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Research Article

Effects of allatostatin on female reproduction in the greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae)

Azam Mikani*

Department of Entomology, Faculty of Agriculture, Tarbiat Modares University, Tehran 14115-336, Iran.

Abstract: The present study investigated the effect of allatostatin (Ast) on the adult female reproductive system of the greater wax moth, *Galleria mellonella* (L.). At first, different concentrations of Ast in the brain- SOG, midgut, ovaries, and fat body of 5 days old adult females were confirmed. Moreover, it was shown that the highest concentration of Ast was observed in brain-SOG. Daily injections of Ast decreased ecdysteroid concentration in the hemolymph and ovaries. Ast injection decreased the expression level of vitellogenin (Vg) genes. Furthermore, it reduced oocyte size. These results showed that Ast has a regulatory role in the reproduction of *G. mellonella* female.

Keywords: *Galleria mellonella*, ecdysteroid, oocyte size, vitellogenin

Introduction

Neuropeptides are important in regulating a wide range of developmental, behavioral, and physiological functions throughout the life cycle of insects. They regulate sleeping, aggression, learning, reproduction, stress responses, memory, and other physiological roles (Nässel and Zandawala, 2019).

Allatostatins (Asts) affect the production of juvenile hormone (JH) in corpora allata. Three families of allatostatin peptides are A-type, B-type, and C-type (Liu *et al.*, 2021). The A-type Asts were first identified in *Diploptera punctata* (Eschscholtz). Later this type of Asts was determined in other insect orders such as Lepidoptera (Audsley *et al.*, 2008). However, further studies showed that Ast-A cannot regulate JH in all insects and is involved in inhibiting starvation in Drosophila. Moreover, Ast-A promotes lipid accumulation in the fat body (Toprak, 2020). AstA is myoinhbitory and inhibited foregut peristalsis in *Helicoverpa virescens* (F.) (Audsley *et al.*, 2008). Competitive ELISA results showed that starvation decreased the AST-A titer in the midgut of *Spodoptera littoralis* (Boisduval), whereas refeeding increased it (Nakhaie Bahrami *et al.*, 2018).

Zhang *et al.* (2021) showed that Ast suppresses gonadotropin production in *Drosophila melanogaster*. Moreover, in mature virgin female insects, the highly sustained activity of sex peptide abdominal ganglion neurons shut off vitellogenesis via activation of the allatostatin (Zhang *et al.*, 2022). It was confirmed that peptides related to Ast inhibit ecdysteroid release from lepidopteran prothoracic glands (Brown *et al.*, 2009). Previously, it was uncovered that Ast-A inhibits muscle contraction and ovarian ecdysteroid biosynthesis of *D. punctate* (Garside *et al*., 2002).

The greater wax moth, *G. mellonella*, is a moth of the family Pyralidae. It is distributed worldwide

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and feeds on wax and pollen, causing bee galleriosis. Here, for the first time, we investigated the effects of Ast on oocyte size, ecdysteroid concentration in hemolymph and ovaries, and the expression of vitellogenin (Vg) in adult female G. mellonella. This is of interest to the scientific community as a model insect (Menard *et al*., 2021).

Materials and Methods

Insect culture

The insect larvae were obtained from an infected honeybee hive in Karaj (35°50′08″N 51°00′37″E), Iran. They were maintained on an artificial diet at 37 ± 2 °C, photoperiod 16 h of light and 8 h of darkness, and relative humidity of 50 ± 5% (Vilcinskas and Matha, 1997). The five-day-old adult female was used for measuring ecdysteroid concentration, oocyte size, and *Vg* gene expression level.

Competitive ELISA

Competitive ELISA was done as described by Sakai *et al*. (2006). The fat body, brain-SOG, midgut, and ovaries of 5 days old adult females were dissected in Tris-buffered saline (TBS; 2.6 mM KCl, 135 mM NaCl, 25 mM Tris–HCl, pH 7.6). All samples were homogenized, centrifuged (5000× g, 15 min, 4 °C), and the supernatant was used as a sample. An Ast- Bovin serum albumin (BSA) conjugate was prepared using dimethyl suberimidate (Aldrich, Switzerland). Microplate reader were coated with Ast- BSA (0.6 µg/mL per well) in 0.05 M sodium carbonate-bicarbonate buffer for 3 h. Then 250 µl of 2% skimmed milk was added to each well and incubated at room temperature (RT) for 1 h. Samples mentioned above or standard peptides (50 µl/well) were added to each well. Then, 50 µl of antibody (1:10000 2% skimmed milk) was added to each well, and the plate was incubated overnight at 4 °C. After three times washing with TBS, 100 µL of secondary antibody (1:1000 TBS) was added to each well and incubated at RT for 1 h. After three times washing with TBS, 100 µl of substrate solution [1 mg/mL ρ-nitrophenylphosphate disodium salt hexahydrate (Sigma, USA) in 10 mM diethanolamine buffer (Sigma–Aldrich, UK), pH 9.5] was added to each well and incubated at RT for 1 h. The reaction was stopped using 50 µl/well of 4 M NaOH. Finally, the absorbance was read at 405 nm with a microplate reader (Epoch, Biotek, USA).

Ast injection

Different concentrations of Ast (ARPYSFGLamide, 1 µl) were injected into newly adult females between the third and fourth segments of the insect's abdomen for four consecutive days. Control insect was injected in the same way with 1 µl of phosphate-buffered saline (PBS; 8.55 mM Na2HPO4, 1.45 mM NaH2PO4, 145 mM NaCl, pH 7.5). On day 5, terminal oocyte size, ecdysteroid concentration in the ovaries and hemolymph, and *Vg* expression level were determined.

Measuring oocyte size

To determine the average size of terminal oocytes located at the base of the ovariole, a piece of millimeter-squared paper was used, according to Van Wielendaele *et al*. (2013). Then the experimental data were analyzed by unpaired *t*-student test.

Ecdysteroid quantification in hemolymph and ovaries

The hemolymph of a newly emerged adult female was extracted for six days (once a day) using a Hamilton syringe (Hamilton Company, USA). 100 µl of methanol was added to each sample and dried using a SpeedVac. On day 7, the ovaries extracted from each adult female were homogenized in the same amount of methanol, followed by centrifugation at 15,000 rpm for 15 min. The supernatant phase was transferred into a new tube, and this step was repeated. The sample was then dried in a Speed Vac machine. Finally, 100 µl of enzyme immunoassay buffer [0.1% Bovine serum albumin (BSA), 0.4 M NaCl, 1 mM Ethylenediaminetetraacetic acid (EDTA), in 0.1 M phosphate buffer] was added to the samples (hemolymph and ovaries) and kept at -20 °C for further experiment.

Ecdysteroid level in the hemolymph and ovaries was quantified using Enzyme

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Immunoassay (EIA). 50 µl of the samples described above were added into EIA plate that contained 100 µl EIA buffer, 50 µl 20E EIA antiserum, and 50 µl 20E 20E-acetylcholinesterase (AchE) tracer and incubated at 4 °C for 18 h. The EIA plate was emptied and washed five times with washing buffer. To develop 200 µl of Ellman's Reagent was added to each well, followed by adding 5 µl tracer. An orbital shaker was used to allow development in the dark for 120 min. 20 E was used as the standard, and each sample had three replicates. Finally the absorbance was checked at 412 nm with a microplate reader (Epoch, Biotek, USA).

qRT-PCR

For total RNA extraction of the fat body, the Trizol solution was used according to the manufacturer's instructions (Molecular Research Center). A NanoDrop system (BioTek, USA) was used to obtain the RNA concentration. Two µg of total RNA was reverse transcribed by MMLV-RT enzyme (Promega, USA) for 1 h at 42 ºC using an oligo (dT) primer. The qRT-PCR was performed to analyze transcript levels of *Vg* using its specific primers. The actin (*PxActin*) was the reference gene (Table 1). PCR conditions were 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 s at 60 °C. Each experiment was replicated three times with three biological and technical replicates.

Table 1 Primer sequences used in this study.

Primer name	Primer sequence (5′–3′)	Reference
Vitellogenin-F	5 ' -GTTGGAGAGCAACATGCAGA-3'	Zaghloul *et al*., 2017
Vitellogenin -R	5 ' -TCGATCCATTCCTTGATGGT-3 '	Zaghloul *et al*., 2017
Actin-F	5 ' -GTAACGAGAGGTTCCGCCCAG-3 '	Zaghloul *et al*., 2017
Actin-R	5 ' -GGGGCCGGACTCGTCGTATTCTTG-3 '	Zaghloul *et al*., 2017

Statistical analysis

The experimental data of oocyte size and ecdysteroid concentration were analyzed by an unpaired *t*-student test. Data of qPCR were analyzed using the $2_T^{-\Delta\Delta C}$ method (Livak and Schmittgen, 2001) and Tukey multiple comparisons. Graphs were prepared with GraphPad Prism (version 8) software.

Results

Ast in various tissues of 5-days-old females

Competitive ELISA showed different concentrations of Ast in the brain- SOG, midgut, ovaries, and fat body of 5 days old adult female. It was revealed that the highest concentration of Ast was observed in brain-SOG (Fig. 1).

Effects of Ast injection on oocyte size

The effect of Ast injections on oocyte size was verified to investigate the possible role of Ast in female reproductive physiology. Daily injections of Ast to newly emerging adult females for 4 days resulted in significantly smaller oocytes. The mean oocyte size for the Ast injection condition (10^{-11} moles) was 2.19-fold as small as the mean value for the control injected only with PBS (Fig. 2).

Effects of Ast injection on ecdysteroid concentration in hemolymph and ovaries

For more analysis of the effect of Ast on female reproductive physiology, the effect of daily injection of Ast on ecdysteroid concentration in the ovaries and hemolymph was investigated using EIA. The results showed that after four days of injection, the concentration of ecdysteroid in the ovaries was significantly lower compared to the control. Moreover, when Ast was injected into the insect (10^{-12} Moles or more), the ecdysteroid levels in the hemolymph were significantly lower compared to the hemolymph of insects injected only with PBS for four days (Fig. 3A, B).

Effect of Ast injections on *Vg* gene expression level

Injection of adult females with Ast (at the concentration of 10^{-13} moles or more) for four days decreased the expression levels of *Vg* gene compared with the control. However, in lower concentrations, the expression level was not statistically different from the control (Fig. 4).

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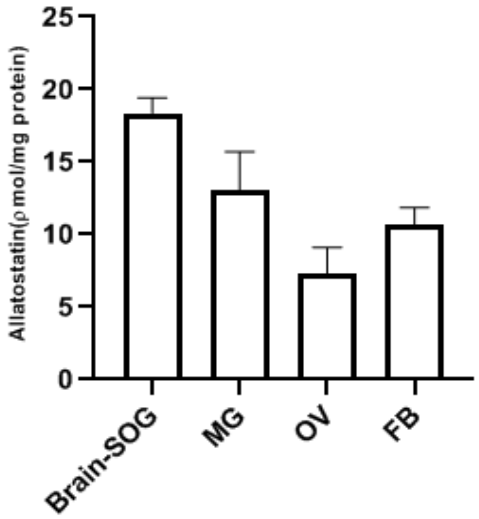

Figure 1 Comparison of allatostatin concentration in various tissues of 5-day-old adult female *G. mellonella* using competitive ELISA. Brain-SOG: Brain-suboesophageal ganglion, MG: Midgut, OV: Ovary, FB: Fat body.

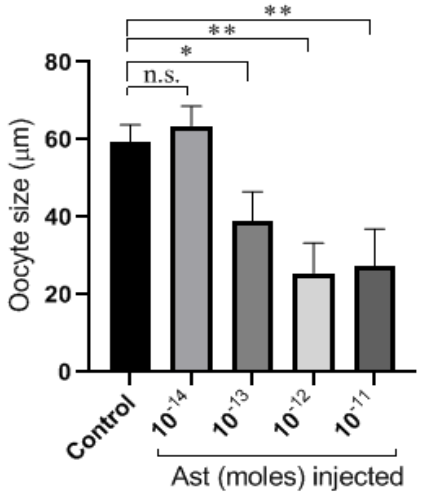

Figure 2 The effect of allatostatin injection on oocyte length in 5-day-old adult females of *G. mellonella*. Twenty newly emerged adult females were injected daily with different concentrations of allatostatin for five days. Control was injected with 1 µl PBS. Significant difference was compared with the control (unpaired *t*-test; *p < 0.05).

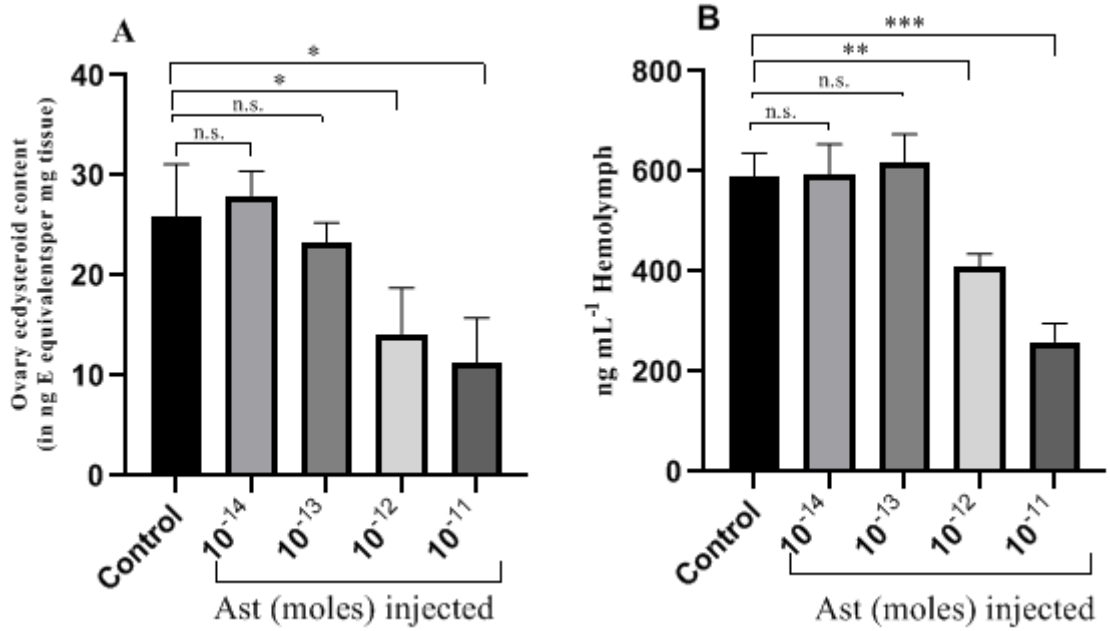

Figure 3 The effect of allatostatin injection on ecdysteroid concentration in the ovaries (A) and hemolymph (B) of twenty 5-day-old adult females of *G. mellonella*. Newly emerged adult females were injected for four days (once every 24 h) with different concentrations of allatostatin using EIA assay. Control was injected with PBS. Significant differences were shown compared with the control (unpaired *t*-test; *p < 0.05).

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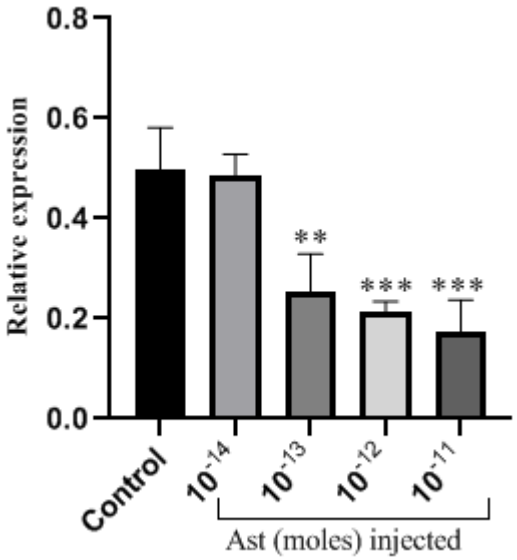

Figure 4 The effect of daily injection of allatostatin on *Vg* gene expression levels using qRT-PCR in adult female *G. mellonella*. Adult females were injected (once every 24 h) with different concentrations of allatostatin for four days. On day 5, the level of *Vg* gene expression level was determined. Actin (*PxActin*) was the reference gene. Asterisks indicate significant differences, (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) analysis of variance was done using Tukey multiple comparisons. n.s. indicates no significant difference.

Discussion

The Asts are well known for their effects on numerous physiological processes, including inhibition of muscle contraction, the inhibition of juvenile hormone III, and their impact on feeding behaviors (Garside *et al.*, 2002; Matsui *et al.*, 2013). In this research, the effects of Ast injection on the female reproductive system in *G. mellonella* were analyzed. Here, the existence of Ast in various tissues was investigated. For the first time, competitive ELISA showed different concentrations of Ast in the brain-SOG, midgut, ovaries, and fat body of adult female *G. mellonella*. Previously, its distribution in the midgut of *S. littoralis* (Nakhaie Bahrami *et al.*, 2018) and *Periplaneta americana* (L.) was shown (Matsui *et al*., 2013). Moreover, Meyering-Vos and Hoffmann (2003) showed the existence of Ast not only in the brain-SOG and midgut but also in the ovaries and fat body of Mediterranean field cricket, *Gryllus bimaculatus* (De Geer).

Several neuropeptides are reported to affect female reproductive physiology. Van Wielendaele *et al*. (2013) indicated that neuropeptide F (NPF) injection increased ecdysteroid titer in the hemolymph and ovaries of *Schistocerca gregaria* (Forsskål). Moreover, it was confirmed that injection of tachykinin-4 increased ecdysteroid titer in the ovaries of *Helicoverpa armigera* (Hübner) (Mikani, 2019). Ecdysteroids are incorporated into the growing oocytes during the vitellogenic period. Some fraction is also leaked into the hemolymph, but these hemolymph ecdysteroids' role is still unknown.

Ecdysteroids are essential factors in the growth of oocytes (Van Wielendaele *et al.*, 2013). Here we confirmed that allatostatin decreased ecdysteroid titer not only in the ovaries but also in the hemolymph. This decrease in ecdysteroid concentration can lead to a reduction in oocyte size. Previously it was confirmed that knockdown of *EcR* gene, which is related to ecdysteroidogenesis, decreased the oocyte size in *Cimex lectularius* (L.) (Gujar and Palli, 2016). It may be possible that a low level of *EcR* led to a decrease in the length of the oocyte.

20E plays an essential role in insect vitellogenesis (Sun *et al.*, 2003). It cannot be excluded that injection of Ast decreased the 20E level, reducing *Vg* gene expression level (Gujar and Palli, 2016).

Based on our results in this paper, we cannot determine Ast's mode of action, but some hypotheses can be proposed. First, the direct effect of allatostatin on ovarian ecdysteroidogenesis or oocyte growth via the impact on ovaries or the fat body cannot be excluded. Another hypothesis is that Ast probably indirectly caused a sequence of effects, such as the effect on food intake, which led to an impact on ecdysteroidogenesis. More investigations will be necessary to clarify the role of Ast in the female reproductive system.

In conclusion, this study, for the first time, provided new insight into the physiological roles

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of Ast in the female reproductive system in *G. mellonella*. It showed that Ast decreases the ecdysteroid titer in hemolymph and ovaries and reduces oocyte size and *Vg* gene expression level. More investigations will be necessary to clarify the exact role of Ast in the female reproductive system in *G. mellonella*.

Conflict of Interest Statement

The author hereby confirms that there is no conflict of interest and ensures no disputes over the ownership of the data presented in the paper.

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اثر آلاتوستاتین روی سیستم تولیدمثلی حشره ماده شبپره موم‌خوار بزرگ *Galleria mellonella* (Lepidoptera: Pyralidae)

اعظم میکانی

گروه حشره‌شناسی‌کشاورزی، دانشکده کشاورزی، دانشگاه تربیت مدرس، تهران، ایران.
پست الکترونیکی نویسنده مسئول مکاتبه: a.mikani@modares.ac.ir
دریافت: ۱۰ تیر ۱۴۰۱؛ پذیرش: ۲۰ شهریور ۱۴۰۱

چکیده: در این پژوهش، اثر آلاتوستاتین روی سیستم تولیدمثلی حشره ماده شبپره موم‌خوار بزرگ *Galleria mellonella* (L.) مورد بررسی قرار گرفت. در ابتدا حضور غلظت‌های مختلف از آلاتوستاتین در بافت‌های مغز، روده میانی، تخمدان‌ها و اجسام چربی حشره ماده پنج روزه مورد تأیید قرار گرفت و مشخص شد که غلظت آلاتوستاتین در بافت مغز بیشتر از بافت‌های دیگر است. تزریق روزانه آلاتوستاتین میزان غلظت اکدیسترویید در همولنف و تخمدان‌ها را کاهش داد. ضمناً این نوروپپتید باعث کاهش اندازه اووسیت‌ها شد. این نوروپپتید میزان بیان ویتلوجنین را نیز کاهش داد. این نتایج نشان داد که آلاتوستاتین در تنظیم تولیدمثل حشره ماده شبپره موم‌خوار بزرگ نقش دارد.

واژگان کلیدی: شبپره موم‌خوار بزرگ، اکدیسترویید، اندازه اووسیت، ویتلوجنین

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