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Embryonic diapause highlighted by differential expression of mRNAs for ecdysteroidogenesis, transcription and lipid sparing in the cricket *Allonemobius socius*

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SUMMARY

Embryos of the ground cricket, *Allonemobius socius*, enter diapause 4–5 days post-oviposition and overwinter in this dormant state that is characterized by developmental arrest. Suppressive subtractive hybridization and quantitative real-time PCR reveal eight candidate genes in pre-diapause embryos that show promise as regulators of diapause entry, when compared with embryos not destined for diapause. Identifications are based both on the magnitude/consistency of differential mRNA abundances and the predicted functions of their products when placed in context of the physiological and biochemical events of diapause characterized in our companion paper. The proteins CYP450, AKR and RACK1 (associated with ecdysteroid synthesis and signaling) are consistently upregulated in pre-diapause, followed by major downregulation later in diapause. The pattern suggests that elevated ecdysone may facilitate onset of diapause in *A. socius*. Upregulation seen for the transcription factors Reptin and TFDp2 may serve to depress transcription and cell cycle progression. Cathpesin B-like protease, ACLY and MSP are three downregulated genes associated with yolk mobilization and/or metabolism that we predict may promote lipid sparing. Finally, embryos that have been in diapause for 10 days show a substantially different pattern of mRNA expression compared with either pre-diapause or embryos not destined for diapause, with the majority of mRNAs examined being downregulated. These transcript levels in later diapause suggest that a number of upregulated genes in pre-diapause are transiently expressed and are less essential as diapause progresses.

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Key words: diapause, metabolic depression, ecdysone, cell cycle, lipid metabolism, qRT-PCR.

INTRODUCTION

Many animals living in environments with predictable periods of harsh environmental conditions spend part of their life cycle in a dormant state known as diapause. Diapause is a preprogrammed form of developmental arrest that allows animals to 'escape' from harsh environmental conditions and may also allow populations to synchronize periods of growth and reproduction with periods of optimal temperatures and adequate water and food supplies (Lees, 1955; Tauber and Tauber, 1976; Denlinger, 1986; Hand, 1991; Hand et al., 2001; Denlinger, 2002). Diapause is endogenously controlled, and this dormancy typically begins well before conditions become too harsh to support normal growth and development. Entry into diapause is widespread among insects and can occur at any stage of the life cycle, although the specific stage at which diapause is initiated depends on the species. Some physiological changes that occur during diapause have been characterized for a variety of species (e.g. Rakshpal, 1962a; Rakshpal, 1962b; Izumigama and Suzuki, 1986; Loomis et al., 1996; Podrabsky and Hand, 1999). However, there have only been a few studies that address the molecular mechanisms that regulate diapause (Flannagan et al., 1998; Blitvich et al., 2001; Jones et al., 2001; Denlinger, 2002; Hong et al., 2006; Kim et al., 2006; Robich et al., 2007), and the majority of these studies have looked at molecular regulation of diapause in larvae, pupae and adults. The molecular regulation of embryonic diapause has received less attention, probably because of the difficulty of extracting the limited amounts of RNA from a developmental stage that has comparatively little tissue and large amounts of protein and lipid. Because of the extreme differences in complexity of tissues in adults, pupae, larvae and embryos, it is unlikely that the molecular regulation of diapause is the same for all life stages. The goal of this study is to reveal genes that may have a role in diapause entry for embryos of the southern ground cricket, *Allonemobius socius* (Scudder). We used PCR-based subtractive hybridization and quantitative real-time PCR (qRT-PCR) to identify genes that are significantly up- or downregulated in embryos that are preparing to enter diapause or that have been in diapause for 10 days.

The only insect embryo for which gene expression during diapause has been studied extensively is the silkworm, *Bombyx mori* (e.g. Dorel and Coulon, 1988; Suzuki et al., 1999; Hong et al., 2006). In this species, the initiation of diapause is accomplished through the action of a 'diapause hormone' which targets the ovaries of females that have received the appropriate cues and initiates the diapause program by altering the carbohydrate composition of the eggs produced (Xu et al., 1995). However, diapause hormone is not widespread among insects; in fact, this peptide has only been found in a few Lepidoptera (Xu et al., 1995). Therefore it is unlikely that the regulation of diapause in this species is universal among insect embryos.

Allonemobius socius is an ideal animal for studying mechanisms that regulate embryonic diapause because adult females can produce either diapause or non-diapause embryos. Although this species is univoltine in the northern part of its range (i.e. has only one generation per year and has an obligate diapause), the species produces two or more generations per year in the southern part of its range and has a facultative diapause (Fulton, 1931; Howard and Furth, 1986; Mousseu and Roff, 1989). By manipulating the environmental conditions experienced by lab colonies of *A. socius*, it is possible to alter the proportion of diapause and non-diapause embryos produced by the population and simultaneously obtain diapause and direct developing, non-diapause embryos. This plasticity permits the direct comparison between diapause and non-diapause embryos that are at a similar developmental stage and thus allows us to separate ontogenetic changes from those that result from the initiation of diapause.

Previous research on the physiological and biochemical characteristics of embryonic diapause in various vertebrate and invertebrate species shows dormancy at this stage is defined by arrest of cell proliferation/development (Nakagaki et al., 1991; Podrabsky and Hand, 2000), depression of protein synthesis (Clegg et al., 1996; Podrabsky and Hand, 2000), increased production of heat shock proteins and chaperones (Clegg, 2005; Qui et al., 2007; Qui and MacRae, 2007) and in many cases by depression of metabolism (Clegg et al., 1996; Podrabsky and Hand, 2000; Loomis et al., 1996); (Reynolds and Hand, 2004 and references therein). Therefore, we hypothesized that there would be significant upregulation of transcripts that encode for cell stress proteins and cell cycle regulators in pre-diapause embryos compared with non-diapause embryos. We also predicted there to be downregulation of genes encoding products required for protein synthesis. *A. socius* appears to be unusual in that there is not an acute metabolic depression during diapause entry at the point when development ceases in the late gastrula stage (4–5 days post-oviposition) (Reynolds and Hand, 2009). However, the ontogenetic increase in respiration observed for the non-diapause embryos is fully blocked during diapause, such that metabolic rate is only 36% of the rate measured for 15 days developing embryos. Thus, we also predicted there to be differences in the mRNA abundance for genes encoding proteins that regulate energy metabolism. This study looks at transcript abundance not only in pre-diapause embryos but also in embryos that have been in diapause for several days. Some transcripts present in pre-diapause embryos may be required for initiating diapause, but not be necessary for maintaining the diapause state. Thus, we expect there to be significant adjustments in transcript abundance as diapause progresses.

MATERIALS AND METHODS

Insect rearing

Allonemobius socius colonies were started in 2002 with eggs provided by Dr Daniel Howard at New Mexico State University, and the colony was supplemented with individuals provided by Dr Jeremy Marshall at Kansas State University in 2006. Nymphs and adults were maintained and fed as described in our companion paper (Reynolds and Hand, 2009). Photoperiod was adjusted to encourage production of either diapause or non-diapause embryos, which were collected as described elsewhere (Reynolds and Hand, 2009).

RNA isolation and cDNA synthesis

Total RNA was isolated from pre-diapause and non-diapause *A. socius* embryos that were reared at 29°C for 2–4 days post-oviposition using RNAwiz reagent (Ambion, Austin, TX, USA). The manufacturer's protocol was followed except for minor changes to help improve recovery from limited starting tissue and to avoid interference from the large amount of yolk. Briefly, approximately 100 eggs (i.e. approximately 30 mg fresh mass) were homogenized in 500 µl of RNAwiz with a Kontes Pellet Pestle driven by a cordless Kontes Pellet Pestle motor (Kimble/Kontes, Vineland, NJ, USA). The homogenate was drawn through an 18 gauge needle to further disrupt the tissue and to improve mixing. After a 15 min incubation at room temperature, a 0.2 volume of chloroform and a 0.1 volume of RNase-free water were added to the homogenate. After an additional 15 min incubation at room temperature the homogenate was centrifuged at 18,000 ***g*** for 30 min at 4°C to separate the RNA from DNA, protein and other cell debris. Total RNA was precipitated from the supernatant with 0.5 volume of RNase-free water and 1 volume of isopropanol and centrifugation at 18,000 ***g***. The resulting RNA pellet was washed with cold 75% ethanol and re-centrifuged. The final RNA pellet was resuspended in water, and the purity and concentration of the RNA were assessed using a Nanodrop 1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Samples were stored at –80°C until used for cDNA synthesis.

To obtain sufficient starting material for the cDNA subtraction, multiple independent isolations (100 embryos each) were performed, and RNA samples were later pooled. Since a single *A. socius* female may produce a mixture of diapause-destined (pre-diapause) and non-diapause embryos, it was necessary to verify the diapause status of the embryos before combining the RNA samples into either pre-diapause or non-diapause batches. To do this, a subset of 15 embryos from each sample was returned to the 29°C incubator and allowed to develop for an additional 10 days (the remaining embryos in each sample where immediately homogenized as described above). After this extra incubation time, embryos were chemically fixed and the chorion was cleared (Hogan, 1959) as described by Reynolds and Hand (Reynolds and Hand, 2009). Embryos within the egg were observed using a Leica MZ7 stereomicroscope, and the number of embryos exhibiting the diapause morphology (see below) was counted. *A. socius* embryos that do not enter diapause typically hatch after about 15 days; as such, after 10 days, non-diapause embryos exhibited a distinctly different morphology than diapause embryos. An embryo was considered to possess the diapause morphology if it was centered in the middle of the yolk, was approximately 0.8 mm long, had a well developed procephalon, and lacked obvious limb development. An RNA sample was categorized as 'pre-diapause' if at least 70% of the embryos from which it was prepared showed the diapause morphology after 15 days. For 75% of all pre-diapause samples, the composition was actually greater than 90% pre-diapause embryos. Twenty-one confirmed pre-diapause samples were pooled to yield 155 µg total RNA. A sample was classified as 'non-diapause' if there were at least 70% non-diapause embryos present; 57% of non-diapause samples were composed of at least 80% non-diapause embryos. Fourteen confirmed non-diapause samples were pooled to yield 100 µg total RNA.

Messenger RNA (mRNA) was isolated from the pooled, total RNA using Oligotex^{TM} beads (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Equivalent quantities (0.5 µg) of mRNA from pre-diapause and non-diapause embryos were then used to synthesize cDNA using a SMART cDNA synthesis kit (Clontech, Mountain View, CA, USA) following the manufacturer's protocol.

Subtractive hybridization

Forward and reverse subtractions were performed using a PCR-based subtractive hybridization kit (Clontech, Mountain View, CA,

USA) according to the manufacturer's protocol. The forward subtraction, intended to isolate genes upregulated in pre-diapause embryos compared with non-diapause embryos, was performed using driver cDNAs generated from pre-diapause mRNA and tester cDNA from non-diapause embryos. The reverse subtraction, to identify genes present only in non-diapause embryos (i.e. downregulated in pre-diapause embryos), was performed using driver from cDNA from non-diapause RNA and tester from cDNA from pre-diapause embryos. Briefly, double stranded (ds) cDNA from tester and driver populations was digested with the restriction enzyme *Rsa*I to remove the oligonucleotides added during SMART cDNA synthesis and to generate short cDNA molecules with blunt ends. The tester cDNA was divided into two pools and each ligated to a different cDNA adaptor. As recommended by the manufacturer's protocol, PCR and gel electrophoresis were used to confirm at least 25% of ds-cDNA strands possessed adaptors (data not shown). In these reactions, primers complementary to the adaptors were used in combination with gene-specific primers for the COX I subunit. COX primers were designed by aligning sequences from several Orthoptera species. The forward primer sequence was 5′-AGCTCCTGATATAGCATTCCCACG-3′ and the reverse sequence was 5′-AGGGCTGTAATACCAAC-GGCTCAT-3′. Two hybridizations were performed to (1) equalize high- and low-abundance molecules and (2) enrich for cDNA molecules unique to the driver population. PCR using nested primers was employed to further amplify only differentially expressed sequences. These products were used to construct forward- and reverse-subtracted libraries as described below. To evaluate the efficiency of the subtractions, we compared the transcript abundance of actin in subtracted and unsubtracted cDNA populations using degenerate primers (forward primer 5′-ACAATGGMT-CYGGWATGTGCAARGCT-3′; reverse primer 5′-CCCAG-TTKGTWACAATWCCRTGCT-3′). Gel electrophoresis of the PCR products showed that the subtraction greatly reduced the amount of *actin*, originally a highly abundant gene, relative to unsubtracted controls.

Sequencing and bioinformatics analysis

The forward and reverse subtracted cDNAs, ligated into pGem vector, were transfected into JM109 competent cells (Promega, Madison, WI, USA). Transformed cells were grown overnight on LB plates containing ampicillin, X-Gal, and IPTG for blue/white selection. For each library, 300–400 white colonies were isolated and grown overnight in LB-ampicillin broth at 37°C. Plasmid DNA was extracted and purified using a Qiaprep miniprep kit (Qiagen, Valencia, CA, USA). Single pass sequencing was carried out using a primer for the sp6 promoter and BigDye terminator chemistry on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were identified by homology to known genes using NCBI Blastx search of the GenBank database. The functions of identified genes were evaluated with the UniProt Knowledgebase (ExPasy proteomic server; http://ca.expasy.org/).

Quantitative real-time PCR analysis

Quantitative real-time PCR (qRT-PCR) was used to assess the relative mRNA abundance in pre-diapause and diapause embryos compared with non-diapause embryos. Total RNA was isolated from pre-diapause and non-diapause embryos that had developed for 2–4 days post-oviposition as described above, and also from diapause embryos that were incubated for 15 days post-oviposition (i.e. 10 days in diapause). As before, independently isolated RNA samples were pooled to obtain sufficient total RNA. All 'pre-diapause' samples for qRT-PCR contained at least 83% confirmed pre-diapause embryos, whereas all 'non-diapause' samples for qRT-PCR contained 80% or more non-diapause embryos. The 'diapause' samples (15 days post-oviposition) contained 100% diapause embryos, because non-diapause embryos could easily be distinguished and removed at this late development point. For each of the three developmental stages, 4 μg of total RNA, was reverse transcribed in an independent 20 μl reaction containing 500 ng of random primers (Integrated DNA Technology, Coralville, IA, USA), 0.5 μmol l^{-1} dNTPs, 1× first strand buffer, 5 mmol l^{-1} DTT, 1 μl RNaseOUT and 200 i.u. of SuperScript III™ Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Incubations were carried out according to the manufacturer's instructions with an additional 5 min incubation at 25°C to promote annealing of the random primers. After a 60 min incubation at 50°C, samples were heated to 70°C for 15 min to inactivate the enzyme.

qRT-PCR was performed with a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR Green mix (Roche, Indianapolis, IN, USA) to which 10 nmol l^{-1} fluorescein (Bio-Rad) was added to allow well-factor correction. The total reaction volume of 20 μl included 300 nmol l^{-1} of primers and 2 μl of template. PrimerQuest software (IDT DNA, Coralville, IA, USA) was used to design primer sequences (see supplementary material Table S1). Cycling parameters were 10 min at 95°C followed by 50 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 30 s. Analysis of melt curves verified that only one product was amplified in each reaction.

Calculations and statistical analysis

mRNA abundance for each developmental stage was evaluated in four to six independent experiments, each with three technical replicates per primer pair. Each experimental plate included three wells with primers for 18S rRNA, the reference gene used for all analyses. After subtracting the baseline value with software provided by Bio-Rad, the cycle threshold (C_t) values were set to give a C_t value of approximately 9.5 for the wells containing the reference gene, which allowed for easy comparison of results across all experiments.

The $2^{-\Delta\Delta Ct}$ method was used to calculate fold change relative to the non-diapause sample and to define relative mRNA abundance for a particular gene of interest in relation to the reference gene, 18S rRNA (Mimmack et al., 2004). Values for fold change in mRNA abundance are given as means ± s.e.m. Under the $2^{-\Delta\Delta Ct}$ method, a fold change of 1 indicates there is no difference in the mRNA abundance for a gene of interest in the experimental group compared with the control group. Rather than arbitrarily select a fold-change cut off to establish significance, we first used the one-way Student's *t*-test to identify genes with a significant change in mRNA abundance (Minitab, State College, PA, USA). Then, to reduce the probability of type I errors from multiple comparisons, a *q*-value was calculated for each hypothesis tested. The *q*-value is a measure based on the false discovery rate (Benjamini and Hochberg, 1995) and is an estimate of the number of false positives expected among the significant tests. *q*-Values were calculated from *P*-values using the QValue plug-in for R Statistical software (Storey and Tibshirani, 2003); a test was considered to be significant when $q \leq 0.015$.

RESULTS

Of the 288 clones sequenced from the upregulated library, 78 had a significant homology with a sequence previously submitted to GenBank; 89% of these were unique transcripts (i.e. only one clone in the library). From the downregulated library, 384 clones were sequenced, and 155 of these ESTs showed significant homology

Table 1. ESTs identified from the upregulated library with significant BLAST matches

Probable homology	Closest GenBank match	Species	E-value*	Identity†
Hsp 20.7	ABC84494	*Locusta migratoria*	1e–52	63%
CHORD containing protein	XP_967567	*Tribolium castaneum*	1e–40	69%
RubV-like 2 (Reptin/TBP interacting protein)	XP_001122537	*Apis mellifera*	3e–43	79%
DNA dependent RNA polymerase III	XP_625037	*Apis mellifera*	2e–77	84%
Pyrroline 5-carboyxlate reductase	XP_974776	*Tribolium castaneum*	9e–30	73%
Male sterility protein (acyl-CoA reductase)	EAT41033	*Aedes aegypti*	2e–39	53%
Ribosomal protein L35Ae	CAJ17420	*Carabus granulatus*	7e–23	66%
Neimann-Pick type C-like	XP_624310	*Apis mellifera*	2e–14	52%
Cytochrome c oxidase VIIc	XP_970390	*Tribolium castaneum*	9e–13	79%
Mitochondrial ribosomal protein L45 (TIMM 44)	EAT35833	*Aedes aegypti*	1e–28	41%
Nedd8	XP_972922	*Tribolium castaneum*	2e–19	98%

*The E-value is an estimate of the number of hits expected by chance; the closer this value is to zero, the higher the significance of the match.
†Identity indicates the percentage of amino acids that are identical in the query *versus* matched sequences.

with sequences in GenBank, with 68% representing unique genes. A total of 33 sequenced ESTs were selected for further analysis with qRT-PCR to quantitatively estimate the abundance of these transcripts in pre-diapause and diapause embryos relative to non-diapause (control) embryos. The selection of these ESTs was based on the predicted functions of their gene products and the likelihood that they could have a role in regulating the known physiological changes that occur in *A. socius* embryos upon entering diapause (Reynolds and Hand, 2009). Eleven of the transcripts were from the upregulated library (Table 1), and 22 were from the downregulated library (Table 2). To facilitate the evaluation, these 33 transcripts were separated into six functional groups.

Stress proteins and chaperones

Eight transcripts were selected that encode proteins known to protect cells from stressful conditions such as extreme temperature or oxidative damage. Pyrroline 5-carboxylate reductase (P5cr) is involved in the biosynthesis of proline, an amino acid associated with increased cold tolerance in *Drosophila* (Misener et al., 2001). This mRNA increased by 58±16% (q=0.006) in pre-diapause embryos and was downregulated by 69±4.4% in diapause (q=0.000) when compared with non-diapause (control) embryos (Fig. 1). In addition, mRNA encoding acyl-CoA delta 9-desaturase (desaturase), which converts saturated fatty acids to monounsaturated fatty acids (MUFAs), was upregulated by 240% (q=0.007) in pre-diapause embryos compared with non-diapause embryos (Fig. 1). There was not a significant change in the abundance of this transcript in diapause embryos compared with non-diapause embryos (q=0.039).

Glyoxalase has been reported to protect cells from oxidative stress (Sommer et al., 2001), and its transcript was downregulated by 32±6.9% (q=0.007) in pre-diapause embryos and 80±4.0% (q=0.00) in diapause embryos. Bax inhibitor-1 (BI-1) is also reported to protect against oxidative stress by inhibiting Bax-induced apoptosis and protecting against cell death induced by heat shock or oxidative stress (Huckelhoven, 2004; Chae et al., 2003). There was no difference in the mRNA abundance of a BI-1 homolog in pre-diapause embryos compared with non-diapause embryos (q=0.064), but an 86±3.0% (q=0.002) reduction in the abundance of this transcript was observed in diapause embryos (Fig. 1).

Table 2. ESTs isolated from the downregulated library with significant BLAST matches

Probable homology	Closest GenBank match	Species	E-value*	Identity†
Heat shock protein 90	AAS45246	*Locusta migratoria*	1e–53	89%
Endoplasmin (Hsp90 family member)	EAT34979	*Aedes aegypti*	5e–52	80%
Heat shock 70 family member	AAO21473	*Locusta migratoria*	1e–36	90%
Cathepsin B-like protease	NP_572920	*Drosophila melanogaster*	1e–59	60%
ATP citrate lyase	EAT44342	*Aedes aegypti*	8e–87	95%
Translation elongation factor 1 gamma	AAL78751	*Locusta migratoria*	3e–44	85%
Fatty acid desaturase	XP_967943	*Tribolium castaneum*	2e–62	76%
Cytochrome P450	AAK57914	*Blattella germanica*	6e–10	40%
Histone2A	XP_307083	*Anopheles gambiae*	8e–48	99%
RACK1	NP_001041703	*Bombyx mori*	7e–72	93%
Eukaryotic translation initiation factor 4 gamma	EAT40334	*Aedes aegypti*	1e–48	60%
Interferon developmental regulator	XP_392883	*Apis mellifera*	5e–39	49%
Spaghetti squash (myosin regulatory light chain)	XP_623372	*Apis mellifera*	4e–35	92%
Bax-inhibitor 1-like protein	ABM55570	*Maconellicoccus hirsutus*	8e–26	80%
Cytochrome c oxidase subunit II	AAU11284	*Allomenobius socius*	2e–39	87%
Arginine kinase	AAT77152	*Periplaneta americana*	1e–63	96%
Transcription factor Dp2 (E2F dimerization partner)	XP_393377	*Apis mellifera*	2e–27	54%
Glyoxalase CG1532-PA	XP_625100	*Apis mellifera*	2e–40	67%
Cytochrome c oxidase subunit IV	AAY66918	*Ixodes scapularis*	2e–05	56%
Mitochondrial processing peptidase	XP_624556	*Apis mellifera*	9e–35	66%
Aldo-keto reductase family member	XP_974785	*Tribolium castaneum*	1e–07	40%
Lipid storage protein CG32645-PB	XP_969264	*Tribolium castaneum*	3e–66	79%

*The E-value is an estimate of the number of hits expected by chance; the closer this value is to zero, the higher the significance of the match.
†Identity indicates the percentage of amino acids that are identical in the query *versus* matched sequences.

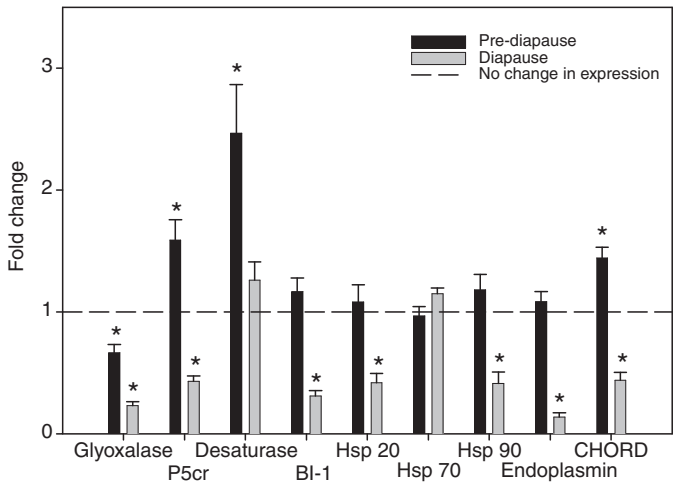

Fig. 1. mRNA expression profiles for genes encoding stress proteins and chaperones in pre-diapause and diapause embryos. Values are the mean ± s.e.m. for 3–6 replicates. Asterisks indicate a significant change in transcript level compared with non-diapause embryos (one-way *t*-test with FDR correction; $q<0.015$). C_t values were corrected for 18S rRNA. The dashed line demarcates a fold change of one, which indicates no change in mRNA abundance.

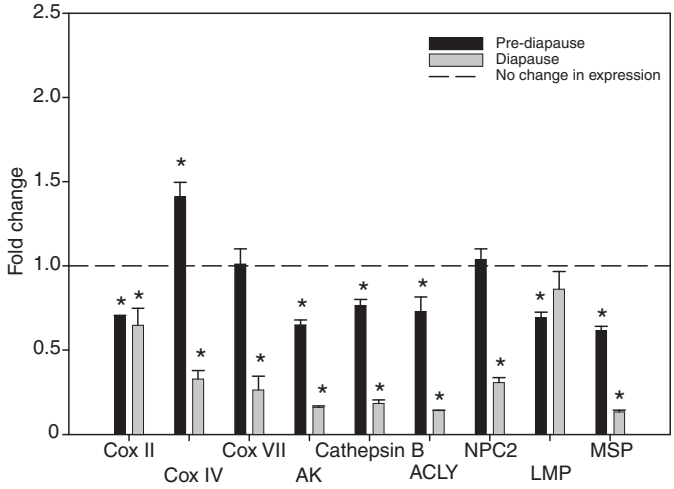

Fig. 2. mRNA profiles for genes encoding proteins involved with energy production and conversion in pre-diapause and diapause embryos. Values are means ± s.e.m. for 3–6 replicates. Asterisks indicate a significant change in transcript abundance compared with non-diapause controls (one-way *t*-test with FDR correction; $q\leq0.015$). C_t values were corrected for 18S rRNA. The dashed line demarcates a fold change of one, which indicates no change in mRNA abundance.

Fig. 1 also illustrates the relative transcript abundance of six heat shock proteins in pre-diapause and diapause embryos. Heat shock protein 20.7 (Hsp20.7), Hsp70, Hsp90, endoplasmin (an Hsp90 variant), and CHORD-containing protein (a homolog of the Hsp90 co-chaperone p23; CHORD) are included in this group. Compared with non-diapause embryos only CHORD showed a significant change in mRNA abundance in pre-diapause embryos, where it was upregulated by 44±8.9% (q=0.006). Comparable levels of Hsp20.7 (q=0.123), Hsp70 (q=0.145), Hsp90 (q=0.058) and endoplasmin (q=0.091) mRNAs were present in pre-diapause and non-diapause embryos. In diapause embryos, Hsp20 and Hsp90 mRNAs were reduced by approximately 60% (q=0.002 and q=0.003, respectively), whereas the abundance of endoplasmin mRNA was reduced by 86±3.0% (q=0.001) compared with non-diapause embryos. The abundance of Hsp70 mRNA was the same in diapause and non-diapause embryos (q=0.021).

Energy production and conversion proteins

Cytochrome *c* oxidase subunits II (COX II), IV (COX IV) and VII (COX VII), and arginine kinase (AK) are involved in the production of ATP; and cathpesin B-like protease, ATP-citrate lyase (ACLY), Niemann-Pick type C_2 like protein (NPC2), lipid metabolism protein (LMP) and male-sterility protein (MSP) are all predicted to have roles in fatty acid and/or lipid metabolism (see Discussion). Compared with non-diapause embryos, the transcript abundances of COX II and AK were reduced in pre-diapause embryos by 31% (q=0.001) and 36% (q=0.003), respectively (Fig. 2). There was no change in the amount of COX VII transcript (q=0.176) in pre-diapause embryos, but the mRNA abundance of COX IV increased 40±8.4% (q=0.006) in this early stage. When mRNA levels in diapause embryos were compared with values for non-diapause controls, it was found that all four of these genes are downregulated by values ranging from about 36% for COX II to 86% for AK (COX II q=0.001, COX IV q=0.001, COX VII q=0.004 and AK q=0.000).

Fig. 2 also shows the mRNA profiles of several genes that are predicted to encode proteins involved in fatty acid and/or lipid metabolism. Several of these transcripts, including cathpesin-B (q=0.003), LMP (q=0.004), ACLY (q=0.013) and MSP (q=0.002), were downregulated by 24–40% in pre-diapause embryos. There was not a significant reduction in the abundance of NPC2 mRNA in pre-diapause embryos (q=0.123) compared with non-diapause embryos. In diapause embryos, four out of five genes in this group were strongly downregulated by amounts ranging from 72% (for NPC2; q=0.001) to 87% (for MSP; q=0.000) compared with non-diapause embryos.

DNA replication, cell cycle regulation and transcription

Developmental arrest is a hallmark feature of embryonic diapause in this species as noted in our companion paper (Reynolds and Hand, 2009), and thus four genes with some role in cell division were evaluated. Histone 2A is part of the nucleosome and is important for chromosome packaging. As seen in Fig. 3 there was a modest but significant 20±2.0% decrease in the amount of histone 2A mRNA (q=0.006) in pre-diapause embryos, and there was an 86±1.0% decrease (q=0.01) in this mRNA in embryos that had been in diapause for 10 days. In *Drosophila spaghetti squash* encodes a cytoplasmic myosin that is required for cytokinesis in dividing cells of (Young et al., 1993; Wheatley et al., 1995). However, the abundance of this transcript was not significantly different in pre-diapause (q=0.09) or diapause embryos (q=0.014) compared with non-diapause embryos.

Transcription factor Dp2 (TFDp2) is a dimerization partner with E2F, a transcription factor known to associate with cyclins and retinoblastoma (Rb) proteins that can inhibit the G_1 to S transition in the cell cycle when Rb is present (Zheng et al., 1999). There was a 99±1.5% increase in TFDp2 (q=0.002) in pre-diapause embryos (Fig. 3), but the abundance of this transcript in diapause embryos was not significantly different from that of non-diapause embryos (q=0.07). Reptin is a protein that can repress transcription through its association with c-Myc (Etard et al., 2005) or histone acyltransferases (Qi et al., 2006). In pre-diapause embryos there was a 76±1.8% increase in Reptin mRNA (q=0.007), and a 71±3.0% drop (q=0.000) was observed in diapause embryos compared with non-diapause embryos.

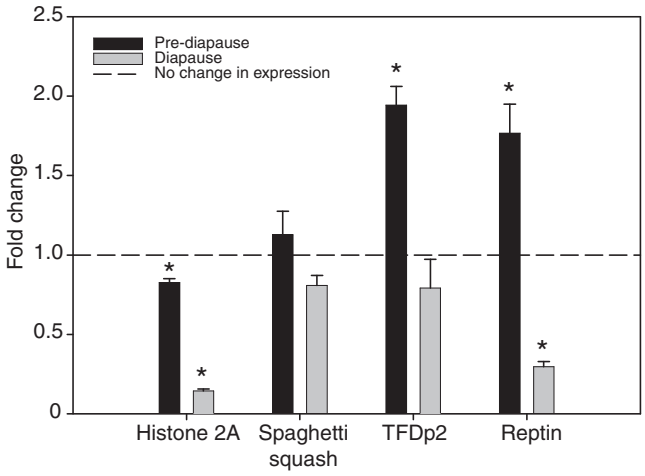

Fig. 3. mRNA profiles for genes encoding proteins that are involved DNA replication, cell cycle regulation in pre-diapause (2–4 days post-oviposition) and diapause embryos compared with non-diapause embryos. Values are means ± s.e.m. for 3–6 replicates. Asterisks indicate a significant change in transcript level compared with non-diapause embryos (one-way *t*-test with FDR correction; $q<0.015$). C_t values were corrected for 18S rRNA. The dashed line demarcates a fold change of one, which indicates no change in mRNA abundance.

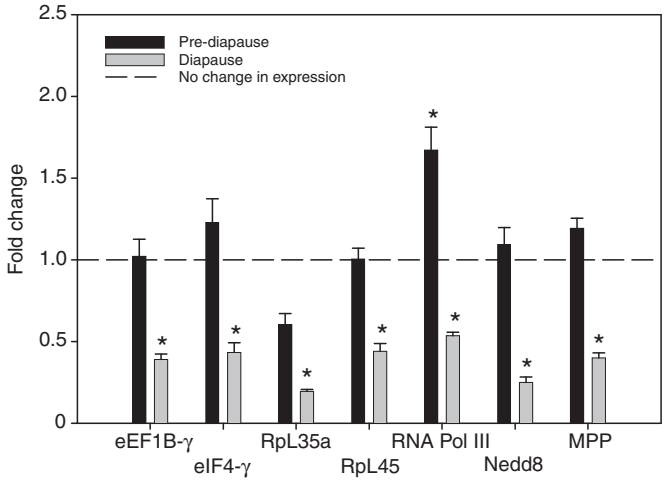

Fig. 4. mRNA profiles of genes encoding proteins involved in transcription, translation and protein processing in pre-diapause and diapause embryos. Values are means ± s.e.m. for 3–6 replicates. Asterisks indicate a significant change in transcript abundance (one-way *t*-test with FDR correction; $q\leq0.015$). C_t values were corrected for 18S rRNA. The dashed line demarcates a fold change of one, which indicates no change in mRNA abundance.

Transcription, translation and post-translational modification

Protein synthesis is an energetically expensive process that has been previously shown to be depressed under conditions of cell stasis and metabolic depression (Hochachka and Guppy, 1987; Hand and Hardewig, 1996; Guppy and Withers, 1999; Storey and Storey, 2007). RNA polymerase III (Pol III), which transcribes small ribosomal RNAs and transfer RNAs that carry amino acids to ribosomes during translation increased almost 70% (q=0.006) in pre-diapause embryos compared with non-diapause embryos (Fig. 4) and decreased 47±2.0% (q=0.000) in diapause embryos. There was a significant decrease in mRNA abundance for only one of the six proteins involved in translation and post-translational modification in pre-diapause embryos (Fig. 4). Ribosomal protein L35a (RpL35a) was reduced approximately 40% (q=0.008). There were no significant changes in the mRNA levels of translation elongation factor 1B-γ (eEF1B-γ; q=0.17), translation initiation factor 4-γ (eIF4-γ; q=0.06), ribosomal protein L45 (RpL45; q=0.18), Nedd8 (q=0.09) or MPP (q=0.013). However, all of the transcripts related to protein synthesis were significantly depressed by 47–80% in diapause embryos compared with non-diapause embryos. eEF1B-γ was reduced by 61±3% (q=0.001), eIF4-γ by 57±6.0% (q=0.004), RpL35a by 80±1.2% (q=0.000), RpL45 by 56±1.2% (q=0.003), Nedd8 by 75±3.0% (q=0.000) and MPP by 60±3.0% (q=0.002).

Endocrine and signal transduction

Fig. 5 illustrates the mRNA abundance of selected signaling molecules in pre-diapause and diapause embryos relative to non-diapause embryos. All of the transcripts evaluated were significantly upregulated in pre-diapause embryos. Cytochrome P450 (CYP450) and aldo-ketoreductase (AKR), which may be involved in ecdysone synthesis, were upregulated 203% (q=0.009) and 70% (q=0.014), respectively. Interferon-related developmental regulator (IFRD), which is homologous to the mammalian genes Tis7 and PC4, and is required for muscle differentiation (Micheli et al., 2005), was 40±13% higher (q=0.009) in pre-diapause embryos. Activated protein kinase c receptor (RACK1), which is reported to have a role in ecdysone signaling (Quan et al., 2006), was moderately, but significantly, upregulated 23±2.1% (q=0.003). In diapause embryos, the abundance of CYP450 was reduced by 50% (q=0.009), and AKR was reduced by 98% (q=0.000) compared with non-diapause embryos. IFRD was reduced by 45±2.2% (q=0.000), and RACK1 was downregulated by 67±1.0% (q=0.000) in diapause embryos compared with non-diapause embryos.

DISCUSSION

Of the genes evaluated in this study, we have shown the transcripts of 11 to be significantly upregulated during pre-diapause in *A. socius* embryos and 9 to be downregulated, when compared with morphologically and chronologically matched non-diapause embryos. Based on the arguments relating to function below, five upregulated genes (encoding CYP450, AKR, RACK1, TFDp2 and Reptin) possess substantial regulatory potential during the entry into diapause. Downregulated genes that hold promise for a significant role in diapause entry are those encoding ACLY, Cathpesin B-like protease and MSP. Diapause embryos, i.e. ones that have been in diapause for approximately 10 days, exhibit a very different expression profile compared with pre-diapause embryos. In diapause embryos, 87% of the genes examined were downregulated and 13% were expressed at the same levels as in non-diapause embryos. It is notable that upregulated genes were not found in these diapause embryos, particularly in light of the fact that late diapause in other insect species is characterized by both up- and downregulated genes (Denlinger, 2002; Robich et al., 2007). One interpretation of our finding for *A. socius* is that genes upregulated in pre-diapause embryos (i.e. those with the potential to regulate diapause entry) are transient and are not required to maintain diapause. It could be argued that an overall depression in transcription (and presumably translation) as diapause proceeds is consistent with a physiological state where energy conservation and developmental arrest are advantageous (Hand and Hardewig, 1996; Hand et al., 2001; Hahn and Denlinger, 2007).

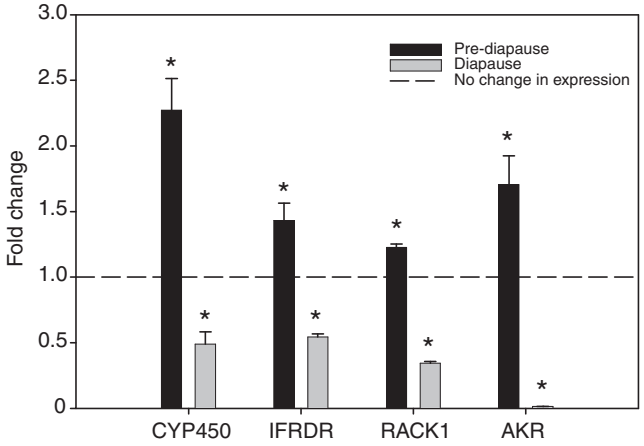

Fig. 5. mRNA profiles of genes encoding endocrine and signaling proteins in pre-diapause and diapause relative to non-diapause embryos. Values are means ± s.e.m., N=3–6. Asterisks indicate a significant change in transcript abundance (one-way t-test with FDR correction; q<0.015). C_t values were corrected for 18S rRNA. The dashed line demarcates a fold change of one, which indicates no change in mRNA abundance.

Stress proteins and chaperones

Numerous studies have shown that animals in diapause are more resistant to environmental insults such as cold stress, heat shock and oxidative damage than con-specific individuals that are not in diapause (e.g. Podrabsky et al., 2001; Rinehart et al., 2006; Hand et al., 2007; Podrabsky et al., 2007). However, upregulation of stress proteins is not a general feature of diapause in *A. socius* because only three of nine transcripts analyzed increased in pre-diapause embryos. Two of the genes that are upregulated in pre-diapause embryos, encoding P5cr and Δ9 desaturase, are also upregulated in some overwintering insects. P5cr is involved in the final step in proline biosynthesis, and this amino acid has been correlated with increased cold acclimation and tolerance in species ranging from crickets to flies (Shimada and Riihima, 1990; Ramlov, 1999; Miesner et al., 2001). P5cr expression and the proline pool are known to be upregulated in cold-adapted *Drosophila melanogaster* (Miesner et al., 2001). Δ9 desaturases are also associated with cold tolerance in a number of species (Tiku et al., 1996; Cossins et al., 2006). The Δ9 desaturases are lipogenic enzymes that produce monounsaturated fatty acids (MUFAs), which are components of membranes and energy storage molecules (Brock et al., 2006). Increasing the proportion of unsaturated fatty acids is correlated with the intrinsic fluidity of the membranes and helps maintain the function of these structures during exposure to low temperatures (Hazel, 1995; Hochachka and Somero, 2002; Michaud and Denlinger, 2006). Since diapause is an overwintering strategy in *A. socius*, we predict that upregulating P5cr and Δ9 desaturase expression is required to guard against damage from low temperatures.

Glyoxylase is reported to protect cells from oxidative damage by enzymatically detoxifying α-oxoaldehydes, which are produced through the degradation of glycolytic intermediates and are known to cause mutations and induce apoptosis (Sommer et al., 2001). Given its protective function, it is surprising that the mRNA for this enzyme is downregulated in pre-diapause and diapause embryos. Perhaps a reduction in glycolytic activity decreases the need for this protection in dormant embryos.

Expression profiles for heat shock proteins such as Hsp70, Hsp90 and Hsp23 have been studied in numerous animals that enter diapause. In general, the expression patterns of these genes are species specific. For example, Hsp70 and Hsp23 are upregulated in brain tissue of *Sarcophaga crassipalpis* whereas Hsp90 is downregulated (Denlinger, 2002). By contrast, in larvae of *Chilo suppressalis*, Hsp90 is upregulated during diapause (Sonoda et al., 2006); and none of the heat shock proteins analyzed show differential expression in diapause larvae of *Lucilia sericata* (Tachibana et al., 2005). In *A. socius*, only CHORD, a homolog of the Hsp90 co-chaperone p23, is upregulated in pre-diapause embryos. There is no change in mRNA abundances of Hsp20.7, Hsp70, Hsp90 or endoplasmin in pre-diapause or diapause embryos compared with non-diapause embryos; thus upregulation of these genes is not a requirement for diapause in this species.

Energy conversion and metabolism

Normally metabolic depression is one defining characteristic of diapause, but for *A. socius* an acute metabolic downregulation does not occur during diapause entry at the point when developmental ceases (Reynolds and Hand, 2009). Instead, the ontogenetic increase in respiration that is observed during development of non-diapause embryos is completely prevented by diapause. Presumably this latter phenomenon would still require a substantial restriction of transcription and translation of selected metabolic genes at the onset of diapause, and thus it was of interest to evaluate differential expression during this transition from growth to maintenance. Certainly other mechanisms including allosteric inhibition and covalent modification could be at work, but for long-term adjustments, we predicted that alterations in mRNA abundance profiles would also occur.

Functionally, the metabolic genes analyzed fall into two groups – genes involved in oxidative phosphorylation and ATP metabolism and genes involved in the interconversion of cellular energy reserves. AK is a phosphagen kinase and is part of the system that buffers ATP levels in insects (Tanaka et al., 2007). Downregulation of transcripts for this enzyme (Fig. 2) is consistent with data that show low levels of ATP in pre-diapause and diapause *A. socius* (Reynolds and Hand, 2009). COX II is one of two subunits forming the catalytic core cytochrome *c* oxidase, the terminal complex in the mitochondrial electron transport chain (Nicholls and Ferguson, 2002), and COX IV and COX VII are required for proper assembly and function of the COX complex in mammals and yeast (Aggler and Capaldi, 1990; Li et al., 2006). With the exception of COX IV, the general trend is a decrease in the amount of mRNA encoding COX subunits in pre-diapause and diapause embryos, a pattern that is in keeping with the respiration profiles described above. The majority of genes involved in inter-conversion of cellular energy reserves are downregulated in pre-diapause embryos. Cathpesin B participates in the digestion of yolk proteins in a number of insects (Medina et al., 1988; Ribolla and De Mianchi, 1995; Cho et al., 1999; Zhao et al., 2005). Downregulation of this gene in *A. socius* (25% in pre-diapause) could slow digestion of the yolk proteins during a time when it is critical to preserve the fuel stores that will be required to complete post-diapause development. Consistent with lipid sparing, ACLY, LMP and MSP (i.e. acyl-CoA reductase) are enzymes that promote fatty acid/lipid usage, and all are downregulated 20–40% in pre-diapause. The trend continues and is more pronounced with Cathepsin B, ACLY and MSP being more strongly downregulated (about 80%) in diapause (Fig. 2B). The NPC2 protein, which is involved in sterol metabolism and homeostasis (Ory, 2004), does not show differential expression in pre-diapause embryos. With this one exception, the data are consistent with conservation of lipids during diapause, a hypothesis supported by previous studies on two Orthoptera species, *Melanoplus*

differentialis (Kaocharern, 1958) and *Aulocara elliotti* (Visscher, 1976), which show accumulation of lipids in diapause embryos (Kaocharern, 1958). Finally, it is notable that ACLY and NPC2 also have been implicated in the synthesis of juvenile hormone and ecdysone, respectively (Noriega et al., 2006; Ioannou, 2007). Both of these hormones are known regulators of diapause in a number of insect species (see Endocrine and signal transduction, below).

DNA replication, cell cycle regulators and transcription factors

Development is arrested 4–5 days post-oviposition when *A. socius* embryos enter diapause (Reynolds and Hand, 2009). Histone 2A, which is downregulated in pre-diapause embryos, is a core histone protein and is essential to the structure of condensed chromatin. Abundance of histone mRNA typically increases only during the DNA synthesis phase of the cell cycle, and mRNA is degraded if DNA synthesis is inhibited (Anderson and Lengyel, 1980; Kaygun and Marzulff, 2005). Downregulation of histone 2A mRNA is consistent with developmental arrest in general and cell cycle arrest in particular.

TFDp2 and Reptin are transcription factors that play a role in the regulation of cell proliferation. TFDp2 (DP) is a dimerization partner with E2F, a transcription factor that can activate or repress the G_1 to S cell-cycle transition (Duronio et al., 1998; Zheng et al., 1999). Targets of this heterodimer include *cyclinE*, *cyclinA*, *cdc2*, *c-myc*, and growth-regulatory proteins. Activity of E2F-DP depends on the presence or absence of a retinoblastoma (Rb) family protein. Rb-E2F-DP complexes arrest the cell cycle at G_1 and thus are characteristic of quiescent cells (Duronio et al., 1998; Zheng et al., 1999). Reptin is an evolutionarily conserved protein that represses transcription through its interactions with c-Myc (Etard et al., 2005) and histone acyltransferases (HATs) (Qi et al., 2006). Because TFDp2 and Reptin are both upregulated almost twofold in pre-diapause embryos, and Reptin is later downregulated in diapause, analysis of the transcript abundance of genes encoding E2F, Rb, c-Myc, HATs and other proteins in these pathways could prove useful for highlighting mechanisms underlying developmental arrest.

Transcription, translation and protein processing

Although protein synthesis is energetically expensive (Hand and Hardewig, 1996) and is depressed during diapause in a number of animals including flies (Joplin and Denlinger, 1989) and fish (Podrabsky and Hand, 2000), genes that encode proteins involved in protein synthesis and post-translational modification are not downregulated in pre-diapause embryos, which is consistent with the lack of acute metabolic depression at the onset of diapause in *A. socius*. Specifically, the mRNA abundance of four genes, *eIF4-γ*, *eEF1B-γ*, *RpL45* and *Nedd8*, is the same in pre-diapause and non-diapause embryos. The exception to this general trend is *RpL35A* which is downregulated by almost 50% in pre-diapause embryos. Overexpression of this gene in Jurkat cells confers resistance to apoptosis-stimulating chemicals (Lopez et al., 2002), but it is unlikely that this protein has a protective role in diapause embryos.

Significant upregulation was observed for two genes, *RNA Pol III* and *MPP*. *Pol III* encodes a polymerase that transcribes 5S ribosomal RNA, tRNAs and other small RNAs. MPP is a protein located in the mitochondrial matrix that removes the leader sequence from proteins targeted to these organelles (Muhopadhyay et al., 2002). Upregulation of these genes suggests an increase in translation and protein processing in pre-diapause embryos rather than the expected downregulation. However, all genes in this category are significantly downregulated during late diapause (Fig. 4).

Endocrine and signal transduction

Diapause in insects is, at least in part, regulated by the endocrine system (Nijhout, 1984; Yamashita and Hasagawa, 1985; Denlinger, 2002). In many species, embryonic diapause results from changes in the titre of ecdysone (e.g. Gharib et al., 1981) and/or juvenile hormone, a sesquiterpenoid (e.g. Visscher, 1976). Three genes thought to be part of the ecdysone signaling and/or biosynthesis pathways, *RACK1*, *AKR* and *CYP450*, show an upregulation of mRNA in pre-diapause embryos and a substantial decrease in mRNA abundance in diapause embryos compared with non-diapause embryos. RACK1, also known as activated protein kinase c receptor, is predicted to be activated by 20-hydroxyecdysone, which results in the phosphorylation and activation of additional components of the ecdysone receptor and, ultimately, stimulation of genes containing an ecdysone response element (Quan et al., 2006). AKR is a member of a protein family that includes more than 120 members with a wide range of physiological functions including polyol synthesis and steroid metabolism (Luccio et al., 2006). AKRs are monomeric proteins with NADPH-dependent catalytic activity. One member of this family is known to be upregulated during diapause initiation in the heteropteran bug, *Pyrrhocoris apterus* (Koštál et al., 2008). Biochemical studies on ecdysteroid metabolism in insects and crustaceans suggest that aldo-ketoreductase enzymes may be involved in this pathway (Maibeche-Coisne et al., 2001; Sieglaff et al., 2005). AKR transcript is upregulated in *Aedes aegypti* ovary at the time ecdysone synthesis peaks, and the authors conclude that early expression of the AKR gene may be required for activation of ecdysteroidogenesis (Sieglaff et al., 2005). CYP450 is part of a large family of enzymes with mono-oxygenase activity. Numerous members of this family are required for ecdysone biosynthesis (Gilbert, 2004; Feyereisen, 2005; Huang et al., 2008) and are known to be expressed in *Drosophila* (Chávez et al., 2000) and *Bombyx* (Horike et al., 2000) embryos. Although CYP450s can have a variety of functions, including the detoxification of xenobiotics, it seems unlikely that a pre-diapause embryo, which is isolated from the environment by a network of extra-embryonic membranes as well as a chorion, would have a need for a detoxification enzyme. Thus we predict that the CYP450 identified in this study has a function in ecdysteroidogenesis. However, further analysis, including 3′ and 5′ RACE to determine the full-length sequence of the gene as well as enzymatic assays to determine the substrate of this enzyme, are required to verify the role of this gene in embryonic development and diapause entry.

It is surprising that the expression of RACK1 (upregulated 1.2-fold), CYP450 (up 2.4-fold) and AKR (up 1.7-fold) is increased in pre-diapause embryos of *A. socius,* because embryonic diapause in insects is typically correlated with a decrease in, or lack, of ecdysone [e.g. *Bombyx mori* (Horike and Sonobe, 1999), *Locusta migratoria* (Tawfik et al., 2002), *Chortoicetes terminfera* (Gregg et al., 1987) and *Oxya yezoensis* (Kidokoro et al., 2006)]. However, the endocrine events associated with embryogenesis are species specific and there is considerable variation in ecdysteroid titres even between species from the same order (Whiting and Dinan, 1988). In addition, previous studies on the endocrine events associated with diapause have looked at species that arrest development at a later point in embryogenesis (e.g. Wardhaugh, 2006). Thus it is possible that diapause is characterized by an increase in ecdysone in *A. socius* rather than a decrease as reported for the species above. For example in *Lymantria dispar*, which enters diapause as a pharate first instar larva, an increase in ecdysone titre is required for diapause induction and maintenance, and a decrease in ecdysone occurs upon diapause termination (Lee and Denlinger, 1996; Lee et al., 1997).

Furthermore, ecdysone has been shown to cause cell cycle arrest in cultured cells from *Aedes albopictus* (Gerenday and Fallon, 2004). The abundances of RACK1, CYP450 and AKR transcripts are depressed at least 50% during diapause in *A. socius*, with AKR being the most dramatically decreased (98% relative to non-diapause embryos). Coupled with its significant upregulation during pre-diapause, AKR has the largest differential expression of any gene in the study – an 85-fold swing between pre-diapause and late diapause. Consequently, it appears that the functions of these gene products are necessary for diapause entry but not for the maintenance of the dormant state. Future studies that quantify the amount of ecdysone, 20-hydroxyecdysone and their conjugates are clearly required to solidify the roles of RACK1, CYP450 and AKR in diapause entry for *A. socius* embryos.

In summary, eight candidate genes have been identified that show promise as regulators of diapause entry in *A. socius* embryos and warrant additional study. Our designations are based both on the magnitude/consistency of differential mRNA expression and our ability to place the projected functions of these genes into rational context by considering the physiological and biochemical events of diapause that we have experimentally characterized for *A. socius* (Reynolds and Hand, 2009). The functional categories into which the products of these genes fall are ecdysteroid synthesis and signaling (CYP450, AKR and RACK1), transcription and cell cycle control (Reptin, TFDp2) and lipid sparing (Cathpesin B, ACYL, MSP). mRNAs for CYP450, AKR and RACK1 are consistently upregulated in pre-diapause, followed by major downregulation later in diapause. This initial upregulation would suggest that elevated ecdysone may facilitate onset of diapause in *A. socius*. Our observed upregulation of Reptin and TFDp2 mRNAs may serve to depress transcription and cell cycle progression. Finally, transcripts for Cathpesin B-like protease, ACLY and MSP are downregulated, which may serve to promote lipid sparing during diapause.

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Table S1. Primers sequences used for quantitative PCR

Primer name	Forward sequence	Reverse sequence
18S Ribosomal RNA	5′-TCTCAGTGCAAGCCGAAGTAGGT-3′	5′-TCCCTTCCATTGCTGGTCGAGATT-3′
Hsp90	5′-AACTGCCCTCCTATCTTCTGGCTT-3′	5′-TCTTCACCTTCCTGCGCATCATCT-3′
Endoplasmin	5′-GGACAGTTTGGCGTTGGCCTTTAT-3′	5′-CAAGGTATTACCACGTGGGTCTTCAG-3′
Hsp20.7	5′-CTCAACAGTCTTCTCAACATTCTTCTTAGG-3′	5′-AGGAACGTCCAATTCCAATCATCC-3′
Hsp70	5′-ACTCCCACACAGGAGTATGTGGTT-3′	5-GTGAACGAACAAGAGTGCGGAGAA-3′
ATP citrate lyase	5′-ACTGCTGATCATGGACCTGCTGTA-3′	5′-AGCACCATCTAGAGCACCACCAAA-3′
Cathpesin-like protease	5′-AGCATGATCCGCAAGATCCCTGAT-3′	5′-CATTCATCGGCTGATGGGTGTTCA-3′
Translation elongation factor-1γ	5′-AAGGCTGGAACCTTTCCAAGAGGA-3′	5′-AGGTGCTCATTGCTGCCTCTTACT-3′
Cytochrome p450	5′-GGACTTGCGTGTTAAGTTAAGCCCAG-3′	5′-ATCACGGGCAGCGTCATCAAAGTA-3′
Histone 2A	5′-TAGACCCGGATGTTGACCCTGTTT-3′	5′-ATTGGAGTTAGCTGGCAATGCTGC-3′
Desaturase	5′-ATGGCCTCTGCGACTTCTATTGGT-3′	5′-TTGTGTGGATCGGCATCAGTCTCA-3′
CHORD containing protein	5′-TAAGGGCTGCACAAGGTCGTATCA-3′	5′-CCTTGACAATTGCTGCAGGTGCTT-3′
Bax Inhibitor	5′-CTTCAGCCTCTGCGTTTACGCTTT-3′	5′-ATCGAGAAGCGTCGAAGTGGTGAT-3′
RACK1	5′-TCTCTTTCCAGCGTGGCCATTAGA-3′	5′-CCTCGAAGCTGTAGAGATTCCGACAT-3′
Nedd8	5′-GGAGGATCGGCTTCTTCATTTGGT-3′	5′-AGCATAGGCAATCTGAGCACGCTA-3′
CG1532-PA/glyoxalase	5′-GGATGGCCCTTTACTGAAGAAGGA-3′	5′-TGCGAGGTAGGCTGAGGTTCATTT-3′
Mitochondrial processing peptidase	5′-ATACAACAGGACGGGCTTCCAAGT-3′	5′-GGCTGGCAAAGTTGGAGATGTTGA-3′
Translation initiation factor 4	5′-AAAGCCTGGGTCTGTGTGATGAGA-3′	5′-TGAGATCCTTGAGGAGCGTGGTTT-3′
IFRD	5′-ATCAGGTGGCTCTCCATCCTCAAT-3′	5′-AGAGGACATGACTCCTCAGTTGGT-3′
TIF	5′-GGTCTGTGTGATGAGACGGATCCAAA-3′	5′-TGAGATCCTTGAGGAGCGTGGTTT-3′
TFDp2	5′-CAGTCTGCTGAGTAGGAGTTAAGCGT-3′	5′-TCCATTTCAGTCACCTGGTGGAAC-3′
RpL45	5′-AGTCGGCGGAGATCTATTGCATGT-3′	5′-GGATTCCAGTGTTTCGAGCCTCTT-3′
eEFtB-γ	5′-AAGGCTGGAACCTTTCCAAGAGGA-3′	5′-AGGTGCTCATTGCTGCCTCTTACT-3′
Spaghetti squash	5′-GGAAATGAGGGCAGTAAGGCACTA-3′	5′-ATCGTGAAGCTCCAATCAAGGGTG-3′
CHORD containing protein	5′-TAAGGGCTGCACAAGGTCGTATCA-3′	5′-CCTTGACAATTGCTGCAGGTGCTT-3′
COX subunit II	5′-TTCCGTCTTCTCGATGTTGACAACCG-3′	5′-CGTCCTGGTGTAGCATCAGATTTAACCC-3′
COX subunit IV	5′-TGCCCGAATCCTTTACTGATGAGC-3′	5′-CCCACTTGGATGTCAATCCATCAAC-3′
COX subunit VII	5′-TTCTTGGAGTCGCTGTGGACATCA-3′	5′-TGGCTCTGGGTTTAGTGCTTCCTT-3′
Arginine kinase	5′-TGCTTCTGTGCACATCAAGGTTCC-3′	5′-AAACTCCACCCTCAGCTTCTGTGT-3′
NPC2	5′-TCATATGTTCCACTGTCCCAGCAAG-3′	5′-AGCTATTTCTTCCCTGGCACTGAC-3′
RpL35	5′-TCCACTTGGTTGGTGTTGAGGTCT-3′	5′-AGATGTTGCATCTACGTCGGCCAT-3′
Reptin	5′-GCCATCTCATTCTCCAGAGCACGATT-3′	5′-TGAGTGGCGTGAAGAAGGGAAAG-3′
Aldo-keto reductase	5′-AGGCCCATGGAGGAACAAGTGAAT-3′	5′-GCCACAAACTGTAATGCCCAAACG-3′
Polymerase III	5′-ATGGACAGAAAGGTGTTACAGGGC-3′	5′-TGATCCACCAAATGCTGTCCCA-3′
Pyrolline carboxylate reductase	5′-TCTCCCAATTCTACGGAGCGCTTT-3′	5′-TGGTATTGGAAACAGGCAAGCACC-3′
Male sterility protein	5′-GGTCCGGCGATACCTCAATAAAGA-3′	5′-TATACTATCACGGGCACCCAAGCA-3′
Lipid metabolism	5′-AAGACGTGTCCAGGGCTTATCGTA-3′	5′-TGTCGGCTGCCATTATGACTACCT-3′