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Upregulation of two actin genes and redistribution of actin during diapause and cold stress in the northern house mosquito, *Culex pipiens*.

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Abstract

Two *actin* genes cloned from *Culex pipiens* L. are upregulated during adult diapause. Though *actins* 1 and 2 were expressed throughout diapause, both genes were most highly expressed early in diapause. These changes in gene expression were accompanied by a conspicuous redistribution of polymerized actin that was most pronounced in the midguts of diapausing mosquitoes that were exposed to low temperature. In nondiapausing mosquitoes reared at 25°C and in diapausing mosquitoes reared at 18°C, polymerized actin was clustered at high concentrations at the intersections of the muscle fibers that form the midgut musculature. When adults 7–10 days post-eclosion were exposed to low temperature (–5°C for 12h), the polymerized actin was evenly distributed along the muscle fibers in both nondiapausing and diapausing mosquitoes. Exposure of older adults (1 month post-eclosion) to low temperature (–5°C for 12h) elicited an even greater distribution of polymerized actin, an effect that was especially pronounced in diapausing mosquitoes. These changes in gene expression and actin distribution suggest a role for actins in enhancing survival of diapausing adults during the low temperatures of winter by fortification of the cytoskeleton.

Keywords

actin; actin distribution; cold tolerance; adult diapause; cytoskeleton

1. Introduction

The cytoskeleton has significant roles in nuclear/cell division, cell signaling, motility and polarity of cells, and cell shape (Amos and Amos, 1991; McIlwain and Hoke, 2005; Ramaekers and Bosman, 2004). Low temperature alterations of the cytoskeleton have been noted in several species of plants and animals, and these changes appear to be critical for low temperature survival. For example, actin filaments and microtubules of tobacco cells exposed to 0°C for a few minutes are depolymerized immediately, and after recovery at 25°C, the filaments and microtubules are repolymerized (Pokorna et al., 2004). In winter wheat (*Triticum aestivum* L.), cold acclimation is achieved by disassembly of the microtubules in response to low temperature (4°C) and the reorganization of the microtubules into a cold-tolerant arrangement (Abdrakhamanova et al., 2003). Cells of homeothermic animals depolymerize most of their microtubules at low temperatures, while poikilotherms, by contrast, frequently assemble

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microtubules at low temperature and thereby prevent depolymerization (Pucciarelli et al., 1997). For example, both the poikilothermic Antarctic fish, *Notothenia coriiceps*, (Detrich et al., 1989) and the Antarctic ciliate, *Euplotes focardii*, (Pucciarelli et al., 1997) undergo microtubule assembly in response to low temperatures. In addition, unique cold-adapted tubulins have been found in some organisms such as the Antarctic ciliate, *E. focardii* (Pucciarelli and Miceli, 2002).

Little is known about cytoskeletal responses of insects to low temperature. Our interest in potential cytoskeletal changes in insects as a low temperature adaptation was prompted by the observation that *actin* is upregulated during adult diapause of the northern house mosquito, *Culex pipiens* (L.) (Robich et al., 2006). In this paper, we report the full length sequences of that actin and an additional actin, both of which are shown to be diapause upregulated. In addition, we use fluorescent staining and confocal microscopy to note changes in actin distribution and abundance in *Cx. pipiens* that have entered diapause and/or have been exposed to low temperature.

2. Materials and Methods

2.1 Insect rearing

Our anautogenous colony of *Cx. pipiens* (Buckeye strain) was maintained at 25°C, 75% R.H., with 15L(light):9D(dark) (Nondiapause, 25°C). Larvae and adults were reared as described by Robich et al. (2006). To induce diapause, the second instar larvae were moved to an environmental room at 18°C, 75% R.H., with 9L:15D (Diapause, 18°C). To eliminate temperature as a variable, a third group of mosquitoes was reared at 18°C, 75% R.H., with 15L:9D (Nondiapause, 18°C). Adults from the diapause 18°C group were provided a sugar source (honey-soaked sponges) for only the 10–13 days after adult eclosion to mimic the absence of a sugar source characteristic of the overwintering period.

2.2 RNA isolation

Total RNA was extracted from a group of 15 females using TRIZOL Reagent (GIBCO BRL, USA) according to the manufacturer's instructions. After washing with 75% ethanol, RNA was stored at -70°C in 100% ethanol. After storage the ethanol was discarded, and 25µl diethylpyrocarbonate (DEPC)-water was added to the RNA pellet. The RNA pellet was heated to 65°C and absorbance was measured at 260nm to quantify RNA by spectrometer (BioSpec-mini, Shimadzu). RNA from females that had broken diapause was collected from individuals that had been in diapause for 2 months at 18°C and then transferred to 25°C for 1 week.

2.3 Cloning and sequencing

The Rapid Amplification of cDNA Ends kit (Invitrogen) was used for 3' and 5' RACE according to the manufacturer's instructions. The initial partial sequence of *actin* (409bp) was obtained by Robich et al. (2006). For 3'RACE, the first-strand cDNA was synthesized from 1µg of total RNA from diapausing adults 7–10 days post-eclosion using a cRNA synthesis primer Adapter Primer (AP) (5'-GGC CAC GCG TCG ACT AGT AC(T)₁₆-3') provided within the kit. Amplification of the target cDNA was performed with a gene-specific primer as the forward primer: *actin* 1(5'-CGG ACA GGT CAT CAC CAT CG-3') and *actin* 2 (5'-AAT CTG CCG GTA TTC ACG AG-3'), and the Abridged Universal Amplification Primer (AUAP) (5'-GGC CAC GCG TCG ACT AGT AC-3') as the reverse primer. The PCR reaction was carried out under the following conditions: the template was first denatured at 94°C for 3min, followed by 35 cycles of amplification (30s at 94°C, 30s at 60°C, and 3min at 72°C) and by extension at 72°C for 7min.

Based on the sequence of the 3'RACE products, the first-strand cDNA for 5'RACE was synthesized using the gene-specific primer 1; *actin 1* (5'-GAG ATC CAC ATC TGC T-3') and *actin 2* (5'-GAC TCG TCG TAT TCC T-3'), followed by purification and TdT tailing of cDNA. The first round of PCR was used to amplify the reverse gene-specific primer 2; *actin 1* (5'-ACA GCG GAA CCG CTC GTT AC-3') and *actin 2* (5'-CAA CGT CGC ACT TCA TGA TCG-3'), and the manufacturer-provided Abridged Anchor Primer (AAP) (5'-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3'). The second round of PCR was performed with the gene-specific primer 3; *actin 1* (5'- CGT AGA TCG GGA CGG TGT GG-3') and *actin 2* (5'-ATG GCA TGG GGC AGA GCA TA-3'), and the Abridged Universal Amplification Primer (AUAP). PCR for the first and the second rounds were carried out by denaturing cDNA at 94°C for 2min, followed by 35 cycles of amplification (30s at 94°C, 30s at 66°C, and 1min at 72°C) and by extension at 72°C for 7min.

The 3' and 5' RACE products were cloned using the TOPO TA Cloning® Kit (Invitrogen), purified using QIAprep Spin Minipreps (QIAGEN), and sequenced at the Plant-Microbe Genomics Facility at Ohio State University using an Applied Biosystem 3730 DNA Analyzer and BigDye™ cycle sequencing terminator chemistry.

2.4 Northern blot hybridization

Total RNA (20µg) was run on a formaldehyde denaturing gel (0.41M formaldehyde, 1.4% agarose). The RNA gel was transferred onto a 0.45µm Hybond-nylon positive membrane (Amersham Bioscience) for 1.5h using a Schleicher and Schuell TurboBlotter system with a DEPC-treated transfer buffer (3M NaCl, 8mM NaOH) designed to inactivate RNase (Plurad and Mabic, 2004). The membrane was neutralized in 1M phosphate buffer and crosslinked on paper soaked with 10x standard sodium citrate (SSC) in a UV crosslinker (Fisher Scientific), as described (Twomey and Krawetz, 1990). The prepared membrane was either stored at -20°C or used immediately for prehybridization.

Digoxigenin (DIG)-labeled *actin* (*actin 1* and *actin 2*) partial cDNA probes, which consisted of approximately 1000bp at the 3'end for *actin 1* and 1200bp at the 3'end for *actin 2*, were prepared from amplified PCR products using RT-PCR, which was carried out under the following conditions: template was first denatured at 92°C for 2min, followed by 35 cycles of amplification (30s at 92°C, 30s at 54°C, and 1min at 72°C) and by extension at 72°C for 1min. *Actin* probes were labeled using 100ng of template DNA and the Dig High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Sciences) following the manufacturer's protocol.

Hybridization using the Dig High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Sciences) was performed overnight at 37°C and immunological detection was performed according to the manufacturer's protocol. The blots were exposed to chemiluminescence films (IsoBioExpress) for 1.5h. To confirm equal loading of RNA, the membranes were stripped and re-probed using DIG-labeled 28S cDNA. Northern blot comparisons were replicated three times.

2.5 Bioinformatics

Sequences obtained from the 3' and 5'RACE were edited in dnaLIMS (dna tools). To identify similar sequences, BLASTn and BLASTx in GenBank (<http://www.ncbi.nlm.nih.gov/>) were used, and BL2seq at Genesteam (<http://xylian.igh.cnrs.fr/bin/bl2seq-guess.cgi>) was used to obtain percent identities. The deduced amino acid sequences were analyzed and multiple sequence alignments were obtained using BLASTp (<http://www.ncbi.nlm.nih.gov/>), the Baylor College of Medicine Search Launcher (<http://dot.imgen.bcm.tmc.edu/seq-util.html>), and Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

2.6 Low temperature treatment and dissection

Nondiapausing adult females 7–10 days post-eclosion and one month post-eclosion (reared at 25°C) and diapausing females (reared at 18°C) were exposed to 0°C or –5°C for 12h using a water bath containing water and glycerol (50:50). Eight females were used for each treatment. Tissues of cold-treated mosquitoes were dissected in insect saline (Ringer's saline: 150mM NaCl, 6.4mM KCl, 1.0mM CaCl₂, 1.0mM MgCl₂, 25mM HEPES, and 5mM glucose, pH 7.0) under a stereo microscope (Wild Heerbrugg M8) and immediately placed on ice. More than 20 mosquitoes per treatment were dissected for microscopy examination.

2.7 Fluorescence staining and microscopy

Tissues were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) containing 36.8mM NaCl, 2.7mM KCl, 8.1mM Na₂HPO₄ and 1.5mM KH₂PO₄, pH 7.3, for 30min at room temperature and washed two or three times in PBS. Tissues were placed on a coverslip in a glass petri dish with 0.1% Triton X-100 in PBS for 10min and washed two or three times in PBS. To reduce nonspecific background staining, fixed tissues were incubated with 1ml PBS containing 100μl of 1% bovine serum albumin (BSA) for 1h at room temperature. Rhodamine phalloidin (Molecular Probes R-415; 300units dissolved in 1.5ml methanol) for red-fluorescent phalloidins was diluted in PBS (5μl in 200μl) and used to detect and label actin. Phalloidin binds at the interface between subunits of F-actin (the polymer form of actin) but does not bind to G-actin (globular actin), thus the stain is effective for detecting the polymerized form of actin (Lodish et al., 2001). The staining solution was placed on a coverslip with 2–3 tissues for 1h at room temperature. The coverslips were kept inside a covered container during the incubation to avoid evaporation. After incubation with the staining solution, tissues were washed 2–3 times in PBS, post-fixed with 2% paraformaldehyde in PBS for 1h, and washed 2–3 times in PBS (Karas et al., 2005). Tissues were air dried, mounted on a glass slide in a mountant (Cytoseal™), and examined immediately or within one day after storage in the dark at 2–6°C.

Specimens were examined on a Zeiss 510 META laser scanning confocal microscope at the Campus Microscope and Imaging Facility at The Ohio State University using a Zeiss Axioskop 10X dry objective, 20X dry objective, and 40X oil objective. ImageJ, software that is available through the National Institutes of Health website (<http://rsb.info.nih.gov/ij>), was used for resizing images and calculating mean pixel intensity. Graphs and a two-way ANOVA were completed using MiniTab (ver.14).

3. Results

3.1 Clone Identification

The full-length cDNA of *actin 1* obtained by RACE is a 1247bp sequence (GenBank accession number **DQ385449**) that encodes 391 amino acids. The open reading frame (ORF) of *actin 1* is 1156bp, from nucleotides 26 to 1156, with a 25bp 5'untranslated region and a 91bp 3'untranslated region including the poly-A tail. The putative polyadenylation signal (AATAAA) was identified at nucleotide positions 1209 to 1214. A multiple sequence alignment of the deduced *actin 1* amino acid sequence with the sequences of other *actins* is shown in Fig.1. The ORF of *actin 1* from *Cx. pipiens pipiens* shares 94% identity with *beta-actin* from a close relative, *Cx. pipiens pallens*, 92% identity with *actin 6* from another mosquito, *Ae. aegypti*, and 91% identity with *beta-actin* from the termite, *Reticulitermes flavipes*.

The full-length cDNA of *actin 2* is a 1564bp sequence (GenBank accession number **DQ322244**) that encodes 375 amino acids. The ORF of *actin 2* is 1128bp, from nucleotides 40 to 1167. The 5'untranslated region is 39bp, and the 3'untranslated region is 397bp including the poly-A tail. The putative polyadenylation signal was identified at nucleotide positions 1518

to 1523. The ORF of *actin 2* shares 97% identity with actin from *Cx. pipiens pipiens*, 94% identity with *actin 5* of *Ae. aegypti*, and 95% identity with *actin 7* of *Drosophila melanogaster* (Fig.2). Also, the ORFs of *actin 1* and *actin 2* share 89% identity, thus indicating that these two *actins* are similar, yet distinctly different.

3.2 Confirmation of diapause-specific actin expression

To generate DIG-labeled probes for northern blot hybridization, we used the partial *actin 1* and *actin 2* cDNA's, which produced bands of 1 and 1.2 Kb, respectively. Northern blot hybridization confirmed the expression patterns of the two actins. Nondiapausing females 7–10 days post-eclosion reared at 25°C were first compared with diapausing females of the same age reared at 18°C (Fig.3). Both *actin 1* and 2 were upregulated at this early stage of diapause. To confirm that these actin expression patterns were related to diapause rather than temperature, we also compared nondiapausing mosquitoes reared at 18°C with diapausing mosquitoes reared at 18°C (Fig.4). These differences (diapause upregulation of *actin 1* and *actin 2*) persisted at 18°C, thus indicating that the upregulation is a function of diapause rather than temperature.

Expression of *actin 1* and *actin 2* continued throughout the three months of diapause, but in both cases, expression was highest in early diapause. In samples collected after diapause was broken the expression levels dropped further, a decline that was especially conspicuous for *actin 1*. Both actins were also expressed 7–10 days and 1 month after adult eclosion in nondiapausing females, but the levels of expression were much lower than in the diapausing mosquitoes. Though *actin 2* could also be detected by northern blot hybridization in nondiapausing mosquitoes two and three months post-eclosion (ND18), expression of *actin 1* was not evident in nondiapausing mosquitoes (ND18) of these ages.

3.3 Changes in actin distribution

Actin in control midguts (nondiapausing mosquitoes reared at 25°C and diapausing mosquitoes reared at 18°C) of early (7–10 days post-eclosion) nondiapausing and diapausing mosquitoes was arranged in a clustered manner, i.e. clumped at intersections of muscle fibers (Fig.5 A, Controls). In the midguts of nondiapausing mosquitoes, actin remained in the clustered arrangement following a 12h exposure to 0°C (Data not shown), but when the mosquitoes were exposed to –5°C for 12h, approximately half of the samples (n = 12) we examined had the clustered actin arrangement, while the other half contained actin that was distributed evenly along the muscle fibers (Fig.5 A, ND25) as previously noted in other mosquitoes (Park and Shahabuddin, 1999; Shahabuddin and Costero, 2000). In the midguts of diapausing mosquitoes, when exposed to 0°C for 12h, half of the samples (n = 12) had the clustered arrangement, while the other half had the distributed arrangement (Data not shown). When diapausing mosquitoes were exposed to –5°C for 12h, all displayed the distributed actin arrangement (Fig.5 A, D18).

Changes were noted as the mosquitoes became older (Fig.5 B). Actin in control midguts (25°C for nondiapausing and 18°C for diapausing mosquitoes) of adults 1 month post-eclosion displayed the clustered pattern (Fig.5 B, Controls). When nondiapausing mosquitoes were exposed to 0°C for 12h, the midgut showed the clustered actin arrangement and when exposed to –5°C for 12h, half of samples (n = 12) showed the clustered arrangement and the other half showed the distributed arrangement (Fig.5 B, ND25). When diapausing mosquitoes were exposed to 0°C for 12h, they showed the clustered actin arrangement, as seen in the controls (Fig.5 B, D18) and when exposed to –5°C the muscle fibers displayed a completely distributed actin arrangement, and the surface area of polymerized actin in the muscle fibers increased dramatically (Fig.5 B, D18).

Malpighian tubules, ovaries, and thoracic muscles were also examined, but no structural changes in actin were noted between nondiapauses and diapause, early and mid diapause, nor in response to low temperature (0°C or -5°C for up to 12h) (Data not shown).

3.4 Statistics

Pixel intensity from sample images, which represents the amount of stained F-actin, was used for statistical analysis using a two-way ANOVA (Fig. 6). Mean pixel intensity was obtained from midgut images of 8 mosquitoes for each condition; a total of 96 images were analyzed. The two dependent variables were diapause and temperature (control, 0°C, and -5°C). Both variables were significant (diapause variable: $F_{1,98}=52.8$, $p<0.001$, temperature variable: $F_{2,98}=68.08$, $p<0.001$), and the interaction between the two variables was also significant ($F_{2,98}=12.80$, $p<0.001$).

4. Discussion

Actin is a highly conserved protein in eukaryotic cells. Three main isoforms (alpha, beta, and gamma) have been reported (Carlini et al., 2000), and they play important roles in a range of cellular functions including muscle contraction, cell motility, cytoskeletal structure, cell division, intracellular transport, and cell differentiation (Herman, 1993). The actin gene family consists of 8 to 44 different genes in plants (Reece et al., 1992), but insects have, at most, six *actin* genes (Fyrberg et al., 1980). We obtained two full-length sequences for *actin* genes from *Cx. pipiens*: *actin* 1, a 1247bp sequence and *actin* 2, a 1564bp sequence. Using northern blot hybridization analysis, we show that the expression patterns of these two genes are related to the diapause program. A comparison of early (7–10 days post-eclosion) nondiapausing mosquitoes reared at 25°C with diapausing mosquitoes reared at 18°C shows that *actin* 1 and *actin* 2 are highly expressed in diapausing mosquitoes. Subsequently, nondiapausing mosquitoes reared at 18°C and diapausing mosquitoes reared at 18°C were compared to determine whether the above differences were due to temperature or the diapause program. Since the differences persisted at 18°C it is evident that the enhanced expression in diapausing mosquitoes is a component of the diapause program rather than a direct response to temperature. *Actin* 1 and *actin* 2 were expressed from early diapause to late diapause, but expression was greatest in early diapause. These genes were also expressed in young nondiapausing adult mosquitoes reared at 18°C, but levels of expression were much lower in the nondiapausing adults.

Little previous work has been done with insects on temperature- or diapause-induced changes in cytoskeletal genes. A brain-specific actin in pharate first-instar larvae of the gypsy moth, *Lymantria dispar*, is expressed during pre- and post-diapause periods but is not expressed during diapause (Lee et al., 1998). Likewise, in the solitary bee, *Megachile rotundata*, an actin is downregulated in pre-diapausing pupae and upregulated in post-diapausing pupae (Yocum et al., 2005). Robich et al. (2006) showed that actin (referred to as *actin* 2 in our current study), is upregulated in early diapause of adult *Cx. pipiens* but is downregulated in late diapause. Another cytoskeletal gene, β -tubulin maintains a stable level of expression during diapause (Robich et al., 2006). Thus, the diapause-specific pattern of actin expression reported in Robich et al. (2006) and here for *Cx. pipiens* is the third diapausing species in which actin expression changes in response to diapause, but it is the first species in which *actins* are specifically upregulated during diapause, suggesting a unique contribution to the function of this mosquito during diapause.

Cytoskeletal components play an important function in the survival of organisms at low temperature. In plant cells, the cytoskeleton contributes to the low temperature response through the disassembly of microtubules that amplify the activity of cold-activated, calcium channels (Thomashow, 2001). Cold hardiness and diapause are important components for

overwintering insects, and these two features are intimately connected in some insects (Denlinger, 1991). Many insects are more resistant to low temperature during diapause than during nondiapausing periods. Flesh flies, *Sarcophaga crassipalpis* and *S. bullata*, are among the insects in which cold hardiness and the diapause program are linked. During pupal diapause, the flies are more cold-hardy than nondiapausing pupae reared at the same temperature (Adedokun and Denlinger, 1984). Also, in the silkworm, *Bombyx mori*, diapausing eggs are more resistant than nondiapausing eggs to cold treatment (Yaginuma and Yamashita, 1986). *Cx. pipiens* is also a species in which cold hardiness is a component of the diapause program, and adult diapause results in a high level of cold hardiness (Rinehart et al., 2006). We are not yet certain of the factors contributing to the increased cold tolerance observed in diapausing adults of *Cx. pipiens*, but the two actins described in this study may make a contribution to the observed enhancement of cold tolerance.

Our results also show dramatic changes in the arrangement of actin in the midgut in response to temperature and diapause. At moderate temperatures (25°C for nondiapausing mosquitoes and 18°C for diapausing mosquitoes) actin is evident throughout the musculature of the midgut, but it is particularly abundant at the intersections of the muscle fibers. We refer to this as the clustered actin arrangement. In response to low temperature, the polymerized actin becomes more dispersed, a condition we refer to as the distributed actin arrangement. The distributed actin arrangement was attained more readily in diapausing than in nondiapausing mosquitoes, especially in adults 7–10 days post-eclosion; approximately half of the diapausing samples displayed the distributed actin arrangement in response to 12h at 0°C, while none of the nondiapausing mosquitoes responded in this manner. The most extreme response was observed in diapausing females 1 month post-eclosion. In these older females, the actin was completely distributed and greatly enhanced after exposure to –5°C for 12h. Thus, the older diapausing adults responded to low temperature by dramatically rearranging the distribution of polymerized actin along the midgut muscle fibers and by greatly increasing the amount of stained surface area in the midgut muscles. The schematic diagram in Fig.5 C summarizes the transition in the arrangement of actin from the clustered state to the fully distributed and enhanced state, as seen in 1 month-old diapausing adults exposed to progressively more severe low temperatures. Since phalloidin specifically stains F-actin, the polymer form of actin, we assume that the conspicuous changes we detect largely represent the conversion of G-actin, the globular form of actin, to F-actin at low temperatures, but our actin mRNA results provide evidence that additional actin is also synthesized by diapausing *Cx. pipiens*.

In Malpighian tubules of the adult mosquito, *Ae. aegypti*, the actin cytoskeleton undergoes active assembly and disassembly related to microvillar growth, intracellular transport, and cell shape after a blood meal has been taken (Karas et al., 2005), thus there is previous evidence for actin changes in adult mosquitoes. In our study, we did not observe changes of actin within the Malpighian tubules, ovaries, or thoracic muscles; only the midgut displayed obvious actin changes in response to low temperature. Specific tissues in different species and different developmental stages have their own distinct responses related to survival at high or low temperature (Krebs and Feder, 1997; Yi and Lee, 2003; Lee et al., 2006), and it appears that the midgut is one of the tissues most responsive to low temperature in *Cx. pipiens*.

The changes in the actin cytoskeleton that we observed in diapausing mosquitoes most likely function to enhance the survival capacity of *Cx. pipiens* at low temperature. The changes we note in the cytoskeleton correlate well with the increased cold tolerance observed in diapausing mosquitoes reared at 18°C (Rinehart et al., 2006).

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CxpiBuck	1	MCDEEIAALVINDNGSGMCKAGFAGDDAPRAVFPSIVGRARHQGVMMVGMGQKDSYVGDEAQ
Aegypti	1	MCDEEVAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMMVGMGQKDSYVGDEAQ
Cxpipal	1	MCDEEVAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMMVGMGQKDSYVGDEAQ
Reticulitermes	1	MCDDDVAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMMVGMGQKDSYVGDEAQ
CxpiBuck	61	SKRGILTLKYP IEHGIVSNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM
Aegypti	61	SKRGILTLKYP IEHGIIINWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM
Cxpipal	61	SKRGILTLMYP IEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKASREKM
Reticulitermes	61	SKRGILTLKYP IEHGIIINWDDMEKIWHHTFYNELRVAPEEHPILLTEAPLNPKANREKM
CxpiBuck	121	TQIMFETFNCPAMYVAIQAVLSLYASGRTAGIVLDSGDGVSTVPIYEGYALPHAAILRLD
Aegypti	121	TQIMFETFNSPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSTVPIYEGYALPHAAILRLD
Cxpipal	121	TQVMFETFNTPAMYVAIQAVLSPYASGRTTGIVLDSGDGVSTVPIYEGYAPQHAILRLD
Reticulitermes	121	TQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSTVPIYEGYALPHAAILRLD
CxpiBuck	181	LAGRDLTDYLMITILTERGYTFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSTLEKS
Aegypti	181	LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAAASSSTLEKS
Cxpipal	181	LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSTLEKS
Reticulitermes	181	LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAAASSSTLEKS
CxpiBuck	241	YELPDGQVITIGNERFRCPETLFPQSF IGMEAGVHETVFNSIKKCDVDIRKDLFANTVL
Aegypti	241	YELPDGQVITIGNERFRCPEALFPQSF LGMESCGIHETVYNSIMKCDVDIRKDLYANTVM
Cxpipal	241	YELPDGQVITIDNERFRCPETLFRPSF IGMEAGVHEKTVFNSIKKCDVDIRKDLFANTV
Reticulitermes	241	YELPDGQVITIGNERFRCPEALFPQSF LGMESCGIHETVYNSIMKCDVDIRKDLYANTVL
CxpiBuck	301	SGGSTMFGGIADRMQRELTALAPPSIKVKIVAPPERKYSVWIGGSILASLSTFQQMWISK
Aegypti	301	SGGTTMYPGIADRMQKEITALAPSTIKIKIIAPPERKYSVWIGGSILASLSTFQQMWISK
Cxpipal	301	LSGGSTMFGGIADRMQRELTALAPPSIKVKIVAPPERKYSVWIGGSILASLSTFQXMWIS
Reticulitermes	301	SGGTTMYPGIADRMQKEITALAPSTIKIKIIAPPERKYSVWIGGSILASLSTFQQMWISK
CxpiBuck	361	QEYDESGPGIVHRKCF
Aegypti	361	EYDESGPGIVHRKCF
Cxpipal	361	KQEYDESGPGIVPRKCF
Reticulitermes	361	QEYDESGPGIVHRKCF

Figure 1.

Multiple sequence alignment of deduced *Cx. pipiens* actin 1 with other insect actins. CxpiBuck: *Cx. pipiens pipiens* Buckeye strain actin 1 (GenBank accession no. **DQ385449**); Aedes: *Ae. aegypti* actin 6 (GenBank accession no. **AAZ31061**); Cxpipal: *Cx. pipiens pallens* beta-actin (GenBank accession no. **AAM43810**); Reticulitermes: *Reticulitermes flavipes* beta-actin (GenBank accession no. **ABA62321**).

CxpiBuck	1	GGR*S*WPGGLQRIYVQGRFCW**RATFYRIFIHRG*SPPAGCYGOYGGQALVRR**GG
Cxpi	1	MDDEEVAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMMVGMGQKDSYVGDEAQ
Aedes	1	MDDEEVAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMMVGMGQKDSYVGDEAQ
Drosophila	1	MDDEEASALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMMVGMGQKDCYVGDEAQ
CxpiBuck	54	VQAWYPHPPEVDRARYHHQLG*YGEDLASHLLQ*AACCPRGAPSPAD*GPPEPQANREKM
Cxpi	61	SKRGILTLKYP IEHGIITNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM
Aedes	61	SKRGILTLKYP IEHGIITNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM
Drosophila	61	SKRGILSLKYP IEHGIITNWDDMEKVWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM
CxpiBuck	111	TQIMFETFNSPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRLD
Cxpi	121	TQIMFETFNSPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRLD
Aedes	121	TQIMFETFNSPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRLD
Drosophila	121	TQIMFETFNSPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRLD
CxpiBuck	171	LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAAASGSIEKS
Cxpi	181	LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAAASTSLEKS
Aedes	181	LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAAASSSLEKS
Drosophila	181	LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAAASTSLEKS
CxpiBuck	231	YELPDGQVITIGNERFRTP EALFQPSFLGMESAGIHETVYTSIMKCDVDIRKDLYANTVL
Cxpi	241	YELPDGQVITIGNERFRCP EALFQPSFLGMESAGIHETVYNSIMKCDVDIRKDLYANTVL
Aedes	241	YELPDGQVITIGNERFRCP EALFQPSFLGMEACGIHETTYNSIMKCDVDIRKDLYANTVL
Drosophila	241	YELPDGQVITIGNERFRTP EALFQPSFLGMESCGIHETVYQSIMKCDVDIRKDLYANNVL
CxpiBuck	291	SGGTTMYPGIADRMQKEITALAPSSIKIKIIAPPERKYSVWIGGSILASLSTFQAMWISK
Cxpi	301	SGGTTMYPGIADRMQKEITALAPSTIKIKIIAPPERKYSVWIGGSILASLSTFQAMWISK
Aedes	301	SGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQAMWISK
Drosophila	301	SGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQAMWISK
CxpiBuck	351	QEYDESGPSIVHRKCF
Cxpi	361	QEYDESGPSIVHRKCF
Aedes	361	QEYDESGPSIVHRKCF
Drosophila	361	QEYDESGPGIVHRKCF

Figure 2.

Multiple sequence alignment of deduced *Cx. pipiens* actin 2 with other insect actins. CxpiBuck: *Cx. pipiens pipiens* Buckeye strain actin 2 (GenBank accession no. **DQ322244**). Cxpi: *Cx. pipiens pipiens* actin (GenBank accession no. **AAV88916**); Aedes: *Ae. aegypti* actin 5 (GenBank accession no. **AAV81972**); Drosophila: *D. melanogaster*, actin 7 (GenBank accession no. **ATFF7**).

Figure 3.

Northern blot hybridizations of *actins* 1 and 2, 7–10 days after adult eclosion in nondiapausing mosquitoes reared at 25°C (ND25) and diapausing mosquitoes reared at 18°C (D18). 28s was used as a control.

Figure 4.

Northern blot hybridization of *actins* 1 and 2 in nondiapausing (ND18) and diapausing mosquitoes (D18) reared at 18°C, at different times after adult eclosion. "Break" refers to mosquitoes that were held in diapause at 18°C for 2 months and then transferred to 25°C for 1 week to terminate diapause.

Figure 5.

Actin distribution in midguts of 7–10 days post-eclosion nondiapausing females of *Cx. pipiens* reared at 25°C (ND25) and diapausing females reared at 18°C (D18) following exposure to -5°C for 12h (A) and in midguts of one month post-eclosion nondiapausing and diapausing mosquitoes following exposure to -5°C for 12h (B). Schematic representation of the changes of actin organization in the midgut of diapausing adults in response to progressively more severe low temperature exposure (C). All images in A and B were taken at same magnification; white scale bar in lower right panel represents 25μm.

Figure 6.

Pixel intensity used to statistically analyze the effect of diapause, low temperature, and their interactions. A two-way ANOVA indicated that the diapause ($F_{1,98}=52.8, P<0.001$) and temperature variables ($F_{2,98}=68.08, P<0.001$) are significant, as well as their interaction ($F_{2,98}=12.80, P<0.001$). Error bars represent standard errors, each $n=8$. ND25: Nondiapausing mosquitoes reared at 25°C. D18: Diapausing mosquitoes reared at 18°C. Early = 7–10 days post-eclosion; Mid = 1 month post-eclosion.