    45
             ****.**.   ..      . **.*.******* ******.***.

D. mel  GRGKRVVTECTISAATLRSVLKTDAKTLVECNKLKNMGGSAMAGSIGGNN   100
B.ger   GRGKSVVCEAIVPADIIKSVLKTSVQALMDVNITKNLIGSAVAGSIGGFN   100
I. para  GRGKSVVCEAIVPAKIVANILKTNVHALVDVNNSKNMVGSAVAGSIGGFN   95
        ****.** * ...* .. ..***....*.. *  **. ***.****** *

D. mel  AHAANMVTAVFLATGQDPAQNVTSSNCSTAMECWAENSEDLYMTCTMPSL   150
B. ger AHAANIVTAIFIATGQDPAQNVGSSNCMTLMEPWGEDGKDLYVSCTMPSI    150
I. para  AHAANIVTAIFLATGQDPAQNVGSSNCMTLMEPWGPTGEDLYISCTMPSI   145
        *****.***.*.**********.**** * ** *. ...***..*****.

D. mel  EVGTVGGGTGLPGQSACLEMLGVRGAHATRPGDNAKKLAQIVCATVMAGE   200
B. ger   EIGTIGGGTVLPPQAACLDMLGVRGANEMCPGENANTLARIVCGTVLAGE   200
I. para  EIGTIGGGTILPPQGTCLEMLGVRGSNIAEPGANASQLAKIVGATVLAGE   195
        *.**.**** ** *..**.******..   **.**. **.** .**.***

D. mel  LSLMAALVNSDLVKSHMRHNRSSIAVNSA-NNPLNVTVSSCSTIS         244
B. ger  LSLMSALAAGHLVKSHMRHNRSSVSTSGS-----EPSTPACKS--         238
I. para  LSLMSALAAGHLVRSHLRHNRSTTLLPDAFDKNKNILVPPCKDKV        240
        ****.**....**.**.*****..   ..     .  ...*..
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Figure 1. Isolation and characterization of *I. paraconfusus HMG-R* cDNA. (*A*) 3′ RACE PCR amplification of templates from thoraces and abdomens of JH III-treated (JH) and acetone-treated (C) adult males. The ~1.2 kb product circled in the control thorax lane was isolated, ligated into pT7Blue plasmid to yield 'ParapT7' and sequenced. (*B*) Alignment of the predicted translation product of ParapT7. The sequence (*I. para*) was aligned with the corresponding portions of HMG-R from the fruit fly (*D. mel*) and the German cockroach (*B. ger*). Perfectly conserved residues are indicated by a '*' below the alignment, similar residues are indicated by a '.'

male *I. paraconfusus* thoracic tissues [21], and we used this information to select tissues for the initial isolation of *HMG-R* cDNA by 3′ RACE PCR. As expected, a strong amplification product was found in templates from thoraces, but not abdomens, although the control thoraces gave the strongest signal, as opposed to the JH-treated tissues (fig. 1A).

The 1.16-kb PCR product was ligated into pT7Blue plasmid to yield the clone ParapT7, and sequenced. It contains a 720-bp open reading frame (ORF) followed by a 424-bp 3′ untranslated region and a poly(A) tract. The sequence has been deposited in GenBank under accession number AF071750. The predicted translation product from the ORF shows very high sequence similarity to HMG-Rs from a variety of organisms when searched against the GenBank database using BLASTP [25]. When aligned with the corresponding portion (approximately 60% of the catalytic domain) of HMG-R from the only other insects from which the sequence has been reported, the fruit fly, *Drosophila melanogaster* [26], and the German cockroach, *Blattella germanica* [27], the beetle sequence is 59% and 75% identical, respectively (fig. 1B).

All Northern blots of poly(A)$^+$ RNA from adult male and female insects show that the ParapT7 insert hybridized with an ~3.2-kb transcript. The mouse β-actin cDNA hybridized with a 1.5-kb transcript, and expression levels of this signal were used to normalize loading variations in different lanes.

Topical application of JH III (5.3 μg) increases *HMG-R* transcript levels in both sexes to a similar degree: ~2.5-fold in males, and ~3.3-fold in females (fig. 2). Both treatment and control expression levels were higher in males compared with females.

We also treated male insects with JH III at doses ranging from 53 ng to 53 μg per insect. Low doses (53 ng–0.53 μg) apparently reduced transcript levels, but higher doses increased transcript levels, up to nearly 5-fold at 53 μg of JH III compared with controls (fig. 3).

A time course experiment, in which poly(A)$^+$ RNA was isolated from thoraces of males dissected 8 or 20 h after treatment with 5.3 μg of JH III showed a general increase in *HMG-R* transcript levels over time as compared with acetone-treated control insects, to a maximum of nearly 10-fold at 20 h, compared with controls (fig. 4).

Discussion and conclusion

The primary structure of eukaryotic HMG-R proteins consists of an N-terminal transmembrane domain separated from the C-terminal catalytic domain by a relatively short hydrophilic linker sequence [2]. While the transmembrane and linker sequences usually vary widely across taxa, the catalytic domain is well conserved. We used this information to design a degenerate primer for 3′ RACE PCR in order to isolate a cDNA for HMG-R from *I. paraconfusus*. Since pheromone precursors are produced in male thoraces [21], and topical JH III treatments induce synthesis [17], we chose cDNA from JH-treated male thoraces as template for our reactions. Curiously, stronger amplifications were observed using template cDNA from control tissues than from JH-treated tissues (fig. 1A). This was consistent over two preparations and multiple PCR reactions. While this apparently contradicts the Northern blot data, it should be noted that the template cDNA was not quantified, and the amplification conditions themselves were not quantitative. We believe that the apparent reduced efficiency of amplification from JH-treated tissues may reflect differences in the internal chemistry of treated and control animals such that RNA isolation and/or cDNA synthesis was affected. This is supported by the fact that we could not recover RNA from insects, which had been fed on *P. radiata* phloem, using the Pharmacia kit (unpublished observations).

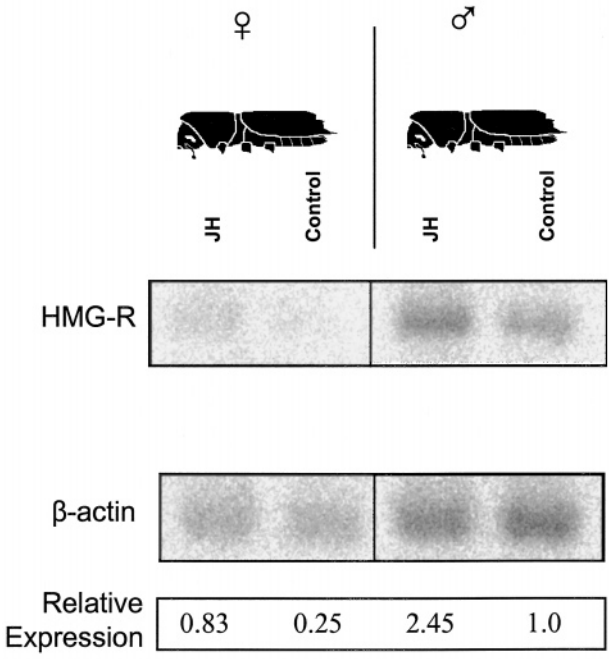

Figure 2. JH III induction of *HMG-R* transcript in male and female *I. paraconfusus*. Northern blot of poly(A)$^+$ RNA isolated from intact insects treated with 5.3 μg JH III (JH) or acetone (Control), hybridized with ^{32}P-labeled ParapT7 (HMG-R), and washed under high stringency. The lower panel shows the hybridization signal for mouse β-actin following washing at moderate stringency. HMG-R transcript levels relative to the control thorax sample are indicated below each lane.

Given the very high BLASTP scores when our PCR fragment is aligned with HMG-Rs in GenBank, and also given the relatively high degree of sequence conservation within the catalytic domains of reported HMG-Rs, we are confident that the PCR fragment isolated, ParapT7, represents approximately the latter one-third of the 3.2-kb *I. paraconfusus HMG-R* transcript.

Adult male beetles treated with ~5 µg of JH III will reliably increase *HMG-R* transcript levels between 2.5- and 10-fold after 20 h. In general, lower induction levels were observed on northern blots of RNA prepared from whole insects compared with RNA prepared from isolated thoraces (2.5- to 3-fold compared with 6.3- to 9.8-fold; this paper and [21]), suggesting that the lower increase in intact insects may be due to the presence of nonthoracic transcripts. Alternatively, the dose-response experiment (fig. 3) suggests that the 5-µg dose is near the lower limit for a reliable response. We used this dose because previous work had shown that it was sufficient to induce de novo pheromone production in *I. pini* [7]. Topical JH III and/or analogue doses reported in the literature to induce pheromone production or wing muscle degeneration in *I. paraconfusus* vary from 0.05 to 50 µg/insect [17, 18, 20]. Also, the amount of pheromone produced by *I. paraconfusus* in response to JH III varies considerably, particularly with the age of the insect, and with the season during which the experiment is performed (F. Lu, personal communication), all of which may be factors influencing the observed response to topical JH application. The dose-response curve (fig. 3) suggests that the highest dose of JH III (≥50 µg/insect) used in this study is not sufficient to induce maximal *HMG-R* transcript accumulation. The apparent reduction in transcript levels at low JH III doses may not be significant; further experiments are required to validate this result.

There are several lines of evidence indicating the regulatory role of *HMG-R* in pheromone biosynthesis: (i) in male *I. duplicatus*, treatment by feeding or JH analogue application combined with the HMG-R inhibitor com-

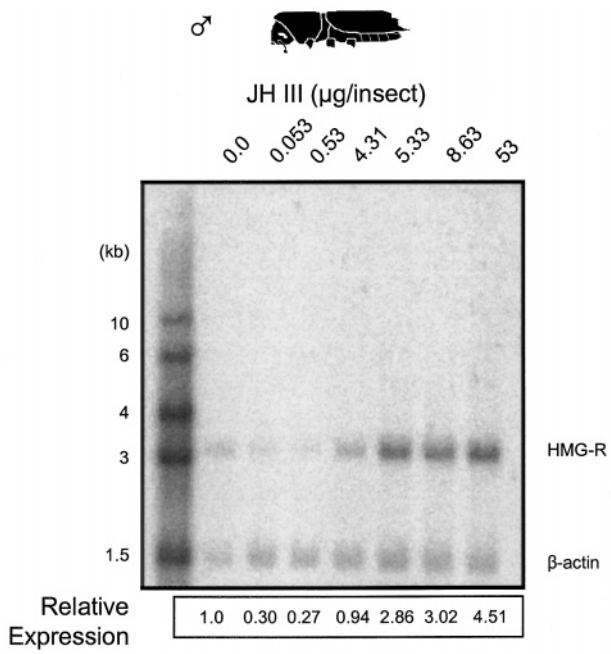

Figure 3. Variation of male *I. paraconfusus HMG-R* transcript levels with JH III dose. Northern blot of poly(A)$^+$ RNA from intact adult males (10 per dose) treated with varying doses of JH III, hybridized with ParapT7 (HMG-R) and β-actin. Size markers (Novagen) are indicated on the left. HMG-R transcript levels relative to control males are indicated below each lane.

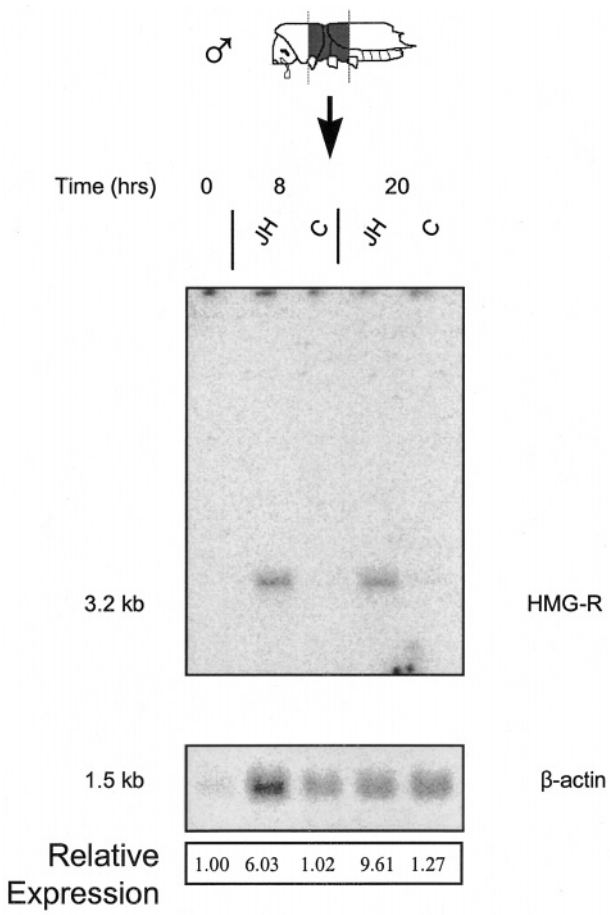

Figure 4. *HMG-R* transcript accumulation over time. Northern blot of poly(A)$^+$ RNA isolated from 25–30 thoraces of JH III-treated (JH, 5.3 µg) or control (C) adult male insects at 0, 8 or 20 h following treatment and hybridized with ParapT7 (HMG-R) and mouse β-actin. HMG-R transcript levels relative to the 0 h control sample are given below each lane.

pactin reduces pheromone production [5, 19]; (ii) in male *I. pini*, JH III stimulates de novo ipsdienol synthesis, likely at an enzymatically catalyzed reaction between acetate and mevalonate [7]; (iii) in male *I. pini*, HMG-R activity levels increase with JH III treatment and feeding (J. Tillman, personal communication); (iv) in male *I. paraconfusus*, pheromone precursors are synthesized in the thorax, the same body region where *HMG-R* transcript accumulates [21]; and (v) in male *I. paraconfusus*, the increase in transcript accumulation over time (fig. 4) parallels the JH analogue-induced increase in pheromone production [17]. Here, we show that *HMG-R* transcript levels in males increase with topical application of JH III in a manner that seems to parallel de novo pheromone biosynthesis [7]. The increase is probably both dose- and time-dependent, though further experiments are planned to determine these correlations more clearly. Whether this increase is due to elevated transcription rates or enhanced transcript stability, or both, and whether JH III acts directly, or further upstream through the release of as yet unknown factors, remains to be determined.

Insect pheromone biosynthesis is regulated by at least three distinct hormone systems. Lepidopteran fatty acid-derived pheromone biosynthesis is often mediated by PBAN (pheromone biosynthesis activating neuropeptide), which alters enzyme activity involved with fatty acid synthesis or modification of the carbonyl group of fatty acids [28]. In the female house fly, *Musca domestica*, ecdysteroids produced in the ovaries regulate hydrocarbon pheromone synthesis by affecting the chain length specificity of fatty acyl-CoA elongation enzymes [29, 30], while coleopteran and blattodean pheromone production is often regulated by JH III [31, 32]. It appears that, at least in *I. paraconfusus*, this regulation occurs in part by increasing *HMG-R* transcript levels. To our knowledge, this is the first report of the apparent regulation of insect pheromone production through increased transcript levels of a key regulatory enzyme.

In addition to its role in development, behavior, female reproduction [33] and response to environmental stress [e.g. 34], it is becoming clear that JH is also important for adult male insects. Miura et al. [35] showed JH-mediated inhibition of cyanoprotein production in the male bean bug, *Riptortus clavitus*. Other studies have shown JH-mediated accumulation of various proteins in male reproductive tissues, including sperm activator peptide in *Danaus plexxipus* [36], trehalases in *Tenebrio molitor* [37] and unspecified proteins in *Melanoplus sanguinipes* [38]. Although not demonstrated, these protein accumulations would presumably be preceded by an increase in the corresponding gene transcripts. Indeed, Ismail et al. [39] observed a JH-mediated increase in RNA synthesis in male *M. sanguinipis* reproductive tissue. Our current study shows specific accumulation of *HMG-R* transcript in response to treatment with juvenile hormone in adult male nonreproductive tissue.

We are extending the preliminary work reported here by looking more closely at *HMG-R* transcript levels and HMG-R activity in both females and males under various conditions. Once we have recovered a full-length HMG-R cDNA, we will continue enzymatic studies, and determine the cellular site of precursor synthesis with in vitro techniques.

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