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Diapause-specific gene expression in the northern house mosquito, *Culex pipiens* L., identified by suppressive subtractive hybridization

Rebecca M. Robich¹, Joseph P. Rinehart², Linda J. Kitchen³, and David L. Denlinger^{*}
Department of Entomology, Ohio State University, 318 West 12th Avenue, Columbus, OH 43210, USA

Abstract

In this study we probe the molecular events underpinning diapause observed in overwintering females of *Culex pipiens*. Using suppressive subtractive hybridization (SSH) we have identified 40 genes that are either upregulated or downregulated during this seasonal period of dormancy. Northern blot hybridizations have confirmed the expression of 32 of our SSH clones, including 6 genes that are upregulated specifically in early diapause, 17 that are upregulated in late diapause, and 2 upregulated throughout diapause. In addition, 2 genes are diapause downregulated and 5 remain unchanged during diapause. These genes can be categorized into eight functional groups: genes with regulatory functions, metabolically-related genes, those involved in food utilization, stress response genes, cytoskeletal genes, ribosomal genes, transposable elements, and genes with unknown functions.

Keywords

Culex pipiens; Mosquito; Adult diapause; Overwintering; Gene expression; SSH

1. Introduction

One of the primary avian vectors of West Nile virus in the northern United States, *Culex pipiens* (L.), enters an adult diapause in late summer and early fall in response to short daylength and low temperature (Eldridge, 1966; Sanburg and Larsen, 1973; Spielman and Wong, 1973). As is the case of most adult diapauses (Denlinger, 1985; 2002), the diapause of *Cx. pipiens* is initiated by a shut-down in the production of juvenile hormone (JH) by the corpora allata (Spielman, 1974). JH levels remain low during diapause preparation, but gradually increase throughout the course of winter until levels comparable to 3-day old nondiapausing females are reached by diapause termination (Readio et al., 1999).

The mosquitoes first appear in overwintering sites such as caves, culverts, and unheated basements (Vinogradova, 2000) as early as August (Service, 1968; Spielman and Wong, 1973; Onyeka and Boreham, 1987) and remain there until spring when environmental conditions again become favorable for development. Only females enter diapause and most

* Corresponding author. Tel.: +1-614-292-6425, Fax: +1-614-292-2180. Email address: denlinger.1@osu.edu

¹Harvard School of Public Health, Department of Immunology and Infectious Diseases, Boston, MA 02115

²SDA, Agricultural Research Service, Red River Valley Station, Fargo, ND 58105

³Ross University School of Veterinary Medicine, West Farm, Saint Kitts, West Indies

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are inseminated prior to entering the hibernation site (Onyeka and Boreham, 1987). In preparation for diapause, females increase their lipid reserves by feeding on plant secretions rich in carbohydrates (Mitchell and Briegel, 1989a; Bowen, 1992), and at this time females rarely, if ever, take a blood meal (Mitchell, 1983; Mitchell and Briegel, 1989b).

Many aspects of diapause in *Cx. pipiens* have been well documented. There is a good database that describes the physiological features of this diapause, its environmental regulators, and the hormonal control mechanism. What is currently lacking is an understanding of its molecular underpinning. Very little is known of these events in any mosquito vector, although such work has been initiated on the embryonic diapause of *Oc. triseriatus*, an important vector of La Crosse encephalitis (Blitvich *et al.*, 2001). In this species, several cDNA fragments have been identified using primers designed to amplify sequences that the La Crosse virus cap-scavenges. Among cDNA fragments that have been identified are a mitochondrial *cytochrome c oxidase subunit*, *18S* and *28S ribosomal RNAs*, *protein disulfide-isomerase*, and several novel transcripts, but it is not yet known if any of these genes are indeed involved in regulation of this diapause or in the regulation of viral transcription.

The molecular events involved in the pupal diapause of the flesh fly are probably the best understood; while many genes are shut down during diapause, a small cluster of genes (approximately 4 %) are diapause upregulated (Denlinger, 2002). Several classes of diapause upregulated genes have been noted, including stress response genes, developmental arrest genes, and genes involved in regulating specific physiological activities that are unique to diapause. Although some genes are turned on at the onset of diapause and remain upregulated until diapause has been broken, others are uniquely expressed only in early or late diapause. For example, *heat shock protein 70* is upregulated throughout pupal diapause in the flesh fly, *Sarcophaga crassipalpis* (Rinehart *et al.*, 2000), while *cystatin* is upregulated only in early diapause (Goto and Denlinger, 2002), and *ultraspiracle* is upregulated only in late diapause (Rinehart *et al.*, 2001). Other genes, such as the cell cycle regulator *proliferating cell nuclear antigen*, are shut down during diapause (Tammariello and Denlinger, 1998).

In this study, suppressive subtractive hybridization (SSH) is used to identify genes that are differentially expressed during the adult diapause of *Cx. pipiens*. Expression patterns are confirmed by northern blot hybridization, and the regulated genes that have been identified are discussed in the context of their possible functional contributions to diapause.

2. Materials and methods

2.1. Insect rearing

An anautogenous colony of *Cx. pipiens* (L.) was established in September, 2000, from larvae collected in Columbus, Ohio (Buckeye strain). The colony was maintained at 25°C, 75% r.h., with a 15hL:9hD daily light:dark cycle. Eggs and first instar larvae were kept under colony conditions until the second instar, and at that time larvae were moved to an environmental chamber at 18°C, 75% r.h., and 15L:9D (nondiapause, 18°C) or placed in an environmental room under diapause-inducing conditions of 18°C, 75% r.h., with a 9L:15D daily light:dark cycle (diapause, 18°C).

Larvae were reared in 5 × 18 × 28 cm plastic containers in de-chlorinated tap water, fed a diet of ground fish food (TetraMin), and maintained at a density of ~250 mosquitoes per pan. Adults were kept in 30.5 cm³ screened cages and provided constant access to water and honey-soaked sponges. Honey sponges were removed from short-day cages 10–13 days after adult eclosion to mimic the absence of sugar in the natural environment during the overwintering period. None of the mosquitoes used in these experiments were offered a blood meal. To confirm diapause status, primary follicle and germarium lengths were measured, and the stage of

ovarian development was determined according to the methods described by Christophers (1911) and Spielman and Wong (1973).

2.2. Suppressive subtractive hybridization

Total RNA was isolated from pools of 20 females by grinding with 4.5 mm copper-coated spheres ("BB's") in 1 ml TRIzol® Reagent (Invitrogen). After homogenization, samples were spun at 12,404 g at 4°C for 10 min, and the supernatant was used for RNA extraction following standard protocol (Chomczynski and Sacchi, 1987). RNA pellets were stored in absolute ethanol at -70°C and dissolved in 30 µl ultraPURE™ water (GIBCO) for use in cDNA synthesis.

Two rounds of suppressive subtractive hybridization (SSH) were performed using the Clontech PCR-Select™ cDNA Subtraction Kit to select for genes upregulated and downregulated in early and late diapause. The first round of SSH consisted of cDNA collected from females in early diapause (short daylength, 18°C, 7–10 days post adult eclosion) as the tester and early nondiapause (long daylength, 18°C, 7–10 days after adult eclosion) as the driver (early diapause – early nondiapause); the reverse selection using early nondiapause as the driver was also performed (early nondiapause – early diapause). The second round of SSH was done using cDNA from late diapausing females (short daylength; 18°C, 56–59 days post-adult eclosion) and early nondiapausing females (long daylength, 18°C, 7–10 days after adult eclosion) for forward (late diapause – early nondiapause) and reverse (early nondiapause – late diapause) selections. Ovarian dissections of the late diapausing females (56–59 days after adult eclosion) indicated that the females were in a late stage of diapause, just prior to diapause termination.

During our initial round of SSH, mRNA was isolated with streptavidin-coupled paramagnetic particles using the PolyATtract® mRNA Isolation System (Promega), and this was directly followed by cDNA synthesis according to standard SSH protocol (Clontech). This yielded a high percentage of clones with identity to fragments of *large ribosomal subunit* RNA. To reduce the abundance of ribosomal RNA during our second round of SSH, cDNA synthesis was performed using the BD SMART™ PCR cDNA Synthesis Kit (BD Biosciences) following standard protocol.

Forward and reverse subtracted libraries were cloned using the TOPO TA Cloning™ Kit (Invitrogen). Transformed plasmids were inserted into competent *Escherichia coli* cells and grown overnight on Luria-Bertani (LB) plates containing X-Gal and ampicillin. For each library, over 100 white colonies were isolated and grown overnight in LB-ampicillin broth at 37°C. Colonies were then purified with QIAprep Spin Miniprep (QIAGEN), run on a 1% agarose gel to determine concentration, and sequenced using the vector internal primer sites (T7 and M13R) at the Ohio State University Plant-Microbe Genomics Facility on an Applied Biosystems 3730 DNA Analyzer using BigDye® Terminator Cycle Sequencing chemistry (Applied Biosystems) following manufacturer's protocol.

2.3. Northern blot analysis

Fifteen micrograms of denatured total RNA samples were separated by electrophoresis on a 1.4% agarose denaturing gel (0.41 M formaldehyde, 1X MOPS-EDTA-sodium acetate). Visualization of ethidium bromide stained rRNA under UV light exposure was used to confirm equal loading. Following the TURBOBLOTTER™ Rapid Downward Transfer Systems protocol (Schleicher and Schuell), the RNA was transferred for 1.5 hours onto a 0.45 micron MagnaCharge nylon membrane (GE Osmonics) using downward capillary action in 3 M NaCl, 8 mM NaOH transfer buffer, followed by neutralization in a 0.2 M phosphate buffer solution and UV crosslinking. The membrane was then air-dried and either stored at -20°C or used immediately for hybridization.

Digoxigenin (DIG)-labeled cDNA probes were developed from genes of interest in our forward and reverse subtracted SSH libraries. PCR was performed on each clone using the SSH nested primers (Clontech PCR-Select™ cDNA Subtraction Kit) according to the following parameters: 94°C for 3 min and 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 2 min, followed by a 7 min extension at 72°C and a 4°C hold. The PCR products were run on a 1% agarose TAE gel and the band of interest was isolated from any remaining vector, extracted with Ultrafree®-DA (Millipore), and re-amplified by PCR. To confirm clone identity, PCR products were sequenced using the forward and reverse nested primers (Clontech) by the methods described above. The cDNAs were individually labeled in an overnight DIG reaction using 100ng of template DNA and the Dig High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Sciences). Probes were stored at -20°C.

Hybridization was carried out overnight followed by stringency washes and immunological detection using the Dig High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Sciences) according to manufacturer's protocol. Blots were then exposed to chemiluminescence film (Kodak Biomax). Each northern was replicated a minimum of three times. To confirm equal transfer of RNA, each membrane was stripped with 0.2 M NaOH/ 0.1% SDS and re-probed using DIG-labeled 28S cDNA, according to manufacturer's instructions.

2.4. Bioinformatics analyses

Sequences were edited and assembled using dnaLIMS (dnaTools) and the Baylor College of Medicine Search Launcher: Sequence Utilities (<http://dot.imgen.bcm.tmc.edu/seq-util/seq-util.html>). Putative sequence identities were determined by BLASTn and BLASTx searches in GenBank (<http://www.ncbi.nlm.nih.gov/>), and the closest match for each sequence was listed in Tables 1 and 2 including % identity, organism, and corresponding accession number. BLAST searches resulting in low percent identities (<40%) and short matches (<30 bp) were considered not significantly similar and are thus listed as "genes with unknown function". If BLASTn results did not produce a high percent identity, BLASTx results were used. Nucleotide sequences were deposited in GenBank and assigned accession numbers listed in Tables 1 and 2.

3. Results

3.1. Early and late diapause subtraction

Our first round of SSH (early diapause – early nondiapause and early nondiapause – early diapause) yielded only 5 unique diapause-upregulated clones from our initial screening of 48 clones, while the rest were identified as fragments of *large ribosomal subunit* RNA (with high identity to *Aedes aegypti large ribosomal subunit* RNA gene, AY431935). New libraries from selected regions of the gel that excluded the putative *large ribosomal subunit* fragments resulted in 13 additional unique clones out of 95 randomly chosen from each forward and reverse subtracted library. The 18 clones obtained in our early diapause and early nondiapause libraries were further analyzed by northern blot hybridization.

Our second round of SSH utilized cDNA from *Cx. pipiens* in late diapause (56–59 days post adult eclosion) and cDNA from nondiapausing females (7–10 days after adult eclosion). When constructing the late diapause forward and reverse subtracted libraries, a slightly different method was employed. Rather than rely on mRNA purification to eliminate ribosomal RNA, the SMART cDNA synthesis kit (BD Biosciences) was used to create full-length enriched cDNA pools, which were then used in the subsequent subtraction procedures. While 10 of these clones had sequences with high similarity to ribosomal RNA, the remaining clones appeared to be of mRNA origin. Out of 95 clones sequenced (80 from the forward-subtracted library

and 15 from the reverse-subtracted library), 22 were unique clones, and all but one were detectable by northern blot hybridization.

3.2. Confirmation by northern blot analysis

Northern blot hybridizations were used to confirm the putative upregulation or downregulation of 40 cDNAs obtained in our early and late diapause subtracted libraries (Figs. 1 and 2). In most cases, northern blot hybridization confirmed the upregulation or downregulation of the cDNAs that were isolated, however, in a few cases northern blots showed a different pattern of expression than that predicted by SSH. All clones produced bands of expected size, as determined by information retrieved from GenBank. Several clones isolated by SSH were undetectable by northern blots, suggesting that a more sensitive technique such as real time PCR will be needed to confirm the diapause expression pattern indicated by SSH.

3.3. Diapause upregulated genes

Of the 18 unique clones from our first round of SSH (early diapause – early nondiapause), 11 were verifiable by northern blot hybridization (Fig. 1). The following genes appear to be upregulated only in early diapause: one gene involved in food utilization (clone no. CpiED-A47, *fatty acid synthase*), a cytoskeletal gene (CpiED-A09, *actin*), one with a metabolic function (CpiED-A07, *putative mitochondrial malate dehydrogenase*), and a ribosomal gene (CpiED-A32, *ribosomal protein L18*). Two genes encoding *cytochrome c oxidase subunit I* (CpiLD-H06) and *large ribosomal subunit RNA* (CpiLD-A01) were obtained from our late diapause library, but northern blots indicated that they are only upregulated in early diapause. In addition, 7 genes are putatively upregulated according to SSH, but these genes were expressed at levels undetectable by northern blot hybridization. These include *putative transcription elongation factor B polypeptide 3 binding protein 1* (CpiED-B03), *putative methoprene-tolerant protein* (CpiED-M01), *selenoprotein* (CpiED-A24), *ribosomal protein 27A* (CpiED-E08), and 3 genes with unknown function (CpiED-A01, CpiED-C09, CpiED-D03).

Of the 22 clones from our late diapause upregulated library, all were verifiable by northern blot hybridization (Fig. 1), except for one clone with unknown identity (CpiLD-G10). Although some clones were expressed in both early and late diapause, others were upregulated only in late diapause. Genes upregulated only in late diapause include three regulatory genes (CpiLD-A38, *ribosomal protein S3A*; CpiLD-L01, *putative ribosomal protein S6*; CpiLD-C04, *ribosomal protein S24*), two stress response genes (CpiLD-A06, *putative aldehyde oxidase*; CpiLD-A11, *small heat shock protein*), a gene with metabolic function (CpiLD-E09, *putative methylmalonate-semialdehyde dehydrogenase*), a ribosomal gene from the endosymbiont *Wolbachia pipientis* (CpiLD-C02, *23S ribosomal RNA*), two transposable elements (CpiLD-D02, *reverse transcriptase*; CpiLD-D11, *similar to miniture inverted-repeat transposable element (Mimo family)*), and 8 genes with unknown functions (CpiLD-B02, CpiLD-B11, CpiLD-D06, CpiLD-F01, CpiLD-F03, CpiLD-F07, CpiLD-H03, and CpiLD-H05). In addition, 2 genes were obtained from our late diapause library but are upregulated in both early and late diapause. These genes encode *cytochrome c oxidase subunit III* (CpiLD-H04) and a gene with unknown function (CpiLD-M43).

3.4. Diapause downregulated genes and genes unchanged in diapause

Only two unique genes were obtained from our first reverse subtracted library (early nondiapause – early diapause) as being downregulated in early diapause: CpiED-A15 encoding *chymotrypsin-like serine protease* and CpiED-A34 encoding *trypsin*. These results were confirmed by northern blot hybridization (Fig. 2); both yielded a strong signal in nondiapausing females, no signal in early diapause, and only a weak signal in late diapause. No unique genes were obtained from our second reverse subtracted library (early nondiapause – late diapause).

In addition, several obtained from our forward and reverse subtracted libraries showed no change in expression levels in all three stages tested (Fig. 2). These include genes encoding *putative poly A binding protein* (CpiED-A18), *ubiquitin/ribosomal protein L40* (CpiED-A29), *cecropin* (CpiLD-A10), *beta tubulin* (CpiED-B06), and *28S large subunit ribosomal RNA* (CpiLD-H09). The consistency of 28S expression in *Cx. pipiens* and other models of diapause (Rinehart *et al.*, 2000) prompted us to use this gene as a control for northern blot hybridizations.

4. Discussion

The results presented here provide some first clues about the molecular events that characterize the adult diapause of *Cx. pipiens*. We have identified 40 genes by suppressive subtractive hybridization and confirmed the following expression patterns by northern blot hybridization: 6 genes are upregulated specifically in early diapause, 17 genes are upregulated in late diapause, and 2 genes are upregulated throughout diapause. In addition, we have identified 2 genes that are diapause downregulated and 5 that remained unchanged during diapause. We have categorized these genes into 8 distinct groupings: regulatory function, food utilization, stress response, metabolic function, cytoskeletal, ribosomal, transposable elements, and genes with unknown functions. Northern blot hybridization has confirmed the expression of 32 of the 40 genes obtained by SSH, while the others are expressed at levels undetectable by this method.

Two of the diapause-upregulated genes we examined in this study (*cytochrome c oxidase subunit III* and an unknown) were upregulated in both our early and late diapause samples. The remainder of the SSH clones were upregulated in either early or late in diapause, but not at both times. Diapause is, of course, a dynamic state, and the distinctions in time of expression of the genes are perhaps highly pronounced here because our “early” sample (7–10 days after adult eclosion) represents an entry phase into diapause when the female would normally be feeding extensively on sugar and actively seeking a hibernaculum, while our “late” timepoint (56–59 days after adult eclosion) represents a time in diapause well after such activities have ceased.

4.1. Regulatory genes

The diapause of *Cx. pipiens*, induced by short daylength, is characterized by a state of inactivity and a dramatically slow rate of ovarian maturation (Radio *et al.*, 1999). Genes regulating these and other molecular events may prove useful in understanding how *Cx. pipiens* can survive in a prolonged inactive state. Certain ribosomal proteins have functions in regulating cell growth and death in addition to their roles in translation (Naora and Naora, 1999). The appearance of three ribosomal proteins in our SSH libraries suggests a possible contribution of these proteins in regulating the reproductive diapause of *Cx. pipiens*. *Ribosomal protein (rp) S3A*, *rpS6*, and *rpS24* are all associated with the 40S ribosomal subunit mRNA binding domain and are involved in the initiation of translation (Takahashi *et al.*, 2002). In *Cx. pipiens*, two of these ribosomal proteins are expressed at low levels in early diapause and all three become highly expressed in late diapause, shortly before diapause is terminated.

The highly conserved gene encoding *rpS3A* is found in high concentration in the ovaries of *Anopheles gambiae* (Zurita *et al.*, 1997) and in the follicular epithelial cells of *Drosophila melanogaster* (Reynaud *et al.*, 1997). Suppression of *rpS3A* in *D. melanogaster* leads to a disruption of the follicular epithelium and an inhibition of ovarian development (Reynaud *et al.*, 1997). Since ovarian development requires high levels of protein synthesis, suppression of this gene likely leads to a disruption in this process. Since arrested ovarian development is a prominent characteristic of *Cx. pipiens* diapause (Spielman and Wong, 1973), the lack of expression of *rpS3A* in early diapause may be key to this developmental arrest. Consequently, its upregulation in late diapause may indicate a resumed competence for egg production prior to diapause termination.

Of equal interest is the low level of expression of *rpS6* in early diapause and its subsequent upregulation in late diapause. This gene is upregulated prior to oogenesis in *Ae. aegypti*; *rpS6* mRNA accumulates 24–48 h after adult eclosion and remains stable until a blood meal initiates its translation which then prompts protein synthesis in the fat body necessary for ovarian development (Niu and Fallon, 2000). Of particular interest to the diapause of *Cx. pipiens* is the fact that suppression of *rpS6* activity has been implicated in other models of developmental arrest. In the encysted embryos of the brine shrimp *Artemia franciscana*, *S6 kinase*, which is required for *S6* activation, shows a rapid accumulation of mRNA 4 h after embryos are placed in hatching conditions, and the active enzyme is detectable within 15 minutes of diapause break (Santiago and Sturgill, 2001). While embryonic diapause differs greatly from an adult diapause, the two models suggest that downregulation of *rpS6* may be a key element in arrested development, and its upregulation may be essential for diapause termination.

The putative upregulation of our clone with high similarity to the *methoprene tolerant protein* gene (*Met*) from *D. melanogaster* is of particular interest because it is possibly a juvenile hormone receptor (Wilson, 2003) or may function as a JH-dependent transcription factor (Miura *et al.*, 2005). The immediate hormonal basis for diapause in *Cx. pipiens* is the absence of juvenile hormone (Readio *et al.*, 1999), thus the upregulation of *Met* during diapause is puzzling because one might have anticipated that, if anything, *Met* would be downregulated at this time. The link between *Met* expression and the juvenile hormone mediation of diapause is unclear at this point, but it raises intriguing scenarios that may need to be considered for future work on the hormonal control of diapause in this species.

In addition, *putative transcription elongation factor B polypeptide 3 binding protein 1* may also have a regulatory function. As a component of the positive transcription elongation factor B complex, its putative upregulation in early diapause is likely involved in signal mRNA processing (Shilatifard, 2004).

4.2. Food utilization

A key characteristic of diapausing *Cx. pipiens* is that they lack the host-seeking response and will not take a blood meal under natural conditions (Mitchell, 1983; Bowen *et al.*, 1988). Although diapausing females can be enticed to take a blood meal if the host-seeking step is bypassed (Mitchell, 1983), most of the blood ingested is ejected within 24 h and blood that remains in the gut is used neither for sequestration of lipid reserves nor for vitellogenesis (Mitchell and Briegel, 1989b). Instead, females are programmed to sequester lipid reserves (Bowen, 1992; Mitchell and Briegel, 1989a) and do so by feeding on plant sources rich in carbohydrates such as nectar and rotting fruits. Our results show the downregulation of two genes encoding the blood digestive enzymes *trypsin* and *chymotrypsin-like serine protease* in early diapause, while the gene encoding the enzyme involved in the conversion of sugars to lipid stores, *fatty acid synthase*, is highly upregulated at this time. The evidence presented here and in Robich and Denlinger (2005) supports the contention that even if a blood meal is taken by diapausing *Cx. pipiens*, they lack the molecular machinery required for blood digestion and are instead programmed to sequester lipid reserves. In late diapause, the accumulation of mRNAs encoding *trypsin* and *chymotrypsin-like* indicates that females are preparing to terminate diapause by regaining competence to digest a blood meal.

4.3. Stress response

Overwintering insects confront harsh environmental conditions including low temperature, varying relative humidity, and invasion by pathogenic organisms. In several insect species, heat shock proteins are highly upregulated upon entry into diapause (Denlinger *et al.*, 2001). These proteins act as molecular chaperones by preventing abnormal protein folding during

environmental stresses such as extreme heat, cold, or desiccation and have also been implicated in playing a role in cell cycle arrest (Feder *et al.*, 1992). In the pupal diapause of the flesh fly *Sarcophaga crassipalpis*, *hsp23* and *hsp70* are developmentally upregulated upon the entry into diapause and remain expressed until diapause has been broken (Rinehart *et al.*, 2000; Yocum *et al.*, 1998), while *hsp90* is downregulated at this time but remains responsive to environmental stress (Rinehart and Denlinger, 2000).

In *Cx. pipiens*, a *small heat shock protein* is upregulated in late diapause, but the upregulation is slight by comparison with the strong upregulation of *hsp23* noted in *S. crassipalpis* (Yocum *et al.*, 1998). A slight elevation of *hsp70* was also noted in the adult diapause of the Colorado potato beetle (Yocum, 2001), but in adults of *Drosophila triauraria*, *hsps* do not appear to be at all upregulated during diapause (Goto and Kimura, 2004). This data, in addition to our results with *Cx. pipiens*, suggests that upregulation of *hsps* is not a major component of the diapause syndrome in adults. Cold stress during diapause, however, can elicit a rapid upregulation of *hsp70* in *Cx. pipiens* (Rinehart *et al.* 2006), thus implying that the stress response remains intact during diapause.

A second stress response gene identified in late diapause is *putative aldehyde oxidase*, which encodes a multifunctional molybdo-flavoenzyme with broad substrate specificity involved in the oxidation of aromatic N-heterocycles and aldehydes (Garattini *et al.*, 2003). Several functions have been proposed for this enzyme including its involvement in catalyzing metabolic pathways, vitamin degradation, and detoxification of environmental pollutants (Gerattini *et al.*, 2003). In addition, *aldehyde oxidase* plays an important role in insecticide resistance in the common house mosquito, *Cx. quinquefasciatus* (Coleman *et al.*, 2002); our clone from *Cx. pipiens* shares 93% identity with *aldehyde oxidase* from *Cx. quinquefasciatus*. In certain insecticide-resistant strains of *Cx. quinquefasciatus*, the *aldehyde oxidase* gene is amplified in conjunction with two resistance-associated esterases, and the enzyme shows high substrate specificity for insecticide oxidation (Hemingway *et al.*, 2000). Although the function of *aldehyde oxidase* in diapausing *Cx. pipiens* is unknown, it is possibly a component of an elevated stress-response system operating during diapause.

An additional stress-response gene, *selenoprotein*, is putatively upregulated in *Cx. pipiens* diapause and may confer protection against environmental stress. In *D. melanogaster*, selenoproteins function as antioxidants and can decrease lipid peroxidation (Morozova *et al.*, 2003), functions that may be especially important in long-lived individuals that are in diapause.

Although *cecropin* and *ubiquitin/rpL40* were obtained from our late diapause upregulated library, northern blot hybridizations indicate that their expression levels remain unchanged in diapause. The immune peptide, *cecropin*, was detectable at very low levels in all stages tested. Further studies are needed to demonstrate if it is upregulated in response to a bacterial infection in overwintering females. The low level of expression is consistent with that observed in other species: Bartholomay *et al.* (2003) demonstrated that *cecropin A* transcripts are not detectable in naïve mosquitoes, but are rapidly transcribed after bacterial inoculation. Likewise, in spite of its isolation from our diapause library, *ubiquitin/rpL40*, a gene associated with protein degradation and stress responses (Esser *et al.*, 2004), was expressed equally in nondiapausing and diapausing mosquitoes, as noted with northern blots.

4.4. Metabolic genes

Four genes with metabolic functions are upregulated during diapause: *putative mitochondrial malate dehydrogenase*, *putative methylmalonate-semialdehyde dehydrogenase*, *cytochrome c oxidase (CO) subunit I* and *COIII*. Two of these, *COI* and *COIII*, are of mitochondrial origin and serve an essential role in aerobic oxidation. Although metabolic rates in insects are typically suppressed during diapause, the metabolic suppression in adult diapauses is not as extensive

as in other stages such as the egg or pupa (Danks, 1987). The upregulation of the two mitochondrial genes, *COI* and *COIII*, in early *Cx. pipiens* diapause may, at first, seem counterintuitive, but *Cx. pipiens* adults are quite active prior to hibernation. From our laboratory observations, it is evident that females programmed for diapause not only feed more extensively on sugar but also have increased flight activity when compared to their nondiapausing counterparts. Under field conditions, females preparing for hibernation actively seek sugar meals (Bowen, 1992) and must locate a suitable hibernaculum. Diapause preparation thus requires considerable energy. Similar results have been noted in the early phase of larval diapause in the Japanese beetle, *Popillia japonica*, where cytochrome oxidase activity actually increases during early diapause (Ludwig, 1953). In *Cx. pipiens*, *COI* expression is depressed in late diapause, while *COIII* transcripts remain high.

The concurrent upregulation of *putative mitochondrial malate dehydrogenase (MDH)* and *putative methylmalonate semialdehyde dehydrogenase* may also be involved in specific metabolic events associated with diapause. *MDH* has been implicated in increased cold tolerance; certain forms of this enzyme function more efficiently at low temperatures (Kim *et al.*, 1999). *MDH* upregulation in conjunction with increased cold tolerance has been observed in organisms as diverse as the channel catfish *Ictalurus punctatus* (Seddon and Prosser, 1997) and the potato *Solanum tuberosum* (Rorat *et al.*, 1997). It is not clear what unique function methylmalonate semialdehyde dehydrogenase, an enzyme involved in amino acid metabolism, may play during diapause.

4.5. Cytoskeletal genes

Our experiments indicate that the expression of certain cytoskeletal genes is affected by diapause: an *actin* is upregulated in early diapause and returns to low levels by late diapause, while a *beta tubulin* is unchanged during diapause. The upregulation of an *actin* in early diapause may reflect the increased flight activity of females preparing for hibernation, but the upregulation of *actin* is in contrast to reports from other species. For example, a brain-specific *actin* is downregulated during the pharate larval diapause of the gypsy moth *Lymantria dispar* (Lee *et al.*, 1998). Data from plant models indicate that *actin* downregulation may contribute to the increased cold tolerance associated with dormancy. In wheat *Triticum aestivum*, actin depolymerizing factor (ADF) accumulation is a major component of cold acclimation (Ouellet *et al.*, 2001). Upon activation, this protein sequesters actin and induces actin depolymerization (Ouellet *et al.*, 2001), and removal of actin from the cytoskeleton increases membrane fluidity and thus increases resistance to cold. In *Cx. pipiens*, however, not only do females actively fly during diapause preparation, but they continue to move around within their hibernaculum during the winter months (Minar and Ryba, 1971; Buffington, 1972).

4.6. Ribosomal genes

In addition to the three ribosomal genes thought to serve regulatory functions (see "Regulatory Genes"), three other ribosomal genes are upregulated in early diapause: *ribosomal protein L18*, *ribosomal protein 27A*, and *large ribosomal subunit RNA*. The fact that two of these ribosomal genes are upregulated in early diapause but downregulated in late diapause (*L18* was undetectable by northern blots) suggests that their function is restricted to the events of early diapause. By contrast, the *23S ribosomal RNA gene* was recovered in late diapause from the obligate intracellular bacteria of *Cx. pipiens*, *Wolbachia*. The strong upregulation of this gene in late diapause indicates that *Wolbachia* is active in late diapausing *Cx. pipiens*. The differential expression of this *Wolbachia* gene in association with the diapause of its host suggests that the diapause status of *Cx. pipiens* may affect development of this bacterial parasite, as demonstrated in *Wolbachia*-infected eggs during the diapause of another mosquito, *Ae. albopictus* (Ruang-areerate *et al.*, 2004).

4.7. Transposable elements

Curiously, two genes encoding fractions of transposable elements, *putative reverse transcriptase* and a gene similar to *miniature inverted-repeat transposable element (Mimo family)*, are upregulated during late diapause in *Cx. pipiens*. Although the function of transposable elements in diapause is unclear, diapause regulation of transposons has also been noted in two other species: two genes encoding retroviral envelope proteins are expressed during the embryonic diapause of *Bombyx mori* (Yamashita *et al.*, 2001), and a gene encoding a retrotransposon is highly expressed in the early pupal diapause of *S. crassipalpis* (Denlinger, 2002). That transposable elements would be diapause upregulated in all three of these species suggests the intriguing possibility of a role for transposable elements in the regulation of diapause.

4.8. Genes with unknown function

Nine genes with unknown functions are upregulated in late diapause, as confirmed by northern blots, and one of these (CpiMD-M43) is also expressed in early diapause. This gene is of particular interest since its high level of expression in early diapause suggests it may play a role in initiating the diapause program.

In summary, this study represents the first large-scale investigation of the molecular aspects of diapause in any mosquito species. By suppressive subtractive hybridization, we have demonstrated the differential regulation of genes specifically involved in early and late diapause and have categorized these genes into several distinct functional groups. Future work will certainly reveal additional genes and possibly additional gene categories that are involved in the diapause of *Cx. pipiens*. We anticipate that these results will prove useful in probing the molecular events that may be common to diapauses in insects representing different taxa and developmental stages. We also anticipate that this type of work will prove helpful in understanding the transseasonal maintenance of West Nile virus in diapausing *Cx. pipiens* and may contribute to an understanding of the dynamic relationships between other pathogens and their vectors during the overwintering season.

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References

- Bartholomay LC, Farid HA, Ramzy RM, Christensen BM. *Culex pipiens pipiens*: characterization of immune peptides and the influence of immune activation on development of *Wuchereria bancrofti*. Molecular and Biochemical Parasitology 2003;130:43–50. [PubMed: 14550895]
- Blitvich BJ, Rayms-Keller A, Blair CD, Beaty BJ. Identification and sequence determination of mRNAs detected in dormant (diapausing) *Aedes triseriatus* mosquito embryos. DNA Sequence 2001;12:197–202. [PubMed: 11762196]
- Bowen MF. Patterns of sugar feeding in diapausing and nondiapausing *Culex pipiens* (Diptera: Culicidae) females. Journal of Medical Entomology 1992;29:843–849. [PubMed: 1404264]
- Bowen MF, Davis EE, Haggart DA. A behavioral and sensory analysis of host-seeking behavior in the diapausing mosquito *Culex pipiens*. Journal of Insect Physiology 1988;34:805–813.
- Buffington JD. Hibernaculum choice in *Culex pipiens*. Journal of Medical Entomology 1972;9:128–132. [PubMed: 5036196]
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry 1987;162:156–159. [PubMed: 2440339]
- Christophers SR. The development of the egg follicle in *Anopheles*. Paludism 1911;2:73–88.

- Coleman M, Vontas JG, Hemingway J. Molecular characterization of the amplified aldehyde oxidase from insecticide resistant *Culex quinquefasciatus*. *European Journal of Biochemistry* 2002;269:768–779. [PubMed: 11846778]
- Danks, HV. Biological Survey of Canada. Terrestrial Arthropods; Ottawa: 1987. Insect dormancy: an ecological perspective.
- Denlinger, DL. Hormonal control of diapause. In: Kerkut, GA.; Gilbert, LI., editors. *Comprehensive insect physiology, biochemistry and pharmacology*. 8. Pergamon Press; Oxford: 1985. p. 353–412.
- Denlinger DL. Regulation of diapause. *Annual Review of Entomology* 2002;47:93–122.
- Denlinger, DL.; Rinehart, JP.; Yocum, GD. Stress proteins: a role in insect diapause?. In: Denlinger, DL.; Saunders, DS., editors. *Insect timing: circadian rhythmicity to seasonality*. Elsevier Science; Amsterdam: 2001. p. 155–171.
- Eldridge BF. Environmental control of ovarian development in mosquitoes of the *Culex pipiens* complex. *Science* 1966;151:826–828.
- Eldridge BF. The effect of temperature and photoperiod on blood-feeding and ovarian development in mosquitoes of the *Culex pipiens* complex. *American Journal of Tropical Medicine and Hygiene* 1968;17:133–140. [PubMed: 5688903]
- Eldridge BF, Bailey CL. Experimental hibernation studies on *Culex pipiens* (Diptera: Culicidae): reactivation of ovarian development and blood-feeding in prehibernating females. *Journal of Medical Entomology* 1979;15:462–467. [PubMed: 544816]
- Esser C, Alberti S, Hohfeld J. Cooperation of molecular chaperones with the ubiquitin/proteasome system. *Biochimica et Biophysica Acta-Molecular Cell Research* 2004;1695:171–188.
- Feder JH, Rossi JM, Solomon J, Solomon N, Lindquist S. The consequences of expressing hsp70 in *Drosophila* cells at normal temperatures. *Genes and Development* 1992;6:1402–1413. [PubMed: 1644286]
- Gao B, Adhikari R, Howarth M, Nakamura K, Gold MC, Hill AB, Knee R, Michalak M, Elliott T. Assembly and antigen-presenting function of the MHC class I molecules in cells lacking the ER chaperon calreticulin. *Immunity* 2002;16:99–109. [PubMed: 11825569]
- Garattini E, Mendel R, Romao MJ, Wright R, Terao M. Mammalian molybdo-flavoenzymes, an expanding family of proteins: structure, genetics, regulation, function and pathophysiology. *Biochemical Journal* 2003;372:15–32. [PubMed: 12578558]
- Goto SG, Denlinger DL. Genes encoding two cystatins in the flesh fly *Sarcophaga crassipalpis* and their distinct expression patterns in relation to pupal diapause. *Gene* 2002;292:121–127. [PubMed: 12119106]
- Goto SG, Kimura MT. Heat-shock-responsive genes are not involved in the adult diapause of *Drosophila triauraria*. *Gene* 2004;326:117–122. [PubMed: 14729269]
- Hemingway J, Coleman M, Paton M, McCarroll L, Vaughan A, DeSilva D. Aldehyde oxidase is coamplified with the world's most common *Culex* mosquito insecticide resistance-associated esterases. *Insect Molecular Biology* 2000;9:93–99. [PubMed: 10672076]
- Henson PM, Bratton DL, Fadok VA. Apoptotic cell removal. *Current Biology* 2001;11:R795–R805. [PubMed: 11591341]
- Johnson S, Michalak M, Opas M, Eggleton P. The ins and outs of calreticulin: from the ER lumen to the extracellular space. *Trends in Cell Biology* 2001;11:122–129. [PubMed: 11306273]
- Kim SY, Hwang KY, Kim SH, Sung HC, Han YS, Cho Y. Structural basis for cold adaptation. *Journal of Biological Chemistry* 1999;274:11761–11767. [PubMed: 10206992]
- Krüger J, Thomas CM, Golstein C, Dixon MS, Smoker M, Tang S, Mulder L, Jones JDG. A tomato cysteine protease required for Cf-2-dependent disease resistance and suppression of autonecrosis. *Science* 2002;296:744–747. [PubMed: 11976458]
- Lee KY, Hiremath S, Denlinger DL. Expression of actin in the central nervous system is switched off during diapause in the gypsy moth, *Lymantria dispar*. *Journal of Insect Physiology* 1998;44:221–226. [PubMed: 12769956]
- Ludwig D. Cytochrome oxidase activity during diapause and metamorphosis of the Japanese beetle (*Popillia japonica* Newman). *The Journal of General Physiology* 1953;36:751–757. [PubMed: 13069678]

- Malik S, Roeder RG. Transcriptional regulation through mediator-like coactivators in yeast and metazoan cells. *Trends in Biochemical Sciences* 2000;25:277–283. [PubMed: 10838567]
- Michalak M, Corbett EF, Mesaeli N, Nakamura K, Opas M. Calreticulin: one protein, one gene, many functions. *Biochemistry Journal* 1999;344:281–292.
- Michalak M, Robert Parker JM, Opas M. Ca^{2+} signaling and calcium binding chaperones of the endoplasmic reticulum. *Cell Calcium* 2002;32:269–278. [PubMed: 12543089]
- Minar J, Ryba J. Experimental studies on overwintering conditions of mosquitoes. *Folia Parasitologica (Prague)* 1971;18:255–259.
- Mitchell CJ. Differentiation of host-seeking behavior from blood-feeding behavior in overwintering *Culex pipiens* (Diptera: Culicidae) and observations on gonotrophic dissociation. *Journal of Medical Entomology* 1983;20:157–163. [PubMed: 6842524]
- Mitchell CJ, Briegel H. Inability of diapausing *Culex pipiens* (Diptera: Culicidae) to use blood for producing lipid reserves for overwinter survival. *Journal of Medical Entomology* 1989a;26:318–326. [PubMed: 2769712]
- Mitchell CJ, Briegel H. Fate of the blood meal in force-fed, diapausing *Culex pipiens* (Diptera: Culicidae). *Journal of Medical Entomology* 1989b;26:332–341. [PubMed: 2769714]
- Miura K, Oda M, Makita S, Chinzei Y. Characterization of the *Drosophila* methoprene-tolerant gene product: juvenile hormone binding and ligand-dependent gene regulation. *FEBS Journal* 2005;272:1169–1178. [PubMed: 15720391]
- Morozova N, Forry EP, Shahid E, Zavacki AM, Harney JW, Kravtsov Y, Berry MJ. Antioxidant function of a novel selenoprotein in *Drosophila melanogaster*. *Genes to Cells* 2003;8:963–971. [PubMed: 14750951]
- Naora H, Naora H. Involvement of ribosomal proteins in regulating cell growth and apoptosis: translational modulation or recruitment for extraribosomal activity? *Immunology and Cell Biology* 1999;77:197–205. [PubMed: 10361251]
- Nasci RS, Savage HM, White DJ, Miller JR, Cropp BC, Godsey MS, Kerst AJ, Bennett P, Gottfried K, Lanciotti RS. West Nile virus in overwintering *Culex* mosquitoes, New York City, 2000. *Emerging Infectious Diseases* 2001;7:742–744. [PubMed: 11585542]
- Niu LL, Fallon AM. Differential regulation of ribosomal protein gene expression in *Aedes aegypti* mosquitoes before and after the blood meal. *Insect Molecular Biology* 2000;9:613–623. [PubMed: 11122470]
- Onyeka JOA, Boreham PFL. Population studies, physiological state and mortality factors of overwintering adult populations of females of *Culex pipiens* L. (Diptera: Culicidae). *Bulletin of Entomological Research* 1987;77:99–111.
- Ouellet F, Carpentier E, Cope MJTV, Monroy AF, Sarhan F. Regulation of a wheat actin-depolymerizing factor during cold acclimation. *Plant Physiology* 2001;125:360–368. [PubMed: 11154343]
- Readio J, Chen MH, Meola R. Juvenile hormone biosynthesis in diapausing and nondiapausing *Culex pipiens* (Diptera: Culicidae). *Journal of Medical Entomology* 1999;36:355–360. [PubMed: 10337107]
- Reynaud E, Bolshakov VN, Barajas V, Kafatos FC, Zurita M. Antisense suppression of the putative ribosomal protein S3A gene disrupts ovarian development in *Drosophila melanogaster*. *Molecular and General Genetics* 1997;256:462–467. [PubMed: 9393444]
- Rinehart JP, Denlinger DL. Heat-shock protein 90 is down-regulated during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*, but remains responsive to thermal stress. *Insect Molecular Biology* 2000;9:641–645. [PubMed: 11122473]
- Rinehart JP, Yocum GD, Denlinger DL. Developmental upregulation of inducible hsp70 transcripts, but not the cognate form, during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *Insect Biochemistry and Molecular Biology* 2000;30:515–521. [PubMed: 10802243]
- Rinehart JP, Cakra-Ireland RA, Flannagan RD, Denlinger DL. Expression of ecdysone receptor is unaffected by pupal diapause in the flesh fly, *Sarcophaga crassipalpis*, while its dimerization partner, USP, is downregulated. *Journal of Insect Physiology* 2001;47:915–921.
- Rinehart JP, Robich RM, Denlinger DL. Enhanced cold and desiccation tolerance in diapausing adults of *Culex pipiens*, and a role for hsp70 in response to cold shock but not as a component of the diapause program. *Journal of Medical Entomology* 2006;43:713–722. [PubMed: 16892629]

- Robich RM, Denlinger DL. Diapause in the mosquito *Culex pipiens* evokes a metabolic switch from blood feeding to sugar gluttony. *Proceedings of the National Academy of Sciences* 2005;102:15912–15917.
- Rorat T, Irzykowski W, Grygorowicz WJ. Identification and expression of novel cold induced genes in potato (*Solanum tuberosum*). *Plant Science* 1997;124:69–78.
- Ruang-areerate T, Kittayapong P, McGraw EA, Baimai V, O'Neill SL. *Wolbachia* replication and host cell division in *Aedes albopictus*. *Current Microbiology* 2004;49:10–12. [PubMed: 15297923]
- Sanburg LL, Larsen JR. Effect of photoperiod and temperature on ovarian development in *Culex pipiens pipiens*. *Journal of Insect Physiology* 1973;19:1173–1190. [PubMed: 4708144]
- Santiago J, Sturgill TW. Identification of the S6 kinase activity stimulated in quiescent brine shrimp embryos upon entry to preemergence development as p70 ribosomal protein S6 kinase: isolation of *Artemia franciscana* p70^{S6K} cDNA. *Biochemistry and Cell Biology* 2001;79:141–152. [PubMed: 11310561]
- Seddon WL, Prosser CL. Seasonal variations in the temperature acclimation response of the channel catfish, *Ictalurus punctatus*. *Physiological Zoology* 1997;70:33–44. [PubMed: 9231374]
- Service MW. Observations on the ecology of some British mosquitoes. *Bulletin of Entomological Research* 1968;59:161–194.
- Shilatifard A. Transcriptional elongation control of RNA polymerase III: a new frontier. *Biochimica et Biophysica Acta* 2004;1677:79–86. [PubMed: 15020049]
- Spielman A. Effects of synthetic juvenile hormone on ovarian diapause of *Culex pipiens* mosquitoes. *Journal of Medical Entomology* 1974;11:223–225. [PubMed: 4851698]
- Spielman A, Wong J. Environmental control of ovarian diapause in *Culex pipiens*. *Annals of the Entomological Society of America* 1973;66:905–907.
- Takahashi Y, Mitsuma T, Hirayama S, Odani S. Identification of the ribosomal proteins present in the vicinity of globin mRNA in the 40S initiation complex. *Journal of Biochemistry* 2002;132:705–711. [PubMed: 12417019]
- Tammariello SP, Denlinger DL. G0/G1 cell cycle arrest in the brain of *Sarcophaga crassipalpis* during pupal diapause and the expression pattern of the cell cycle regulator, proliferating cell nuclear antigen. *Insect Biochemistry and Molecular Biology* 1998;28:83–89. [PubMed: 9639874]
- Vinogradova, EB. *Culex pipiens pipiens* mosquitoes: taxonomy, distribution, ecology, physiology, genetics, applied importance and control. Pensoft Publishers; Sofia: 2000.
- Wilson, TG. Methoprene-tolerant, a bHLH-PAS gene essential for insect endocrinology. In: Crews, ST., editor. *PAS proteins: regulators and sensors of development and physiology*. Kluwer Academic; Boston: 2003. p. 109–132.
- Yamashita, O.; Shiomi, K.; Ishida, Y.; Katagiri, N.; Niimi, T. Insights for future studies on embryonic diapause promoted by molecular analyses of diapause hormone and its action in *Bombyx mori*. In: Denlinger, DL.; Giebultowicz, J.; Saunders, DS., editors. *Insect timing: circadian rhythmicity to seasonality*. Elsevier Science; Amsterdam: 2001. p. 145–152.
- Yocum GD. Differential expression of two HSP70 transcripts in response to cold shock, thermoperiod, and adult diapause in the Colorado potato beetle. *Journal of Insect Physiology* 2001;47:1139–1145. [PubMed: 12770192]
- Yocum GD, Joplin KH, Denlinger DL. Upregulation of a 23 kDa small heat shock protein transcript during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *Insect Biochemistry and Molecular Biology* 1998;28:677–682. [PubMed: 9755478]
- Zurita M, Reynaud E, Kafatos FC. Cloning and characterization of cDNAs preferentially expressed in the ovary of the mosquito *Anopheles gambiae*. *Insect Molecular Biology* 1997;6:55–62. [PubMed: 9013255]

Diapause Upregulated Genes

Fig 1.

Northern blot hybridization of *Cx. pipiens* upregulated SSH clones. Clone ID and putative identities are listed above each blot. ND = nondiapausing females (18°C, long daylength; 7–10 days after adult eclosion), ED = females in early diapause (18°C, short daylength; 7–10 days after adult eclosion), LD = females in late diapause (18°C, short daylength; 56–59 days after adult eclosion). Each lane contains 15 µg of total RNA pooled from 20 females. Equal loading was confirmed by northern blot hybridization with a 28S cDNA probe.

Diapause Downregulated Genes

Genes Unchanged in Diapause

Fig 2.

Northern blot hybridization of *Cx. pipiens* diapause downregulated genes and genes unchanged in diapause. Clone ID and putative identities are listed above each blot. ND = nondiapausing females (18°C, long daylength; 7–10 days after adult eclosion), ED = females in early diapause (18°C, short daylength; 7–10 days after adult eclosion), LD = females in late diapause (18°C, short daylength; 56–59 days after adult eclosion). Each lane contains 15 µg of total RNA pooled from 20 females. A 28S cDNA probe was used to confirm equal loading.

Table 1

Diapause upregulated genes from *Cx. pipiens*, isolated by suppressive subtractive hybridization. Percent identities, organisms, and accession nos. were retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>). In the blast column, “n” and “x” indicate putative identities obtained through a nucleotide (BLASTn) and/or translation (BLASTx) query, respectively. Confirmation by northern blot is indicated by an “X” in early (18°C, short daylength; 7–10 days post adult eclosion) and late (18°C, short daylength; 56–59 days post adult eclosion) diapause, ED and LD, respectively.

clone	size	acc. no.	putative identity	blast	% identity, organism, accession no.	ED	LD
<i>regulatory</i>							
CpiLD-A38	469	DQ401432	ribosomal protein S3A	n	91%, <i>Aedes aegypti</i> , AY431319		X
CpiED-B03	356	DQ401433	putative transcription elongation factor B	x	40%, <i>Mus musculus</i> , XP_143238		
CpiLD-L01	296	DQ401434	polypeptide 3 binding protein I	n	97%, <i>Aedes aegypti</i> , AY431732		X
CpiED-M01	70	DQ401435	putative ribosomal protein S6	n	100%, <i>Anopheles gambiae</i> , DQ303468		X
CpiLD-C04	295	DQ401436	putative methoprene-tolerant protein	n	98%, <i>Aedes aegypti</i> , AY064700		X
<i>food utilization</i>							
CpiED-A47	230	AY958428	ribosomal protein S24	n	85%, <i>Armigeres subalbatus</i> , AY441061	X	
<i>stress response</i>							
CpiED-A24	306	DQ401437	fatty acid synthase	n	87%, <i>Anopheles gambiae</i> , AF457547		X
CpiLD-A06	1,515	DQ401438	selenoprotein	n	93%, <i>Culex pipiens quinquefasciatus</i> , AF202953		X
CpiLD-A11	483	DQ401439	putative aldehyde oxidase	x	49%, <i>Sarcophaga crassipalpis</i> , AAC63387		X
<i>Cytoskeletal</i>							
CpiED-A09	408	DQ322244	small heat shock protein	n	95%, <i>Culex pipiens pipiens</i> , DQ023309	X	
<i>metabolic function</i>							
CpiED-A07	116	DQ401440	Actin	n	92%, <i>Aedes aegypti</i> , AY433139		X
CpiLD-E09	1,055	DQ401441	putative mitochondrial malate dehydrogenase	x	88%, <i>Drosophila melanogaster</i> , Q7KW39		X
CpiLD-H04	805	DQ360493	semialdehyde dehydrogenase	n	87%, <i>Aedes aegypti</i> , AY431617	X	X
CpiLD-H06	912	DQ360492	cytochrome c oxidase subunit III	n	92%, <i>Culex tarsalis</i> , AF425847	X	X
<i>ribosomal</i>							
CpiED-A32	177	DQ401442	cytochrome c oxidase subunit I	n	86%, <i>Aedes aegypti</i> , AY431545		X
CpiED-E08	105	DQ401443	ribosomal protein L18	x	100%, <i>Aedes aegypti</i> , AY432783		X
CpiLD-A01	231	DQ401444	ribosomal protein 27A	n	98%, <i>Aedes aegypti</i> , AY431935		X
CpiLD-C02	324	DQ401445	large ribosomal subunit RNA	n	97%, <i>Wolbachia pipiens</i> , U23710		X
<i>transposable elements</i>							
CpiLD-H09	291	DQ401446	<i>Wolbachia</i> 23S ribosomal RNA, endosymbiont of <i>Cx. pipiens pipiens</i>	n	87%, <i>Uranotaenia lowii</i> , AF417833		X
CpiLD-D02	897	DQ401447	28S large subunit ribosomal RNA	x	43%, <i>Aedes aegypti</i> , CA196711		X
CpiLD-D11	889	DQ401448	putative reverse transcriptase	n	90%, <i>Culex pipiens</i> , AF217612		X
<i>genes with unknown function</i>							
CpiED-A01	859	DQ401449	similar to miniture inverted-repeat transposable element (Mimo family)	n,x	no significant similarity		X
CpiED-C09	306	DQ401452	putative diapause upregulated unknown	x	46%, <i>Anopheles gambiae</i> , EAL40389		X
CpiED-D03	614	DQ401453	putative diapause upregulated unknown	x	66%, <i>Anopheles gambiae</i> , EAA10746		X
CpiLD-B02	312	DQ401450	late diapause upregulated unknown	x	42% Plasmodium yoelii yoelii, XP_727512		X
CpiLD-B11	841	DQ401451	late diapause upregulated unknown	n,x	no significant similarity		X
CpiLD-D06	1,047	DQ401454	late diapause upregulated unknown	n,x	no significant similarity		X
CpiLD-F01	445	DQ401455	late diapause upregulated unknown	n,x	no significant similarity		X
CpiLD-F03	311	DQ401456	late diapause upregulated unknown	x	65%, <i>Anopheles gambiae</i> , XP_317882		X
CpiLD-F07	250	DQ401457	late diapause upregulated unknown	n,x	no significant similarity		X
CpiLD-G10	519	DQ401458	putative diapause upregulated unknown	n	94%, <i>Aedes aegypti</i> , AY432235		X
CpiLD-H03	133	DQ401459	late diapause upregulated unknown	n,x	no significant similarity		X

clone	size	acc. no.	putative identity	blast	% identity, organism, accession no.	northern	
						ED	LD
CpiLD-H05	398	DQ401460	putative diapause upregulated unknown	n,x	no significant similarity		X
CpiLD-M43	561	DQ401461	diapause upregulated unknown	n,x	no significant similarity	X	X

Diapause downregulated genes from *Cx. pipiens*, isolated by suppressive subtractive hybridization, and genes unchanged in diapause. Percent identities, organisms, and accession nos. were retrieved from GeneBank (<http://www.ncbi.nlm.nih.gov/BLAST/>). In the blast column, “n” and “x” indicate putative identities obtained through a nucleotide (BLASTn) and/or translation (BLASTx) query, respectively. Confirmation by northern blot is indicated by an “X” in nondiapause (18°C, long day/length; 7–10 days post adult eclosion) and early diapause (18°C, short day/length; 7–10 days post adult eclosion), and late diapause (18°C, short day/length; 56–59 days post adult eclosion).

Table 2

clone	size	acc. no.	putative identity	blast	% identity, organism, accession no.
Diapause Downregulated Genes					
<i>food utilization</i>					
CpiED-A15	294	AY958427	chymotrypsin-like serine protease	x	45%, <i>Aedes aegypti</i> , AAX56968
CpiED-A34	264	AY958426	trypsin	n	98%, <i>Culex pipiens pallens</i> , AY034060
Genes Unchanged in Diapause					
<i>regulatory</i>					
CpiED-A18	292	DQ401462	putative poly A binding protein	n	91%, <i>Aedes aegypti</i> , AY431644
<i>stress response</i>					
CpiED-A29	295	DQ401463	ubiquitin/ribosomal protein L40	n	94%, <i>Aedes albopictus</i> , AY826155
CpiLD-A10	333	DQ401464	cecropin	n	79%, <i>Culex pipiens pipiens</i> , AY189808
<i>cytoskeletal</i>					
CpiED-B06	241	DQ401465	beta tubulin	n	89%, <i>Drosophila melanogaster</i> , NM_166356