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A coordinated expression of biosynthetic enzymes controls the flux of juvenile hormone precursors in the corpora allata of mosquitoes

Marcela Nouzova^{¶,φ}, Marten J. Edwards^{†,φ}, Jaime G. Mayoral[¶], and Fernando G. Noriega[¶]

[¶]Department of Biological Sciences, Florida International University, Miami, Florida

[†]Department of Biology, Muhlenberg College, Allentown, PA

Abstract

Juvenile hormone (JH) is a key regulator of metamorphosis and ovarian development in mosquitoes. Adult female *Aedes aegypti* mosquitoes show developmental and dynamically regulated changes of JH synthesis. Newly emerged females have corpora allata (CA) with low biosynthetic activity, but they produce high amounts of JH a day later; blood feeding results in a striking decrease in JH synthesis, but the CA returns to a high level of JH synthesis three days later. To understand the molecular bases of these dynamic changes we combined transcriptional studies of 11 of the 13 enzymes of the JH pathway with a functional analysis of JH synthesis. We detected up to a 1000-fold difference in the levels of mRNA in the CA among the JH biosynthetic enzymes studied. There was a coordinated expression of the 11 JH biosynthetic enzymes in female pupae and adult mosquito. Increases or decreases in transcript levels for all the enzymes resulted in increases or decreases of JH synthesis; suggesting that transcript changes are at least partially responsible for the dynamic changes of JH biosynthesis observed.

JH synthesis by the CA was progressively increased *in vitro* by addition of exogenous precursors such as geranyl-diphosphate, farnesyl-diphosphate, farnesol, farnesal and farnesoic acid. These results suggest that the supply of these precursors and not the activity of the last 6 pathway enzymes is rate limiting in these glands. Nutrient reserves play a key role in the regulation of JH synthesis. Nutritional deficient females had reduced transcript levels for the genes encoding JH biosynthetic enzymes and reduced JH synthesis.

Our studies suggest that JH synthesis is controlled by the rate of flux of isoprenoids, which is the outcome of a complex interplay of changes in precursor pools, enzyme levels and external regulators such as nutrients and brain factors. Enzyme levels might need to surpass a minimum threshold to achieve a net flux of precursors through the biosynthetic pathway. In glands with low synthetic activity, the flux of isoprenoids might be limited by the activity of enzymes with low levels of expression

Keywords

Aedes aegypti; mosquito; juvenile hormone; precursors; flux

Correspondence to: Fernando G. Noriega, Department of Biological Sciences, Florida International University, 11200 SW 8th ST. Miami, FL 33199, USA. Telephone: (305)-348-6632. Fax: (305)-348-1986, noriegaf@fiu.edu.

^φBoth authors contributed equally to this work.

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INTRODUCTION

Juvenile hormone (JH) is a critical hormonal regulator in insects (Goodman and Granger, 2005). JH is part of a transduction system that assesses nutritional information and regulates reproduction in mosquitoes (Noriega, 2004). Oogenesis in mosquitoes is a nutrient-limited process, triggered only if sufficient reserves are available (Briegel, 1990). Mosquito larvae often encounter suboptimal conditions that result in a high variability in the size and reproductive potential of the adult females (Nasci, 1990; Feinsod and Spielman, 1980; Tun-Lin et al., 2000; Caroci et al., 2004). Newly eclosed females must have some mechanism to restrain ovary development and a hormonal control system activated by suitable nutritional stimuli is very appropriate. Consequently adult female *Aedes aegypti* mosquitoes show very dynamic changes in JH titers; the JH level increases during the first day after adult emergence, remains high in sugar-fed females, and rapidly falls after a blood meal (Shapiro et al., 1986). JH titer in female adult mosquitoes is primarily determined by the rate at which the *corpora allata* (CA) synthesize JH (Li et al, 2003a). The CA biosynthetic activity of the newly emerged female will increase only if teneral nutritional reserves are high or the female obtains these nutrients by feeding on nectar. *Aedes aegypti* females are micropredators and need a blood meal to complete oogenesis. Blood feeding results in a striking decrease in JH synthesis, but the CA returns to a high level of JH synthesis three days later (Li et al, 2003a). The biosynthetic pathway of JH III in mosquitoes involves 13 discrete enzymatic steps organized in an obligatory sequence (Fig. 1). The early steps of JH III biosynthesis follow the mevalonate pathway to form farnesyl pyrophosphate (FPP) (Belles et al., 2005). During the late steps, FPP is transformed sequentially to farnesol, farnesal, farnesoic acid, methyl farneosate and JH III (Belles et al., 2005). Studies of JH biosynthesis in *Bombyx mori* showed a relationship between JH biosynthesis and expression of most JH biosynthetic enzymes in the CA (Kinjoh, et al., 2007, Ueda et al., 2009); these studies also pointed that the transcripts for most JH enzymes are highly enriched or exclusively expressed in the CA. Our goal is to understand how the activity of the 13 enzymes and the flux of precursors in this enzymatic pathway interact with allatoregulatory factors or nutritional signaling pathways to influence JH synthesis. In these studies we analyzed the changes in the expression of the genes encoding 11 of the 13 JH biosynthetic enzymes in female pupae and adult *A. aegypti* mosquitoes under normal and nutrient-deficient conditions. We also studied the effect of perturbing specific precursor pools on JH synthesis. Our results suggest that a coordinated expression of genes encoding biosynthetic enzymes plays a role in regulating the flux of JH precursors in the CA of mosquitoes.

MATERIALS AND METHODS

Chemicals

(*E,E*)-farnesoic acid and (*E,E*)-methyl farneosate, GPP, FPP, farnesol and farnesal were purchased from Echelon (Salt Lake City, UT) JH III (79% purity) was purchased from Scitech (Prague, Czech Republic).

Insects

Aedes aegypti of the Rockefeller strain were reared at 28°C and 80 % relative humidity under a photoperiod of 16 h light: 8 h dark. Mated adults were offered a cotton pad soaked in 3% sucrose solution. We will refer to the cotton pad sucrose-fed adults as sugar fed. Four-day-old female mosquitoes were fed pig blood equilibrated to 37 °C, and ATP was added to the blood meal to a final concentration of 1mM immediately before use as previously described (Noriega et al., 1999).

Nutrient-deficient mosquitoes

small nutrient-deficient mosquitoes were produced as previously described (Caroci et al., 2004); briefly, larvae were reared in pans (23 cm × 35 cm × 13 cm) containing 1 liter of distilled water, with the following amounts of a 10 % solution of bovine liver powder (ICN, Aurora, OH) diet: 0.75 ml on day 1, 3, 5 and 7. Under these rearing conditions, most larvae pupate at day 8. Only those females with wing length under 2.5 mm were used.

Quantitative real-time PCR (qPCR)

total RNA was isolated using RNA-binding glass powder as previously described (Noriega and Wells, 1993). Contaminating genomic DNA was removed using the DNA-free™ kit (Ambion, Austin, TX). Reverse transcription was carried out using an oligo dT priming method according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA). Real-time PCR was performed with a 7300 Real Time PCR System using TaqMan® Gene Expression Assays together with TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA). PCR reactions were run in triplicate using 1 µl of cDNA per reaction in a 20 µl volume according to manufacturer recommendations for Custom TaqMan® Gene Expression Assays. Standard curves to quantify relative gene copy number were made from serial dilutions of plasmids containing the mosquito genes (300,000; 30,000; 3,000; 300; 30 copies of a plasmid per reaction). Real-time data were collected by the 7300 System SDS Software and analyzed in Microsoft Excel. Transcript levels were normalized with rpl32 transcript levels in the same sample. Each RT-PCR data point is average of two independent biological replicates.

The primer probes sequences and accession numbers for the housekeeping gene 60S ribosomal protein L32 and for the different enzyme genes are included in table S1.

Corpora allata in vitro radiochemical assay for JH synthesis

the corpora allata-corpora cardiaca complexes (CA-CC) were isolated as previously described (Li et al., 2003a). Rates of MF and JH III biosynthesis were estimated using an *in vitro* radiochemical assay (Feyereisen and Tobe, 1981; Feyereisen, 1985), as previously modified (Li et al., 2003a; 2003b). Briefly, CA-CC complexes were incubated for 4h in fresh medium containing ³H-labeled methionine. After extraction and separation by thin-layer chromatography, the MF and JH III bands were removed, placed into scintillation cocktail and assayed for ³H. The quantity of MF and JH III produced was calculated from the specific activity of the ³H-labeled methionine in the medium. The effect of increasing the pool of precursors on MF and JH III synthesis was tested by adding the precursor directly into the CA-CC incubation medium.

Statistical analysis

statistical analysis of the data was performed by *t*-test using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). The results were expressed as mean ± SEM, and considered significantly different at *P* < 0.05.

RESULTS

A 1000-fold difference was detected in the levels of mRNA expression among the JH biosynthetic enzymes in the CA

Quantitative real-time PCR was used to analyze the levels of expression and tissue specificity of mRNAs encoding 11 of the 13 JH biosynthetic enzymes at the peak of JH synthesis, 24 h after adult eclosion. There was a 1000-fold difference in the levels of mRNA expression in the CA among the enzymes that were analyzed. Transcripts for the two last

enzymes, juvenile hormone acid methyl transferase and cytochrome P 450 methyl farnesoate epoxidase were the most abundant (Fig. 2, empty bars). There were 6 enzymes with moderate levels, 3-hydroxy-3-methyl-glutaryl-CoA synthase, 3-hydroxy-3-methyl-glutaryl-CoA reductase, mevalonate kinase, phosphomevalonate decarboxylase, isopentenyl diphosphate isomerase and farnesyl diphosphate synthase (Fig. 2, striped bar). Three enzymes presented low levels of expression, acetoacetyl-CoA thiolase, phosphomevalonate kinase and farnesol dehydrogenase (Fig 2, filled bar). The two additional enzymes, farnesyl diphosphate pyrophosphatase and farnesal dehydrogenase remain uncharacterized in *A. aegypti*.

Six tissues were used to analyze the transcript tissue specificity of the JH enzymes: CA, brain (BR), fat body (FB), midgut (MG), Malpighian tubules (MT) and ovaries (OV). Relative expression of transcripts encoding JH biosynthetic enzymes were highly enriched in the CA (Fig. 3). The second highest relative expression for most of the enzymes was found in the ovaries. The three enzymes with the lowest transcript levels in the CA (acetoacetyl-CoA thiolase, phosphomevalonate kinase and farnesol dehydrogenase) showed the highest relative expression levels in other tissues. In fact all the JH enzymes were expressed in the 6 evaluated tissues; with the higher number of transcripts for most of them in the CA (especially for juvenile hormone acid methyl transferase and methyl farnesoate epoxidase). Transcript levels are expressed as the ratio JH enzyme/ribosomal proteins. The CA has a very low cell number when compared to other tissues and therefore shows remarkable differences in those ratios that can be misinterpreted as strict specificity in the CA.

Coordinated expression of JH biosynthetic enzymes in female pupae and adult mosquito

Analyses of developmental changes in mRNA levels for the 11 JH biosynthetic enzymes studied showed common trends that correlated well with JH synthesis levels (Fig. 4). Transcript levels in early pupae were very low (the pupal stage lasts 48 h and the age of the pupae is expressed as hours before adult eclosion). Steady-state mRNA levels increased in the pupae during the last 6 hours before adult eclosion and newly emerged females had significantly higher mRNA levels for all enzymes except farnesol dehydrogenase (Fig. 4). The maximum relative mRNA levels for all the enzymes were always detected in sugar-fed females 24 h after adult eclosion. After this peak, transcript levels for all of the enzymes showed a tendency to decrease as sugar-fed females get older. Twenty-four hours after blood feeding we detected a remarkable reduction of transcript levels for all the enzymes (an average 76% decrease); this decrease was followed by a significant increase by 48 h after blood feeding (Fig. 5)

CA of nutritionally-deficient mosquitoes show reduced mRNA expression for the JH biosynthetic enzymes

Nutritionally-deficient adult mosquitoes were generated by limiting the amount of food available to the larvae (Caroci et al., 2004). The synthesis of JH and the steady-state levels of mRNAs encoding JH biosynthetic enzymes in the CA of well nourished (large) and nutrient-deficient (small) females were compared 24 h after adult eclosion. JH synthesis was decreased in small mosquitoes (Fig. 6A). The transcript levels of 10 of the 11 enzymes analyzed showed significantly reduced expression in the CA of small mosquitoes (Fig. 6B). As an average transcript levels were reduced approximately 50% in the nutrient-deficient mosquito.

Increasing the size of five JH precursor pools progressively raised JH synthesis

Corpora allata with low JH biosynthetic activity dissected immediately after adult eclosion (Fig. 7A) or 24 h after blood feeding (Fig. 7C), as well as CA with high JH biosynthetic

activity dissected from sugar-fed females 72 h after eclosion (Fig. 7B) were stimulated *in vitro* with 5 different JH precursors; geranyl-diphosphate (GPP), farnesyl-diphosphate (FPP), farnesol (FOL), farnesal (FAL) and farnesoic acid (FA). The effect of precursors on JH synthesis was analyzed using the *in vitro* radiochemical assay. Methylation of FA results in the formation of radioactive MF, which is further transformed into radioactive JH III; therefore the sum of MF and JH III represents the endogenous catalytic activity of JHAMT and epoxidase (Fig. 1). The addition of exogenous JH III precursors to the incubation media had a significant stimulatory effect on the synthesis of JH. These stimulations were observed in CA dissected from adult female mosquitoes independently of their spontaneous JH biosynthetic activity; although the rates of stimulation were significantly lower in CA with low JH biosynthetic activity. As a general rule we observed that the effect of the five tested precursors was progressive, namely the closer the precursor to the end of the pathway (JH III), the greater the stimulatory effect observed.

We also evaluated whether CA dissected from female pupae could be stimulated by addition of precursors. Glands were dissected from female pupae 24, 6 and 2 hours before adult eclosion and stimulated with FA *in vitro*. The addition of FA had no effect on JH synthesis by CA dissected from early pupae (-24h). The precursor had low stimulatory effects on glands dissected at 6 and 2 h before adult eclosion (Fig. 8).

DISCUSSION

JH levels must be modulated to enable the normal progress of development and reproductive maturation in mosquitoes (Klowden, 1997). Changes in JH titers in female adult *A. aegypti* mosquito are very dynamic (Shapiro et al., 1986). The CA needs to adjust its synthetic activity to generate these dynamic changes (Li et al., 2003a). Part of this regulation involves coordinated changes in the transcription of the genes encoding the JH biosynthetic enzymes in pupae and adult female mosquitoes.

Transcription of the genes encoding JH biosynthetic enzymes is synchronously activated before adult eclosion

Insect metamorphosis is a developmentally regulated process controlled by hormones. Once the larva has reached its species-specific size JH titer decreases allowing pupation and metamorphosis to proceed (Riddiford, 2008). To assure a successful completion of metamorphosis, JH synthesis is suppressed during pupae development; indeed transcript levels for the 11 enzymes analyzed were very low in the early pupae. The mRNA steady state levels for all genes encoding JH biosynthetic enzymes increased sharply during the last 6 hours before adult eclosion. Newly eclosed females had significantly higher mRNA levels. At the same time, JH titers (Shapiro et al., 1986) and spontaneous CA synthetic activity (Li et al., 2003a) are very low in newly eclosed females. Remarkably, the synthesis of JH can be rapidly activated if the CA of the newly eclosed females is stimulated with several JH precursors indicating that the enzymatic activities to process those precursors are already present at the time of adult eclosion.

JH synthesis and ovary development are activated only if nutrients are available

After adult eclosion, changes in mRNA levels for the 11 JH biosynthetic enzymes studied showed common trends that correlated well with CA activity. Transcript levels and JH synthesis increased during the first day after eclosion. This initial rise in JH after eclosion is essential for the female's reproductive maturation; it signals that ecdysis of the adult has finished and reproductive processes should begin (Klowden, 1997). Factors released from the brain induce the CA to synthesize enough JH to activate reproductive maturation only when nutrients are appropriate (Caroci et al., 2004). Decapitation at eclosion that interrupts

the connection between the CA and the brain prevents the increase of JH synthesis, suggesting that the brain plays a key role sensing the nutritional status and activating JH synthesis (Hernandez-Martinez et al., 2007). Teneral reserves are utilized to initiate previtellogenic ovarian development in mosquitoes. Nutritionally deficient females having emerged with low teneral reserves had reduced transcript levels for the genes encoding JH biosynthetic enzymes; small females have lower JH synthesis and reduced previtellogenic development (Caroci et al., 2004). For the female mosquito a tight regulation of JH titers is vital. Allocation of nutritional reserves for ovarian development is life-threatening if resources are not sufficient (Hernandez-Martinez et al., 2007).

The flux of precursors determines JH synthesis rates

The rate of JH synthesis, expressed in our studies as fmol per pair of glands per hour, refers to the number of molecules that transit the synthetic pathway from the time they are taken up by the first enzyme as the initial substrate (Acetyl-CoA) until they are released by the last enzyme as the end product (JH III) (Fig. 1). The rate of JH synthesis is controlled by the rate of flux of isoprenoids in the pathway, which is the outcome of a complex interplay of changes in precursor pools, enzyme levels and external regulators (Fig. 9). It has previously described that the modulation of the flux of isoprenoid precursors throughout the synthetic pathway changes the levels of JH synthesis (Schooley and Baker, 1985). These changes in biosynthetic rates are always initiated by an external alteration which sets into motion changes in some enzymes and fluxes (Cascante et al., 1995). Discussion on “control” or “regulation” of biosynthetic pathways normally focuses on the question of which individual enzymes are controlling the flux in a pathway (Kacser and Burns, 1973). Flux is a systemic property, and questions of its control cannot be answered by looking at the different enzymatic steps in isolation. If we would like to understand how regulators modify JH synthesis, it is important to know their effect on the changes in the levels of all enzymes and precursor pool sizes.

The JH synthetic pathway involves 13 discrete enzymatic steps organized in an obligatory sequence. Each product represents the substrate for the next “downstream” enzyme. Enzymes are connected by metabolite pools that are common to them, for example farnesol is the product of the FPP-pyrophosphatase activity and the substrate for farnesol dehydrogenase. The pools are in fact the *links* in the system interactions, therefore pool concentrations and fluxes (which are flows into and out of pools) are critical variables in JH regulation. Experimental manipulation of individual precursor pool concentrations differentially affected the rates of JH synthesis. The experimental results showed that the effect of addition of the five tested precursors was progressive, namely the closer the precursor to the end of the pathway (JH III), the greater the stimulatory effect observed. The “progressive” effect can be due to many factors; such as the rate of penetration into the CA cells, the rate of degradation of GPP and FPP and subsequent re-phosphorylation, the size of the endogenous pool, etc.

The spontaneous synthesis of JH in CA dissected from both sugar-fed and blood-fed females was always markedly stimulated by the addition of precursors to the medium, demonstrating that the supply of these precursors and not the activity of the last 6 enzymes in the pathway is rate limiting in these glands. Exogenous late precursors were efficiently utilized by the mosquito CA to nullify the effect of rate-limiting factors at earlier steps in JH synthesis.

The system *sensitivity* to changes in the size of a precursor pool indicates the control importance of this enzymatic step in the final flux and can be experimentally tested. If the level of farnesol dehydrogenase is low but still most of the farnesol is present in the enzyme-bound pool, the addition of exogenous precursor might not have a significant effect on the rate of JH synthesis. We observed the contrary; an increase in the farnesol pool size resulted

in up to a 6 fold stimulation in the rates of MF + JH synthesis. This information can be used to understand how *elastic* this particular enzymatic step is in its response to a pool change. In a non-stimulated CA the free-farnesol pool appears to be small and most of the mass of farnesol could be present as part of the pool bound to farnesol dehydrogenase. Increasing the farnesol pool to 40 μ M apparently was enough to saturate the farnesol dehydrogenase; because further increasing the pool size to 200 μ M mostly increased the size of the free farnesol pool and had limited additional stimulatory effect on JH synthesis (Supplementary Fig. 1).

Unfortunately, the effect of increasing the farnesol pool could not be evaluated directly by looking at changes in the farnesol pool, but only by changes in pool sizes for two compounds (MF and JH III) upstream in the pathway. We recently reported a Direct Analysis in Real Time (DART) mass spectrometry approach that allows the rapid simultaneous evaluation of the levels of the last 5 metabolites in the JH pathway (Navare et al., 2010). This technique could be used in the future to evaluate more accurately changes in individual pool sizes. Adding farnesol to the incubation media had also limited stimulatory effect when compared with the addition of FA, suggesting that the levels of farnesol dehydrogenase enzymatic activity might also be low.

Although control of fluxes tends to be distributed among all enzymes in a pathway rather than confined to a single rate-limiting enzyme, the extent of control can differ widely between enzymes of a synthetic pathway (Kacser and Burns, 1973). In glands with low synthetic activity, the flux of isoprenoids might become *sensitive* to minimal thresholds of enzymes with low levels of expression such as acetoacetyl-CoA thiolase, phosphomevalonate kinase and farnesol dehydrogenase. Under these conditions any of these enzymes could become rate limiting or “bottleneck”.

Having the last 6 enzymes of the JH synthetic pathway readily available all the time might be critical for rapid dynamic changes in JH synthesis in response to nutritional changes or blood feeding. Evidence for the dynamic regulation comes from the observation that spontaneous and stimulated rates of CA activity were very different. The addition of FA resulted in a 7.5 and 16.1 fold induction of MF + JH synthesis in gland dissected from sugar fed females at 0h and 72h respectively; as well as a 15 fold increase in CA dissected 24h after a blood meal. It has been postulated that in a synthetic pathway containing numerous enzymes, almost all the enzymes will appear to be “in excess”, in the sense that individual quantities or activities can be considerably reduced without appreciable effect on the flux (Kacser and Burns, 1973). Stimulation with exogenous precursors has been reported for the CA of many insect species and it seems that having an excess of enzymes is common in most insects studied (Feyereisen et al., 1984; Gadot and Applebaum, 1986). In the CA of the cockroach *Diploptera punctata* 3-hydroxy-3-methyl-glutaryl-CoA reductase and 3-hydroxy-3-methyl-glutaryl-CoA synthase activities were not always closely linked to the rate of spontaneous JH synthesis (Feyereisen and Farnsworth, 1987; Couillaud and Feyereisen, 1991). Sutherland and Feyereisen (1992) showed in *D. punctata* that inhibiting the 3-HMG reductase activity by a third has a moderate inhibition of JH synthesis (less than 15%), indicating that this enzyme is in excess and has a low control coefficient on JH synthesis.

There was a coordinated expression of JH biosynthetic enzymes in female pupae and adult mosquito. A comprehensive analysis a several of the JH biosynthetic enzymes has only been done in *B. mori* (Kinjoh, et al., 2007; Ueda et al., 2009); their studies showed that transcripts levels for 8 enzymes of the mevalonic pathway and juvenile hormone acid methyl transferase are expressed in a highly coordinated manner during the 4th and 5th instar larvae as well as in pupae and adult. It seems that evolution has selected mechanisms that regulate

transcription of JH biosynthetic enzymes in insects by affecting the entire synthetic pathway rather than individual pathway components. Positive correlations between JH synthesis and transcripts levels for the JH biosynthetic enzymes suggest that a coordinated regulation in the transcription of the genes encoding JH biosynthetic enzymes is at least partially responsible for the changes of JH biosynthesis in the CA of mosquitoes; that suggest the existence of common transcription factors regulating the enzymes of the entire pathway and stress the need to search for similar regulatory regions in the promoters of the genes encoding all these enzymes.

We are aware that transcript levels might not exactly reflect the levels of enzymatic activities. For example, in the synthesis of cholesterol, the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase is regulated at 3 levels by addition of the inhibitor mevastatin: an 8-fold increase in transcription, a 5-fold increase in translation and a 5-fold decrease in degradation speed; that gives a total increase in 200-fold (Goldstein and Brown, 1990). Nevertheless, we would like to propose that transcripts levels need to surpass a minimal threshold to ensure a significant flux of precursors to sustain a sensible rate of JH synthesis. We observed that when all enzyme concentrations simultaneously increased or decreased by a factor, JH synthesis (or the flux) increased or decreased by a similar factor (Supplementary Fig. 2). The changes in the transcript levels of the enzymes we described seem to be enough to modify the flux of precursors and therefore to modify the rate of JH synthesis.

These variations in enzyme levels during cycles of CA activity are responsible for the *constitutive responses* previously described for the control of JH synthesis (Unnithan et al., 1998). Additional regulatory mechanisms are most likely in place. Flux of metabolites into other synthetic pathways cannot be ruled out, especially in the early steps (mevalonate pathway); other aspects such as compartmentalization of the enzymatic steps might add an additional level of complexity. Experiments performed by Sutherland and Feyereisen (1996) provided strong evidence that *D. punctata* CA glands inhibited with allatostatin-A (AS-A) were prevented from using glucose or amino acids to synthesize JH, but free to utilize acetate, i.e., AS-A was inhibiting steps in the glucose or amino acid (mitochondrial) incorporation pathway but not the acetate (cytoplasmic) incorporation pathway. Results from the *D. punctata*-AS-A model confirm that compartmentalization of the precursor pools and enzymatic steps is important and suggest that a major target of AS-A is either the transport of citrate across the mitochondrial membrane and/or the cleavage of citrate to yield cytoplasmic acetyl-CoA (Sutherland and Feyereisen, 1996). Quantitative proteomics, with the development of new mass spectrometry (MS)-based techniques to detect, identify and quantify minute amounts of proteins, should open the opportunity to fill the current gap of knowledge between transcriptional studies and enzymatic activities (Walther and Mann, 2010; Ning et al., 2011).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Scheme of juvenile hormone biosynthesis

Precursors are in bold and connected by arrows. Enzymes are in italic. Numbers before the enzyme refers to the position in the pathway. Abbreviations for the enzymes are between brackets.

Fig. 2. Transcript levels for the JH biosynthetic enzymes in CA of sugar-fed adult female 24h after eclosion

Enzymes were grouped in three categories: Low abundance (filled bars), moderate abundance (striped bar) and high abundance (empty bar). Numbers before the enzyme refers to the position in the pathway. Enzyme mRNA are expressed as copy number of enzyme mRNA/ 10,000 copies of rpL32 mRNA. Each RT-PCR data point is average of two independent biological replicates of 10 CAs. Enzyme abbreviations are as in Fig. 1.

Fig. 3. Tissue specific expression of JH biosynthetic enzyme mRNAs

All tissues were dissected from three-day old sugar-fed females. CA: corpora allata; BR: brain; FB: fat body; MG: midgut; MT: Malpighian tubules and OV: ovaries. Enzymes mRNAs are expressed as copy number / 10,000 copies of rpL32 mRNA. Each RT-PCR data point is average of two independent biological replicates of 10 tissue samples. Enzyme abbreviations are as in Fig. 1.

Fig. 4. Developmental expression of JH biosynthetic enzyme mRNAs

Expression of JH biosynthetic enzymes mRNAs in CA of pupae 24h (-24) and 6h (-6) before adult eclosion; newly emerged adult female (0) and sugar-fed females 24h (24) and 96h (96) after adult eclosion. Enzyme mRNA are expressed as copy number of enzyme mRNA/10,000 copies of rpl32 mRNA. Each RT-PCR data point is average of three independent biological replicates of 10 CAs. Box shows JH biosynthesis values at the same developmental stages expressed as fmol/pair CA/hour. Enzyme abbreviations are as in Fig. 1.

Fig. 5. Effect of blood-feeding on the expression of JH biosynthetic enzyme mRNAs
 Females were blood-fed and CAs were dissected at 0, 24 and 48 hours after blood feeding. Enzyme mRNAs are expressed as percentages of the maximum value measured for the corresponding enzyme (in most cases at 0h). Bars represent the means $\pm$ S.E.M. of three independent replicates of groups of 10 CA. (unpaired *t*-test; ****P* $\leq$ 0.001). Box shows JH biosynthesis values at the same times, expressed as fmol/pair CA/hour. Enzyme abbreviations are as in Fig. 1.

Fig. 6. Expression of JH biosynthetic enzyme mRNAs and JH synthesis in nutrient-deficient mosquitoes

A) JH biosynthesis was evaluated *in vitro* in CA dissected 24h after emergence from large (L) and small mosquitoes (S). Each bar represents the means $\pm$ SEM of 5-10 independent determinations of individual CA expressed as fmol/pair CA/hour. **B)** Enzyme mRNAs from large mosquitoes (empty bar) and small nutrient-deficient mosquitoes (filled bar) are expressed as percentages of the maximum value measured for the corresponding enzyme. Each RT-PCR data point is average of two independent biological replicates of 10 CA. (unpaired *t*-test; * $P \leq 0.05$, ** $P \leq 0.01$). Enzyme numbers are as in Fig. 1.

Fig. 7. Stimulation of MF + JH synthesis by exogenous addition of precursors

JH biosynthesis was evaluated in CA that were incubated *in vitro* in culture medium alone (control) or with the addition of 200 μ M of individual precursors: geranyl-diphosphate (GPP), farnesyl-diphosphate (FPP), farnesol (FOL), farnesal (FAL) and farnesoic acid (FA). **A)** CA dissected from newly emerged females (0h); **B)** CA dissected from sugar-fed females 72h after eclosion; and **C)** CA dissected from females 24 h after blood feeding. MF: methyl farnesate, JH: Juvenile hormone. Bars represent the means $\pm$ S.E.M. of at least three independent replicates of incubations of groups of two CA. Values labeled with the different letters denote significant differences (unpaired *t*-test $P \leq 0.05$).

Fig. 8. Synthesis of JH by CA dissected from female pupae and newly eclosed females
 JH biosynthesis was evaluated *in vitro* in CA dissected from female pupae 24h (-24), 6h (-6) and 2h (-2) before adult eclosion. 0 = newly emerged adult female. CA were incubated in control medium (grey bar) or stimulated with 200 μ M of FA (black bar). Bars represent the means $\pm$ S.E.M. of at least three independent replicates of incubations of groups of two CA. (unpaired *t*-test; ****P* $\leq$ 0.001).

Fig. 9. A schematic representation of our working model for the control of the flux of precursors in the JH biosynthetic pathway

Precursor pools (Acetyl-CoA, S2, etc) are represented by circles and connected by arrows. E: Enzymes are followed by a number that refers to the position in the pathway (enzyme numbers are as in Fig. 1). Regulatory factors are affecting both precursor pool sizes and enzymatic activities (e.g., AS-C: allatostatin C).