Published in final edited form as:

J Insect Physiol. 2014 May ; 64: 48–53. doi:10.1016/j.jinsphys.2014.03.005.

Suppression of allatotropin simulates reproductive diapause in the mosquito *Culex pipiens*

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Abstract

The cessation of juvenile hormone (JH) production is a key endocrine event that halts ovarian development and hence initiates diapause in females of the mosquito, *Culex pipiens*. The shutdown in endocrine activity of the *corpora allata* (CA), the source of JH, was manifested in the smaller size of CA in females reared under short daylengths (diapause) compared to those reared under long daylengths (nondiapause), as well as in low expression of the mRNA encoding allatotropin, the neuropeptide that promotes JH biosynthesis in the CA. Genes encoding both allatotropin and allatostatin were identified in *C. pipiens*, but only expression levels of allatotropin differed in the two types of females. Knockdown of allatotropin mRNA using RNA interference in females programmed for nondiapause resulted in a cessation of ovarian development akin to diapause. This arrest in development could be reversed with an application of JH. Our results thus suggest that suppression of allatotropin is a critical link in regulating the shutdown of the CA during diapause.

Keywords

Allatotropin; Allatostatin; Diapause; Juvenile hormone; *Culex pipiens*

1. Introduction

A shutdown in the production of juvenile hormone (JH) is one of the unifying features of adult diapause (Denlinger et al., 2012). In the mosquito *Culex pipiens* this shutdown in JH production (Readio et al., 1988; Readio et al., 1999), is manifested in arrested ovarian development, enhanced stress resistance, fat storage and sugar gluttony (Bowen, 1992;

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Data deposition: The sequences reported in this paper have been deposited in the Genbank database (accession nos. *C. pipiens* allatotropin, KF781633; allatostatin, KF781634)

Mitchell and Briegel, 1989; Robich and Denlinger, 2005; Sanburg and Larsen, 1973; Sim and Denlinger, 2008; Sim and Denlinger 2013a). The central role of JH in endocrine regulation of ovarian development is evident not only from the shutdown of JH synthesis by the *corpora allata* (CA), but also by the fact that a topical application of JH will restore ovarian development in diapausing females of *C. pipiens* (Spielman 1974; Sim and Denlinger, 2008). Thus, the fact that JH directly controls ovarian development in *C. pipiens* suggests that the CA, the endocrine glands that synthesize JH, are finely regulated as a component of the diapause syndrome.

The neuropeptides best known for mediating activity of the CA are allatotropins (AT), neuropeptides that stimulate production of JH, and allatostatins (AS), neuropeptides known to inhibit JH biosynthesis in numerous insects (Hoffmann et al., 1999; Goodman and Granger, 2005). AT, first isolated from *Manduca sexta* (Kataoka et al., 1989), are also present in mosquitoes (Veenstra and Costes, 1999). AS, originally isolated from cockroaches (Woodhead et al., 1989; Tobe, 1980), are also widely distributed in other insects. Three families of AS are recognized: type A, YXFGL-amide allatostatins (AS-A) reported first from cockroaches; type B, W₂W₉ allatostatins (AS-B) isolated from crickets, locusts and stick insects; and type C PISCF-allatostatins found first in Lepidoptera but now known from other orders as well (Bendena et al., 1999; Gilbert et al., 2000; Goodman and Granger, 2005). In *A. aegypti* AT functions to stimulate JH synthesis in the CA (Li et al., 2003) and AS-C inhibits JH synthesis in the CA (Li et al., 2004, 2006); AS-C appears to be the only AS of importance to *A. aegypti* (Li et al., 2004).

AT and AS-C are thus prime candidates to be involved in regulating the diapause response in *C. pipiens*. In this report we identify the genes encoding AT and AS-C in *C. pipiens* and demonstrate that transcript levels of AT, but not AS-C, are significantly lower in diapausing females than in their nondiapausing counterparts. We also show that knocking down AT in nondiapausing females with RNA interference mimics the ovarian arrest characteristic of diapause.

2. Material & Methods

2.1 Insect Rearing

The stock colony of *C. pipiens* (Buckeye Strain) was reared at 25°C and 75% relative humidity under a 15 h light: 9 h dark (L:D) photoperiod, as previously described (Robich and Denlinger, 2005); adults were provided a 10% sucrose solution and fed chicken blood using an artificial membrane system. When larvae reached the second instar, rearing containers were placed under one of two environmental conditions: nondiapausing (ND) adults were generated by rearing at 18 °C, 75% RH, and 15:9 L:D. To induce diapause (D), mosquitoes were reared at 18 °C, 75% RH, and 9:15 L:D. To confirm diapause status, primary follicle and germarium lengths were measured, and the stage of ovarian development was determined according to methods described (Christophers, 1911).

2.2 Measurements of follicle and *corpora allata* size

Follicles and CA were dissected from diapausing and nondiapausing females one week after adult eclosion. Tissues were placed in a drop of saline solution, dissected with a needle, and

examined at 200 and 400 fold magnifications (Zeiss Axioskop, Thornwood, NY). Samples were then analyzed with an Olympus SZH-ILLD light microscope with an attached DP72 12.8 megapixel digital camera and DP2-TWAIN software (Olympus Corp, Center Valley, PA). Lengths of 10 follicles and the CA were calculated for 11–12 individuals. A Student's T-test was used to distinguish differences in the sizes of these two tissues in diapausing and nondiapausing females.

2.3 Identification of *Culex allatotropin* and *allatostatin* sequences

To retrieve sequences of *C. pipiens* allatotropin (AT) and allatostatin (AS-C), sequences of *Aedes aegypti* AT and AS-C were utilized in discontinuous MEGA-BLAST searches on the *C. quinquefasciatus* genome database (<http://cpipiens.vectorbase.org/Tools/BLAST/>). cDNAs containing AT and AS-C coding regions were amplified using the following primers (5'–3'): AT, AAGGTCCTGTTCGTGGTGAT and AGAACAAGTCGTTGCTGTCG; AS-C, AACCTCCCCC GTCATGTT and GTTGTACCGGTTGCGAAGTC.

2.4 Transcript levels of allatotropin and allatostatin in diapausing and non-diapausing mosquitoes using qRT-PCR

To compare transcript levels of AT and AS-C in early diapause and in nondiapausing mosquitoes, total RNA samples were extracted with Trizol (Invitrogen, CA) from three batches of 15 adult female mosquitoes 1, 3, 6 and 10 days after eclosion. To remove genomic DNA contamination, RNA samples were treated with 1.0 µl DNase I following the manufacturer's instructions (50–375 units/µl; Invitrogen, CA). For reverse transcription, 5 µg total RNA was reverse transcribed with Superscript III RNase H-reverse transcriptase (Invitrogen). qRT-PCR was performed using an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA), as described previously (Sim et al., 2005; Sim et al., 2007). Briefly, standard curves were generated for each transcript tested using 10-fold serial dilutions of plasmids which included each AT and AS-C fragment, ranging from 100 to 0.01 pg per reaction. All reactions were performed in triplicate in a total volume of 20 µl containing 10 µl of SYBR Green PCR Master Mix, 300 nmol of each primer at the following conditions: 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. Ribosomal protein L19 (RpL19) (FJ2666017) was used as an internal control. Sequences of gene specific qRT-PCR primer sets are listed accordingly (5'–3'): qAT, TCTGCTGTACACGTCC AGT and CCCGTATCGAACGTGGTATT; qAS-C, AACCTCCCCCGTCATGTT and GTTGTACCGGTTGCGAAGTC; qRpL19, CGCTTTGTTTGATCGTGTGT and CCAATCCAGGAGT GCTTTTG. Statistical significance of differences in the expression of individual genes was determined using a Student's t-test between the relative transcript values derived from the diapause and non-diapause mosquitoes across three replicates for each gene; a P-value less than 0.05 was considered to be a significant change in transcript level.

2.5 dsRNA of the allatotropin gene preparation and injection into adult females

dsRNA for *C. pipiens* AT (dsAT) and β -galactosidase (ds β -gal) genes were prepared using the MEGascript T7 transcription kit (Ambion, Austin, TX), as previously described (Sim et

al., 2007), and T7 primers are listed accordingly (5'–3'): dsAT, TAATACGACTCACTATAGGGCTG TGTTCGGTTCTGCTCTG and TAATACGACTCACTATAGGGAGAACAAGTCGTTGCTG TCG; ds β -gal, TAATACGACTCACTATAGGGGTCGCCAGCGGCACCGCGCCTTTC and TAATACGACTCACTATAGGGCCGGTAGCCAGCGCGGATCATCGG Each PCR-derived fragment was sequenced and BLASTed against the *C. quinquefasciatus* genome database (<http://cpipiens.vectorbase.org/Tools/BLAST/>) to validate redundancy of the sequence and to confirm unique sequences. In knockdown experiments, ~ 0.5 μ l dsRNA of *Culex* AT (2.0 μ g/ μ l) or ~ 0.5 μ l dsRNA of β -galactosidase (ds β -gal, 2.0 μ g/ μ l) was injected into the thorax of cold-anesthetized females using a microinjector (Tritech Research, Los Angeles, CA). Thus, females were injected with ~1.0 μ g dsAT or ds β -gal (control).

2.6 RNAi efficiency evaluation using qRT-PCR

qRT-PCR of the dsRNA injected mosquitoes was carried out as above and as previously described (Sim and Denlinger, 2008). To evaluate RNAi efficiency, primers were used to amplify endogenous AT. Ribosomal protein large subunit 19 (RpL19) from *C. pipiens* was used to normalize AT transcript levels in females injected with either dsAT or ds β -gal.

2.7 Follicle assay following dsAT and juvenile hormone treatment

ND females, within a day after adult eclosion, were injected in the thorax with dsAT or ds β -gal (control). Each treated cohort was kept in an 8 cm diameter $\times$ 12 cm cage. Cotton soaked in a 10 % sucrose solution was provided 1 hour after the dsAT or ds β -gal injection. Juvenile hormone III (Sigma-Aldrich, MO) was used to evaluate the mosquito's response after dsAT injection. Females injected with dsAT were then topically treated on day 1 with serial dilutions of juvenile hormone III (5, 50, 500 ng/♀) diluted in 0.5 μ l acetone. Caged females were placed at 18 °C, 75 % R.H., 15: 9 h L:D, and ovaries were assessed one week after injection. Ovaries were dissected in a drop of saline solution, disrupted with a needle, and examined at 200 and 400 fold magnifications (Zeiss Axioskop, Thornwood, NY). Mean follicle length for each female was calculated from measurements of 10 follicles, and data were collected from 15–29 individuals. An ANOVA test was used to distinguish differences in follicle sizes.

3. Results

3.1 Development of *corpora allata* is halted in diapausing females

Ovaries and CA from adult females reared either under the ND photoperiod (L:D 15:9) or D photoperiod (L:D 9:15) at 18°C were dissected 7–10 days after adult eclosion. Both the ovarian follicles and CA from nondiapausing females were significantly larger than those from diapausing adults (Fig. 1. A–D). One week after adult eclosion, CA from nondiapausing females were approximately twice as large as those from diapausing females reared at short daylength (Fig. 1E).

3.2 *C. pipiens* allatotropin (AT) and allatostatin (AS-C)

C. pipiens AT and AS-C sequences (accession nos. *C. pipiens* allatotropin, KF781633; allatostatin, KF781634) were identified via BLAST alignment searches of known *Aedes*

aegypti sequences against the genome of *C. quinquefasciatus*, a sister species of *C. pipiens*. cDNA fragments of *C. pipiens* AT (503 bp) share highest identities (89%) with AT from a closely related mosquito, *Aedes aegypti*. The 352-bp cDNA fragment of *C. pipiens* AS-C was not detected in predicted transcripts of the *C. quinquefasciatus* genome, but aligned with 3 regions of supercontig 3.9; 48897–48761, 54861–54741 and 43816–43720. AS-C cDNA shares 85% identity with allatostatin C-type from *Ae. aegypti* and 84% identity to allatostatin 2 sequence from *Anopheles gambiae*.

3.3 Transcript levels of AT and AS-C in diapausing and nondiapausing females of *C. pipiens*

Expression patterns of the AT and AS-C genes in nondiapausing and diapausing *C. pipiens* were obtained using qRT-PCR. mRNA expression patterns of the gene encoding AT showed that expression was high in nondiapausing females relative to diapausing females at eclosion and reached its highest level on day 6 after eclosion (Fig. 2A). In contrast, mRNA expression patterns of the gene encoding AS-C were not significantly different between nondiapausing and diapausing females (Fig. 2B). Reduced expression of the AT gene in adult females in response to short daylength (destined to diapause) suggests that this gene may control the diapause phenotype by failing to stimulate JH III synthesis in the CA.

3.4 Halt of ovarian development in nondiapausing females by dsAT and rescue by JH

dsRNA efficiency was first assessed by qRT-PCR. In contrast to the relatively high expression of AT mRNA in ds β -gal injected mosquitoes, only traces of AT mRNA were detected in dsAT injected, nondiapausing females (Fig. 3A), indicating that injection of dsAT successfully inhibited expression of the AT gene. The ubiquitous ribosomal protein L19 (RpL19), which expresses a uniform, moderate copy number in all cells, was used as a loading control to normalize transcript levels (Sim and Denlinger 2009) and showed no difference in expression 3 and 6 days post injection (Fig. 3B), thus supporting the conclusion that the low expression levels of the AT gene were related to the knockdown effect of dsRNA rather than variation in sample loading.

Seven days post-injection, primary follicles in untreated nondiapausing females and ds β -gal injected controls were nearly 70% larger than follicles in diapausing females reared at short daylength (Fig. 4). By contrast, follicle length was greatly reduced following injection of dsAT into nondiapausing females and was not significantly different from that observed in diapausing females, thus knockdown of AT mRNA in nondiapausing females mimicked the diapause response.

When ovarian status was monitored using the standard classification for diapause (Spielman and Wong, 1973) few untreated and ds β -gal control, nondiapausing females were in the diapausing category 7–10 days post injection. In contrast, a diapause-like status was highly prevalent in nondiapausing mosquitoes injected with dsAT and was maintained at a level similar to that typically observed in diapausing mosquitoes (Fig. 4)

As demonstrated above, ovaries of dsAT-injected nondiapausing females halted development in a state simulating diapause (Fig. 4). This arrest could be reversed with

juvenile hormone, as indicated by the increase in length of primary follicles in dsAT-injected females following a topical application of juvenile hormone III (Fig. 4).

4. Discussion

The JHs dictate many aspects of insect growth, metamorphosis, and reproductive physiology and are intimately involved in regulation of adult reproductive diapause. Production of these sesquiterpenoids, secreted by the CA, is consistently suppressed at the onset of adult diapause in the mosquito *C. pipiens* (Readio et al., 1988; Denlinger and Armbruster, 2014), as well as in other insects that diapause in the adult stage (Denlinger et al., 2012). In *C. pipiens*, removal of the CA or decapitation in adult females interrupts development of the ovary, and development can be restored by reimplantation of active CA or by treatment with JH (Spielman, 1974). This suggests that inactivation of the CA in adult females mimics a key aspect of the diapause syndrome, ovarian arrest in the previtellogenic stage. JH III is the dominant JH in *C. pipiens*, and in nondiapausing females the titer of JH III rapidly increases during the first week after adult emergence (Readio et al., 1988). By contrast, if females of *C. pipiens* are raised under short daylengths, the JH titer is low, the diapausing females are not receptive to a blood meal, and, after mating, she will seek a protected environment in which to overwinter (Denlinger and Armbruster, 2014). Size of the CA is not universally a reliable indicator of gland activity, but during diapause in *C. pipiens* the small size of the CA correlates with a period of low JH production, as noted in this study, as well as in our previous work (Sim and Denlinger, 2008).

In this study, a comparison of AT transcript levels between females reared in long daylengths (nondiapause) and short daylengths (diapause) revealed a major difference in these two groups. Expression of the gene encoding AT was quite low for the diapause females while levels were much higher in the nondiapause group, at least for the first 3–6 days after adult eclosion. These results mirror the JH titer distinctions observed between diapause and nondiapause females of *C. pipiens*: while the JH titer is elevated at this time in nondiapause females, the titer remains low in diapause (Readio et al., 1999). These results are thus consistent with the hypothesis that suppression of AT transcripts contributes to the blocking of JH synthesis in diapausing females of *C. pipiens*. When transcripts of the AT gene were suppressed by RNAi in nondiapausing females immediately after adult eclosion, ovarian development was also halted, and the ovaries mimicked the phenotype of adult females reared under short daylength (diapause). This suppression could be reversed by the topical application of JH III, further indicating that the AT knock-down was exerting its effect by impairing JH synthesis.

In contrast to AT, our experiments do not suggest a role for AS-C in shutting down the CA in association with diapause. Expression levels of AS-C mRNA were equivalent in diapausing and nondiapausing females. Although mRNA levels are a good predictor of the levels of the end products, differences in hemolymph peptide titers can also be determined postranscriptionally or by regulation at the site of release. The hemolymph titers of neither the AT nor AS-C peptides in relation to diapause are currently known.

We previously demonstrated that another neuropeptide, insulin-like peptide 1 (ILP-1), is also suppressed in diapausing females, and its knock-down by RNAi also yields a halt in ovarian development at the same previtellogenic stage (Sim and Denlinger, 2009; Sim and Denlinger 2013b). Thus, multiple hormonal factors appear to contribute to JH regulation during diapause in *C. pipiens*. Whether AT and ILP-1 both act directly on the CA or if these two neuropeptides act on different targets in the regulatory pathway of CA stimulation and/or JH production remains unknown. AT play multiple functions beyond their classic role in regulating the CA (Goodman and Granger, 2005; Hernandez-Martinez et al., 2007), thus we cannot discount the possibility that suppression of AT during diapause may also contribute to other features of the diapause syndrome.

How environmental signals of daylength are translated into the diapause program still remains largely a black box. Although evidence that the clock genes play a role in deciphering the distinction between long and short daylengths is growing (Meuti and Denlinger, 2013), we know little about how such information is integrated within the brain to eventually evoke the diapause response. The results we present here indicate that any comprehensive pathway for diapause in *C. pipiens* is likely to involve suppression of AT, leading to a shutdown in JH production.

Acknowledgments

This work was supported in part by National Institutes of Health grant 2R56-AI058279 to DLD and CS.

Abbreviations

AT	allatotropin
dsAT	double strand RNA of <i>C. pipiens</i> allatotropin
AS-C	allatostatin
dsβ-gal	double strand RNA of β -galactosidase
qRT-PCR	quantitative real-time PCR
RNAi	RNA interference
ND	nondiapause
D	diapauses
JH	juvenile hormone

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- Higher levels of allatotropin (AT) mRNA are present in nondiapausing females than in diapausing females of *Culex pipiens*.
- Allatostatin (AS-C) mRNA levels are not altered by diapause.
- RNAi knockdown of AT in nondiapausing females mimics the diapause status of the ovaries.
- The knockdown effect can be reversed with an application of juvenile hormone III.

Fig. 1.

Morphological distinctions in ovarian follicles and *corpora allata* between nondiapausing (ND) and diapausing (D) females of *Culex pipiens*. Ovarian follicles from (A) ND females and (B) D females one week after adult eclosion show the cessation of development in D females. *Corpora allata* from (C) ND females and (D) D females dissected one week after adult eclosion, showing that the CA are larger and exhibit increased cell counts in ND females (scale bar, 50 Hm). (E) Mean $\pm$ s.d. length of *corpora allata* in ND and D females dissected one week after adult eclosion, n = 11–12 females.

Fig. 2. Quantitative real-time PCR showing expression of AT and AS-C in *C. pipiens* females at 1, 3, 6 and 10 days after adult eclosion. ND (■) = programmed by long daylength for nondiapause, D (◇) = programmed by short daylength for diapause. Both groups were maintained at 18°C. Bars (mean ± s.d.) with asterisk(s) indicate significant differences at *P=0.05 and **P=0.001, T-test. Ribosomal protein large subunit (RpL19) was used to confirm equal loading. Data are expressed as mean transcript level ± s.d.; n=3 groups of 10 individuals each.

Fig. 3.

RNA interference efficiency of dsAT in *C. pipiens*. (A) AT transcript levels in females injected with dsAT (white bars) were compared with the dsβ-gal controls (black bars). Expression levels were measured by qRT-PCR at 3 and 6 days after RNAi injection and normalized using a Ribosomal protein large subunit 19 (RpL19) as a loading control. (B) RpL19 transcript levels in females injected with dsAT (white bars) were compared with the dsβ-gal controls (black bars). Expression levels were not significantly different between treated and control groups at $P=0.05$, T-test.

Fig. 4.

Juvenile hormone rescue of ovarian development in AT knocked-down mosquitoes that were programmed by long daylength for nondiapause. Mean $\pm$ s.d. length of ovarian follicles in dsAT injected ND females that subsequently received graded doses of JH III. $n = 15$ – 29 females for each treatment. Bars with the same letters were not significantly different at $P=0.05$, ANOVA.