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Metabolic Analysis Reveals Changes in the Mevalonate and Juvenile Hormone Synthesis Pathways Linked to the Mosquito Reproductive Physiology

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Abstract

Juvenile hormone (JH) regulates reproductive maturation in insects; therefore interruption of JH biosynthesis has been considered as a strategy for the development of target-specific insecticides. The *corpora allata* (CA) from mosquitoes is highly specialized to supply variable levels of JH, which are linked to ovarian developmental stages and influenced by nutritional signals. However, very little is known about how changes in JH synthesis relate to reproductive physiology and how JH synthesis regulation is translated into changes in the CA machinery. With the advent of new methods that facilitate the analysis of transcripts, enzymes and metabolites in the minuscule CA, we were able to provide comprehensive descriptions of the mevalonic (MVA) and JH synthesis pathways by integrating information on changes in the basic components of those pathways. Our results revealed remarkable dynamic changes in JH synthesis and exposed part of a complex mechanism that regulates CA activity. Principal component (PC) analyses validated that both pathways (MVAP and JH-branch) are transcriptionally co-regulated as a single unit, and catalytic activities for the enzymes of the MVAP and JH-branch also changed in a coordinate fashion. Metabolite studies showed that global fluctuations in the intermediate pool sizes in the MVAP and JH-branch were often inversely related. PC analyses suggest that in female mosquitoes, there are at least 4 developmental switches that alter JH synthesis by modulating the flux at distinctive points in both pathways.

Keywords

Juvenile hormone; mosquito; biosynthesis; *corpora allata*; metabolomics; *Aedes*

1. Introduction

Juvenile hormone (JH) controls insect female reproductive physiology (Goodman and Cusson, 2012). The *corpora allata* (CA) interfaces between the brain and reproductive

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tissues, producing JH at rates proportional to female nutrient reserves (Clifton and Noriega, 2011; Clifton and Noriega, 2012; Perez-Hedo et al., 2014). In *Aedes aegypti* mosquitoes, four stages can be defined in the development of the ovaries: females emerge with 40 μm immature previtellogenic follicles that grow into 100 μm mature previtellogenic oocytes in the next 24-48 h. Oocytes remain in a dynamic “state of arrest”, and will enter vitellogenesis after a blood meal (Hagedorn, 1974; Klowden, 1997) (Fig. 1). JH directly controls nutrient allocation into the ovaries in the previtellogenic phases, and indirectly influences the fate of vitellogenic follicles after a blood meal (Clifton and Noriega, 2011; Clifton and Noriega, 2012; Noriega, 2004).

JH is synthesized through the mevalonate pathway (MVAP), an ancient metabolic pathway present in the three domains of life (Lombard and Moreira, 2010), responsible for the synthesis of many essential molecules required for cell signaling, membrane integrity, energy homeostasis, protein prenylation and glycosylation (Goldstein and Brown, 1990; Holstein and Hohl, 2004; McTaggart, 2006; Vranova et al., 2013). The MVAP consists of a main trunk followed by sub-branches that generate a diverse range of biomolecules. Insects lack the cholesterol-synthetic branch present in vertebrates (Belles et al., 2005), but in the CA the MVAP branches into the synthesis of JH. The main trunk of the MVAP consists of multiple enzymatic steps through which acetyl-CoA is gradually transformed into the 5-carbon compound isopentenyl-pyrophosphate (IPP), and later on to the 15-carbon molecule farnesyl-pyrophosphate (FPP) (Klowden, 1997). In the CA of mosquitoes, FPP is sequentially transformed to farnesol (FOL), farnesal (FAL), farnesoic acid (FA), methyl farnesoate (MF) and JH III (hereafter, JH) (Nouzova et al., 2011) (Fig. 2).

Regulation of CA activity occurs at three different levels (Applebaum et al., 1991; Unnithan and Feyereisen, 1995): developmental maturation to synthesize JH which normally proceeds in conjunction with developmental changes, such as the transition from pupa to adult (Goodman and Cusson, 2012), long-term modulation such as variations in enzyme levels during cycles of CA activity (Applebaum et al., 1991; Nouzova et al., 2011) and short term responses such as the inhibition of JH synthesis by allatostatins (Unnithan and Feyereisen, 1995; Li et al., 2003a). In all these instances, the rate of JH biosynthesis 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 (Li et al., 2004; Li et al., 2003a, 2003b; Li et al., 2006; Nouzova et al., 2011; Nyati et al., 2013; Rivera-Perez et al., 2013). A coordinated expression of most JH biosynthetic enzymes has been previously described in mosquitoes and silkworm (Kinjoh et al., 2007; Nouzova et al., 2011; Ueda et al., 2009). Increases or decreases in transcript levels for all the enzymes are generally concurrent with increases or decreases in JH synthesis (Kinjoh et al., 2007; Nouzova et al., 2011; Rivera-Perez et al., 2013; Ueda et al., 2009). Previous studies have proposed that regulation of JH synthesis occurs upstream of the acetyl-CoA pool (Sutherland and Feyereisen, 1996), as well as by rate limiting bottlenecks at different enzymatic steps in the pathway, including the activities of HMG-CoA reductase (Kramer and Law, 1980; Monger and Law, 1982), farnesol dehydrogenase (Mayoral et al., 2009), farnesal dehydrogenase (Rivera-Perez et al., 2013) or juvenile hormone acid methyltransferase (Shinoda and Itoyama, 2003; Minakuchi et al., 2008; Sheng et al., 2008). Studies in several insects

demonstrated that exogenous additions of precursors stimulate JH biosynthesis in a developmental-dependent mode (Feyereisen et al., 1981a; Feyereisen et al., 1981b; Nouzova et al., 2011), indicating that as a general rule, enzymes of the JH pathway are in excess. However, studies evaluating how changes in metabolite levels and enzyme activities influence the flux of precursors on the JH synthesis pathway and how these changes relate to the different stages of reproductive physiology are lacking. With the development of new strategies that facilitate the analysis of transcripts, enzymatic activities and metabolites in the minuscule CA of insects (Nouzova et al., 2011; Nyati et al., 2013; Rivera-Perez et al., 2012; Rivera-Perez et al., 2013); it is now possible to provide comprehensive descriptions of the MVA and JH pathways by integrating information on changes in the three basic components of those pathways. In the present studies, we integrated the analysis of changes in mRNA levels for the 13 enzymes of the MVA and JH-branch pathways at 10 different developmental or physiological states during pupae and adult female mosquito, with studies of the catalytic activities of 8 of the enzymes at 5 critical times in pupae and adult female and finally, we measured changes in all the intermediates of the MVAP from acetyl-CoA to FPP, as well as all metabolites of the JH-branch during 9 distinct physiologically-relevant time points in female pupal and adult stages. We studied the complex mechanisms that control CA activity and revealed that the MVAP is well adapted for delivering adjustable levels of isoprenoid precursors to the JH synthesis metabolic branch, with both pathways playing key roles on the regulation of JH titers and reproduction in insects.

2. Materials and Methods

2.1. Insects

A. aegypti from the Rockefeller strain were reared as previously described (Nouzova et al., 2011).

2.2. JH synthesis assays

JH III quantification by High Performance Liquid Chromatography coupled to a Fluorescent Detector (HPLC-FD), and mass spectrometry characterization of metabolites were done as described by Rivera-Perez et al. (2012).

2.3. Analysis of CA metabolites

MVAP and JH-branch metabolites were extracted from CA samples, labelled with fluorescent tags and quantified by HPLC-FD as previously described (Rivera-Perez et al., 2012). Mevalonate, mevalonate-5P, mevalonate-5PP and FA were tagged with AABD-SH. Acetyl-CoA, acetoacetyl-CoA, HMG-CoA and farnesal were tagged with DBD-H. Isoprenol, geraniol and farnesol were labelled with BD-COCl. For the quantification of IPP, GPP and FPP, compounds were first converted to alcohols by treating CA extracts with alkaline phosphatase 2 h at 30 °C (270 µl 0.1 M Tris-HCl pH 8.0, 10 µl 0.25 M MgCl₂ and 0.5 µl of alkaline phosphatase [2 U/mg]). The efficiency of conversion of phosphates into alcohols was 94%. MF was quantified after converting it into FA by treatment with H₂SO₄ (150 µl 8 M H₂SO₄, sonicated 3 min and incubated at 70 °C for 10 min). The endogenous values of FA were measured and subtracted. The efficiency of conversion of MF into FA was 88%. Efficiencies of conversions were tested in triplicate experiments using 100 fmols

of standards. Similar efficiencies were obtained when biological samples were used. Standard curves for all metabolites were constructed (Fig. S1). JH III acid (JHA) was prepared following the protocol described by Goodman and Adams (1984) and its identity confirmed by ESI-MS. JHA was tagged with AABD-SH. JHA was readily detected in hemolymph samples but not found in CA extracts.

2.4. Enzymatic activities

For each assay, 10 glands were dissected, homogenized and analyzed for particular enzymatic activities using specific buffers (Table S1). The reaction products were analyzed by HPLC-FD.

2.5. Statistical analysis

Data were analyzed for statistical differences using GraphPad Prism (San Diego). The results were expressed as means $\pm$ S.E.M. Significant differences ($p < 0.05$) were determined with one-way ANOVA followed by Tukey's test. Principal component analyses (PCA) were performed using the software R Studio version 0.97.551.

3. Results

3.1. There is a vast and short lived increase of JH synthesis 12 h after adult emergence

In vitro biosynthesis of JH by CA was measured at different physiological states in pupae and adult females using a recently developed HPLC-FD protocol (Rivera-Perez et al., 2012). Changes in JH synthesis were dynamic and revealed a complex regulation of CA activity linked to the reproductive physiology of the mosquito (Fig. 1). Our present detailed studies, performed at a higher temporal resolution than earlier investigations of the mosquito CA, exposed a previously undetected peak of maximum JH synthesis 12 h after adult emergence. This sharp increase of JH synthesis conveys information on teneral nutritional reserves and provides a signal for the previtellogenic maturation of the ovaries (Noriega, 2004). The process of "activation" of CA was very fast and short lived. Juvenile hormone synthesis increased from 10 fmol/h/CA to almost 100 fmol/h/CA in 2 h, and decreased to less than 40 fmol/h/CA in the next 2 h, remaining at this relatively high and constant rate until 24 h after emergence. However, in these mosquitoes that fed a restricted diet (3% sugar), by 48-72 h JH synthesis decreased to a low 12 fmol/h/CA triggering the resorption of ovarian follicles (Clifton and Noriega, 2011). Finally, at 24 h after blood feeding there was a significant decrease in JH synthesis that is critical for the completion of the vitellogenic development of the first batch of eggs and the activation of the previtellogenic development of follicles for the second gonotrophic cycle (Klowden, 1997; Li et al., 2003a).

3.2. Transcripts levels and enzyme capacities are coordinated in the CA

We constructed a heat map from previous transcriptional studies of the genes encoding the 13 enzymes included in the MVA and JH pathways at 10 different developmental or physiological stages during pupae and adult female mosquito (Nouzova et al., 2011; Nyati et al., 2013; Rivera-Perez et al., 2013) (Fig. 3). There is a coordinated expression of the 13 genes, with relative high expression in sugar-fed and blood-fed females. Principal component analysis (PCA) provided an unbiased determination of which transcripts vary the

most across groups, and validated that mRNA levels correlated at any individual time point analyzed, showing that one single component was enough to explain the variation among all transcripts (73.61%) ($p < 0.0074$) (Fig. S2). On the other hand, PCA of the multiple developmental times surveyed for a particular transcript clearly discriminated the active CA of adult female (PC1: 79.94%) from the inactive CA of the pupae (PC2: 11.23%) ($p < 0.00060$) (Fig. S3). All together these results suggest that increases or decreases in transcript levels for all the enzymes are generally concurrent with increases or decreases in JH synthesis, as well as that both pathways (MVA and JH branch) are transcriptionally co-regulated as a single unit.

We developed “*in vitro*” enzymatic assays to measure the capacity of the CA to metabolize MVAP and JH-branch intermediates. With the inclusion of appropriate cofactors (Table S1), CA extracts were capable of *de novo* synthesis of MVAP or JH-branch metabolites from precursors. Eight selected enzymes were evaluated using CA homogenates. HMG-CoA synthase and JHA methyl transferase showed the highest activities (in the nanomolar range), while the activities of the additional six enzymes were in the femtomolar range. The enzyme capacities for the five developmental times assessed for each enzymatic activity are shown in Fig. S4. A heat map representation revealed dynamic changes in the activities of the enzymes of the MVAP and JH-branch during the mosquito gonotrophic cycle (Fig. 4). Changes in enzymatic activities correlated well with the changes in transcript levels. Catalytic activities for the enzymes of the MVAP and JH-branch largely changed in a coordinate fashion. PCA of the enzymatic activities for a particular time point revealed that activities for all the enzymes were highly correlated, and one single component was enough to explain the variation observed (86.29%) ($p < 5.3 \times 10^{-61}$) (Fig. S5). PCA of the multiple developmental times surveyed for a particular enzymatic activity clearly discriminated two groups, the first principal component (PC1) accounted for 48.58% of the total variation, and included CA with reduced biosynthetic activities from -24 h pupae, 0 h and 24 h after blood feeding. On the other hand, the second principal component (PC2) covered glands with higher biosynthetic activities. The latter component included CA from 12 and 24 h sugar-fed females and explained 34.34% of total variation ($p < 0.0074$) (Fig. S6).

3.3. Global fluctuations in the metabolite pool sizes in the MVA and JH pathway are inversely related

A HPLC-FD protocol allowed the detection and quantification of all intermediates of the MVAP, from Acetyl-CoA to FPP, as well as all metabolites of the JH-branch. This method was used to analyze isoprenoid intermediate profiles during nine distinct physiologically-relevant time points in female pupae and adults (Fig. 5). These results are summarized in a heat map that reveals dynamic changes in the precursor pool sizes in the MVAP and JH-branch during the mosquito gonotrophic cycle (Fig. 6). Analysis of the pathways changes in the heat map were performed vertically, indicating the changes of all metabolites for a particular time or physiological condition. Vertical analyses revealed remarkable modifications in the fluxes in the MVAP and JH-branch during the first 24 h after emergence (Fig. 7). A 10-fold increase in JH synthesis rates by 12 h caused a striking depletion of all MVAP metabolite pools, with some of them replenished by 24 h. Horizontal analyses of the heat map reveal the changes of one metabolite under several different

physiological conditions, uncovering fluctuations in levels of a particular intermediate which expose potential changes in specific enzymatic capacities. PCA of the multiple metabolites surveyed for a particular time point discriminated 2 groups: the first component (PC1) correlated positively with the precursors of the JH-branch (25.45% of total variation), while the PC2 (23.36% of total variation), correlated positively with the precursors of the MVAP ($p < 0.0024$) (Fig. S7). When MVAP metabolite concentrations were high, late JH precursor levels were reduced and vice versa. As a general trend, increases in the flux into JH III were linked to increases in the size of precursor pools in the JH-branch and associated with decreases in the pools in the MVAP.

When the metabolic profiles of the nine time points studied were decomposed by PCA, the first two PCs accounted for 41% of the variation in the data ($p < 0.00065$). The developmental stages comprising the first principal component (PC1) were 48, 72 and 96 h sugar-fed females and accounted for 24.91% of the total variation in the experiment. Developmental stages included in the second principal component (PC2) were 0, 12 and 24 h sugar-fed females and accounted for 16.52% of total variation. Plotting the PCA yielded a cluster of four groups of developmental stages physiologically distinct from one another: group 1: -24 h pupae (inactive CA), group 2: 0, 12 and 24 h sugar-fed females (active CA), group 3: 48, 72 and 96 h sugar-fed females (modulated CA) and group 4: -6 h pupae and 24 h blood-fed female (suppressed CA) (Fig. 8). The presence of MF and absence of JHA in CA extracts confirms that in mosquitoes the pathway follows the sequence FA-MF-JH III and not FA-JHA-JH III.

4. Discussion

4.1. Dynamic changes in JH synthesis reveal a complex regulation of CA activity linked to the reproductive physiology of the mosquito

Nutrient availability is central to female mosquito reproductive physiology (Noriega, 2004). Three sources of nutrients provide energy and building blocks for the three distinct phases of ovarian development in *A. aegypti*. Reserves acquired during preimaginal stages are partially consumed during previtellogenesis, nectar-feeding adds nutrients during the ovarian resting stage, and later a blood meal triggers vitellogenesis (Briegel, 1985; Briegel, 1990; Klowden, 1997; Noriega, 2004; Zhou et al., 2004a, 2004b) (Fig. 1). JH directly controls nutrient allocation into the ovaries in the previtellogenesis and ovarian resting stage phases, and indirectly influences the fate of follicles during the vitellogenesis stage after a blood meal (Clifton and Noriega, 2011; Clifton and Noriega, 2012).

Analysis of the time-dependent metabolite changes delimited four distinct nutritional-dependent CA physiological conditions that we named: inactive, active, modulated and suppressed CA. Flux control is most likely different for each of these four CA phases, with different enzymes targeted for regulation. Indeed, our results indicate that at specific times different enzymatic activities are modified and these modifications in the activity of particular enzymes are reflected by changes in the corresponding pools of isoprenoid intermediates. We have previously described that transcript levels for most of the JH biosynthetic enzymes are very low in early pupae (Nouzova et al., 2011). Consequently JH synthesis rates were undetectable (below 0.5 fmol/h/CA) in pupae 24 and 12 h before adult

eclosion (Fig 1). However, in the last 6-8 hours before adult emergence transcript levels for the biosynthetic enzymes commence to rise. The pupal CA thus becomes “competent” and starts to synthesize JH (Nouzova et al., 2011). Although the CA of the newly emerged female is fully competent, for the next 10-11 h it synthesizes relatively low levels of JH (10 fmol/h/CA) (Fig. 1). Decapitation during these first 12 h of imaginal life prevents increases of JH synthesis, suggesting that the brain plays a key role sensing the nutritional status and stimulating CA activity (Hernandez-Martinez et al., 2007). Only when preimaginal reserves are appropriate would the brain command the CA to synthesize enough JH to activate previtellogenic reproductive maturation (Caroci et al., 2004; Hagedorn et al., 1977). This activation seems to be a developmentally regulated “all-or-nothing” process that requires a general increase in flux efficiency and therefore concurs with increases of enzymatic activities in the MVAP and JH-branch. Later, during the ovarian resting stage, female mosquitoes are capable of fine-tuning of the MVAP and JH-branch fluxes at rates that are proportional to the supply of acetyl units acquired by sugar-meal (Perez-Hedo et al., 2014). In our mosquitoes that were fed a restricted diet, JH synthesis was significantly reduced by 48 h (Fig 1). Furthermore, intermediate concentrations in the JH-branch were progressively diminished and conversely MVAP metabolite pools were replenished. By 96 h the JH-dependent previtellogenic maturation of the ovaries has been completed and changes in metabolite pool sizes in the MVA and JH pathways reflected a broad metabolic switch to the resting stage. Accordingly, females at this stage need to adjust their reproductive output by follicular resorption based on the reserve levels supplemented by sugar-feeding (Clifton and Noriega, 2011; Clifton and Noriega, 2012; Perez-Hedo et al., 2014). Finally, at 24 h after blood feeding there was an active suppression of CA activity with significant decreases in enzyme transcripts and activities (Li et al., 2003a; Nouzova et al., 2011). After blood feeding major reductions in the sizes of late precursor pools in the JH branch (FA and MF) assured a quick and effective decrease in JH synthesis, critical for the appropriate completion of oogenesis (Klowden, 1997). These studies lead us to hypothesize that regulation of the CA activity is quite different in the previtellogenesis, ovarian resting stage and vitellogenesis phases, and consequently the strategies for flux regulation are unique in each case.

4.2. An integrated view of flux control regulation in the MVAP and JH-branch

Effective physiological control of biosynthetic pathways is often shown to involve simultaneous multisite modulation through action on a number of enzymes (Fell and Thomas, 1995; Kacser and Burns, 1973). Improved flux efficiency is particularly important for multi-enzyme pathways with sub-branches such as the MVAP. Although the mosquito CA cells are highly specialized to synthesize JH, the FPP produced by the MVAP can also be shunted to other metabolic branches for the synthesis of critical molecules such as ubiquinone, dolichol or prenylated proteins (Belles et al., 2005). Branch point regulation is an important mechanism controlling carbon flow in the MVAP. Sesquiterpene cyclases and squalene synthetases regulate carbon flux into different branches in plant cells (Vogeli and Chappell, 1988), while squalene synthase and prenyltransferases are important branch point enzymes in mammalian systems, where the higher affinity of prenyltransferases for FPP prioritize the shunting into this branch (Faust et al., 1979).

Consequently, understanding flux control in the MVAP and JH-branch requires answering some of the following questions: 1) Is the activity of both pathways always coordinated? 2) Is the flux into JH specifically regulated at the level of the MVAP, the JH-branch, or at both stages? 3) From a functional point of view, do we have a single JH pathway or a trunk (MVAP) coupled with a JH-branch?

What do the study of CA transcript, enzyme activities and metabolites tell us about the coordination of MVAP and JH-branch activities? On one hand, PCA analyses suggest that both pathways (MVAP and JH-branch) are transcriptionally co-regulated as a single unit, and catalytic activities for the enzymes of the MVAP and JH-branch also changed in a coordinate fashion in the “active” and “inactive” CA. On the other hand, analysis of intermediate pools revealed a different profile. PCA analysis indicated that global changes in the precursor pools in the MVAP and JH-branch are not functioning as a unit, but behave inversely. Remarkably, when the CA was very active MVAP intermediate pools were completely depleted, implying that most of MVAP precursors were channeled into the JH-branch. These results suggest that during the peak of synthesis, the activity of the enzymes on the JH-branch are controlling the flux in the synthesis of JH, indicating that although CA cells are using the MVAP to synthesize additional metabolites that are important for various biological processes, when necessary, the production of JH supersedes the trafficking of FPP into other branches of the MVAP.

Different strategies could be used by the CA to balance pathway fluxes, including modulating the expression levels of individual enzymes through transcriptional regulation (Kinjoh et al., 2007; Nouzova et al., 2011) and improving turnover activities of rate-limiting enzymes through post-transcriptional control or substrate channeling (Lombard and Moreira, 2010; Percival and Zhang, 2011; Rauthan and Pilon, 2011). Studies in plants have emphasized the importance of compartmentalization in the control of terpene biosynthesis (Wu et al., 2006), results that are not consistent with the traditional view of isoprenoid metabolism occurring in a homogeneous environment with intermediates mixing freely and accessible to successive or competing enzymes. Domains of the endoplasmic reticulum or some other cytoskeletal structure must be dedicated to the assembly of unique metabolons, and genes coding for enzymes making up these specific metabolons must have similar transcriptional networks to coordinate expression of the metabolic unit (Newman and Chappell, 1999).

Pathway efficiency increases if metabolites are synthesized at lower enzyme expression levels. Thus, overall flux is high while limiting the accumulation of intermediates and without introducing excessive energy requirements (Fendt et al., 2010). Efficient adjustments of JH synthesis could be hypothetically achieved through a minimization of metabolite concentration at a given flux. In this case, *in vivo* substrate metabolite concentrations are much lower than the K_m of the enzymes that catalyze their reactions (Fendt et al., 2010). Consequently, catalytic enzyme capacities are in excess and substrate metabolite concentrations limit the reaction rates. This paradigm is supported by numerous experiments showing that the spontaneous rate of JH synthesis is always markedly lower than the maximum inducible rate observed when CA are stimulated with precursors or activators (Tobe and Pratt., 1975; 1976). Addition of precursors in the media where CA are

incubated could increase the rate of JH synthesis by up to 20 fold (Gadot and Applebaum, 1986; Couillaud et al., 1988; Feyereisen and Farnsworth, 1987; Piulachs and Couillaud, 1992; Nouzova et al., 2011).

In the CA some MVAP precursor pools are small and might be highly controlled, with regulation imposed by feedback mechanisms mediated by FPP operating in the downstream portions of the pathway; for instance, the increase in mevalonate at 48 h might be the result of a negative feedback of late MVAP precursors (GPP, FPP) on the activity of phosphomevalonate kinase, as previously described for terpene homeostasis in mammals (Hinson et al., 1997). In addition, the large accumulation of acetyl-CoA at 96 h suggests a change in CA physiology to adjust to the nutrient condition in the resting stage. The interpretation of CA metabolite pool changes is inherently challenging. The increase of an intermediate pool size could be the result of a rise in the capacity of the enzyme producing the precursor, as well as a decrease in the activity of the enzyme consuming the precursor; for example the accumulation of FAL in the CA of blood-fed females is caused by a decrease in farnesal dehydrogenase activity, and it is reflected by reductions in the pools of FA and MF (Rivera-Perez et al., 2013). In addition, there are examples of a reversal of the flux in the MVAP and its branches. Kinase activities converting FOL back into FPP were demonstrated in *Nicotiana tabacum* cell cultures (Thai et al., 1999), while a reductase activity that converts FAL back into FOL was previously described in human fibroblasts (Rizzo and Craft, 2000), as well as in the CA of mosquitoes (Rivera-Perez et al., 2013). Although rate limiting bottlenecks have been proposed at single specific steps in both the MVAP and JH-branch in the CA of different insects (Kramer and Law, 1980; Mayoral et al., 2009; Monger and Law, 1982; Sheng et al., 2008; Sutherland and Feyereisen, 1996), our results suggest that there are multiple regulatory points and they change in different physiological stages. Further studies will be necessary to unveil what are the enzymes that restrict the flux into JH III at specific physiological conditions. Analysis of the heat maps can help to generate hypothesis that can be tested using the HPLC-FD tools developed. In addition, comprehensive CA transcript analysis by RNAseq might reveal common types of regulation for some of the enzymes and help to identify conserved response elements in the various genes.

In summary, our studies suggest that the MVAP is well adapted for delivering adjustable levels of isoprenoid precursors to the JH synthesis branch playing a key role in the nutrient-dependent regulation of JH titers. We identified four physiological phases, defined as inactive, active, modulated and suppressed CA, where JH synthesis is altered by gating the flux at distinctive points in both pathways. The present analysis is already teaching us that metabolic signatures can highlight potential regulatory points in the JH synthesis pathway that may be target for mosquito control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Changes in juvenile hormone (JH) synthesis reveal a complex regulation of CA activity.
- Adjustments in specific enzymes alter the flux in the mevalonate (MVA) and JH pathways.
- Enzymes in the MVA and JH synthesis pathways are transcriptionally co-regulated as a unit.
- Changes in intermediate pool sizes in the MVA and JH pathway are inversely related.
- Metabolic signatures highlight bottlenecks in the JH synthesis pathway.

Fig. 1. JH biosynthesis and ovarian development in female mosquitoes

Top panel: representative images of the progression of ovary development from emergence to 24 h after blood feeding. The inset in 96 h shows the lipid content of follicles from females fed 3% sugar (top) and 20% sugar (bottom). Colors for the panels match colors for the CA physiological phases described in the bottom panel. Bottom panel: JH biosynthesis by CA dissected from pupa, sugar-fed and blood-fed adult females. Hours represent times before (pupa) and after adult emergence (sugar-fed), or after blood feeding (BF). Y axis: JH biosynthesis expressed as fmol/h. Bars represent the means $\pm$ SEM of three independent replicates of three groups of 3 CA. Colors represent the four distinct CA physiological phases identified: inactive CA (blue), active CA (black), modulated CA (green) and suppressed CA (red).

Fig. 2. Mevalonic pathway and JH synthesis branch metabolites and enzymes

Enzyme names abbreviations: Acetoacetyl-CoA thiolase: Thiolase; HMGCoA synthase: HMGS; HMG-CoA reductase: HMGR; Mevalonate kinase: MK; Phosphomevalonate kinase: PMK; Diphosphomevalonate decarboxylase: PPM-Dec; IPP isomerase: IPPI; FPP synthase: FPPS; Farnesyl pyrophosphatase: FPPase; Farnesol dehydrogenase: FOL-SDR; Farnesal dehydrogenase: FALDH; Juvenile hormone acid methyltransferase: JHAMT and Methyl farnesoate epoxidase: EPOX.

Fig. 3. Heat map representation of JH biosynthetic enzyme mRNAs levels in CA extracts
Top: physiological stages are described as hours relative to adult emergence (0h) or blood feeding (BF). Right side: enzyme names as described in Fig. 2. Colors from white to red represent increases of transcript levels as percentages of the maximum value.

Fig. 4. Heat map representation of JH biosynthetic enzyme activities in CA extracts

Physiological stages are described as hours relative to adult emergence (0h) or blood feeding (BF). Colors from white to red represent increases of activity as percentages of the maximum value. Absolute values are shown in the Fig. S4. Enzymes names are as described in Fig. 2.

Fig. 5. Changes in MVAP and JH-branch metabolite pool sizes in CA
MVAP and JH-branch metabolite profiles during nine distinct physiologically-relevant time points in female pupae and adults. Bars represent the means $\pm$ SEM of three independent replicates of three groups of 3 CA. Physiological stages are described as hours relative to adult emergence (0h) or blood feeding (BF). Significant differences ($p < 0.05$) were determined with one-way ANOVA followed by Tukey's test.

Fig. 6. Heat map representation of MVAP and JH-branch metabolite pool sizes in CA
 Top: physiological stages are described as hours relative to adult emergence (0h) or blood feeding (BF). Right side: metabolites. Colors from white to red represent increases of metabolite pool sizes as percentages of the maximum value. Absolute values are shown in the Fig. 5.

Fig. 7. Changes in CA metabolic profiles during the first 24 h after adult emergence
Physiological stages are described as hours relative to emergence (0h). Bars represent the means $\pm$ SEM of two independent replicates of three groups of 3 CA.

Fig. 8. Principal Component Analysis of developmental changes in CA metabolite pool sizes
 Colors represent developmental or physiological stages: pupae (blue), sugar-fed females (black) and blood-fed (red). Numbers are hours relative to adult emergence (0h) or blood feeding (BF). Groups significantly different from each other ($p < 0.05$) are clustered inside circles. Round dot line: inactive CA (-24h). Solid line: active CA (0h, 12h and 24h). Long dash dot: modulated CA (48h, 72h and 96h). Dash line: suppressed CA (-6h and 24BF).

Fig. 9. Schematic representation of the four CA physiological conditions in reproductive female mosquitoes

The four CA phases and corresponding stages are: inactive (early pupae), active (12-24 h sugar-fed females), modulated (48-96 h sugar-fed females) and suppressed (24 h blood-fed females). JH synthesis: the color and direction of the arrows reflect: low levels (arrows down and red), high levels (arrows up and black) or variable levels (arrow up and down). Changes in transcripts, activities and metabolites: the direction of the arrows reflects the trend of the changes (increases: up and decreases: down). The size of the arrow reflects the magnitude of the changes. Limiting factor: hypothetical critical factor limiting CA activity.