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20-hydroxyecdysone stimulation of juvenile hormone biosynthesis by the mosquito *corpora allata*

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Abstract

Juvenile hormone III (JH) is synthesized by the *corpora allata* (CA) and plays a key role in mosquito development and reproduction. JH titer decreases in the last instar larvae allowing pupation and metamorphosis to progress. As the anti-metamorphic role of JH comes to an end, the CA of the late pupa (or pharate adult) becomes again “competent” to synthesize JH, which plays an essential role orchestrating reproductive maturation. 20-hydroxyecdysone (20E) prepares the pupae for ecdysis, and would be an ideal candidate to direct a developmental program in the CA of the pharate adult mosquito. In this study, we provide evidence that 20E acts as an age-linked hormonal signal, directing CA activation in the mosquito pupae. Stimulation of the inactive brain-*corpora allata*-*corpora cardiaca* complex (Br-CA-CC) of the early pupa (24 h before adult eclosion or –24 h) *in vitro* with 20E resulted in a remarkable increase in JH biosynthesis, as well as increase in the activity of juvenile hormone acid methyltransferase (JHAMT). Addition of methyl farnesoate but not farnesoic acid also stimulated JH synthesis by the Br-CA-CC of the –24 h pupae, proving that epoxidase activity is present, but not JHAMT activity. Separation of the CA-CC complex from the brain (denervation) in the –24 h pupae also activated JH synthesis. Our results suggest that an increase in 20E titer might override an inhibitory effect of the brain on JH synthesis, phenocopying denervation. All together these findings provide compelling evidence that 20E acts as a developmental signal that ensures proper reactivation of JH synthesis in the mosquito pupae.

Graphical Abstract

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AUTHOR CONTRIBUTIONS

MA, MN, CRP and FGN developed the concepts and approaches. MA, MN and CRP performed experiments. MA, MN, CRP and FGN did data analysis. MA, MN, CRP and FGN prepared and edited the manuscript prior to submission.

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Keywords

Juvenile hormone; mosquito; biosynthesis; *corpora allata*; 20-hydroxyecdysone

INTRODUCTION

Periodic pulses of ecdysteroids (20E) and juvenile hormone (JH) orchestrate the timing of organism-wide developmental transitions in insects (Yamanaka et al., 2013; Riddiford, 2012). Juvenile hormone (JH) delays metamorphosis until larvae have attained an appropriate stage and size. At that point, a drop in JH secretion permits a metamorphic molt (Riddiford, 2012; Smykal et al., 2014). JHs are synthesized by the *corpora allata* (CA), a pair of endocrine glands with neural connections to the brain (Tobe and Stay, 1985). In *Aedes aegypti* mosquitoes the CA is inactive for most of the duration of the pupal stage (Nouzova et al., 2011; Rivera-Perez et al., 2014). As the anti-metamorphic role of JH comes to an end, the CA of the late pupa (or pharate adult) is reactivated and becomes “competent” to synthesize JH, which plays an essential role in coordinating reproductive maturation. (Klowden, 1997).

The activation of the CA after metamorphosis in mosquitoes is a fascinating and tightly regulated developmental process. The initiation of JH synthesis in pharate adult mosquito is partitioned into two temporally discrete steps: first a process of maturation during the last 6–8 hours of pupal stage prepares the CA gland to start synthesizing low amounts of JH (Rivera-Perez et al., 2014). At eclosion, a 2-fold increase in JH synthesis brings JH synthetic rates to a value of 15–20 fmol/CA/h, a threshold level required for the maximal expression of an early-posteclosion JH-dependent gene cluster (Zou et al., 2013; Riddiford, 2013). The two-step activation guarantees that a proper rise of JH synthesis concurs with adult eclosion. So we can define the activation of JH biosynthesis as a process “associated” to ecdysis, and its correct timing is critical. Factors involved in the initiation and scheduling of the ecdysis sequence, such as 20-hydroxyecdysone (20E) and ecdysis triggering hormone (ETH), are ideal to time CA activation with molt (Žit' an and Adams, 2012; Adams et al., 2013). We have previously defined the critical role of ETH acting as an allatotrophic regulator of JH biosynthesis (Areiza et al., 2014).

Circulating levels of 20E in female mosquitoes increase in the last hours before adult eclosion (Supplemental Fig. 1), corresponding well with increases of synthesis and titer of JH (Margram et al., 2006; Rivera-Perez et al., 2014; Hernandez-Martinez et al., 2015); as well as increases of transcripts and activity of juvenile hormone acid methyl transferase (JHAMT), a key enzyme that transforms farnesoic acid (FA) into methyl farnesoate (MF) (Areiza et al., 2014). To further test the notion that 20E mediates the temporal control of CA maturation, we assayed its ability to trigger the premature activation of the CA of early pupae (24 h before adult eclosion or – 24 h). Indeed, *in vitro* stimulation with 20E of brain-*corpora allata-corpora cardiaca* complexes (Br-CA-CC) dissected from early pupae resulted in a significant increase of JH synthesis.

We observed that CA from early pupae exhibited high levels of FA but little MF and undetectable levels of JH. Correspondingly, addition of FA failed to stimulate JH synthesis by the Br-CA-CC complexes dissected from early pupae, while addition of MF resulted in a significant increase of JH synthesis. Consistent with this finding, stimulation with 20E resulted in an increase in the activity of JHAMT. We next assessed whether the brain modulate JH synthesis in early pupae. Separation of the CA from the brain (denervation) resulted in a sharp increase in JH synthesis; suggesting that the brain inhibits JH synthesis in early pupa. All together these results suggest that an increase in 20E titer in late pupae is sufficient to activate JH biosynthesis by overriding the inhibitory effect of the brain.

MATERIALS AND METHODS

Insects

A. aegypti of the Rockefeller strain were reared at 28 °C and 80% humidity as previously described (Nouzova et al., 2011). Female pupae were collected at 30 min intervals as they molted from fourth instar larvae into the pupal stage. The duration of the pupal stage was determined to be 50 h (Areiza et al., 2014).

Chemicals

20-hydroxyecdysone (20E) was provided by Sigma (St. Louis, MO). Stock of aqueous solutions of 20E were prepared at a concentration of 10^{-5} M and stored in aliquots at –80 °C. Farnesoic acid (FA) and methyl farnesoate (MF) were purchased from Echelon Biosciences (Salt Lake city, UT).

Dissections of corpora allata complexes

Female mosquito pupae were cold-anesthetized and *corpora allata* complexes were dissected in *Aedes* physiological saline (APS) (138 mM NaCl, 8.4 mM KCl, 4 mM CaCl₂, 2 mM MgCl₂, 12 mM NaH₂PO₄, 12 mM Na₂HPO₄ and 42.5 mM sucrose) as previously described (Li et al., 2003a). Unless otherwise noted, preparations were of intact *corpora allata-corpora cardiaca* complexes connected to the brain and head capsule, and are denoted as Br-CA-CC complexes. “Denervated” CA-CC complexes are CA-CC separated from the brain (Nouzova et al., 2012).

Testing the effect of 20E on JH synthesis

Br-CA-CC complexes were dissected and incubated in the presence of different concentrations of 20E (10^{-5} to 10^{-7} M) at 32°C for 2 h in 150 µl of tissue culture media M-199 (Lavallete, NJ, USA) containing 2% Ficoll, 25 mM HEPES (pH 6.5) and 50 µM methionine. Controls were not treated with the hormone. Biosynthesized JH III was labelled with a fluorescent tag and analyzed by reverse phase high performance liquid chromatography coupled to a fluorescent detector (HPLC-FD) as previously described (Rivera-Perez et al., 2012).

Analysis of CA metabolites

Metabolites (FA and MF) were extracted from CA samples, labelled with fluorescent tags and quantified by HPLC-FD as previously described (Rivera-Perez et al., 2012; Rivera-Perez and Noriega, 2014). MF was quantified by treating the sample with sulfuric acid, to convert MF into FA, which is derivatized with 4-Acetamido-7-mercapto-2,1,3-benzoxadiazole (AABD-SH). The endogenous levels of FA were subsequently subtracted from the sulfuric acid treated sample to obtain the MF values. Briefly, glands (10 CA) were dissected in APS, and transferred into a Eppendorf tube containing 100 µL of APS solution, afterwards 150 µL of H₂SO₄ (8M) were added. The CA were sonicated for 3 min and incubated at 70°C for 10 min. The FA formed was extracted by the addition of 500 µL of hexane, samples were vortexed for 5 min and centrifuged at 14,000 rpm for 10 min. The organic phase, containing the FA was recovered with a syringe and filtered it into a new glass tube, the solution was finally dried under N₂ atmosphere for further HPLC analyses.

HPLC was performed using a Dionex Summit System (Dionex, Sunnyvale, CA) equipped with a 680 HPLC pump, a TCC 100 column oven, a UV 170U detector and an UltiMate 3000 fluorescence detector connected in series and a Chromeleon software version 6.8 SR10. Samples were reconstituted in 40 µL acetonitrile, and 20 µL of 10 mM AABD-SH, 20 µL 5 mM of triphenylphosphine (TPP) and 20 µL of 5 mM 2,2'-dipyridyl disulfide (DPDS) were added. Vials were allowed to stand for 15 min at room temperature. Aliquots of the reaction mixture were injected into the HPLC. Derivatized products were recorded at 368 nm excitation and 524 nm emission. Standard curves were constructed for FA and analyzed as previously described (Rivera-Perez et al., 2012).

Juvenile hormone acid methyltransferase and farnesal dehydrogenase activity assays

CA-CC were dissected in APS and transferred to 100 µl of Tris-HCl buffer (50 mM, pH 7.4), sonicated in a water bath sonicator for 3 min, placed on ice for 1 min and centrifuged (13,000 g, 10 min, 4 °C). Supernatants (crude extract) were collected. Juvenile hormone acid methyltransferase (JHAMF) activity in crude extracts was tested by adding FA to a final concentration of 50 µM, and S-adenosyl methionine (SAM) to a final concentration of 10 µM. Farnesal dehydrogenase (FALDH) activity was measured in glycine buffer pH 9.5, with 2 mM NAD⁺ and 50 µM farnesal (FAL) (Rivera-Perez et al., 2013). Samples were incubated for 2 hours in a water bath at 37 °C. Reactions were stopped by adding 500 µl of hexane. Samples were vortexed for 1 min and centrifuged (13,000 g, 10 min, 4 °C). The organic phase was recovered and filtered into a new Eppendorf tube. Lastly, samples were dried with N₂ and stored at -20 °C until analyzed. Conversion of FAL into FA (FALDH

activity) and FA into MF (JHAMT activity) were determined by HPLC (Rivera-Perez et al., 2014).

Statistical analysis

Statistical analyses were performed using the GraphPad Prism Software (San Diego, CA, USA). The results are expressed as means $\pm$ S.E.M. Significant differences ($p < 0.05$) were determined with a one tailed students t-test performed in a pair wise manner or by one-way ANOVA followed by Tukey's test.

RESULTS

20E stimulates JH synthesis by Br-CA-CC complexes dissected from "early pupae"

JH biosynthesis by Br-CA-CC complexes dissected from pupa -24 h before eclosion (early pupae) was significantly stimulated by *in vitro* treatment with 20E (Fig. 1). A series of 20E concentrations ranging from 10^{-5} to 10^{-7} M were tested. Only a concentration of 10^{-6} M prompted a significant increase of JH biosynthesis, while concentrations of 10^{-5} or 10^{-7} M were ineffective (Fig. 2).

To assess if the *in vitro* stimulatory effect of 20E was reversible, intact Br-CA-CC complexes were first incubated with or without 20E for a 2 h period. JH synthesis was significantly stimulated by 20E (Fig. 3A). Afterwards, control and 20E-treated Br-CA-CC complexes were rinsed in fresh medium and incubated once more for 2 h in the presence or absence of 20E. While control glands that were never exposed to 20E remained inactive, those glands exposed to 20E during the first 2 h incubation period continued synthesizing high levels of JH independently of the presence or absence of 20E during the second incubation period (Fig. 3B).

20E stimulates JHAMT activity in the CA of early pupae

There are remarkable changes in the metabolic profiles of JH precursors and the activities of JH biosynthetic enzymes in the CA of early and late mosquito pupae (Rivera-Perez et al., 2014). We have detected high levels of most metabolites corresponding to the late step of JH synthesis in the CA of the early pupae, including FA (220 fmol/CA), but little MF (5 fmol/CA) and undetectable levels of JH (Fig. 4A). Correspondingly we have found significant activities of most of the enzymes of the JH-branch, including farnesal dehydrogenase (FALDH) that converts FA into MF (2500 fmol/CA/h), but not detectable activity of JHAMT (Fig. 4B).

To assess the effect of addition of exogenous precursors on JH synthesis, Br-CA-CC were dissected from early pupae (-24 h) or late pupae (0 h) and incubated *in vitro* in the presence of FA and MF. The addition of these two late JH precursors to the incubation media had different stimulatory effects on the synthesis of JH by the Br-CA-CC complexes dissected from early or late pupae. While MF efficiently increased JH synthesis when added to both glands, FA only stimulated JH synthesis when added to the Br-CA-CC complexes dissected from late pupae (Fig 5).

The results of these experiments imply that the CA from early pupa (–24 h) lacks JHAMT activity and is unable to convert FA into MF, but presents epoxidase activity and efficiently metabolizes MF into JH. To test this hypothesis Br-CA-CC were dissected from early pupae (–24 h) and incubated *in vitro* for 2 h in the presence of 20E. Ecdysteroid-treatment resulted in a significant increase in JHAMT activity (Fig. 6). Although 20E induces increases in JHAMT activity and JH biosynthesis, transcripts levels of JHAMT were not upregulated by 20E in our *in vitro* conditions (Supplemental Fig. 2); suggesting that the increase of JHAMT activity might be a posttranscriptional event.

Denervation activates JH synthesis in early pupa

To determine whether the brain modulates JH synthesis in early pupae (–24 h), we measured *in vitro* JH biosynthesis by denervated glands (CA-CC). Denervation resulted in a sharp increase in JH synthesis (Fig. 7); suggesting that the brain inhibits JH synthesis in early pupa.

DISCUSSION

The biosynthesis of JH-III in *A. aegypti* involves 13 sequential enzymatic steps. The early steps follow the mevalonate pathway up to the formation of farnesyl diphosphate (FPP); in the late steps, FPP is transformed sequentially to farnesol, farnesal, FA, MF and ultimately JH III (Rivera-Perez et al., 2014). 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 transcripts, enzyme activities and metabolites (Nouzova et al., 2011; Rivera-Perez et al., 2014). Molecules responsible for the activation, modulation and suppression of JH synthesis in female mosquitoes include ETH (Areiza et al., 2014), allatostatin-C (AST-C) (Nouzova et al., 2015), insulin (Perez-Hedo et al, 2012, 2013) and allatotropin (AT) (Li et al., 2003b). The activities of these factors are linked to developmental and nutritional signals (Noriega 2014). JH is therefore an important part of a transduction mechanism that connects developmental changes and nutritional status with the activation of specific physiological events during reproduction (Noriega, 2004; Clifton and Noriega, 2011, 2012).

We have earlier reported that brain-*corpora allata-corpora cardiaca* complexes (Br-CA-CC) dissected from mosquito pupae up to 10 h prior to adult eclosion do not synthesize detectable amounts of JH, and are insensitive to ETH stimulation (Areiza et al., 2014). On the contrary, addition of ETH to Br-CA-CC complexes dissected from late pupa ranging from 8 to 2 h before eclosion show significantly higher levels of JH biosynthesis and JHAMT activity (Areiza et al., 2014).

Our studies are unraveling a scheme of interconnected molecular events explaining how 20E and ETH instruct the timing of CA maturation and activation in late pupae of mosquitoes. Pharate adult mosquitoes exhibit marked temporal changes in JH synthesis and JHAMT activity, which match well with 20E dynamics. The genes encoding ETH and ETH receptors (ETHR) are also under tight regulation by 20E (Žit' an and Adams, 2012). That suggested that an increase in 20E titer might provide a timing cue to turn on the activating program in CA in the late pupae. We tested this hypothesis in a series of experiments. Indeed, 20E

produced precocious increases in JH biosynthesis. Therefore, manipulating 20E titer was sufficient to trigger CA maturation.

Remarkably, the stimulatory effect of 20E occurred in a narrow concentration range that might mimic the 20E rise in the late pupae; this surge of steroids regulates ETH receptor expression in the mosquito CA providing the gland with the competence to respond to ETH (Areiza et al., 2014), as well as increases ETH levels in Inka cells (Dai and Adams, 2009; Areiza et al., 2014). On the other hand, declining steroid levels are required for secretory competence of Inka cells (Kingan et al., 1997; Kingan and Adams, 2000). Small concentration windows of rising and declining 20E levels provide a molecular framework to explain how systemic hormonal control coordinates tissue specific programs of differentiation with developmental timing.

In contrast to a JH modulator such as AST-C that exerts a strong, rapid and reversible *in vitro* inhibition of JH synthesis that can be overridden by removing the peptide (Nouzova et al., 2015), the *in vitro* stimulatory effect of 20E was irreversible and could not be overturned by washing away the hormone. Nutritional modulators such as AST-C and insulin control the availability of precursors, such as cytoplasmic acetyl-CoA that sustains JH synthesis in the CA of mosquitoes, without affecting the synthetic potential of the CA (Nouzova et al., 2015). On the contrary, developmental regulators such as ETH and 20E, tend to modulate the activity of key enzymes like JHAMT (Areiza et al., 2014). Enzyme levels need to surpass a minimum threshold to achieve a net flux of precursors through the JH biosynthetic pathway; an increase in JHAMT enzymatic activity is critical for the increases in JH biosynthesis in pharate adult and newly eclosed females (Rivera-Perez et al., 2014; Areiza et al., 2014). *In vitro* incubation of Br-CA-CC with 20E was sufficient to induce a remarkable increase in JHAMT activity. The increase of 20E titer in late pupae might act as a checkpoint that commits the CA irreversibly. There are many examples of physiological processes irreversibly initiated by 20E (Stieper et al., 2008).

We have previously described stimulatory and inhibitory effects of brain factors in mosquitoes. Denervation prevents a 10-fold activation of JH synthesis that occurs 12 h after adult eclosion (Hernandez-Martinez et al., 2007; Rivera-Perez et al., 2014). In contrast, denervation causes a significant increase in JH synthesis in sugar-fed and blood-fed females (Li et al., 2004). Distinct neural modulators likely mediate these effects. For example, AST-C and AT are present in the brain of *A. aegypti* (Hernandez-Martinez et al., 2005). They both modulate JH synthesis *in vitro* (Li et al., 2004; Li et al., 2006) and their receptors are expressed in the CA (Mayoral et al., 2010; Nouzova et al., 2012).

Studies on *Manduca sexta* larvae also suggested that 20E controls JH synthesis acting indirectly via the Br-CC. Dose-response analyses revealed that 20E had a stimulatory effect on JH synthesis by the CA, but only when the glands were complexed with the brain-*corpora cardiaca* (Whisenton et al., 1985; 1987). We observed that denervation resulted in a remarkable activation of JH synthesis in early pupae; suggesting that the activity of the CA is in fact actively repressed by the brain. These results suggest an evolutionary conserved mechanism of regulation of JH biosynthesis by ecdysteroids.

20E has been proposed as an important regulator of the transcription of JH biosynthetic enzymes in *Bombyx mori* (Gu and Chow, 1996; Kaneko et al., 2011). Ecdysteroid signaling in insects is transduced by a hetero-dimer of ecdysteroid (EcR) and ultraspiracle (USP) nuclear receptors (Hill et al., 2013), and both receptors are expressed in the CA of the mosquito pupae (Supplemental Fig. 3). The fact that isolated CA-CC were able to synthesize JH without addition of 20E, argue for a brain-dependent effect of 20E on JH synthesis, with the steroid overriding the allatostatic role of the brain. Although we cannot rule out a direct effect of 20E on the CA, the fact that denervation phenocopies the result of 20E stimulation prevented us from testing a direct effect of the hormone on isolated CA-CC preparations.

Conclusions

Taking advantage of the recent development of a HPLC-FD protocol that allows the measurement of JH and its precursors, as well as the enzymatic activities of the biosynthetic enzymes, we set out to test the hypothesis that 20E plays a key role on the reactivation of the CA in the late pupae of mosquitoes. Our *in vitro* approaches combined with the analyses of precursors and enzymes in the isolated CA provided evidence that:

- The CA of early pupae has a sizeable amount of FA, but it is deficient in JHAMT activity and MF.
- Treatment with 20E stimulates JHAMT activity that catalyzes the conversion of FA into MF.
- The stimulatory effect of 20E is irreversible and occurred only in a narrow concentration range,
- 20E acts as a “derepressor”, overriding the inhibitory action of the brain.

Before the rise of 20E titers in late pupa, the CA activation program has been progressively assembled, but it is kept on hold through expression of a brain repressor. The increase of the 20E titer provides temporal cues for the execution of a CA maturation program.

Determining the mechanism by which 20E overrides the inhibitory effect of the brain is an exciting question that remains to be answered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- 20-hydroxyecdysone (20E) stimulates juvenile hormone (JH) synthesis by the *corpora allata* (CA) of the early pupae.
- The CA of early pupae has a large amount of farnesoic acid (FA), but little methyl farnesoate (MF)
- The CA of early pupae is deficient in juvenile hormone acid methyl transferase activity (JHAMT).
- Treatment with 20E stimulates JHAMT activity that catalyzes the conversion of FA into MF.
- The stimulatory effect of 20E is irreversible and occurred only in a narrow concentration range,
- 20E acts as a “derepressor”, overriding the inhibitory action of the brain.

Fig. 1. Effect of 20E on JH synthesis by Br-CA-CC complexes from “early pupae”

JH biosynthesis by Br-CA-CC complexes incubated for 2 h without (control) and with (+20E) addition of 20E (10^{-6} M) was assayed *in vitro* using the HPLC-FD detection method. Each data point represents the mean $\pm$ S.E.M. of 25 independent determinations of groups of 4 Br-CA-CC complexes dissected from -24 h pupae. Asterisks denote significant differences (unpaired t-test; *** P = 0.001).

Fig. 2. Dose-response effect of 20E

The effect on JH synthesis of 20E concentrations ranging from 10^{-5} to 10^{-7} M were tested on Br-CA-CC complexes dissected from -24 h pupa. Each data point represents the mean $\pm$ S.E.M. of at least 25 independent determinations of groups of 4 Br-CA-CC complexes.

Fig. 3. The stimulatory effect of 20E is irreversible

Br-CA-CC were incubated for two consecutive 2 h periods. **A)** During the first 2 h, Br-CA-CC complexes were incubated either in the presence or absence of 20E (10^{-6} M). After 2 h, glands were rinsed in fresh culture medium. **B)** During the second 2 h period, control and previously treated glands were incubated in fresh culture medium with and without 20E. Each data point represents the mean $\pm$ S.E.M. of three independent determinations of three Br-CA-CC complexes. Asterisks denote significant differences (unpaired t-test; *** P 0.001). Different letters above the columns indicate significant differences among treatments (ANOVA $P < 0.05$, with Tukey's test for multiple comparison).

Fig. 4. Metabolite pool sizes and enzymatic activities in CA of the early and late pupae
A) Relative levels of endogenous concentrations of FA and MF, and JH biosynthesis rates in CA of early (- 24 h) and late (0 h) pupae. **B)** Farnesal dehydrogenase (FALDH) and juvenile hormone acid methyl transferase (JHAMT) enzymatic activities in in CA extracts of early (- 24 h) and late (0 h) pupae. Bars represent the means $\pm$ SEM of three independent replicates of three groups of 5 CA.

Fig. 5. Effect of stimulation with precursors on JH biosynthesis

The effect of addition of precursors on JH biosynthesis was evaluated on Br-CA-CC complexes incubated in culture medium M-199 alone (Control) or with the addition of 200 μ M of FA or MF. **A)** Br-CA-CC dissected from early pupae (-24 h). **B)** Br-CA-CC dissected from late pupae (0 h). Each data point represents the means $\pm$ S.E.M. of 3 independent biological replicates of three Br-CA-CC complexes. Different letters above the columns indicate significant differences among treatments (ANOVA $P < 0.05$, with Tukey's test for multiple comparison).

Fig. 6. Effect of 20E on JHAMT enzymatic activity

Br-CA-CC complexes were incubated for 2 h without (Control) and with (+20E) addition of 20E (10^{-6} M). Consequently CA-CC extracts were prepared and JHAMT activity was measured. Each data point represents the mean $\pm$ S.E.M. of three independent determinations of five CA complexes. Asterisk denotes significant difference (unpaired t-test; *** $P = 0.001$).

Fig. 7. Effect of denervation on JH synthesis

Br-CA-CC and CA-CC complexes were dissected from early pupae (– 24 h) and incubated *in vitro* for 2 h. JH biosynthesis was assayed using the HPLC-FD detection method. Each data point represents the mean $\pm$ S.E.M. of 3 independent determinations of 4 complexes. Asterisk denotes significant difference (unpaired t-test; *** $P < 0.001$).