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Inhibition of juvenile hormone synthesis in mosquitoes by the methylation inhibitor 3-deazaneplanocin A (DZNep)

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ABSTRACT

Juvenile hormone (JH), synthesized by the *corpora allata* (CA), controls development and reproduction in mosquitoes through its action on thousands of JH-responsive genes. These JH-dependent processes can be studied using tools that increase or decrease JH titers *in vitro* and *in vivo*. Juvenile hormone acid methyl transferase (JHAMT) is a critical JH biosynthetic enzyme. JHAMT utilizes the methyl donor S-adenosyl-methionine (SAM) to methylate farnesoic acid (FA) into methyl farnesoate (MF), releasing the product S-adenosyl-L-homocysteine (AdoHcy), which inhibits JHAMT. S-adenosyl-homocysteine hydrolase (SAHH) catalyzes AdoHcy hydrolysis to adenosine and homocysteine, alleviating AdoHcy inhibition of JHAMT. 3-deazaneplanocin A (DZNep), an analog of adenosine, is an inhibitor of SAHH, and an epigenetic drug for cancer therapy. We tested the effect of DZNep on *in vitro* JH synthesis by CA of mosquitoes. DZNep inhibited JH synthesis in a dose-response fashion. Addition of MF, but not of FA relieved the inhibition, demonstrating a direct effect on JHAMT. *In vivo* experiments, with addition of DZNep to the sugar ingested by mosquitoes, resulted in a dose-response decrease in JH synthesis and JH hemolymphatic titers, as well as expression of early trypsin, a JH-dependent gene. Our studies suggest that DZNep can be employed to lower JH synthesis and titer in experiments evaluating JH-controlled processes in mosquitoes.

1. Introduction

Juvenile hormone (JH), synthesized by the *corpora allata* (CA), controls development and reproduction in mosquitoes (Goodman and Cusson, 2012). Microarray and RNA sequencing data have revealed that extensive portions of the mosquito genome are positively or negatively regulated in response to changes in JH titer (Zou et al., 2013; Saha et al., 2016). To study processes that are controlled by JH, researchers need tools that can increase or decrease JH titers *in vitro* and *in vivo*. JH titers in insects can be easily increased by topical application of JH analogs (JHA), such as methoprene (Clifton and Noriega, 2012). On the other hand, purposely decreasing JH titers has been more difficult. Nevertheless, some efficient anti-juvenile hormones compounds have been described. Precocenes are efficient inhibitors of CA activity (anti-allatotropins) in certain insect species (Bowers et al., 1976; Bowers and Martinez-Pardo, 1977). They generate anti-JH effects by triggering necrosis of the CA (pro-allatocidins) (Pratt et al., 1980). In addition, JH degradation by JH esterase (JHE) plays an important role in lowering JH titers (Goodman and Cusson, 2012); consequently recombinant JHE (rJHE) has been employed as an anti-JH agent in several insects (Philpott and Hammock, 1990; Bonning et al., 1997; Edgar et al., 2000).

A critical enzyme in JH synthesis is juvenile hormone acid methyltransferase (JHAMT); originally described in *Bombyx mori* (Shinoda and Itoyama, 2003), it was later characterized in several insect species, including the mosquito *Aedes aegypti* (Mayoral et al., 2009). Insect JHAMTs are S-adenosylmethionine-dependent MTs (SAM-MTs), using the methyl donor S-adenosylmethionine (SAM or AdoMet) to methylate farnesoic acid (FA) into methyl-farneosate (MF), which is later epoxidized to JH III (Fig. 1). AdoMet-dependent methyltransferase reactions release the strong product inhibitor S-adenosyl-L-homocysteine (AdoHcy), as a by-product of the reaction. S-adenosyl-L-homocysteine hydrolase (SAHH) catalyzes the reversible AdoHcy hydrolysis to adenosine and homocysteine, relieving AdoHcy inhibition of JHAMT

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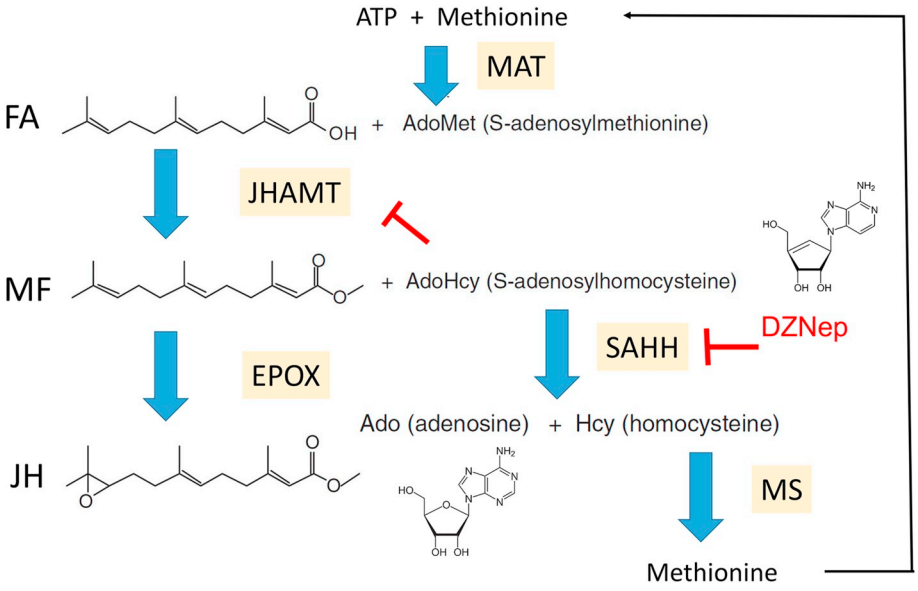

Fig. 1. JHAMT and SAHH roles in JH synthesis: Schema of the last two steps of JH III biosynthesis, with details on the effects of DZNep. JHAMT catalyzes the transfer of a methyl group from AdoMet to FA, to produce MF and AdoHcy. MF is converted to JH III by the action of an epoxidase (EPOX). AdoHcy inhibits JHAMT activity. SAHH is responsible for the reversible hydration of AdoHcy into adenosine (Ado) and homocysteine (Hcy). DZNep inhibits SAHH activity as an adenosine analog. Hcy is methylated to methionine by a methionine synthase (MS). Methionine adenosyltransferase (MAT) creates AdoMet by reacting methionine and ATP.

(Fig. 1) (Glazer et al., 1986). 3-deazaneplanocin A (DZNep), an analog of adenosine, is a competitive inhibitor of SAHH. It has been previously reported that DZNep has a detrimental effect on development, fecundity and survivorship of anopheline mosquitoes (Sharma et al., 2015). Increase in mortality and decrease in size were observed in immature mosquitoes exposed to DZNep; while adult female *Anopheles gambiae* showed decreases in fecundity. Juvenile hormone plays key roles in the regulation of these mosquito developmental and reproductive processes that were affected by DZNep treatment. In the present studies, we investigated if the effects of DZNep were mediated through changes in JH titer. We observed that DZNep inhibited JH synthesis in female adult *Ae. aegypti* in a dose-response fashion. Addition of MF, but not of FA relieved the inhibition, demonstrating a direct effect on JHAMT. *In vivo* experiments, with addition of DZNep to the sugar ingested by mosquitoes, resulted in a dose-response decrease in JH synthesis and JH hemolymphatic titers, as well as expression of early trypsin, a JH-dependent gene. Our studies suggest that DZNep can be employed to lower JH synthesis and titer in experiments evaluating JH-controlled processes in mosquitoes.

2. Materials and methods

Insects: *Ae. aegypti* of the Rockefeller strain were reared at 28 °C and 80% humidity as previously described (Rivera-Perez et al., 2014). Adult mosquitoes were offered a cotton pad soaked in a 3% sucrose solution.

DZNep inhibitor: DZNep-HCl was purchased from Tocris (MN, USA). Stock solutions (10 mM) were prepared dissolving DZNep in water. Stock solutions were aliquoted and stored at −20 °C. For experiments evaluating JH synthesis *in vitro*, stock solutions were diluted to the final concentration with M199 medium. For experiments evaluating the effect of feeding *in vivo*, DZNep was dissolved in sucroses.

Dissections of corpora allata complexes and JH biosynthesis assay: Adult female mosquitos were cold-anesthetized and brain-*corpora allata–corpora cardiaca* complexes (BR-CA-CC) were dissected and incubated at 32 °C for 2 h or 4 h in 150 µl of tissue culture media M-199 (Gibco, Grand Island, NY, USA) containing 2% Ficoll, 25 mM HEPES (pH 6.5) and methionine (100 µM) (Nouzova et al., 2011). Biosynthesized JH was analyzed by HPLC-MS/MS as previously described (Ramirez et al., 2016). Stimulation of JH synthesis was performed with 200 µM farnesoic acid (FA) or 200 µM methyl farnesoate (MF) (Echelon; Salt Lake City, UT).

Hemolymph collection: Hemolymph of adult female mosquitoes was obtained by perfusion as previously described (Hernandez et al., 1999). Fine needles were made from 100-µl micro-glass capillary tubes using a pipette puller P-30 (Sutter Instrument, Novato, CA), and mounted in a pipette pump (Drummond, Broomall, PA). Needles were inserted manually through the neck membrane into the thoracic cavity, and insects were perfused with 20 µl of a "bleeding solution" of phosphate-buffered saline (PBS) (100 mM NaCl, 25 mM NaHCO3, pH 7.2. The hemolymph was obtained from a small tear made laterally on the intersegmentary membrane of the last abdominal segment. The first drop of perfused hemolymph was collected directly into a glass silanized tube (Thermo Scientific) placed on ice.

JH extraction and quantification: 10 µL of 6.25 ppb of heavy deuterated JH III analog (JH III-D3) in acetonitrile were added to each sample, followed by 600 µL of hexane. Samples were vortexed for 1 min, and spun for 5 min at 4 °C and 2000 g. The organic phase was transferred to a new silanized vial, dried under nitrogen flow and stored at −20 °C. Dried extracts were re-suspended in 50 µl of acetonitrile, vortexed 1 min, and transferred to a new silanized vial with a fused 250 µL insert. JH quantifications by high performance liquid chromatography coupled to electrospray tandem mass spectrometry (HPLC-MS/MS) were done as previously described by Ramirez et al. (2016). Briefly, we employed a HPLC-MS/MS workflow based on multiple reaction monitoring (MRM) using the two most abundant fragmentation transitions: 267- > 235 (primary) and 267- > 147 (secondary). In order to accurately quantify the amount of JH III present in the hemolymph or produced by the BR-CA-CC complexes, JH III-D3 was utilized as an internal standard to normalize recoveries during the sample preparation, extraction and analysis steps. An extraction recovery of 55% or more was routinely observed regardless of the analyte concentration (Ramirez et al., 2016).

Quantitative Real-Time PCR (q-PCR): Total RNA was isolated from female abdomens using Norgen Biotek's total RNA purification kit. Total RNA was treated with DNase I according to Norgen Biotek's instructions (Thorold, ON, Canada). Reverse transcription of RNA was carried out using the Verso cDNA Synthesis Kit (Thermo Fisher, Waltham, MA). The expression of the early trypsin gene (ET) was quantified by real-time PCR performed in a 7300 Real-Time PCR System using TaqMan® Gene Expression Assays together with TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Reactions were run in triplicate in a 20 µl volume. Accession number and primer probe sequences for ET are included in Table S1.

Statistical analysis: Statistical analyses were performed using the GraphPad Prism Software (San Diego, CA, USA). The results are expressed as means ± S.E.M. Significant differences ($p < 0.01$) were

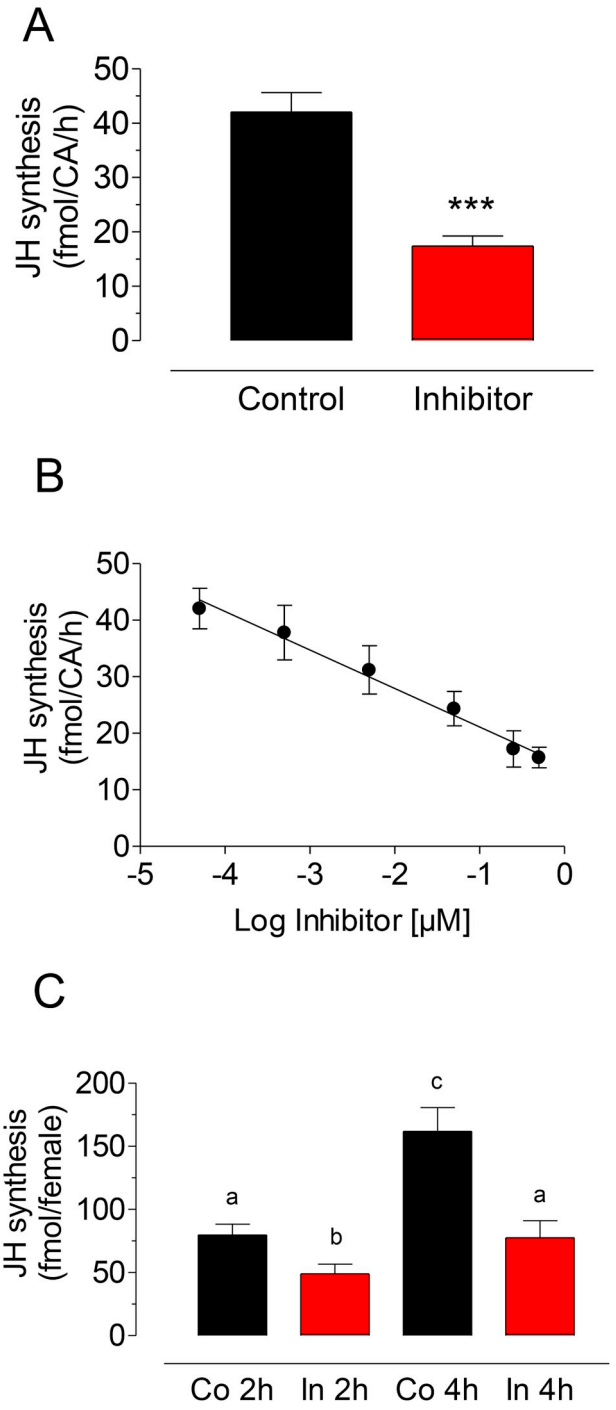

Fig. 2. DZNep inhibits JH synthesis *in vitro* in a dose-dependent mode: A) Addition of DZNep (0.5 μM) to the incubation media resulted in a significant inhibition of JH synthesis. Bars represent the means ± S.E.M. of 15 independent replicates of incubations of groups of 4 BR-CA-CC complexes. Asterisks denote significant differences (unpaired *t*-test; ***P ≤ 0.001). **B)** Six different DZNep concentrations were tested *in vitro* (0.00005, 0.0005, 0.005, 0.05, 0.25 and 0.5 μM). JH synthesis rates decreased in a dose response manner (r^2: 0.9865, P < 0.0001). Points represent the means ± S.E.M. of 4–15 independent replicates of incubations of groups of 4 BR-CA-CC complexes. **C)** The dynamics of inhibition were studied by comparing JH synthesis *in vitro* by CA that were incubated for 2 h or 4 h in the absence (Co) or presence of DZNep (0.5 μM) (In). Glands incubated with DZNep for 4 h still showed increases in JH synthesis when compared to those incubated with DZNep for 2 h. Bars represent the means ± S.E.M. of 8 independent replicates of incubations of groups of 4 BR-CA-CC complexes. Different letters above the bars indicate significant differences among treatments (One way ANOVA, p < 0.05, with Tukey's test of multiple comparisons).

determined with a one tailed students t-test performed in a pair wise manner or by one-way ANOVA followed by Tukey's test.

3. Results

3.1. DZNep inhibits JH synthesis in vitro in a dose-dependent mode

Brain-*corpora allata-corpora cardiaca* complexes, dissected from sugar-fed female adult mosquitoes, were incubated for 4 h in the presence or absence of DZNep (0.5 μM). The amounts of JH synthesized by the CA were evaluated by HPLC-MS/MS. Addition of the inhibitor resulted in a highly significant decrease in JH synthesis (Fig. 2A). When six different DZNep concentrations were tested *in vitro* (0.00005, 0.0005, 0.005, 0.05, 0.25 and 0.5 μM), JH synthesis rates decreased in a dose response manner (r^2: 0.9865, 0, < 0.0001) (Fig. 2B). To further understand the dynamics of DZNep inhibition, we compared total JH synthesis *in vitro* by CA that were incubated for 2 or 4 h in the presence or absence of DZNep. Incubations for 2 h resulted in a 39% decrease in JH synthesis compared to controls; CA incubated in DZNep for 4 h showed a 63% rate of inhibition compared to the amount of JH synthesized by controls; but still showed an increase when compared to those incubated in DZNep for 2 h (Fig. 2C).

3.2. Addition of DZNep to the sugar meal resulted in a decrease in JH synthesis and JH hemolymphatic titer

Female adult mosquitoes were offered a cotton pad soaked in a 3% sucrose solution in the presence or absence of DZNep (1 mM). Three days later, the amount of JH synthesized by the CA *in vitro* was evaluated by HPLC-MS/MS. Addition of the inhibitor to the sugar-meal resulted in a significant decrease in JH synthesis (Fig. 3A). JH synthesis rates decreased in a dose response manner (Supplemental Fig. 1). In addition, hemolymph was collected from the same females, and JH titers evaluated. Addition of DZNep to the sugar-meal resulted in a significant decrease in JH hemolymphatic titers (Fig. 3B). When DZNep was fed to adult females for six days, the inhibition of JH synthesis, as well as the decrease in JH titers were similar to those observed after feeding DZNep for three days (Supplemental Fig. 2).

We tested the hypothesis that the effect of DZNep was transient, and the inhibition could be reversed by discontinuing the oral administration of the compound. Groups of females were offered a cotton pad soaked in a 3% sucrose solution in the presence or absence of DZNep (1 mM) for three days. Females exposed for three days to DZNep synthesized only 25% of JH when compared with controls. After three days of receiving DZNep, some females were offered a sucrose solution for another three days. Females that were exposed for three days to DZNep, and then received just sugar for additional three days showed JH synthesis levels similar to those of controls fed sugar for 6 days (Fig. 4); revealing that JH inhibition could be reversed by removing DZNep from the diet.

3.3. DZNep decreases JH synthesis through inhibition of JHAMT activity

To explore the mechanisms of action of DZNep, we tested the hypothesis that the compound was decreasing JH synthesis by interfering with the ability of JHAMT to methylate FA into MF. Addition of MF (200 μM) to the incubation media (Fig. 5A), relieved the inhibition, indicating a direct effect of DZNep on JHAMT activity. On the other hand addition of FA (200 μM) increased JH and MF synthesis in control glands, but failed to increase JH and MF synthesis on DZNep-treated glands to the same levels of control samples (Fig. 5B) (Supplemental Fig. 3).

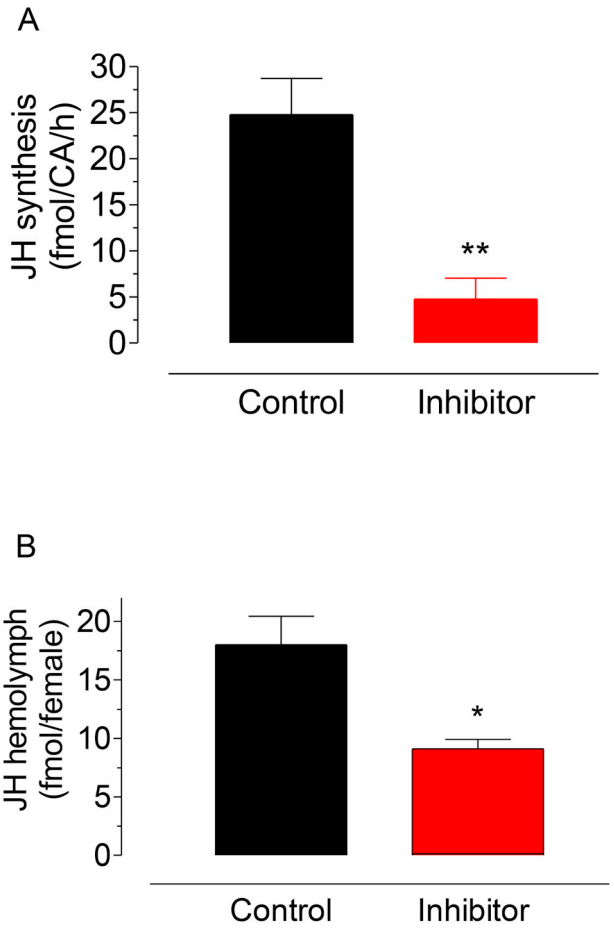

Fig. 3. Addition of DZNep to the sugar meal resulted in a decrease in JH synthesis and JH hemolymphatic titer. A) Newly emerged adult female mosquitoes were offered a cotton pad soaked in a 3% sucrose solution in the presence or absence of DZNep (1 mM). Three days later, BR-CA-CC complexes were dissected and JH synthesis was evaluated. Bars represent the means ± S.E.M. of 4 independent replicates of incubations of groups of 5 BR-CA-CC complexes. Asterisks denote significant differences (unpaired *t*-test; **P ≤ 0.01). **B)** Hemolymph was collected from the same animals, and JH titer was evaluated. Bars represent the means ± S.E.M. of 4 independent replicates of hemolymph from groups of 5 females. Asterisks denote significant differences (unpaired *t*-test; *P ≤ 0.05).

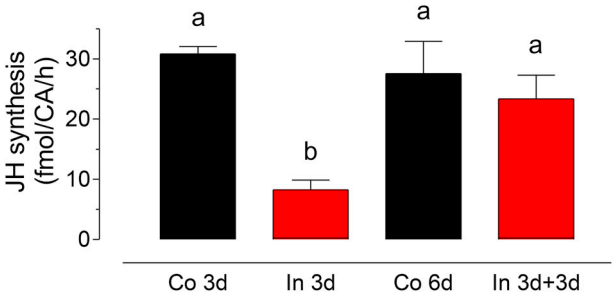

Fig. 4. JH inhibition could be reversed by removing DZNep from the diet. Groups of females were offered for three days a cotton pad soaked in a 3% sucrose solution in the absence (Co 3d) or presence of DZNep (1 mM) (In 3d). Some females were offered a sucrose solution for 6 days (Co 6d). To evaluate the effect of removing DZNep from the diet, some females after three days of receiving DZNep, were offered a sucrose solution for another three days (In 3d +3d). Bars represent the means ± S.E.M. of 4 independent replicates of incubations of groups of 4 BR-CA-CC complexes. Different letters above the bars indicate significant differences among treatments (One way ANOVA, $p < 0.05$, with Tukey's test of multiple comparisons).

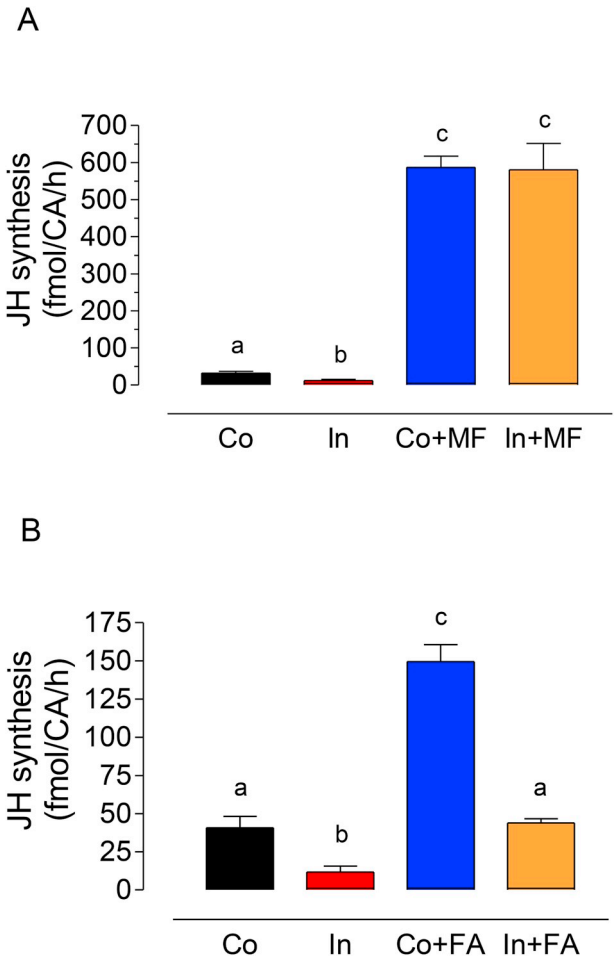

Fig. 5. DZNep decreases JH synthesis through inhibition of juvenile hormone acid methyl transferase activity: A) JH synthesis by BR-CA-CC stimulated with MF (200 μM). Co: control, In: with DZNep (5 μM), Co + MF: control + MF, In + MF: DZNep (5 μM) + MF. **B)** JH synthesis by BR-CA-CC stimulated with FA (200 μM). Co: control, In: with DZNep (5 μM), Co + FA: control + FA, In + FA: DZNep (5 μM) + FA. Bars represent the means ± S.E.M. of 4 independent replicates of incubations of groups of 4 BR-CA-CC complexes. Different letters above the bars indicate significant differences among treatments (One way ANOVA, $p < 0.05$, with Tukey's test of multiple comparisons).

3.4. DZNep decreased the expression of early trypsin, a well-characterized JH target gene

We tested the hypothesis that addition of DZNep to the sugar meal would result in a decrease in the expression of JH-dependent genes. We analyzed the effect of feeding for three days a 3% sucrose solution in the presence or absence of DZNep (1 mM) on the expression of early trypsin (ET), a well-characterized JH target gene. Quantitative Real-Time PCR was used to evaluate the expression of ET on abdomens of control and DZNep-treated females. A highly significant decrease on ET mRNA levels was consistently observed in abdomens from DZNep-treated females (Fig. 6A). When different concentrations of DZNep were used, the decrease of ET expression was dose-dependent (Supplemental Fig. 4). After three days of receiving DZNep, some females were offered a sucrose solution for another three days. Females that were exposed for three days to DZNep, and then received just sugar for additional three days showed ET mRNA levels similar to those of controls fed sugar for 6 days (Fig. 6B), revealing that the detrimental effect of DZNep on ET mRNA expression could be reversed by removing the inhibitor from the diet.

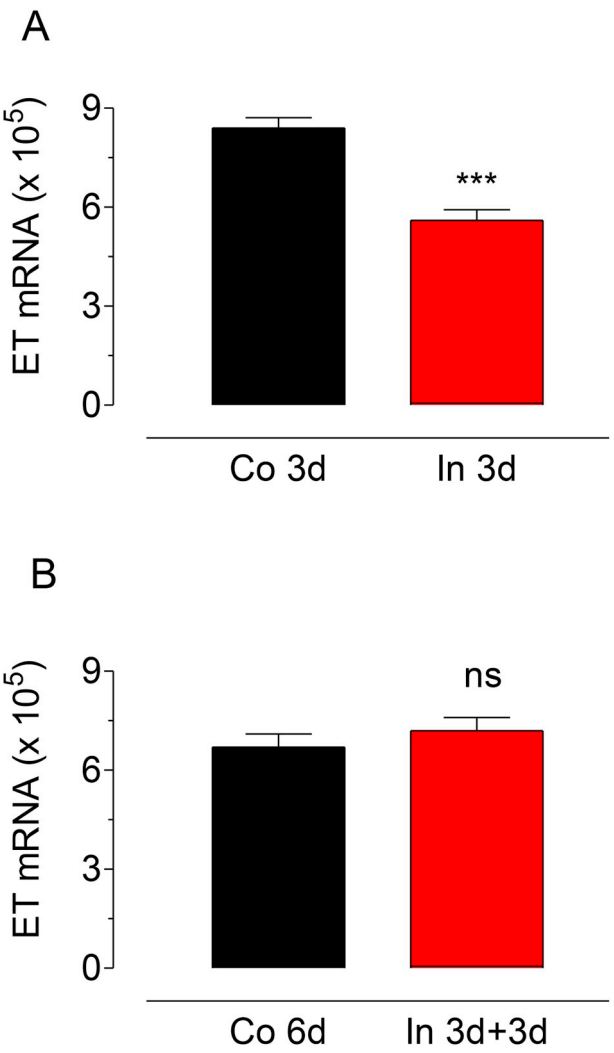

Fig. 6. DZNep decreased the expression of the early trypsin gene. A) Groups of females were offered for three days a cotton pad soaked in a 3% sucrose solution in the absence (Co, black bar) or presence of DZNep (1 mM) (In, red bar). Early trypsin mRNA levels in abdomens of treated and untreated females were measured by q-PCR. **B**) To evaluate the effect of removing DZNep from the diet on ET expression, some females after three days of receiving DZNep, were offered just a sucrose solution for another three days (In 3d + 3d), while controls received sucrose for six days (Co 6d). Bars represent the means ± S.E.M. of 8 independent replicates of individual abdomens. Asterisks denote significant differences (unpaired *t*-test; ***P ≤ 0.001). ns: no significant differences. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

The biological functions of methylation are wide ranging, and include biosynthesis, metabolism, detoxification, signal transduction, protein sorting and repair, and nucleic acid processing (Martin and McMillan, 2002). Most methylation reactions are catalyzed by S-adenosylmethionine-dependent MTs (SAM-MTs). The reaction transfers a methyl group from the donor S-adenosylmethionine (SAM) to a variety of cellular substrates, including lipids, proteins, nucleic acids, and other cellular metabolites. The addition of a methyl group to biologically active molecules such as hormones, neurotransmitters, lipids, proteins and nucleic acids causes a change in the physicochemical properties and activities of these molecules. Methylation consumes SAM and releases the byproduct S-adenosylhomocysteine (AdoHcy), which is further metabolized to homocysteine that can be remethylated to methionine by the enzyme methionine synthase (MS) (Brosnan and Brosnan, 2006). Methionine adenosyltransferase (MAT) produces SAM by reacting methionine and ATP, ending the SAM regeneration cycle (Fig. 1).

In addition to having a direct effect on SAHH, which causes an increase in AdoHcy with the consequent inhibition of JHAMT activity, DZNep could affect JH synthesis by preventing the replenishment of the SAM pool (Fig. 1). The SAM pool inside CA cells is small, and rapid consumption of modest CA methionine pools have been described for *Ae. aegypti* (Li et al., 2003), *Schistocerca gregaria* (Tobe and Pratt, 1974) and *Diploptera punctata* (Feyereisen et al., 1984).

We would like to understand what factors could affect the dynamics of DZNep inhibition of JH synthesis or titer in mosquitoes. Most SAHH inhibitors are adenosine analogs that irreversibly bind to the enzyme (Zhang and Zheng, 2016). In rats, the kinetics of SAHH inhibitors effects *in vivo* varied in a dose-dependent manner. Higher concentrations resulted in faster (1 h) and longer lasting (24 h) effects (Converso et al., 2014). We also observed a very significant decrease of JH synthesis after 4 h *in vitro* incubations. The magnitude and duration of the decreases of JH titers observed in experiments *in vitro* and *in vivo* might depend on multiple factors; including the concentration and stability of DZNep, the sizes of the CA homocysteine, methionine and MF pools, the half-life and turnover of SAHH and JHAMT proteins, etc.

Pre-incubation of the CA complexes with DZNep did not completely abolish JH synthesis. We would like to hypothesize that new JHAMT and SAHH proteins might be constantly synthesized, increasing the pools of active enzymes. Our *in vivo* experiments revealed that the effect of DZNep was transient, and the inhibition could be reversed by discontinuing the oral administration of the compound. This observation has important implications when designing experiments to study JH-dependent processes.

Oral ingestion of DZNep resulted in a significant decrease on ET mRNA levels, which was reversed by discontinuing the oral administration of the compound. We believe that the efficacy of the modulation of expression of different JH-dependent genes by DZNep might be dissimilar. Genes like ET, which are highly expressed and very sensitive to changes in JH titer (Noriega et al., 1997; Edgar et al., 2000), might respond in a different fashion than JH-dependent genes that are stimulated by more subtle changes in JH titer (Zou et al., 2013). JHAMT is a well conserved protein in different insect species (Defelipe et al., 2011); playing always a critical role on JH synthesis. Although it was not tested in the current studies, we would like to postulate that DZNep should have an inhibitory effect on JHAMT activity in other insect species. However, if the compound is going to be used to modulate JH titer *in vivo*, or JH synthesis *in vitro*, the doses and experimental conditions will have to be optimized on a one-to-one basis, depending on the insect, the gene, or the hypothesis to be tested.

Some SAHH inhibitors are cytotoxic after long-term exposure (Converso et al., 2014). Because the goal of these studies was to evaluate the effect of DZNep on JH synthesis and titer, as well as to test if these effects were the results of inhibition of JHAMT activity in the CA, we did not thoroughly evaluate the effect of DZNep on mosquito's fitness. Exposing newly hatched first instar larvae consistently resulted in high pupal mortality, preventing them to reach the adult stage (results not shown). It is important to emphasize that DZNep is an inhibitor of all SAM-MTs, and therefore should have a broad effect on multiple processes, besides JH synthesis. Potential phenotypes affecting development and reproduction could be the result of disturbing other processes, and cannot be attributed solely to disruption of JH synthesis.

5. Conclusions

Our studies suggest that DZNep can be employed to lower JH synthesis and titer in experiments evaluating JH-controlled processes in mosquitoes. It can be orally administrated with the food, and represents an efficient tool to purposely decrease JH titers, and transiently reduce

the expression of JH-dependent genes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.103183.

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