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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.

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, 1999), is manifested in arrested ovarian development, enhanced stress resistance, fat storage and sugar gluttony (Bowen, 1992; Mitchell and Briegel, 1989; Robich and Denlinger, 2005; Sanburg and Larsen, 1973; Sim and Denlinger, 2008, 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, W_2W_9 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 *Aedes 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

Abbreviations: AT, allatotropin; dsAT, double strand RNA of *C. pipiens* allatotropin; AS-C, allatostatin; dsβ-gal, double strand RNA of β-galactosidase; qRT-PCR, quantitative real-time PCR; RNAi, RNA interference; ND, nondiapause; D, diapause; JH, juvenile hormone.

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

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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. Materials & 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 1 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 *A. aegypti* AT and AS-C were utilized in discontinuous MEGA-BLAST searches on the *Culex 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, AACCTCCCCCGTCATGTT and GTTGTACCGGTTCGGAAGTC.

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, 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,TCTGCTGTCACACGTCCAGT and CCCGTATCGAA CGTGGTATT; qAS-C, AACCTCCCCCGTCATGTT and GTTGTACCG GTTCGGAAGTC;qRpL19,CGCTTTGTTTGATCGTGTGT and CCAATCC AGGAGTGCTTTTG. 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 (dsβ-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, TAATACGACTC ACTATAGGGCTGTGTTCGGTTCTGCTCTG and TAATACGACTCACTAT AGGGAGAACAAGTCGTTGCTGTCG;dsβ-gal, TAATACGACTCACTAT AGGGGTCGCCAGCGGCACCGCGCCTTTC and TAATACGACTCACTA TAGGGCCGGTAGCCAGCGCGGATCATCGG. 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 μl dsRNA of *Culex* AT (2.0 μg/μl) or ~0.5 μl dsRNA of β-galactosidase (dsβ-gal, 2.0 μg/μl) was injected into the thorax of cold-anesthetized females using a microinjector (Tritech Research, Los Angeles, CA). Thus, females were injected with ~1.0 μg dsAT or dsβ-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 dsβ-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 dsβ-gal (control). Each treated cohort was kept in an 8 cm diameter × 12 cm cage. Cotton soaked in a 10% sucrose solution was provided 1 h after the dsAT or dsβ-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 and 500 ng/♀) diluted in 0.5 μl acetone. Caged females were placed at 18 °C, 75% RH, 15:9 h L:D, and ovaries were assessed 1 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 to 29 individuals. An ANOVA test was used to distinguish differences in follicle sizes.