METABOLISM AND GENE EXPRESSION DURING EMBRYONIC DIAPAUSE IN ARTHROPODS

A Dissertation

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For Matt, Alison and Kaitlin.
Throughout this entire process Matt's love and support have been without measure.
I truly could not have completed this journey without him.
Kaitlin and Alison have only been part of this pursuit for the last two years,
but they have been a greater source of motivation than they will ever know.

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ABSTRACT

Arthropods may enter diapause to escape environmental insult. Diapause is an endogenously controlled dormant state defined by developmental arrest and species-specific physiological changes (e.g., metabolic depression and upregulation of compounds that protect cell structure and function). Although physiological changes have been documented for a number of species in diapause, biochemical and molecular regulation of diapause remains largely unexplained.

Aerobic metabolism in diapause, *Artemia franciscana,* embryos is reduced up to 92 % compared with post-diapause embryos. Differences in isolated mitochondria are insufficient to account for respiratory depression because mitochondria in diapause embryos are structurally similar to mitochondria in post-diapause embryos. Respiratory control ratios and P:O flux ratios of mitochondria from diapause embryos are equal to or higher than those of mitochondria from post-diapause embryos. State 3 and state 4 respiration rates on pyruvate are equivalent in the two stages, and mitochondria isolated from diapause embryos show a moderate, 15-27 % reduction with succinate. Cytochrome c oxidase activity is 53 % lower in diapause embryos, but the minimal impact on mitochondrial respiration appears to be due to the 31 % excess of COX capacity in these embryos.

Allonemobius socius embryos enter diapause 3-4 d post-oviposition as indicated by their morphology and DNA embryo^{-1}. There is not an acute downregulation of metabolism during diapause in this species. Diapause embryos consume O_2 at the same rate as morphologically similar non-diapause embryos. Diapause and non-diapause embryos exhibit unusually high [AMP]/[ATP] and low [ATP]/[ADP] during early embryogenesis, suggesting that these embryos may be hypoxic early in development. However, superfusing 3 d embryos with O_2 enriched air only partially relieves the hypoxic state, which indicates the unusual energy status is an ontogenetic feature not fully explained by oxygen limitation.

Subtractive hybridization and qPCR identified 6 genes predicted to regulate diapause entry in *A. socius*. Reptin, TFDp2, CYP450, AKR are significantly upregulated in pre-diapause embryos, and ACLY and Capthesin B-like protease are downregulated compared to non-diapause embryos. The need for genes upregulated in pre-diapause embryos appears to be transient as these genes are substantially downregulated 10 d after diapause entry. Taken together, these studies provide an integrative examination of mechanisms underlying diapause entry in arthropods.

CHAPTER 1

INTRODUCTION

Organisms ranging from bacteria and fungi (Soussman and Douthit 1973; Henis 1987) to plants (Bewley 1997; Footitt and Cohn 2001), invertebrates (Loomis et al. 1996; Bishop et al. 2002) and even vertebrates (Podrabsky and Hand 1987; Boutilier and St-Pierre 2002; Epperson and Martin 2002) survive adverse environmental conditions by becoming dormant. Dormancy can take a wide variety of forms, but in general, dormant states are classified as either quiescence or diapause. Quiescence is primarily characterized by metabolic depression and is a direct response to changes in the environment. Quiescence begins as soon as conditions become unfavorable and ends immediately once environmental conditions improve. Dehydration, anoxia, and low temperature are examples of conditions that lead to quiescence (Stuart et al. 1998; Jones et al. 2001; Eads and Hand 2003a, b). In contrast, diapause is an endogenously controlled form of dormancy that is characterized by developmental arrest as well as a suite of species-specific physiological changes, which may include decreased metabolism and increased production of protective molecules (e.g., glycerol or heat shock proteins) that preserve cellular structure and function (Clegg 1965; Storey and Storey 1990; Denlinger 2002; Lee et al. 2002). Animals enter diapause well in advance of the harsh conditions, in response to cues, such as changes in photoperiod or food quality, that signal a future change in the environment (Lees 1955; Denlinger 1986). They will remain in this state even when conditions are adequate to support normal growth and metabolism. Animals in diapause resume development only after they are exposed to appropriate diapause termination cues; low temperatures and dehydration are examples of

ecologically relevant factors that can release animals from diapause (Rakshpal 1962; Drinkwater and Crowe 1987; Footit and Cohn 1995; Koštál 2006).

Diapause is particularly widespread among the arthropods (Lees 1955; Hand 1991) and has been studied in copepods (Hairston 1995; Romano et. al 1996), *Daphnia* (Stross and Hill 1965), brine shrimp (Drinkwater and Crowe 1987), spider mites (Ito and Saito 2006), and a variety of insects (e.g., Jungreis 1978; Denlinger 1985, 2002). Even though diapause in arthropods has been a popular research topic during the last 50 years, and a large number of papers have been published on this topic, the mechanisms that regulate diapause remain elusive. The primary goal of the research presented in this dissertation is to provide additional understanding of the mechanisms that control metabolic depression and developmental arrest during embryonic diapause. Keeping with August Krogh's principle, which states that "for a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied," (Krogh 1929; Krebs 1975), two species were selected for my studies on embryonic diapause. *Artemia franciscana* (Kellogg) is ideal for studying metabolic depression because it is easy to obtain the large amounts of embryos required for biochemical and bioenergetic studies. *Allonemobius socius* (Scudder) is well suited for studies on the molecular regulation of diapause because it is simple to obtain diapause and non-diapause embryos that are morphologically similar, so changes that occur because of diapause can be separated from ontogenetic changes. The remainder of this Introduction will focus on aspects of development and diapause that are relevant to these two species.

Metabolic Downregulation during Diapause in *Artemia franciscana* Embryos

The brine shrimp, *Artemia franciscana,* is an anostracan crustacean known to inhabit hypersaline bodies of water such as the Great Salt Lake, Utah, and the San Francisco Bay, CA. In response to changes in photoperiod, population density, and/or salinity, ovoviviparous females switch to oviparous reproduction and produce encysted embryos (cysts) that enter diapause (Berthelemy-Okazaki and Hedgecock 1987). Diapause in this species is characterized by developmental arrest at the gastrula stage (approximately 4,000 cells) and high levels of trehalose in the embryo (Clegg 1965). However, the hallmark characteristic of diapause in this species is extreme metabolic depression. Although the metabolic rate of newly released embryos is comparable to that of actively developing embryos, oxygen consumption drops to barely detectable levels within 5 d (Drinkwater and Clegg 1991; Clegg et al. 1996), in spite of environmental conditions that are adequate to support much higher metabolic rates.

Reducing metabolic activity during diapause is advantageous for a number of reasons. First, nutrient reserves can be extended by reducing energetically expensive processes (Hand and Hardewig 1996; Hahn and Denlinger 2007). Such conservation is essential for embryos that develop outside the female since the only fuels available to complete development are the stores of carbohydrates, proteins, and lipids provisioned by the mother during oogenesis (Forte et al. 2002; Němec 2002). Because diapause effectively increases the time required to complete embryonic development, metabolic depression ensures the availability of nutrients to fuel post-diapause development. Second, reducing the activity of the mitochondria through metabolic depression prevents cellular damage from reactive oxygen species (Stuart

and Brown 2006), and has the potential to maximize post-diapause fitness and fecundity (Hahn and Denlinger 2007).

Metabolic downregulation has been studied extensively in *A. franciscana* embryos under anoxia (Hand 1990; Kwast and Hand 1996; Covi and Hand 2005; Covi et al. 2005; Clegg 2007), and many aspects of metabolic downregulation under these conditions are well understood. Specifically, under anoxia metabolic depression occurs through intracellular acidification (Busa et al. 1982; Clegg et al. 1995; Kwast et al. 1995; Covi and Hand 2005; Covi et al. 2005) and is correlated with a coordinated reduction in energy producing (i.e., oxidative phosphorylation) and energy consuming (i.e., transcription, translation, and ion pumping) processes (Hand 1998 and references therein). However, there is less information on the metabolic depression of normoxic, fully hydrated diapause embryos for this species. As noted above, there are fundamental differences between anoxia-induced quiescence and diapause, and the mechanisms underlying metabolic depression are expected to be different for these two types of dormancy.

Metabolic depression has been observed in studies of a number of species with embryonic diapause (e.g., Rakshpal 1962; Sonobe and Nakamura 1991, Romano et al. 1996; Naylor 1999). However, these authors made little attempt to correlate their observations with underlying mechanisms; thus the phenomenon is still not well understood. Chapter 2 of this dissertation focuses on the proximal cause of metabolic depression in diapause embryos of *A. franciscana* collected from the Great Salt Lake, UT. Specifically, I tested the hypothesis that metabolic depression in this species is the result of intrinsic changes in the mitochondria. Support for this hypothesis comes from previous studies on aestivating snails which correlate changes in the phospholipid composition of mitochondrial membranes with a decrease in the

rate of oxygen consumption (Stuart et al. 1998). In addition, two studies on insects that enter diapause provide evidence that mitochondria are degraded during this dormant state (Kukal et. al. 1989; Levin et al. 2003).

Embryonic Development and Diapause in *Allonemobius socius*

While diapause *A. franciscana* embryos are an ideal organism for studying metabolic depression during diapause, their usefulness in gene expression studies has some practical limitations. This is largely because non-diapause embryos develop within the female's brood sac while diapause embryos are released into the environment. Not only is it tedious to surgically remove large numbers of non-diapause embryos from the female, but it is difficult to match diapause embryos with ontogenetically identical non-diapause embryos. Furthermore, there are likely fundamental differences (i.e., quantity and quality of sugars provisioned by the female; Clegg 1965) between embryos that develop within the female versus those that are encapsulated and released into the environment. *A. socius* is a species that is more suited to studies on the molecular regulation of embryonic diapause because it is possible to measure transcript abundance in diapause and non-diapause embryos that are morphologically similar. This matching of diapause and non-diapause embryos makes it possible to separate changes that result from diapause from otherwise "normal" ontogenetic changes.

A. socius is a small ground cricket found in the eastern portion of the United States. Populations that occur in the northern portion of its range are univoltine (i.e, have one generation per year with an obligatory diapause). However, southern populations of this species are bivoltine, and diapause is facultative. Typically, the fist generation each year matures during the summer and produces embryos that develop without entering diapause.

Individuals in this second generation mature in the fall, and females produce embryos that survive through the winter by entering diapause (Roff 1980; Mousseau and Roff 1989; Mousseau and Dingle 1991; Olvido et al. 1998; Heustis and Marshall 2006). In a laboratory setting, this plasticity can be manipulated in order to obtain both diapause and non-diapause embryos. Females reared under long day conditions produce non-diapause embryos that typically hatch 15 d post-oviposition, and females reared under short day conditions produce a high percentage of embryos that enter diapause and remain dormant for several months. Even though a number of researchers have taken advantage of the plasticity of diapause induction in this species and have published studies on the evolutionary significance of diapause (e.g., Olvido et al. 1998; Fedorka et al. 2004; Heustis and Marshall 2006; Winterhalter and Mousseau 2007), the studies described in Chapters 3 and 4 are unique in their focus on the biochemical and molecular mechanisms of diapause in this species. Chapter 3 examines morphological and biochemical aspects of diapause, and Chapter 4 builds on this foundation by focusing on differential gene expression in pre-diapause and diapause embryos compared to non-diapause embryos.

There is already a large body of research on embryonic diapause in a variety of other Orthoptera species (e.g., Rakshpal 1962a, b; Browning 1965; Izumigama and Suzuki 1986; Tawfick 2002). However, the majority of these studies simply identify abiotic factors that induce diapause or document physiological or biochemical changes that occur upon diapause entry. There has been little attempt to uncover mechanisms that regulate the observed changes. In addition, each study cited above examines a different species; there are no integrative studies on diapause in a single species. Taken together, Chapters 3 and 4 are a

comprehensive examination of embryonic diapause in a single species through specific research goals as described below.

Research Aims of This Dissertation

The primary goal of the research presented in this dissertation is to begin provide a comprehensive picture of the biochemical and molecular aspects of diapause in arthropod embryos. Chapter 2 focuses on the role of mitochondria in metabolic depression in *A. franciscana* embryos. To test the hypothesis that metabolic downregulation is associated with intrinsic changes in the mitochondria, I used electron microscopy to compare the morphology of mitochondria in diapause and post-diapause embryos. I also examined the function of mitochondria isolated from these two stages. Specifically, I measured state 3 and state 4 respiration rates with pyruvate or succinate as the metabolic fuel and used these measures to calculate respiratory control ratios (RCRs) and P:O flux ratios. I also measured the amount and activity of cytochrome c oxidase, the terminal enzyme of the electron transport chain.

Chapters 3 and 4 take an integrative approach to understanding embryonic diapause by investigating both biochemical (Chapter 3) and molecular (Chapter 4) aspects of diapause in a single species. In Chapter 3, I compared the morphology of direct developing, non-diapause embryos and *bona fide* diapause embryos to establish a timeline for the entry into diapause. I also quantified DNA embryo^{-1}, measured oxygen consumption rates and adenylate status of developing and diapause embryos for 15 d post-oviposition.

Chapter 4 focuses on the molecular regulation of diapause entry. Specifically, I used a PCR-based subtractive hybridization technique to isolate and identify transcripts that are up- or downregulated in pre-diapause and diapause embryos. Quantitative real-time PCR

(qPCR) was used to verify differential expression and quantify the relative expression levels of select genes in pre-diapause, diapause, and non-diapause embryos. The goal of this chapter was to identify candidate genes involved in the initiation and maintenance of the diapause state, and thereby, gain a better understanding of the molecular basis for the physiological changes observed in Chapter 3.

CHAPTER 2

DIFFERENCES IN ISOLATED MITOCHONDRIA ARE INSUFFICIENT TO ACCOUNT FOR RESPIRATORY DEPRESSION DURING DIAPAUSE IN *ARTEMIA FRANCISCANA* EMBYOS[1]

Introduction

Some animals inhabiting environments with pronounced seasonal variation survive extreme physical conditions by entering diapause (Lees 1955; Denlinger 1986; Hand 1991). Diapause is a specific type of dormancy that is genetically programmed into the life cycle and is controlled by endogenous factors (Lees 1955; Jungreis 1978; Denlinger 1985; Denlinger 2002). Once in diapause an animal will remain dormant until the appropriate termination cues are received, even though environmental conditions may be adequate to support normal growth and metabolism (Tauber and Tauber 1976; Drinkwater and Crowe 1987). Diapause is characterized by developmental arrest and a variety of species-specific physiological changes, which may include decreased metabolism and increased production of protective molecules, such as glycerol or heat shock proteins, that preserve cellular structure and function (Clegg 1965; Loomis et al. 1996; Denlinger 2002). Physiological changes that occur during diapause have been characterized for a number of species (for example see Rakshpal 1962a, b; Izumigama and Suzuki 1986; Loomis et al. 1996; Podrabsky and Hand 1999; Denlinger 2002). However, only a few studies have addressed the biochemical and cellular mechanisms that lead to the observed changes. The goal of this paper is to identify factors that may regulate aerobic metabolism during diapause in embryos of the brine shrimp

[1] Data in this chapter previously published as J.A. Reynolds and S.C. Hand. 2004. Differences in isloated mitochoncria are insufficient to account for respiatory depression during diapause in *Artemia franciscana* embryos. Physiological and Biochemical Zoology, 77: 366-377. Included with permission from University of Chicago Press.

Artemia franciscana Kellogg, an animal that is remarkable in its ability to reduce aerobic metabolism by as much as 97% during diapause even when oxygen pressure, hydration levels, and temperatures are sufficient to support much higher metabolic rates (Clegg et al. 1996). Specifically we evaluated the oxidative capacity and other bioenergetic characteristics of mitochondria from diapause embryos and compared these features to those of post-diapause embryos in order to gain insight into potential mechanisms that are involved in the downregulation of respiration.

A. franciscana is an anostracan crustacean that inhabits hypersaline bodies of water such as the Great Salt Lake, UT. In response to changes in photoperiod, population density, and/or salinity, ovoviviparous females switch to oviparous reproduction and produce encysted embryos (cysts) that enter diapause (Berthelemy-Okazaki and Hedgecock 1987). Development is arrested at the gastrula stage (approximately 4,000 cells) before the embryo is released from the female's ovisac. Other diapause specific changes that occur before the embryo is released from the female include increased production of trehalose and a decrease in the amount of glycerol (Clegg 1965). Metabolic depression in this species is not directly coupled to developmental arrest. When the encysted embryos are initially released into the water column, they consume oxygen at a rate comparable to that of actively developing embryos (Drinkwater and Clegg 1991). Oxygen consumption then decreases to barely detectable levels over the next 4-5 days (Clegg et al. 1996). The cysts will remain in diapause until they are activated by the appropriate termination cues. Dehydration and exposure to low temperatures are two examples of ecologically relevant factors known to activate or release embryos from diapause (Drinkwater and Crowe 1987; Lavens and Sorgeloos 1987).

A number of studies have examined metabolic processes in post-diapause embryos (e.g., Schmitt et al. 1973; Drinkwater and Clegg 1991; Kwast and Hand 1996; Vallejo et al 1996; Hand 1998; Eads and Hand 2003a,b). However, only a few published studies have addressed metabolic regulation in embryos that are in diapause (Clegg 1965; Drinkwater and Crowe 1987; Van der Linden et al. 1988; Clegg et al. 1996). One reason for the lack of information on diapause embryos is due to the difficulty in obtaining sufficient quantities of diapause embryos to perform biochemical assays. Commercially available cysts are dehydrated, and thus the majority are no longer in diapause. To alleviate this problem, we collected large quantities of diapause cysts from the surface of the Great Salt Lake, UT during multiple fall seasons.

In this study, we examined the hypothesis that changes in the intrinsic oxidative capacity of mitochondria contribute to metabolic downregulation during diapause. Specifically we used oxygen consumption measurements to estimate rates of state 3 and state 4 respiration with either pyruvate or succinate as a substrate. These data, in turn, were then used to examine metabolic efficiency through P: O flux ratios and respiratory control ratios (RCRs). Finally, we examined the activity of cytochrome *c* oxidase (COX) and its possible contribution to metabolic downregulation. The results suggest that metabolic downregulation in diapause *A. franciscana* embryos is not due to loss of oxidative capacity of isolated mitochondria. Rather, metabolic depression appears to result from substrate limitation or active inhibition of oxidative phosphorylation in vivo.

Materials and Methods

Reagents

Sucrose was purchased from Pfanstiehl (Waukegan, Illinois) and chemicals for transmission electron microscopy were from Electron Microscopy Sciences (Fort Washington, PA). All other reagents used were the highest quality available and were purchased from either Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich (St. Louis, MO).

Artemia franciscana Embryos

All embryos originated from the Great Salt Lake, UT. Encysted, diapause embryos were collected during the fall of 2000, 2001, and 2002. The embryos were washed and stored in 0.25 M NaCl with gentamycin sulfate (50 μg/ml) and Nystatin (100 units/ml). Prior to each assay, diapause embryos were incubated in artificial seawater (Instant Ocean™; Aquarium Systems, Mentor OH, USA; 35 ppt) with shaking at room temperature for 4 days to allow individuals not in diapause to hatch; larvae and empty shells were removed. Periodically, the viability of diapause embryos was estimated using a method modified from Lavens et al. (1986) and Clegg et al. (1996). Briefly, unhatched embryos (cysts) were dried at ambient temperature and humidity for up to two weeks. The dried cysts were treated with 3 % hydrogen peroxide with 0.4 M NaCl for approximately 30 min. After rinsing with 0.25 M NaCl, the cysts were incubated as described above. After 4 d the percentage hatching was quantified by counting at least 300 unhatched cysts and free-swimming nauplii. The number of nauplii divided by the total number counted (i.e. unhatched cysts + nauplii) gave the viability, which typically ranged from 70-90 %.

Activated (post-diapause) embryos from the Great Salt Lake were purchased in the dehydrated state from Sander's Brine Shrimp Company (Ogden, UT) in 1999 or 2001 and

were stored at -20° C. Before use, these embryos were hydrated in 0.25 M NaCl at 0° C for at least 4 h. The embryos were then allowed to develop under normoxic conditions at room temperature for 8 h.

Isolation of Mitochondria

Mitochondria were isolated essentially as described by Kwast and Hand (1993). Prior to homogenization, the cysts were dechorionated by treatment with antiformin solution (1 % hypochlorite, 0.4 M sodium hydroxide, and 60 mM sodium carbonate) for 15-20 min at room temperature, followed by three rinses with ice-cold 0.25 M NaCl. Embryos were then incubated in ice-cold 1 % (w/v) sodium thiosulfate for 5 min and were rinsed two times with cold 0.25 M NaCl. Finally, embryos were incubated for 3-5 minutes in cold 40 mM hydrochloric acid prepared in 0.25 M sodium chloride, followed by three rinses with 0.25 M NaCl. Dechorionated cysts were homogenized at 0^{o} C in four volumes of isolation buffer consisting of 0.5 M sucrose, 1 mM EGTA, 0.5 % (w/v) bovine serum albumin (fatty acid free, fraction V), 20 mM HEPES, and 150 mM KCl, titrated to pH 7.5 with 1.0 M KOH. The homogenate was centrifuged (4^{o} C) at 1000 x *g* for 10 min, and the resulting supernatant was centrifuged at 9000 x *g* for 15 min. This pellet was washed by resuspension in the starting volume of isolation buffer followed by centrifugation at 9000 x *g*. The final mitochondrial pellet was resuspended in isolation buffer to a final concentration of approximately 8 mg protein/ml as determined using a modified Lowry protein assay (Peterson 1977).

Oxygen Consumption Measurements

Respiration measurements on isolated mitochondria (Kwast et al. 1995) were made using polar graphic oxygen sensors (model 1302), oxygen meters (model 781) and glass respiration chambers (RC 350) from Strathkelvin Instruments (Glasgow, Scotland). Changes in the

partial pressure of O_2 were digitally recorded with Datacan V acquisition software (Sable Systems, Las Vegas Nevada, USA). Briefly, 30-60 µl of mitochondrial suspension (240-480 µg of mitochondrial protein) was added to respiration medium at a final volume of 1.5 ml. Respiration medium consisted of 0.5 M sucrose, 150 mM KCl, 20 mM KH_2PO_4, 1 mM EGTA, 2 mM $MgCl_2$, 0.5 % (w/v) BSA (fatty acid free, fraction V), and 5 µM rotenone, when succinate was used as the substrate, titrated to pH 7.5 with 1.0 mM KOH. After temperature equilibration at 25° C, 15 µl substrate (final concentrations, 15 mM pyruvate with 0.5 mM malate, or 5 mM succinate) was added to the chamber and state 2 respiration was recorded for several minutes. State 3 respiration was initiated by adding 45-75 µl ADP (final concentration, 150-250 µM). State 4 respiration was recorded for at least 5 min once ADP was depleted. Respiration data were analyzed using DatGraf software (Oroboros, Innsbruck, Austria). Oxygen concentration in the chamber (c_{O2}) was calculated from pO_2 measurements based on O_2 solubility in the respiration medium at 25º C and ambient barometric pressure. Oxygen flux (JO_2, pmol O_2 per sec per ml) was calculated as the time derivative of c_{O2}. Corrections were made for consumption of oxygen by the electrode and back diffusion of oxygen into the chamber, the exponential time constant of the oxygen sensor, and transient changes in c_{O2} resulting from the injection of the solutions. Respiratory control ratios (RCR) and P:O flux ratios were calculated for isolated mitochondria. RCRs were calculated as $J_{O2\ (state\ 3)} / J_{O2\ (state\ 4)}$. P:O flux ratios were calculated as $0.5\ \Delta\ c_{ADP} / \Delta\ c_{O2\ (total)}$, where $\Delta\ c_{ADP}$ is the total change in ADP concentration (µmol ADP added; assuming complete conversion to ATP) and $\Delta\ c_{O2}$ is the total change in oxygen concentration during state 3 respiration.

Oxygen consumption by whole embryos was measured at 25° C with 30 mg of hydrated cysts suspended in 1.5 ml of respiration medium consisting of 0.25 M NaCl with 50 μg/ml gentamycin sulfate. Instrumentation, software and oxygen flux corrections were the same as with isolated mitochondria.

Respiration with TMPD and Ascorbate

In isolated mitochondria, excess COX capacity is estimated by comparing state 3 respiration (e.g., with succinate and rotenone) to the oxygen flux measured in the presence of an artificial electron donor for cytochrome *c* (N,N,N',N'-tetramethyl-*p*-phenylenediamine dihydrochloride, TMPD) and with antimycin A added to block the electron transport chain upstream at complex III. Reduced TMPD is regenerated with ascorbate during the assay. Thus cytochrome *c* is maintained in a highly reduced state by electron donation from TMPD, thereby providing an *in organello* estimate of the maximal reaction velocity of COX. The ratio of this value to state 3 respiration on succinate gives the excess COX capacity. Respiratory measurements of mitochondria isolated from post-diapause embryos were performed essentially as described above, except that a high resolution Oxygraph with DatLab acquisition and analysis software (Oroboros Instruments, Innsbruck, Austria) were used. First, state 3 respiration with succinate was measured in the presence of 1 mM ATP, 2 mM ADP, 5 mM succinate, and 0.5 μM rotenone in respiration medium. Then 2.5 μM antimycin A, 500 μM TMPD, and 2 mM ascorbate were added in rapid succession and the oxygen flux recorded. Background corrections were made for the autoxidation of TMPD, ascorbate and cytochrome *c*; the rate of auto-oxidation is dependent on oxygen concentration (cf. Gnaiger and Kuznetsov, 2002).

Assay for Cytochrome *c* Oxidase (COX) Activity

COX activity in isolated mitochondria was measured by following the rate of oxidation of cytochrome *c* spectrophotometrically at 550 nm as previously described (Hoffmann and Hand 1990). Reduced cytochrome *c* was prepared by adding trace amounts of sodium dithionite to a 1 mM stock solution of cytochrome *c* prepared in nitrogen saturated, 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. Residual dithionite and its breakdown products were removed by gel sieving with Sephadex G-25. Assays were performed at 25 ºC in a reaction volume of 1 ml. Mitochondria were preincubated for 2 min in a medium of low osmotic pressure consisting of 100 mM potassium phosphate buffer (pH 6.0); 50 μM reduced cytochrome *c* was added to initiate the reaction. Enzyme units were calculated using an extinction coefficient for cytochrome *c* of 18.5 mM/cm (reduced – oxidized). Osmotic compromise of the outer membrane during the above preincubation was confirmed by measuring an unchanged enzyme activity when preincubation in deionized water was used instead of buffer.

Quantification of Mitochondrial Cytochromes

Selected mitochondrial cytochromes were quantified in preparations of mitochondria isolated from diapause and developing embryos using a method modified from Suarez et al. (1999). Briefly a suspension of isolated mitochondria was diluted to approximately 8 mg protein per ml with water and 0.1 mM lauryl maltoside. Dithionite-reduced minus air-oxidized difference spectra from 500 – 650 nm were obtained with a Cary 100 spectrophotometer using baseline correction. The quantity of cytochromes *c*, c_1, *b*, and aa_3 were calculated from equations published by Schneider et al. (1980) and expressed as micromoles. Specifically:

[cytochrome *c*] = $49.3(\Delta A_{550-535}) - 27.9(\Delta A_{554-540}) + 15.8\ (\Delta A_{563-577})$

[cytochrome c_1] = -16.6($\Delta A_{550-535}$) + 51.1 ($\Delta A_{554-540}$) – 12.7 ($\Delta A_{563-577}$) – 3.5($\Delta A_{605-630}$)

[cytochrome *b*] = 3.8 ($\Delta A_{550-535}$) – 4.2 ($\Delta A_{554-540}$) – 44.7 ($\Delta A_{563-577}$) + 1.4 ($\Delta A_{605-630}$)

[cytochrome *a*] = 2.4($\Delta A_{554-550}$) – 0.4($\Delta A_{563-577}$) + 76.1($\Delta A_{605-630}$)

Since COX contains two identical heme *a* species (*a* and a_3), the quantity of COX was calculated as half the amount of cytochrome *a*. All cytochrome values were normalized per mg of mitochondrial protein in the preparation.

Electron Microscopy

Diapause and developing embryos were prepared for election microscopy by fixation in a solution of 2 % glutaraldehyde, 1 % osmium tetroxide prepared in 0.1 M sodium cacodylate buffer (pH 6.8). The cyst wall is permeable only to water and low-molecular weight gasses (Clegg and Conte 1980). Therefore the cyst wall was nicked with an insect pin (size 00) to allow the fixative to enter. Prior to nicking the cyst wall, embryos were soaked in ice-cold 2.0 M sucrose for 20 min to reduce the internal turgor pressure of the embryo and prevent extrusion and damage of tissue that would otherwise occur when the cyst was punctured. After fixation the cysts were washed with deionized water, stained with 0.05 % uranyl acetate, and dehydrated in a graded ethanol series. Cysts were embedded in LR White embedding medium and sectioned on an MT5000 ultramicrotome. Thin sections were stained with Reynolds' lead citrate and then observed and photographed with a JOEL 100CX transmission electron microscope.

Statistical Analyses

All statistical analyses were performed using Minitab™ release 12 for Windows (Minitab, State College, PA, USA). One-way ANOVA was used to test for significant differences between collection years and developmental stage. Fisher's LSD was used to test for

significant differences between pairs. Two-tailed, Student's T test was used to test for differences between state 3 respiration with succinate vs. state 3 respiration with TMPD and ascorbate.

Results

Oxygen Consumption in Whole Embryos

Oxygen consumption rates of field collected *A. franciscana* embryos from the Great Salt Lake (GSL) that are in diapause are significantly lower than those of post-diapause GSL embryos that have been developing for 8 h (ANOVA $F = 209.5$, d.f. = 4, $P < 0.001$). Under our measurement conditions of normoxia and full embryo hydration, the aerobic metabolic rates for diapause embryos are depressed by as much as 92 % relative to those for post-diapause embryos with active metabolism (Fig. 2.1). It is appropriate to note that the metabolic rates for diapause embryos also vary significantly across collection years as indicated.

Mitochondrial Morphology and Quantification

Electron micrographs of mitochondria *in situ* (Fig. 2.2) indicate that diapause embryos possess mitochondria that are intact, well differentiated and contain distinct cristae. The mitochondria are structurally similar to those of post-diapause embryos that have been developing for 8 h. Because impermeability of the cyst wall makes uniform fixation of tissue difficult and the serial sectioning required for morphometric analysis problematic (see Materials and Methods), we chose to use an alternative approach for estimation of the mitochondria content of tissues. Table 2.1 shows the yield of mitochondrial protein from diapause embryos to be equivalent to that of post-diapause embryos (ANOVA $F = 1.72$, D.F. = 2, $P = 0.21$). Based on the recovery of cytochrome *c* oxidase activity in the mitochondrial

fraction, the percentage of total mitochondria isolated from a given embryo preparation ranged from 26 to 34 %. More importantly, there was no significant difference in this extraction efficiency for mitochondria originating diapause versus post-diapause embryos (P = 0.32). Thus the mitochondrial protein data suggest that quantities of mitochondria contained in diapause and post-diapause embryos are indistinguishable.

There is no consistent pattern across collection years in the content of mitochondrial cytochromes of diapause and post-diapause mitochondria (Fig.2.3). The total cytochrome content of diapause embryos across two collection years bracketed that measured for post-diapause embryos. Thus differences in cytochrome content of mitochondria do not explain the consistently lower respiration rates observed for diapause versus post-diapause embryos

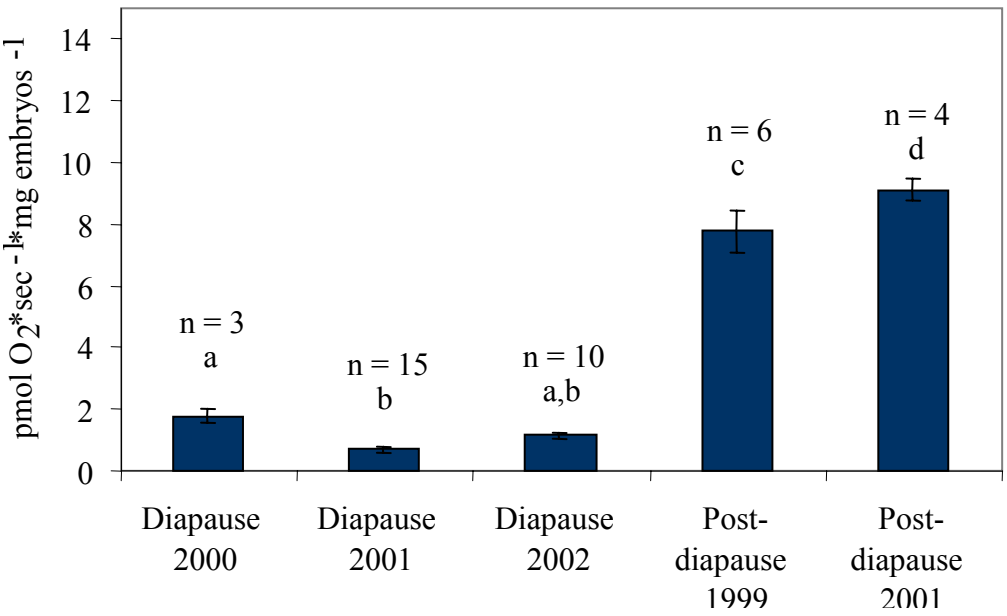

Figure 2.1. Oxygen consumption by encysted embryos of *A. franciscana*. Respiration rates of embryos in diapause are significantly depressed compared to post-diapause embryos that have developed at room temperature for 8 h. Bars represent mean ± SEM ; n = the number of independent recordings for each group. Bars with the same letter are not significantly different (Fisher's LSD = 0.05).

Table 2.1. Yield of mitochondria protein extractable from diapause and post-diapause embryos.

	Protein Yield
Diapause 2001 (n = 4)	3.73 ± 0.46
Diapause 2002 (n = 4)	2.45 ± 0.40
Post-diapause (n = 10)	3.81 ± 0.47

Units are mg mitochondrial protein·g hydrated embryos^{-1}.
Values are mean ± SEM; n = number of independent mitochondrial isolations.

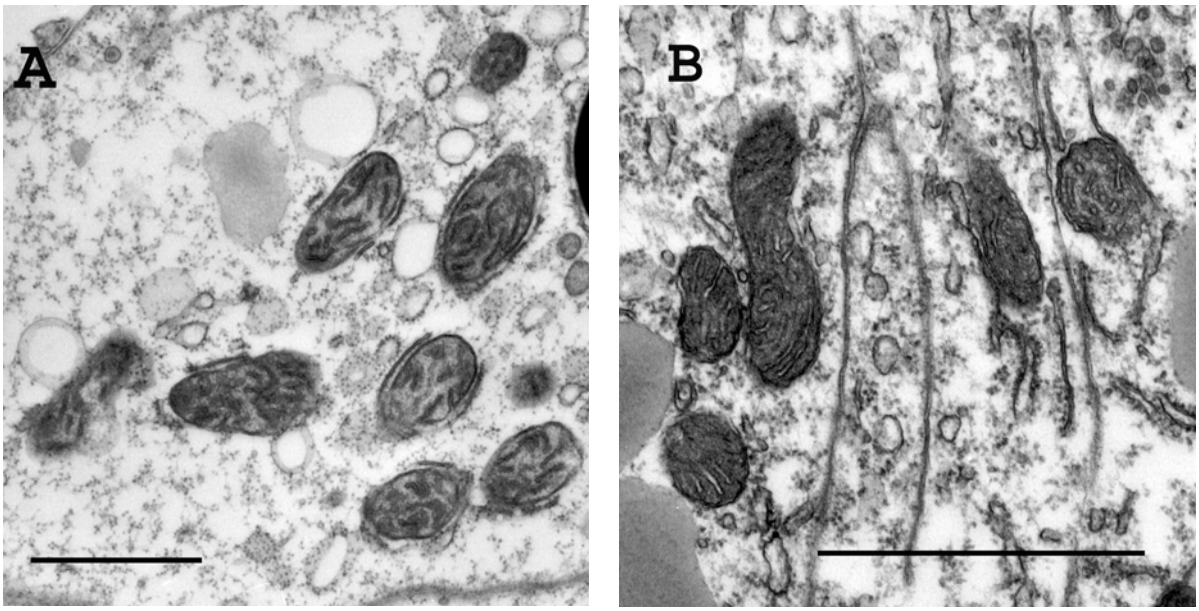

Figure 2.2. Electron micrographs of mitochondria *in situ* indicate that mitochondria of diapause embryos (A) are well developed and structurally similar to mitochondria from post-diapause embryos that have developed at room temperature for 8 h (B). Bars = 1 μm.

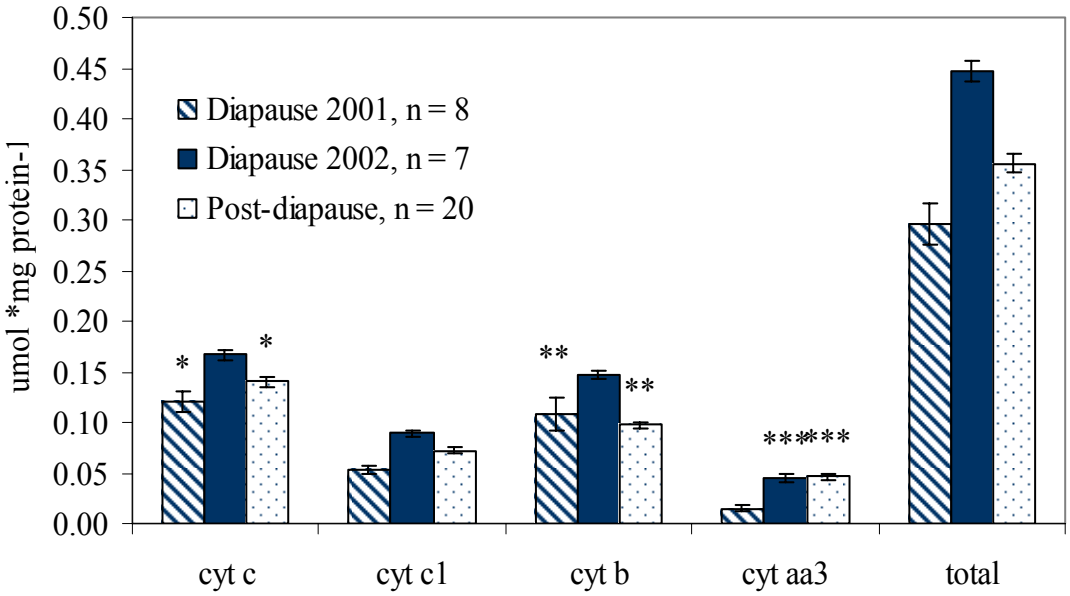

Figure 2.3. Cytochrome content of mitochondria isolated from diapause and post-diapause embryos. Post-diapause embryos have developed at room temperature for 8 h. Bars represent mean ± SEM. There are significant differences between collection years and developmental stages observed for cytochrome *c* (ANOVA F = 6.46, d.f. = 2, P < 0.01), cytochrome c_1 (ANOVA F = 8.51, d.f. = 2, P = 0.001), cytochrome *b* (ANOVA F = 7.69, d.f. 2, P < 0.01), and cytochrome aa_3 (ANOVA F = 14.50, d.f. = 2, P < 0.01). Bars with the same symbol are not significantly different (Fisher's LSD P = 0.05). The total amount of mitochondrial cytochromes was significantly greater in diapause embryos collected in 2002 than in mitochondria from either 2001 diapause embryos or post-diapause embryos that had developed aerobically for 8 h (ANOVA F = 13.16, d.f. = 2, P < 0.01).

Mitochondrial Respiration

As shown in Table 2.2, state 3 respiration rate of mitochondria isolated from diapause embryos is equivalent to that of mitochondria from post-diapause embryos when pyruvate (with malate) is used as the substrate ($P = 0.48$). The same is true for state 4 respiration ($P = 0.93$) and for the respiratory control ratio (RCR, $P = 0.75$). Respiration rates of mitochondria from *A. franciscana* embryos were substantially higher when succinate was used as the substrate instead of pyruvate. State 3 respiration of mitochondria isolated from diapause embryos collected in 2002 was not significantly different from mitochondria from post-diapause embryos (Fig 2.4A). Modest reductions of 25 and 15 %, respectively, were observed in state 3 respiration for mitochondria of diapause embryos collected in 2000 and 2001. Similarly, state 4 respiration rates of mitochondria isolated from diapause embryos collected in 2002 were not significantly different from those of mitochondria isolated from post-diapause embryos, but state 4 respiration rates were 27 or 19 % lower in mitochondria isolated from 2000 or 2001 diapause embryos (Fig. 2.4B). As seen with pyruvate, the RCR of mitochondria from diapause embryos exhibit strong ADP-stimulation when succinate is used as a substrate. The RCRs of mitochondria isolated from diapause embryos collected in 2000 or 2001 were equivalent to those from post-diapause embryos, while mitochondria isolated from diapause embryos collected in 2002 had RCRs that were significantly higher than all other groups (Fig. 2.5). Thus, overall, the capacity for oxidative phosphorylation of mitochondria from diapause and post-diapause embryos is very similar.

The P:O flux ratio is an estimate of the amount of ATP produced per atomic oxygen consumed and is an indicator of the efficiency of oxidative phosphorylation. The P:O flux ratio using pyruvate is close to the expected value of approximately 2.5 for mitochondria

from diapause and post-diapause embryos (Table 2.2), and there is not a significant difference between mitochondria from diapause and post-diapause embryos (P = 0.58). When succinate was used as the substrate, the P:O flux ratio was slightly below the expected value of 1.5 (Fig. 2.6), and again there was no significant difference between states (P = 0.07).

COX Enzyme Activity

Cytochrome *c* oxidase (COX) activity in mitochondria isolated from diapause embryos collected in 2001 is reduced by approximately 47 % compared to mitochondria isolated from post-diapause embryos (Table 2.3), which is a far greater drop than the modest reduction seen in state 3 respiration for diapause mitochondria. The reduction in COX activity appears to be due to a reduction in the actual amount of cytochrome aa_3 present (Table 2.3). Figure 2.7 shows there to be significant correlation between the amount of cytochrome aa_3 and COX activity (P = 0.009). In the subsequent collection year (2002), COX activity is not significantly reduced during diapause, even though metabolic depression in diapause embryos was dramatic in both years (Fig 2.1).

Excess COX Capacity

For various mammalian mitochondria, the reaction velocity of COX exceeds the maximum pathway flux attainable through the respiratory chain (e.g., Gnaiger et al. 1998), but whether or not this pattern exists in invertebrate mitochondria is unknown. If excess COX capacity is present for *A. franciscana* mitochondria, this feature could explain our observations above that flux through the respiratory chain appears to be buffered to a significant degree from the measured decreases in COX activity and amount. Based on measurements with TMPD and ascorbate in the presence of antimycin A (see Materials and Methods), we estimate there is

31% excess COX capacity in *A. franciscana* embryos (Table 2.4). In other words, the maximum capacity for electron transport through complex IV as measured with TMPD is greater than that needed to satisfy the overall respiratory flux stimulated by succinate and ADP.

Table2.2. Respiration with pyruvate (plus malate) by mitochondria isolated from diapause and post-diapause embryos.

	Diapause[a] (n = 8)	Post-diapause (n = 10)
State 3[b]	279 ± 8.5	267 ± 13.5
State 4[b]	48.5 ± 4.0	47.0 ± 3.3
RCR	5.95 ± 0.09	5.80 ± 0.26
P:O Flux Ratio	2.57 ± 0.13	2.68 ± 0.12

[a]Mitochondria were isolated from diapause embryos collected in 2001.
[b]Units are pmol $O_2 \cdot sec^{-1} \cdot mg\ protein^{-1}$. Mean ± SEM; n = independent measurements from one mitochondrial isolation.

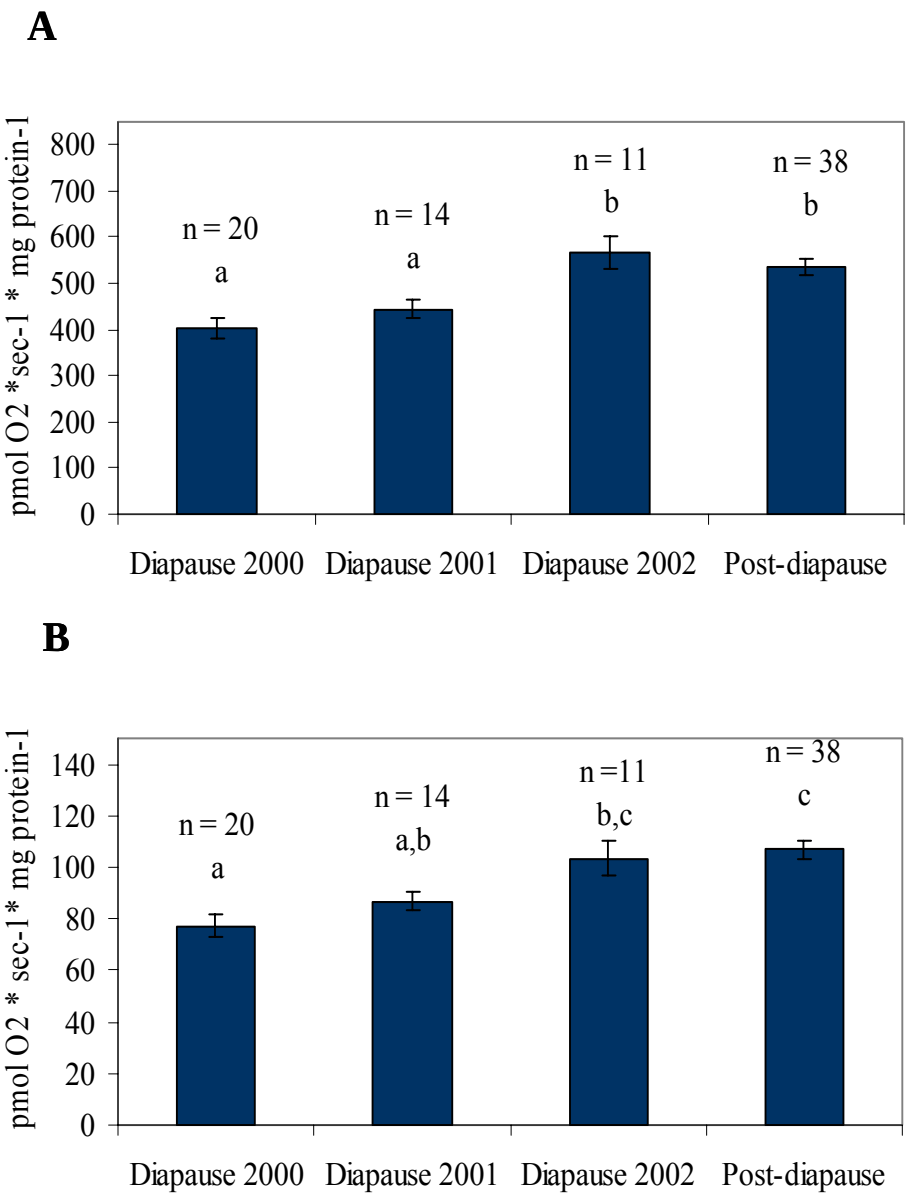

Figure 2.4. State 3 (A) and state 4 (B) respiration with succinate and rotenone for mitochondria isolated from diapause and post-diapause embryos. State 3 respiration rates are reduced in mitochondria isolated from diapause embryos collected in 2000 and 2001 compared to those of mitochondria from 2002 diapause embryos or post-diapause embryos (ANOVA $F = 10.07$, d.f. = 3, $P < 0.01$). State 4 respiration rates are also reduced in mitochondria from 2000 and 2001 embryos compared to 2002 and post-diapause embryos (ANOVA $F = 9.92$, d.f. = 3, $P < 0.01$). Bars represent mean ± SEM; n is the number of independent recordings from 3 (Diapause 2000), 2 (Diapause 2001 and 2002) or 5 (post-diapause) independent mitochondrial preparations. Bars with the same letter are not significantly different (Fisher's LSD = 0.05).

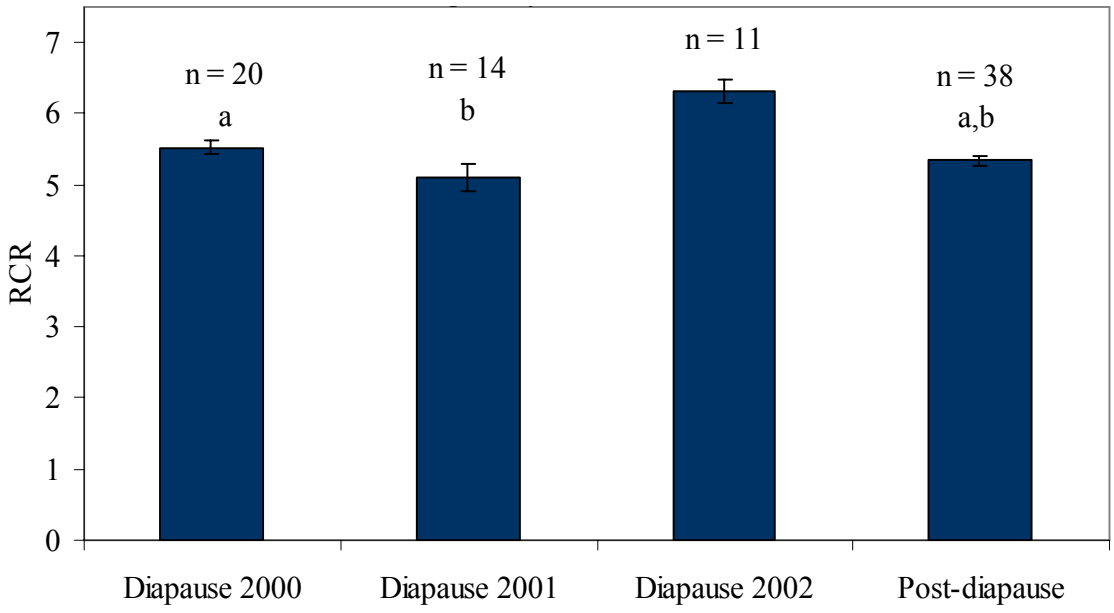

Figure 2.5. Respiratory control ratios (RCRs) with succinate and rotenone for mitochondria isolated from diapause and post-diapause embryos. RCRs were calculated from state 3 and state 4 respiration measurements (25 °C, pH 7.5). ANOVA $F = 12.86$, d.f. = 3, $P < 0.01$. Mean ± SEM; n = the number of independent recordings from 3 (diapause, 2000), 2 (diapause, 2001 and 2002) or 5 (post-diapause) independent mitochondrial preparations. Bars with the same letter are not significantly different (Fisher's LSD = 0.05).

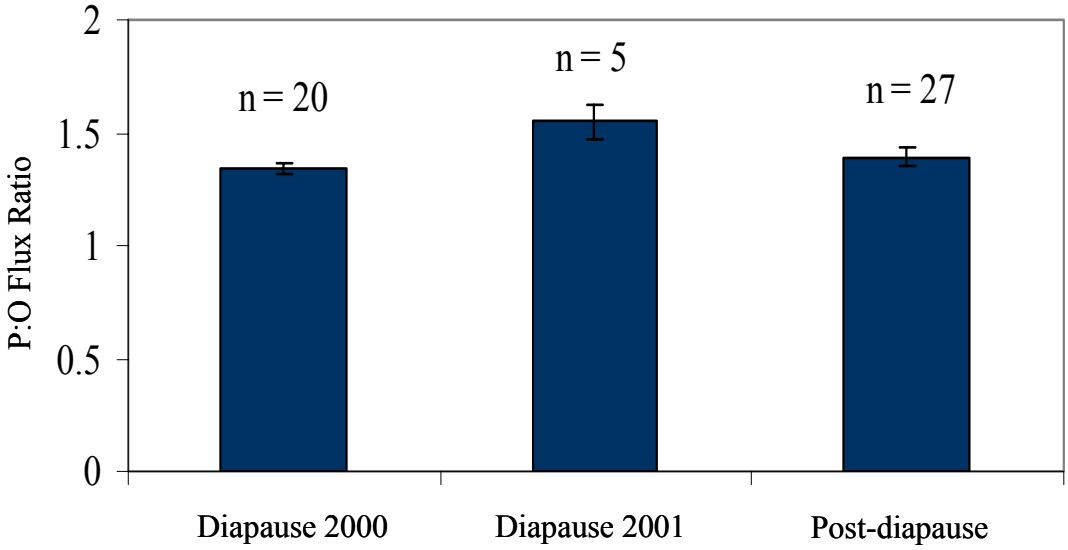

Figure 2.6. P:O flux ratios with succinate and rotenone for mitochondria isolated from diapause and post-diapause embryos (25 °C, pH 7.5). There is no significant difference between treatment groups (ANOVA F = 2.84, d.f. = 2, P = 0.068). Mean ± SEM. N for each group is the number of independent respiration measurements.

Table 2.3. COX activity and amount of cytochrome aa_3 in mitochondria isolated from diapause and post-diapause embryos[a]

		COX Activity (Enzyme Units/mg protein)[b]	**Amount of Cytochrome aa_3** (μmol/mg protein)[d]
Diapause, 2001	n = 17	0.70 ± 0.04	0.015 ± 0.003
Diapause, 2002	n = 11	1.33 ± 0.03[c]	0.044 ± 0.005[e]
Post-diapause	n = 38	1.49 ± 0.07[c]	0.046 ± 0.004[e]

[a]Values are mean ± SEM.
[b]Enzyme activity varies significantly between groups ($F = 34.65$, d.f. = 2, $P < 0.001$)
[c]COX activity is not significantly different (Fishers LSD $P = 0.005$)
[d]The amount of cytochrome aa_3 varies significantly between groups ($F = 14.05$, d.f. = 2, $P<0.001$)
[e]Amount of cytochrome aa_3 is not significantly different between these (Fishers LSD $P = 0.005$

Table 2.4. Excess capacity of cytochrome *c* oxidase (COX) in mitochondria isolated from post-diapause embryos (8 h)

State 3 Respiration [a] (succinate)	**State 3 Respiration** [a] (TMPD-Asc.)	**Excess COX Capacity** (TMPD-Asc./succinate)
252 ± 3.7[b]	331 ± 13.3[b]	1.31 ± 0.05

[a]Units are pmol $O_2 \cdot sec^{-1} \cdot ml^{-1}$
[b]Means are significantly different (Student's $T = -5.66$, d.f. = 8, $P < 0.005$)
Values are mean ± SEM, where n = 8 independent measurements for one mitochondrial isolation.

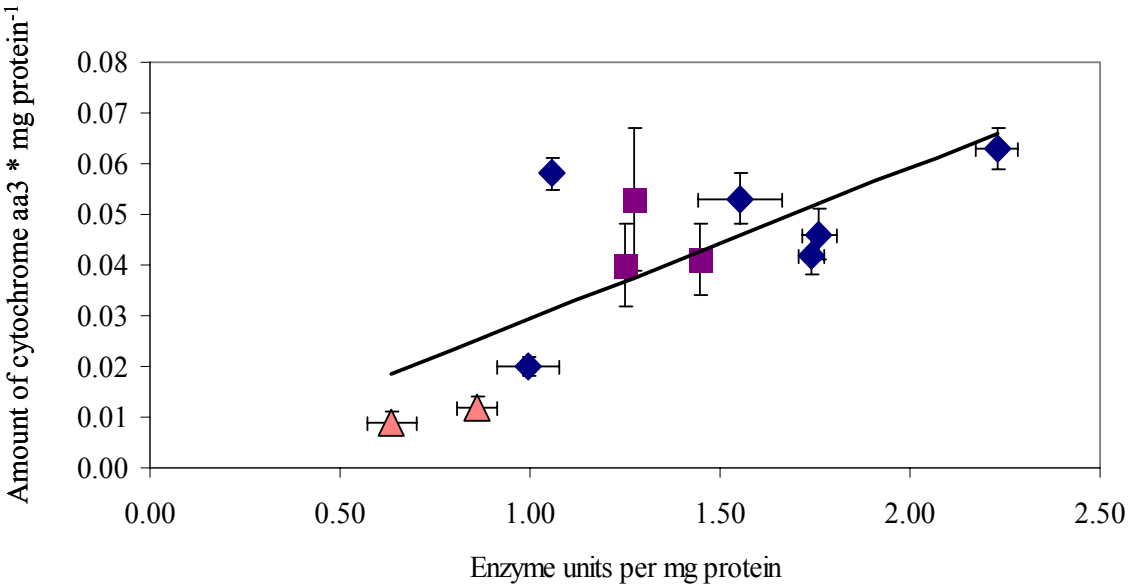

Figure 2.7. Cytochrome *c* oxidase activity is significantly correlated with the amount of cytochrome aa_3 (r = 0.74, P = 0.01). Triangles represent values for mitochondria isolated from diapause embryos in 2001; squares, diapause embryos in 2002; diamonds, post-diapause embryos. Each point represents the means ± SEM for enzyme activity and amount of cytochrome aa_3 for an independent mitochondrial isolation. Where bars are absent, the SEM is less than the size of the symbol.

Discussion

Under conditions that promote normal metabolism (i.e., normoxia, full hydration), the oxygen consumption rates for diapause *A. franciscana* embryos field-collected from the Great Salt Lake are reduced by at least 86 % compared to those of embryos that are actively developing. In this study, we have shown that changes in the properties of isolated mitochondria are not sufficient to explain the large decrease in aerobic metabolism observed for whole *A. franciscana* embryos that are in diapause. Diapause embryos appear to have mitochondria that are well developed as judged by electron microscopy and are similar in quantity to those of post-diapause embryos, based on the fact that amounts of mitochondrial protein isolated from these two stages are equivalent. The total amount of mitochondrial cytochromes per unit mitochondrion from diapause embryos is greater than or equal to the amount in post-diapause embryos (Fig. 2.3). Thus, the quantities of many mitochondrial proteins are similar in diapause and post-diapause embryos. This contrasts with a recent study on metabolic depression during diapause in the goldenrod gall fly, *Eurosta solidaginis* and the Arctic wollybear caterpillar, *Gynaephora goenlandica*, in which Levin et al. (2003) measured the quantity of mitochondrial (mt)DNA and suggested the reduction in oxygen consumption in these animals is due to a decrease in the amount of mtDNA and, therefore, the quantity of mitochondria. Second, a modest decrease in both state 3 and state 4 respiration rates was seen with succinate as the substrate but not with pyruvate. The reduction with succinate is insufficient to explain the high degree of metabolic depression observed in intact embryos. Third, the reduction in COX activity seen for mitochondria from diapause embryos collected in 2001 had no impact on pyruvate respiration. Any potential influence on succinate respiration is disproportionately smaller that the actual depression

seen in COX activity, consistent with our measurement of excess COX capacity in *A. franciscana* mitochondria (see below). Finally, ADP-stimulated respiration (RCRs) and the phosphorylation efficiency of the mitochondria (P:O flux ratios) are equivalent for mitochondria isolated from diapause and post-diapause embryos. Taken together these data suggest that the downregulation of respiration in diapause *A. franciscana* embryos does not result from measurable changes in isolated mitochondria, but rather may be due to active inhibition or substrate limitation of aerobic metabolism in vivo.

Oxygen consumption by mitochondria is often categorized phenomenologically. State 3 respiration is a measure of ADP-stimulated oxygen consumption; it is the maximum rate of oxidative phosphorylation and depends on the flux of protons from the intermembrane space through the ATP synthase into the matrix. State 4 respiration, measured in the absence of ADP, is oxygen consumption that is not linked to ATP production but is due to uncoupled proton flux across the inner membrane attributable partly to proton leak (Nicholls and Ferguson 2002). Evidence from metabolically depressed frogs and estivating snails suggests that both state 3 and state 4 respiration are coordinately downregulated when these animals enter a hypometabolic state (Bishop et al. 2002; Boutilier and St-Pierre 2002). In addition, recent studies suggest uncoupled proton flux is decreased in mitochondria when oxidative phosphorylation is limited by oxygen availability (Gnaiger et al. 2000). The modest reductions in state 3 and state 4 respiration (on succinate) that we measured for mitochondria isolated from diapause *A. franciscana* collected in 2001 (Fig. 2.4) are qualitatively consistent with these studies. These reductions appear to be coordinated during diapause because the ratio of these oxygen fluxes (RCRs) of mitochondria from 2000 and 2001 diapause embryos is not significantly different from that of mitochondria from post-diapause embryos (Fig.

2.6). However, this reduction in mitochondrial respiration is not observable across all collection years (Fig. 2.6) even though the depressed oxygen consumption for whole embryos is readily apparent. The moderate changes in oxygen flux of isolated mitochondria do not appear to be a prerequisite for metabolic downregulation in vivo**.**

The reduction observed in state 3 and state 4 respiration for mitochondria from diapause embryos depends on the substrate chosen as an electron donor for the electron transport system (ETS). Oxidation of pyruvate results in the production of NADH and passage of electrons through NADH dehydrogenase (Complex I), while electrons produced through the oxidation of succinate enter the ETS through succinate dehydrogenase (Complex II). Compared to mitochondria isolated from post-diapause embryos, state 3 and state 4 respiration rates of mitochondria from diapause are reduced when succinate (with rotenone) is used as the substrate (Fig. 2.4), but not with pyruvate (Table 2). One interpretation is that entry of electrons through Complex II may simply be compromised in mitochondria isolated from diapause embryos. Alternatively, the higher absolute rate of electron flux promoted by succinate (versus pyruvate) may cause a decrease in respiration in diapause mitochondria to become apparent due to partial restriction in transfer capacity at another site in the chain. A more thorough analysis of the entire ETS will be required to resolve this question and identify specific sites of potential control (e.g., Murphy 2001).

We observed a reduction in cytochrome *c* oxidase activity in mitochondria isolated from diapause embryos collected in 2001 compared to those from post-diapause embryos. A positive correlation between COX activity and the amount of cytochrome aa_3 in mitochondrial preparations from diapause and post-diapause embryos suggests the reduction in enzyme activity to be the result of a decrease in the amount of enzyme present rather than

to inhibition of the enzyme (Fig. 2.7). Studies on the regulation of COX have suggested this enzyme may exert limited control over oxidative phosphorylation in mammals via allosteric mechanisms (Villani and Attardi 1997; Kadenbach et al. 2000). Lowered COX activity is correlated with metabolic depression during estivation in two species of snails (Stuart et al. 1998; Bishop et al. 2002). In particular, Bishop et al. (2002) showed there were reductions in COX activity in mitochondria isolated from hepatocytes of metabolically depressed *Helix aspera*, and these authors suggest that metabolic downregulation in these cells is linked to decreased COX activity.

It may be problematic to assume that a reduction in overall COX activity will have a direct impact on oxygen flux in the mitochondrion, because of the observation that the kinetic capacity of COX often exceeds the maximum rate for ADP-coupled respiration (i.e., excess COX capacity). Studies on mammalian mitochondria suggest that COX capacity exceeds the level necessary to sustain state 3 respiration by up to twofold (Gnaiger et al. 1998). Davey et al. (1998) have shown that COX activity in mitochondria isolated from rat brain can be inhibited by as much as 70% before there is a significant reduction in respiration and ATP production. Excess COX capacity provides the basis for the low flux control coefficient of this enzyme typically seen in various tissues (Kuznetsov et al. 1996; Villani et al. 1998; Gnaiger et al. 1998 Rossignol et al. 1999). The data we present here on the oxidative properties of mitochondria isolated from diapause *A. franciscana* embryos are consistent with the concept of excess COX capacity, and our direct measurements indicate an excess COX capacity of 31% for mitochondria isolated from post-diapause embryos (Table 2.4). Thus, if the reduction in COX activity is responsible for any of the metabolic depression during diapause in *A. franciscana* embryos, the effect is likely attenuated due to

excess COX capacity, since we observed a 47 % reduction in COX activity in mitochondria isolated from 2001 diapause embryos (Table 2.3) but only a 15 % reduction in state 3 respiration on succinate (Figure 2.4). While excess COX capacity of 31% for *A. franciscana* mitochondria is not as large as is often seen for mammalian mitochondria, the value is fully consistent with the lower oxygen affinity of these mitochondria compared to rat liver mitochondria (Gnaiger et al. 2000); higher excess COX capacity is associated with greater oxygen affinity (Gnaiger et al. 1998). The above analysis is particularly germane because our rough calculations suggest that respiration of intact, post-diapause embryos is operating close to the state 3 oxygen fluxes measured for isolated mitochondria (for succinate, 550 pmol·s^{-1}mg mitochondrial $protein^{-1}$). [We typically isolate about 3.8 µg mitochondrial protein·mg hydrated $embryo^{-1}$ with a recovery of approximately 30% (see Results section). Consequently, there should be about 12.7 µg mitochondrial protein per mg of hydrated embryo available for respiration in vivo. Thus, 12.7 µg mitochondrial protein·mg $embryo^{-1}$ X 550 pmol O_2 ·s^{-1}·1000 µg isolated mitochondrial $protein^{-1}$ = 7 pmol·s^{-1}·mg hydrated $embryo^{-1}$ as a projected rate for in vivo respiration. This value is similar to our measured value of 8.5 pmol O_2 ·s^{-1} ·mg hydrated active $embryo^{-1}$.]

While noteworthy, it is not overly surprising that we found some differences in the degree of metabolic depression for diapause embryos across collection years, as well as in the properties of their respective isolated mitochondria. Variations in abiotic factors (e.g., temperature and salinity) and biotic factors (e.g., the quantity and quality of food available to reproducing females) can affect the metabolism and physiology of *A. franciscan*a embryos (Clegg and Conte 1980; Lavens et al. 1986; Drinkwater and Clegg 1991). For example, we observed the salinity of the Great Salt Lake to be higher in 2002 than in 2001 (130 ppt versus

110 ppt; Reynolds, Covi and Hand, unpublished observations); this change may have contributed to observed differences in metabolic rates of diapause embryos collected in these two years. Similarly, although respiration rates of field collected diapause embryos from the Great Salt Lake were greatly depressed, the reduction during diapause reported by Clegg et al. (1996) was even larger for *A. franciscana* embryos originating from the San Francisco Bay salterns. Not only were the geographical origins of *A. franciscana* different, but also the collection methods possible at the San Francisco Bay salterns makes it likely that these embryos were more synchronous.

In summary, while we do observe a modest reduction in the oxidative capacity of mitochondria isolated from *A. franciscana* embryos that are in diapause, the differences we observed are not sufficient to account for the amount of metabolic depression observed in whole embryos. Future studies will focus on the possibility that oxidative phosphorylation is actively inhibited in vivo by unidentified chemical factors that are removed when mitochondria are isolated from the normal cellular milieu. For example, metabolic properties of mitochondria isolated from post-diapause embryos could be evaluated in the presence of cellular extracts from diapause *A. franciscana* embryos. A second possibility is that oxygen consumption in vivo in diapause embryos is limited by substrate availability, for example, through the arrest of glycolytic flux. Previous studies on metabolic depression under anoxia in *A. franciscana* have shown that flux through the glycolytic pathway is severely restricted at multiple sites (Carpenter and Hand 1986a; 1986b; Hand and Carpenter 1986; Rees et al. 1989).

CHAPTER 3

BIOCHEMICAL INDICATORS OF METABOLIC POISE DURING EMBRYONIC DEVELOPMENT AND DIAPAUSE OF THE CRICKET, *ALLONEMOBIUS SOCIUS*

Introduction

The life cycles of many animals inhabiting regions with pronounced seasonal variation in environmental conditions include a period of dormancy known as diapause. Diapause is a preprogrammed form of developmental arrest that allows animals to "escape" from harsh environmental conditions and may also permit animals 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). Diapause is endogenously controlled, and the entry into diapause begins even while conditions are adequate to support normal development. In addition to developmental arrest, diapause is characterized by a species-specific suite of physiological changes that typically include decreased metabolism and increased production of proteins that guard from a variety of environmental stressors including extreme temperatures and oxidative stress (Denlinger 2002; Lee et al 2002; Clegg 1965). While a number of papers have reported the physiological and/or biochemical characteristics of embryos that are in diapause (Rakshpal 1962a, b; Izumigama and Suzuki 1986; Podrabsky and Hand 1999, 2000; Podrabsky et al. 2001; Reynolds and Hand 2004), or of animals that have received diapause terminating cues and have resumed development (Yaginuma and Yamashita 1999), limited information exists about preparatory events associated with entry of embryos into diapause. The southern ground cricket, *Allonemobius socius* (Scudder), is an ideal animal for studying mechanisms that regulate embryonic diapause because adult females can produce either diapause or non-diapause embryos. This

plasticity permits direct comparison between diapause embryos and non-diapause embryos that are of a similar developmental stage and allows one to separate ontogenetic changes from those that result from the initiation of diapause. The goal of the present study is to characterize the bioenergetics of embryogenesis and diapause entry in this species. Specifically, I examined the rate of aerobic metabolism in non-diapause and diapause embryos to determine whether metabolic depression accompanies diapause in this species. I also examined the adenylate status of diapause and non-diapause embryos as a function of developmental time. If an elevation in [AMP] occurs during diapause entry, it could serve as a cellular signal for metabolic arrest via AMP-activated protein kinase (cf. Podrabsky and Hand 1999).

A. socius is a small ground cricket found throughout eastern North America. In the northern portion of its range, this cricket is univoltine (i.e., has only one generation per year) and has obligate diapause while in the southern portion of its range this species produces two or more generations per year and diapause is facultative (Fulton 1931, Howard and Furth 1986, Mousseu and Roff 1989). Each year, the first generation matures during the spring months. When the first generation matures, the adults produce a second generation of offspring that develop directly without entering diapause. These individuals hatch as nymphs after 15-20 d of development, and they become sexually mature during the late summer and early fall. In response to changing day lengths and, to a lesser extent, cooler temperatures that occur during this part of the year, adults of the second generation produce high numbers of progeny that enter diapause as embryos and remain in this suspended state though the winter (Bradford and Roff 1997).

By simply manipulating the rearing environment of the females, it is possible to alter the proportion of diapause and non-diapause eggs produced. Specifically, Huestis and Marshall (2006) and Olvido et al. (1998) have shown that females reared under long day conditions (16:8 L:D) produce a high proportion of non-diapause embryos, while rearing females under short day conditions (12:12 L:D) increases the proportion of diapause embryos. With sufficiently large colonies, it is possible to obtain large numbers of diapause or non-diapause embryos simultaneously. Recent studies have examined the quantitative genetic basis for plasticity in diapause induction (Roff and Bradford 2000) and the relationship between maternal behavior and diapause incidence (Olvido and Mousseau 1998; Heustis and Marshall 2006). I am unaware of any work describing physiological or biochemical aspects of diapause in this species. Similar information on embryos is also surprisingly scarce for Orthoptera in general, even though embryonic diapause is common among crickets and grasshoppers.

My results indicate that diapause in *A. socius* is primarily characterized by developmental arrest, which occurs 4-6 d post-oviposition. There is no significant metabolic arrest accompanying the entry into diapause, although the ontogentic increase in metabolism is blocked during diapause. [AMP]:[ATP] ratio is remarkably high in early embryos of this species. However, elevated [AMP] does not appear to be a feature of diapause because both diapause and non-diapause embryos exhibit high [AMP]:[ATP] ratios. Instead, elevated [AMP]:[ATP] ratios are due, in part, to a limited oxygen supply within the egg. Superfusing embryos with oxygen enriched air significantly decreases [AMP]. Such an atypical set point for biochemical indicators of metabolic poise is an ontogenetic feature of this embryo and only partially explained by oxygen availability.

Materials and Methods

Animals

A. socius colonies were established with individuals provided by Dr. Daniel Howard at New Mexico State University that were collected during 2001 and 2002. Nymphs were kept in 20 x 13 cm plastic boxes with moistened cotton and food. They were maintained at room temperature with a 14:10 (L: D) photoperiod. Upon reaching adulthood, males and females were transferred to 38 L glass aquaria. Approximately 50 adults were kept in each tank. They were provided with water and food *ad libitum*. Both nymphs and adults were fed Fluker's™ Cricket Food that was purchased from Fluker's Cricket Farm (Baton Rouge, LA) and was supplemented with dry cat food to supply additional protein. Adults were maintained at either 22°C with a short-day photoperiod (12:12, L:D) to encourage production of diapause embryos or 28°C with a long day photoperiod (16:9, L:D) to encourage production of non-diapause embryos (Olvido et al. 1998 and Heustis and Marshall 2006). Plastic vials containing moistened cheese cloth were provided as an oviposition site. Eggs were collected every 24 h and incubated at 29°C.

Developmental profiles for a given feature (e.g., respiration rate, DNA quantity, adenylate status, etc.) were generated for diapause and non-diapause embryos by taking measurements at 24 h intervals for the first 10 d of embryonic development. Additional measurements at 15 d post-oviposition were made to further characterize the biochemical and metabolic state of diapause embryos.

Staging of Embryos

To determine the age at which *A. socius* embryos enter diapause, individuals incubated at 29°C were sampled at 24 h intervals for up to 5 days. Embryos were fixed, chemically

cleared (Hogan 1956), and observed inside the egg. Briefly, embryos were incubated in water at room temperature for 30 min. After a 45 min incubation in a mixture of chloroform, glacial acetic acid, and 100 % ethanol (2:2:1) at 37 °C, they were transferred to a solution of glycerol and 70% ethanol (1:1) and incubated at room temperature 18-20 h. Embryos were observed with a Leica dissecting microscope (Leica Microsystems, Wetzlar Germany), and images were digitally recorded with a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI). The length of the embryo, the width of the procephalon, and the position of the embryo within the egg were recorded. These morphological features were compared to those of *bona fide* diapause embryos fixed 30 d post-oviposition.

Metabolic Rate

Oxygen consumption was measured at 29 °C with a Gilson Differential Respirometer (Gilson Medical Electronics; Middleton Wisconsin, USA). Approximately 100 embryos were placed in 15 ml respiration flasks with moistened filter paper. A filter paper wick with 3M KOH in the side arm of the flask served as a CO_2 trap. After temperature equilibration, changes in the volume of O_2 were monitored every 60 min for 200-300 min. Respiration rate was estimated by plotting the μL O_2 consumed as a function of time. The change in μL O_2 per minute was adjusted for standard temperature and pressure; data were expressed in pmol O_2 min^{-1} $embryo^{-1}$.

Heat dissipation was monitored at 29 ºC with a Thermal Activity Monitor (model 2277; LKB, Sweden). Approximately 100 embryos were placed in a 5 ml stainless steel ampoule with a moistened piece of filter paper; ampoules were made air-tight with stainless steel screw caps and Teflon sealing rings. Using oxygen consumption measurements (see previous section), I determined there to be sufficient air space within the ampoule to preclude

oxygen limitation during the experimental time course. Recordings were obtained in a twinned configuration with a reference ampoule without embryos. The difference in heat dissipation between the experimental and reference ampoules was recorded and analyzed using Digitam Software (Thermometric AB, Sweden). Heat dissipation was averaged over a 3 h collection period. Calorimetric/respirometric (CR) ratios (kJ mol O_2^{-1}) were calculated from oxygen consumption and heat dissipation data recorded independently, yet simultaneously, from embryos collected at the same time from the same colony of females (Podrabsky and Hand 1999). Theoretical oxycaloric equivalents were taken from Gnaiger (1983) and Widdows (1987).

Metabolite Extraction

Perchloric acid extracts were prepared as described by Podrabsky and Hand (1999). Approximately 200-300 embryos were pulverized in liquid nitrogen and then homogenized in 500 µl of ice cold 1M perchloric acid containing 5 mM EDTA. The homogenate was centrifuged at 10,000 x g for 10 min at 4 °C. The supernatant was neutralized with 5 M K_2CO_3 and centrifuged at 10,000xg for 15 min at 4°C to remove perchlorate salts. Supernatants were stored at -80 ºC until analyzed for metabolites as described below. The acid-insoluble pellet was resuspended in 10% perchloric acid for DNA quantification.

Biochemical Analyses

DNA was quantified using a diphenylamine assay described by Giles and Myers (1965, modified from Burton 1956). Briefly, perchloric acid insoluble pellets, resuspended in 10 % perchloric acid, were incubated at 75°C for 30 min and centrifuged at 10,000 x g at 4 °C for 15 min. A 150 µl sample of supernatant was combined with 150 µl of 4% diphenylamine (prepared in glacial acetic acid) and 7.5 µl of 1.6 mg/ml acetaldehyde made in water. Highly

polymerized DNA (bovine calf thymus) prepared in 10 % perchloric acid was used as the standard. After incubating samples and standards at 30°C for 18 h in a 96-well microtiter plate, color development was measured at 595 nm using a SPECTRAmax Plus microplate reader (Molecular Devices, Sunnyvale CA). Corrections were made for non-specific color development and turbidity by subtracting the absorbance at 700nm from the absorbance at 595 nm.

Adenine nucleotides were quantified in acid extracts by reverse phase chromatography with a method similar to that described by Menze et al. (2005). Prior to analysis, extracts were filtered through a 0.45 μm Nanosep MF Centrifugal Device (Pall Corporation, East Hills, New York, USA) to remove remaining perchlorate salts. AMP, ADP, and ATP were separated using a 4.6 mm x 25 cm reversed phase column (Synergy 4 μm Hydro RP 80A, Phenomenex, Torrance, CA) with a Dionex HPLC system (Sunnyvale, CA) that included a PDA-100 photodiode array detector, GP-50 gradient pump, AS50 auto-sampler set at 4°C, and thermal compartment set at 30°C. The starting buffer was composed of 50 mM potassium phosphate buffer (pH 5.0), 10 mM tetrabutylammonium hydrogen sulfate (TBS), and 1.5% acetonitrile (v/v). A linear gradient of acetonitrile (1.5% to 25%) was initiated 15 min after the start of the run.

Peaks were identified by comparison with retention times of standards, as well as by analyzing peak spectra from a recorded 3D-field with Chromeleon™ software (Dionex, Sunnyvale, CA; Fig. 3.5). The concentration of nucleotides was determined from measurement of the peak area at 260 nm. Calibration curves were linear for the range assayed.

Lactate was determined enzymatically as described by Bergmyer (1974). Perchoric acid extracts were assayed in glycine/hydrazine buffer (0.43 M glycine and 0.34 M hydrazine, final concentrations) that contained 2.75 mM NAD. Reactions (2.9 ml volume) were initiated by the addition of 55 U of lactate dehydrogenase. The change in absorbance at 340 nm was followed for at least 40 min. The concentration of lactate was calculated with a molar extinction coefficient of 6.22 (Bergmeyer 1974).

Hypoxia and Hyperoxia Treatments

To test the hypothesis that *A. socius* embryos experience tissue hypoxia early in development under normal rearing conditions, 3 d embryos were exposed to one of three experimental oxygen regimes: normoxia (compressed air, O_2 concentration = 20 %), hyperoxia (compressed air supplemented with pure O_2, final concentration = 40 %) or mildly hypoxia (compressed air mixed with N_2, final O_2 concentration = 10 %). Embryos were incubated at 29 °C for 24 h. They were placed in a small plastic weighing boat enclosed in a container with a small amount of water to prevent dehydration. The container was then superfused with the desired gas mixture at a rate sufficient to replace the air volume every 3 min. Gas mixtures and air flow were regulated by a mass flow controllers and mass flow meter (Tylan FC-260 and RO-28, respectively; Mykrolis Corporation, Billerica, MA). These three treatment groups were compared to control embryos incubated under standard rearing conditions (see above) without perfusion. To verify the oxygen levels present under control conditions, an air-tight syringe was used to draw air from triplicate vials containing 3 d embryos. Samples were injected into a Hewlett Packard 5890 II gas chromatograph (Palo Alto, CA) equipped with a molecular sieve column (1/8 inch diameter, 5 ft long) and a

thermal conductivity detector maintained at 50 °C during the analysis. Percent O_2 was calculated as the ratio of peak areas for O_2 and N_2.

At the end of the incubations, embryos were pulverized at liquid nitrogen temperatures and then homogenized in perchloric acid as previously described. Neutralized extracts were analyzed for either adenylates or lactate as described above.

Normalization of Data and Statistical Analysis

Biochemical data collected for developing embryos can be difficult to normalize because the amount of metabolically active tissue increases over time, but it is not necessarily proportional to the mass of the egg due to changes in amounts of yolk present. The problem is even more complicated for Orthopteran embryos because they absorb water during embryogenesis (e.g. Browning 1965; Yoder, 1992). As seen in Figure 3.1, the wet weight of *A. socius* embryos destined to enter diapause increases dramatically after 3 d post-oviposition. However, there is very little increase in dry weight during the same period, based on measurements of embryos dried to constant weight at 90° C. With these considerations in mind, the data were either normalized per embryo or per µg DNA.

One-way ANOVA and Student's T-test were performed with MiniTab software (MiniTab Inc., State College, PA). For both tests, results were considered significant if $P \leq 0.05$. Fisher's LSD was used to test for significance, *a posteriori*, for significant ANOVA.

Results

Developmental Arrest

A. socius embryos enter diapause early in development at what Tanaka (1984, 1986a) described as "Stage 2". At this morphological stage, embryos appear as elongated dumbbells with no obvious appendages and only rudimentary segmentation. Because the position of the

embryo relative to the yolk changes throughout development in a predictable manner and is important for determining the age of the embryo (Lees 1955; Gillott 2005), the chorion was chemically cleared and the embryos examined *in situ* as shown in Figure 3.2. Diapause embryos, fixed 30 d post-oviposition, are approximately 0.79 mm long and the procephalon has an average width of 0.22 mm. The embryo at this stage is found at the center of the egg and is surrounded by yolk (Fig. 3.2 B).

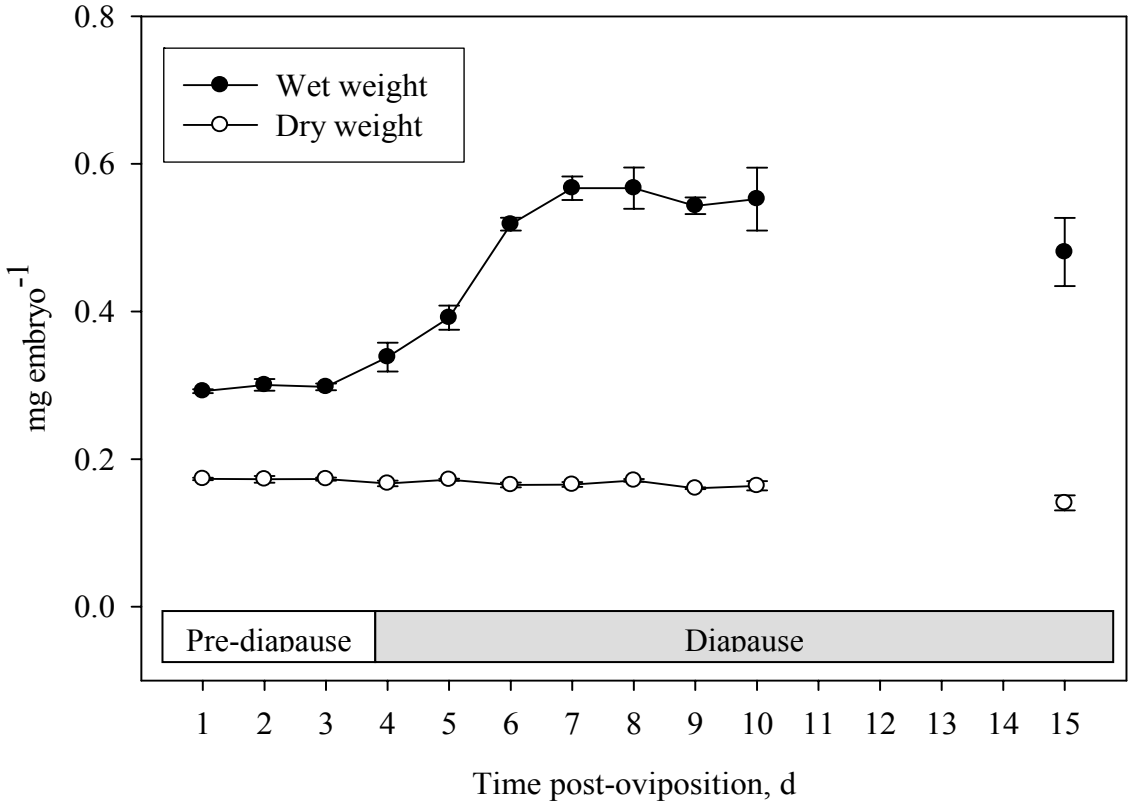

Figure 3.1. Weight of embryos as a function of developmental time. Mean ± SEM, N = 3 for each time point. The although there is little change in the dry weight (mg embryo^{-1}) during the first 15 d of development, wet weight increases dramatically beginning after day 3. The data shown are for embryos destined to enter diapause, but a similar pattern is seen for non-diapause embryos (data not shown).

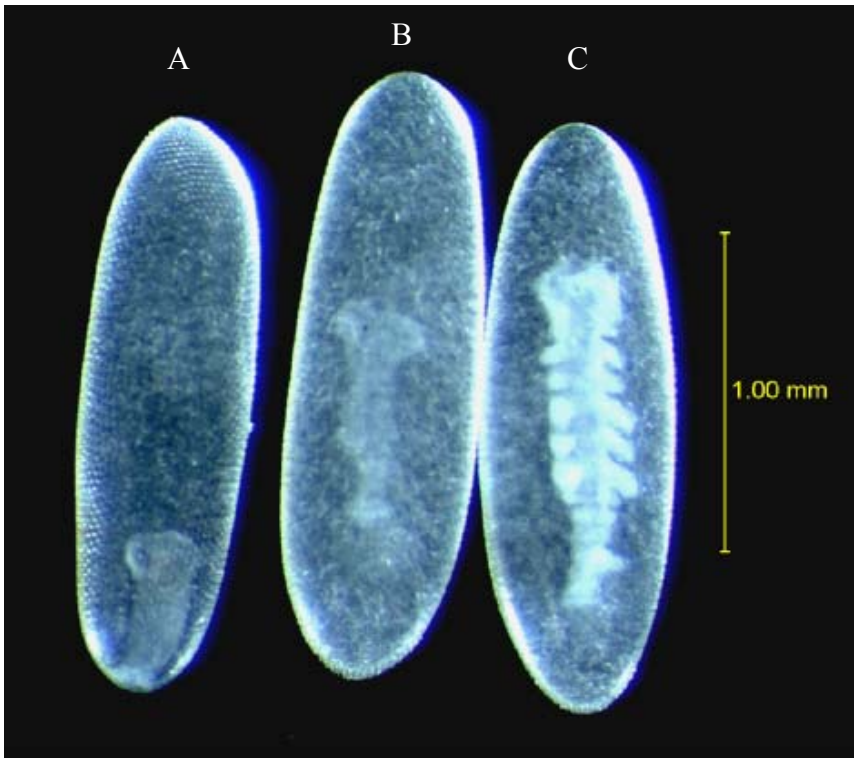

Figure 3.2. Morphology of *A. socius* embryos. Diapause embryos are morphologically similar to embryo 'B', which is moderately dumbbell shaped. This embryo has migrated from the posterior end of the egg to the center and is surrounded by yolk (compare to 'A'). In addition, embryos in diapause lack limb buds (compare to 'C').

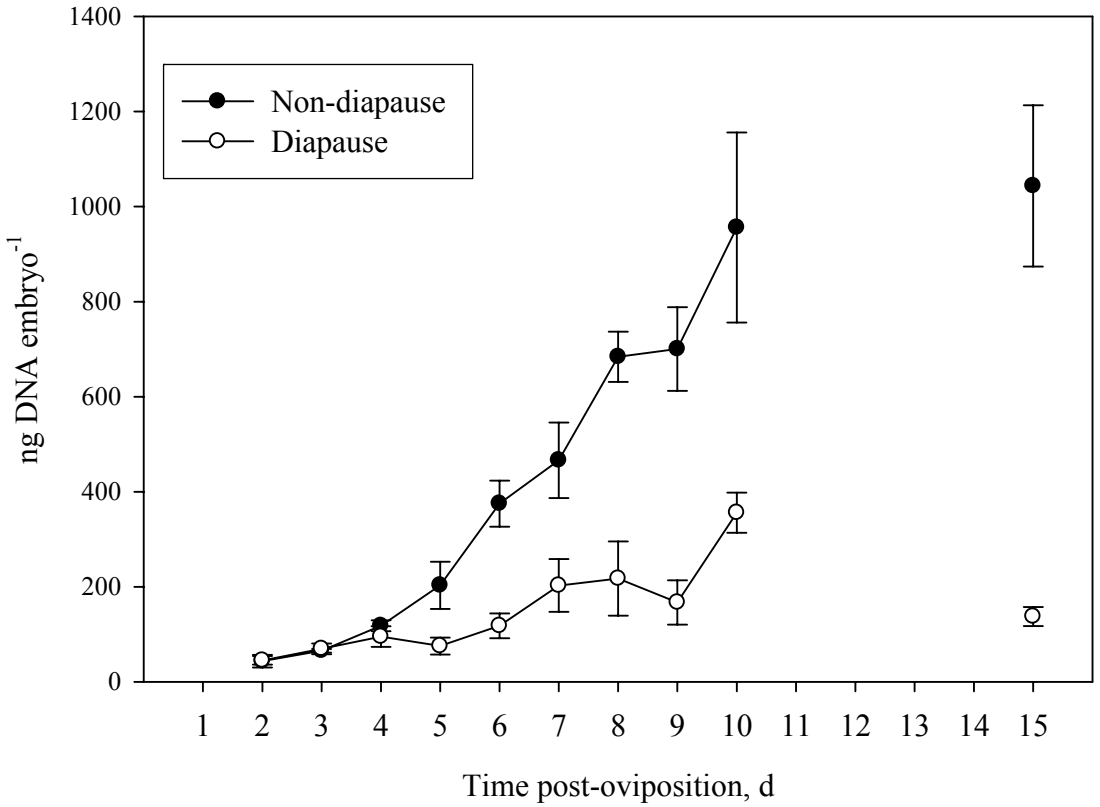

Figure 3.3. Total DNA per embryo as a function of developmental time in non-diapause and diapause embryos. Mean ± SEM, n = 3-6 samples of 200-300 embryos for each time point.

DNA content is an indirect measure of cell proliferation and can be used to precisely confirm the time point at which development is arrested upon diapause entry. In non-diapause embryos, DNA content (ng DNA embryo^{-1}) gradually increases during the first four d post-oviposition (Fig. 3.3).Beginning on day 5, there is a steep increase from 203 ng DNA embryo^{-1} to 950 ng DNA embryo^{-1} at 10 d. For embryos programmed to enter diapause, there is a likewise gradual increase in DNA during the first 4 d post-oviposition. As these embryos enter diapause, there is not a significant increase in DNA content over the next 10 d. Diapause embryos at 5 d post-oviposition contain the same amount of DNA as those at 10 d

(Fishers LSD, P = 0.05). Furthermore, non-diapause embryos at 6 d have significantly more DNA per embryo than those in diapause (Student's T test T = 4.34, d.f. = 4, P = 0.012). Thus, comparison of DNA content provides a quantitative, unambiguous, means by which to pinpoint developmental arrest and the entry into diapause, which for *A. socius* occurs 4-5 d post-oviposition.

Metabolic Rate

Oxygen consumption by non-diapause embryos increases significantly over time (ANOVA F = 7.56, d.f. = 11, P < 0.001) from 19.8 ± 0.57 pmol O_2 min^{-1} $embryo^{-1}$ (mean ± SEM) shortly after oviposition to 124 ± 8.95 pmol O_2 min^{-1} $embryo^{-1}$ at 15 d post-oviposition (Fig. 3.4). While there is an overall increase in aerobic metabolism, there is a notable decrease at 6 d post-oviposition and again at 9 d. These decreases may be due to peaks in ecdysone metabolism and/or intensive cellular differentiation (Sláma 2000).

The oxygen consumption rate for diapause embryos of *A. socius* measured at 15 d post-oviposition is 45.3 ± 3.9 pmol O_2 min^{-1} $embryo^{-1}$ (mean ± SEM, n = 22; far right bar, Fig. 3.4). This value is not significantly lower than that for non-diapause embryos at 4 d and 5 d, which are at a similar morphological stage (Fisher's LSD P = 0.05). Thus, there is not a detectable metabolic depression upon diapause entry. However, the aerobic metabolism in 15 d diapause embryos is only 36% of the rate measured for 15 d non-diapause embryos (Fisher's LSD P = 0.05). This comparison clearly shows that the ontogenetic increase in metabolism observed in non-diapause embryos is blocked during diapause.

To estimate aerobic metabolism per unit amount of metabolically active cells, the O_2 consumption rate was expressed per ng DNA. As seen in the insert of Fig. 3.4, oxygen consumption per unit DNA is highest early in development when there is relatively little

embryonic tissue present. As the amount of DNA per embryo increases (over 20-fold from day 2 to day 15), the mass-specific oxygen consumption decreases, which is consistent with allometric scaling of metabolic rate and body size.

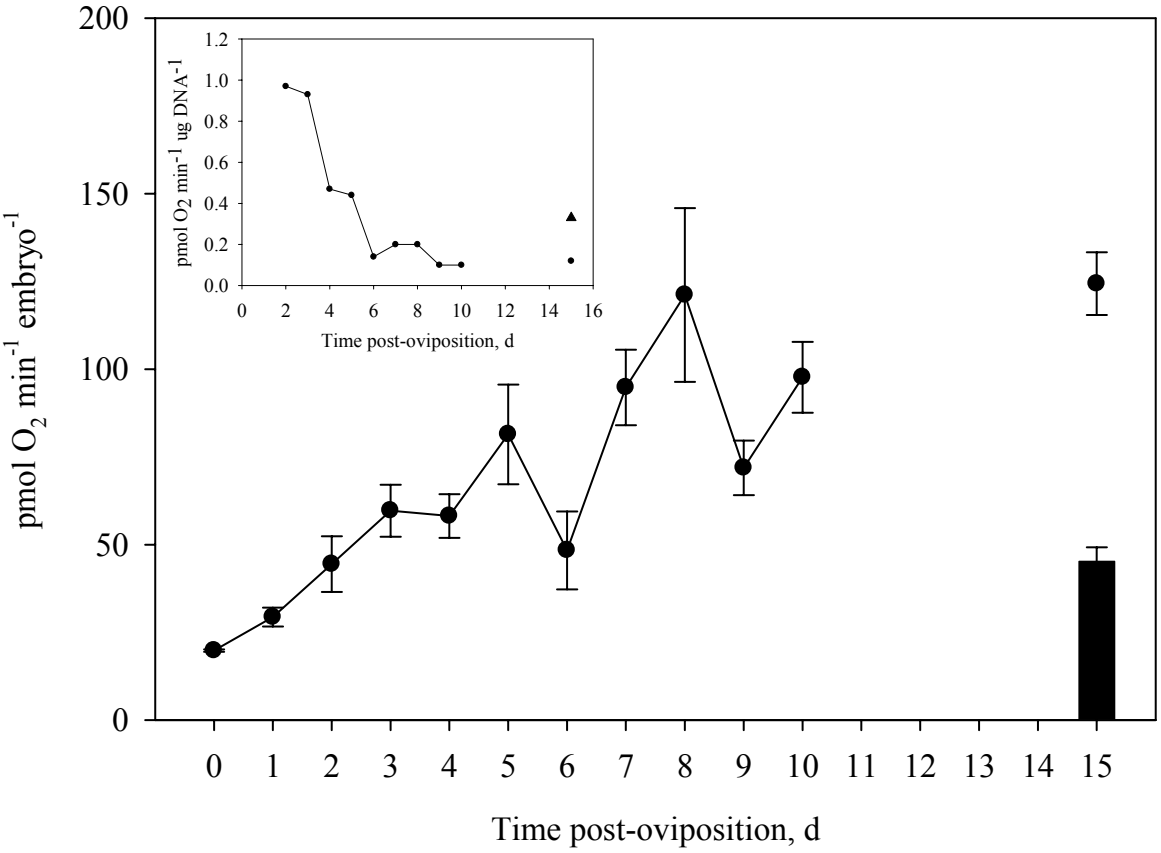

Figure 3.4. Respiration rate of *A. socius* embryos as a function of time post-oviposition. Line represents O_2 consumption rate per embryo of non-diapause embryos during the first 15 d of development (mean ± SEM; N = 3-12 samples of 100 embryos for each time point). Bar indicates respiration rate of diapause embryos 15 d post-oviposition (mean ± SEM, n = 22). Inset shows oxygen consumption per μg of DNA for non-diapause (circles) and diapause (triangle) embryos.

Table 3.1. Calorimetric/Respirometric Ratios of non-diapause embryos measured at 3 and 7 d post-oviposition (mean ± SEM).

Days Post-oviposition	N	CR ($kJ\ mol^{-1}\ O_2$)
3	3	353 ± 39.3
7	4	333 ± 32.3

There is not a significant difference in the ratio of heat production to oxygen consumed (CR ratio) by non-diapause embryos 3 and 7 d post-oviposition (Student's T test, T = -0.40, P = 0.7; Table 3.1). It is notable that these values are well below the expected value of -450 kJ mol^{-1} O_2 for the oxycaloric equivalent for mixed-substrate respiration under fully aerobic conditions. One interpretation is that these embryos are recovering from a previous exposure to hypoxia or anoxia (Hand, 1999; see Discussion, below).

Adenylate Status

The total quantity of adenylates in *A. socius* embryos (ATP+ADP+AMP; pmol adenylates μg DNA^{-1}) is highest soon after oviposition, and decreases as a function of developmental time (Fig. 3.6A). This pattern is seen for both non-diapause embryos (ANOVA F = 13.15, d.f. = 9, P < 0.001) and for embryos that enter diapause (ANOVA F = 4.65, d.f. = 9, P = 0.001). In non-diapause embryos, the general trend holds true for all adenylate species (Fig. 6B) with significant decreases observed for ATP (ANOVA F = 4.67, d.f. 9, P = 0.001), ADP (ANOVA F = 25.66, d.f. = 9, P < 0.01), and AMP (ANOVA F=12.58, d.f. = 9, P < 0.001).

In diapause embryos (Fig. 6C), there is a significant decrease in ADP (ANOVA F = 6.20, d.f. = 9, P < 0.001) and AMP (ANOVA F = 4.47, d.f. = 9, P = 0.002); however, there is not a significant change in the level of ATP (ANOVA F = 1.54, d.f. = 9, P = 0.194).

In both non-diapause and diapause destined embryos, the amount of AMP is remarkably high early in development, relative to the other adenylates, as seen in Figures 3.6B and 3.6C. This AMP status corresponds with an unusually high [AMP]/ [ATP] ratio; at 2 d post-oviposition the [AMP]:[ATP] ratio is 3.5 ± 0.08 (mean ± SEM) for non-diapause embryos and 4.5 ± 0.56 for diapause embryos (Fig. 3.7A). The [AMP]:[ATP] ratio decreases significantly with time after oviposition in both non-diapause (ANOVA F = 8.50, d.f. = 9, P < 0.001) and diapause embryos (ANOVA F = 4.14, d.f. = 9, P = 0.003). The ratio reaches a steady-state of about 0.5 after 6 d post-oviposition in non-diapause embryos, and 0.7 in diapause embryos after 7 d.

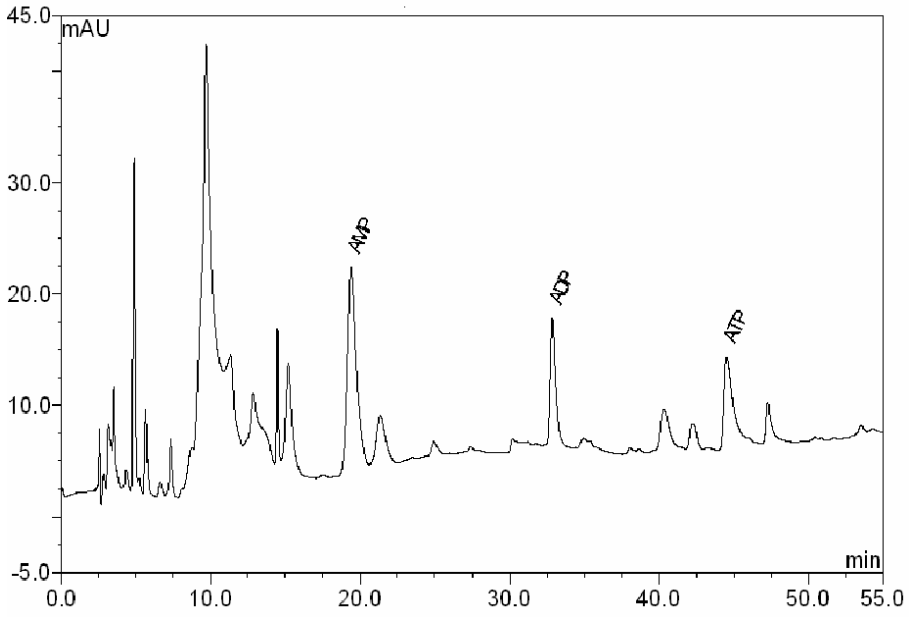

Figure 3.5. Representative HPLC chromatogram for separation and quantification of adenine nucleotides. Adenylates in perchloric acid extracts were separated using a reverse phase column with a potassium phosphate buffer (pH 5)/acetonitrile mix as the mobile phase (see Materials and methods for details).

At 4 d post-oviposition, the [ATP]:[ADP] ratio is low in both diapause and non-diapause embryos (Fig 7B). [ATP]: [ADP] begins to increase, beginning 5 d post-oviposition, in both non-diapause (ANOVA $F = 7.05$, d.f. = 9, $P < 0.001$) and diapause embryos (ANOVA $F = 4.55$, d.f. $P = 0.001$). However, the total increase is greater for non-diapause embryos than for diapause embryos. In non-diapause embryos, the [ATP]:[ADP] ratio increases 85 % over 15 d of embryonic development, primarily due to increased quantity of ATP that begins after 4-5 d of development. In embryos destined to enter diapause, the [ATP]:[ADP] ratio increases 73 %, from 0.87 to 3.22, during the first 15 d post-oviposition. In these embryos the increase is primarily due to a decrease in [ADP] rather than a change in [ATP] (Fig. 6B). Even at the latest chronological stages evaluated for either non-diapause or diapause embryos, the [ATP]:[ADP] ratio is abnormally low for tissues respiring under normoxic conditions.

The adenylate status of *A. socius* embryos depends in part on the availability of oxygen in the environment in which they are incubated. As seen in Figure 3.8A, the [ATP]:[ADP] ratio increases as a function of oxygen percentage (ANOVA $F = 7.21$, d.f. = 3, $P = 0.006$). Embryos incubated under hyperoxic conditions (superfused with 40 % O_2) or normoxic conditions (superfused with 20 % O_2) have higher [ATP]:[ADP] ratios than embryos incubated under hypoxic (superfused with 10% O_2) or control (not superfused) conditions (Fisher's LSD $P = 0.05$). The percentage of oxygen measured for air samples collected from control, non-superfused vials containing 3 d embryos was 20 ± 0.33 (mean ± SEM, n = 3).

Embryos exposed to hyperoxia exhibit a significant 37% decrease in [AMP]:[ATP] compared to control embryos (ANOVA $F = 4.69$, d.f. = 3, $P = 0.024$; Fisher's LSD $P = 0.05$).

However, there is no difference in [AMP]:[ATP] ratios among embryos incubated in 10 %, 20 % or 40 % O_2 (Fishers LSD P = 0.05). It is appropriate to note, however, that the improvement in adenylate status seen with elevated oxygen does not result in adenylate ratios that are typical for normoxically respiring tissues, and thus the abnormal ratios are not fully explained by oxygen limitation but rather are an intrinsic feature of these embryos (see Discussion section).

Anaerobic End-product

The adenylate data suggest that *A. socius* embryos may experience some degree of tissue hypoxia during early development. Thus the whole-embryo concentration of lactate, a known anaerobic end product in insects (Wegener 1993; Hoback et al, 2000; Hoback and Stanley 2001), was measured in non-diapause embryos. Between 3 and 7 d post-oviposition, lactate (nmol per μg DNA) decreases 84 % in non-diapause embryos (Student's T-test, T = 5.86, d.f. = 3, P = 0.001; Fig. 3.9). Embryos incubated for 24 h under normoxia (20 % O_2), hyperoxia (40 % O_2), or mild hypoxia (10 % O_2) did not exhibit any significant change in the quantity of lactate present compared to controls (20.33% O_2) (ANOVA F = 1.02, d.f. = 3, P = 0.421). Thus large lactate stores are present in these embryos during early development, but the lactate levels are not affected by short-term (24 h) incubation in elevated oxygen

Discussion

I have shown in this study that embryos of the ground cricket, *Allonemobius socius,* enter diapause approximately 4 d post-oviposition when development is arrested; a significant depression of aerobic metabolism does not accompany the entry into diapause in this species. However, the ontogenetic increase in O_2 consumption observed for non-diapause embryos is blocked in diapause embryos.

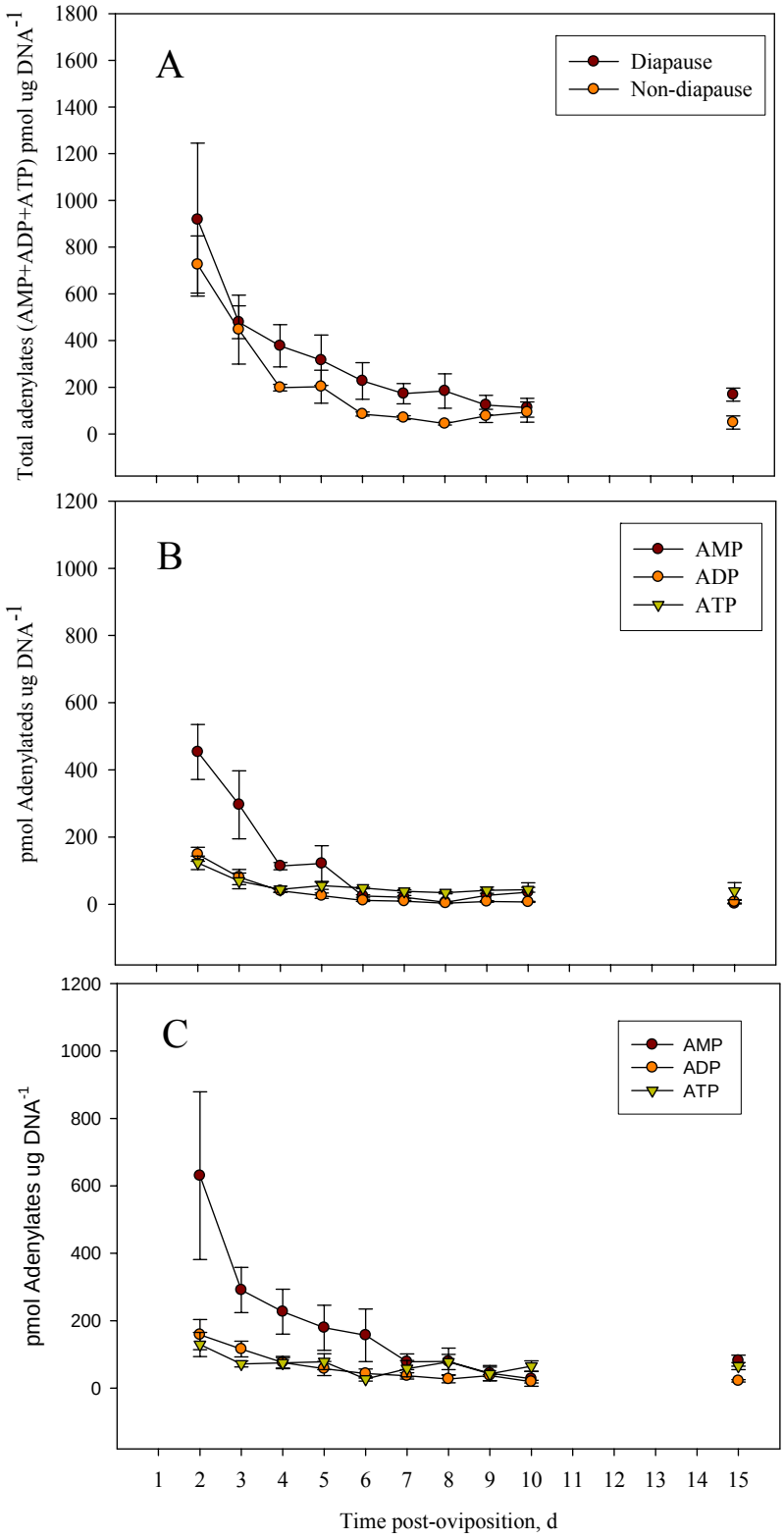

Figure 3.6. Adenine nucleotides in *A. socius* embryos as a function of developmental time. (A) Total quantity of adenylates (ATP+ADP+AMP) per µg DNA^{-1} as a function of time in non-diapause and diapause embryos. ATP, ADP, and AMP in non-diapause (B) and diapause (C) embryos as a function of developmental time. Mean ± SEM, N = 3-6 samples of 200-300 embryos for each time point

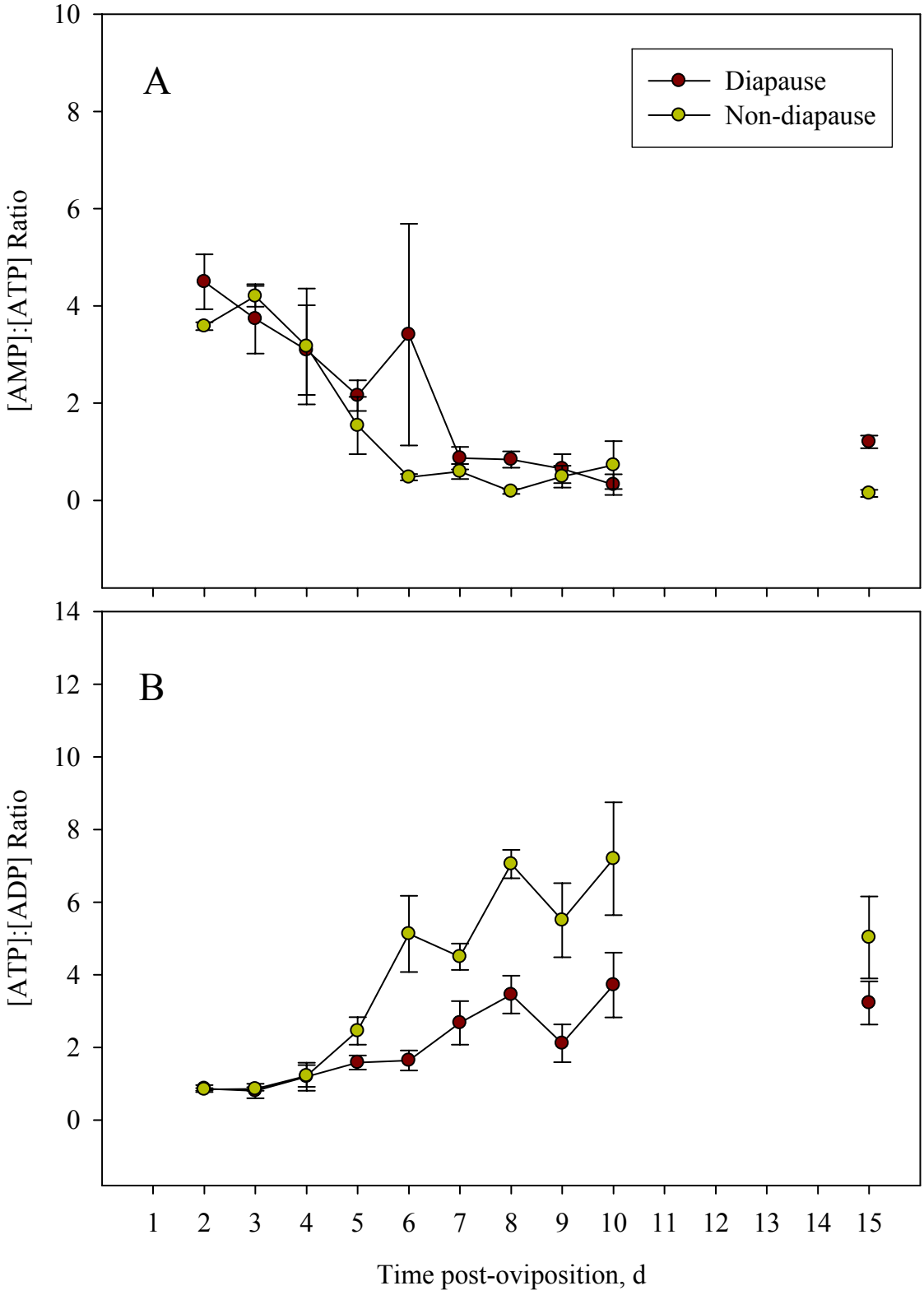

Figure 3.7. A) [AMP]:[ATP] ratios and B) [ATP]:[ADP] ratios of non-diapause and diapause embryos as a function of developmental time. Mean ± SEM, N = 3-6 samples of 200-300 embryos for each time point.

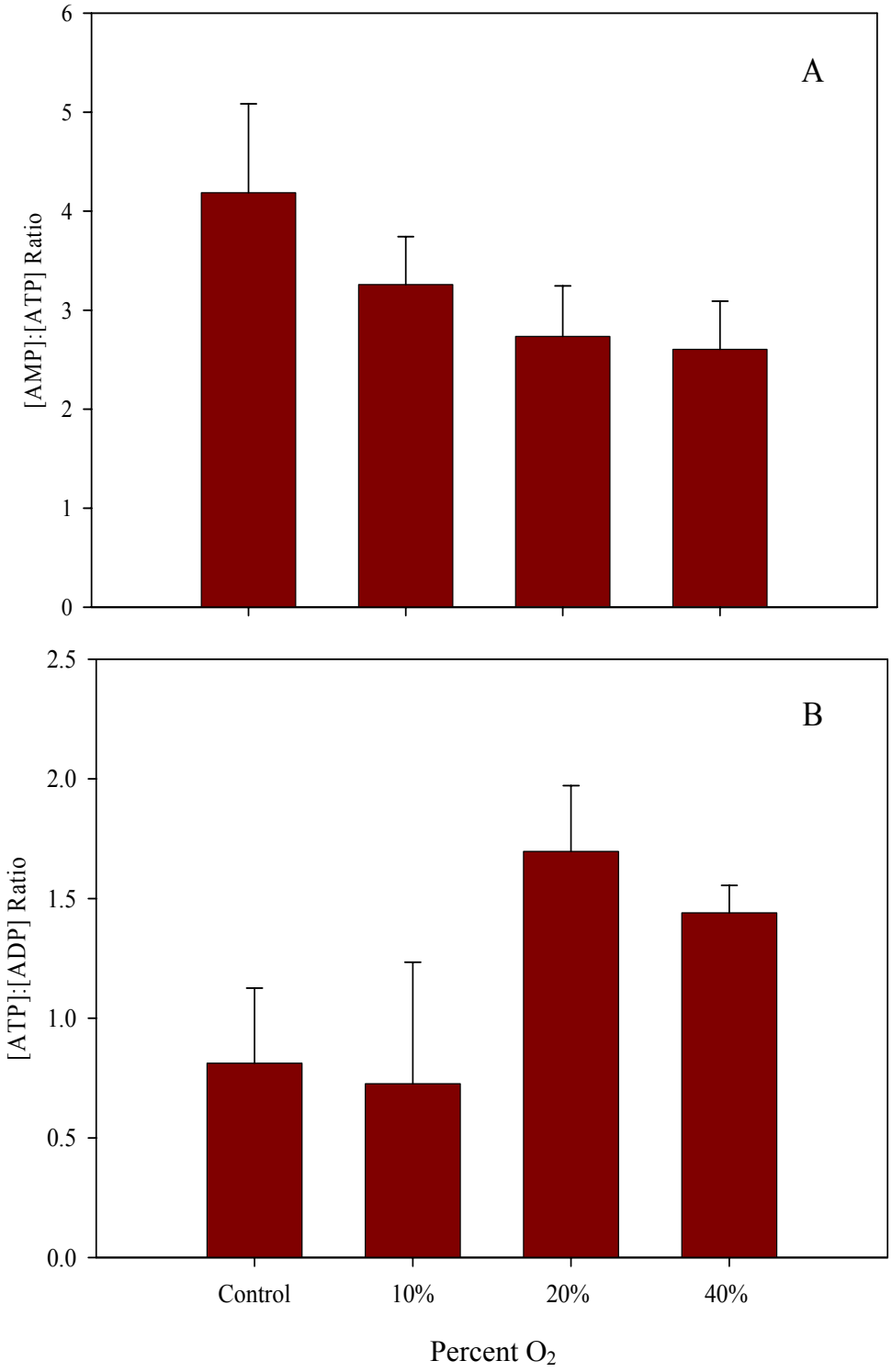

Figure 3.8. (A) [AMP]:[ATP] ratios and (B) [ATP]:[ADP] ratios of 3 d non-diapause embryos incubated for 24 h under hyperoxic (40% O_2), normoxic (20% O_2) or mildly hypoxic (10% O_2) conditions. Control embryos were embedded in moist cheese cloth and incubated under normoxia but without perfusion of the incubation vials (20.33% O_2). Mean ± SEM, N=3-4 samples of 200-300 embryos.

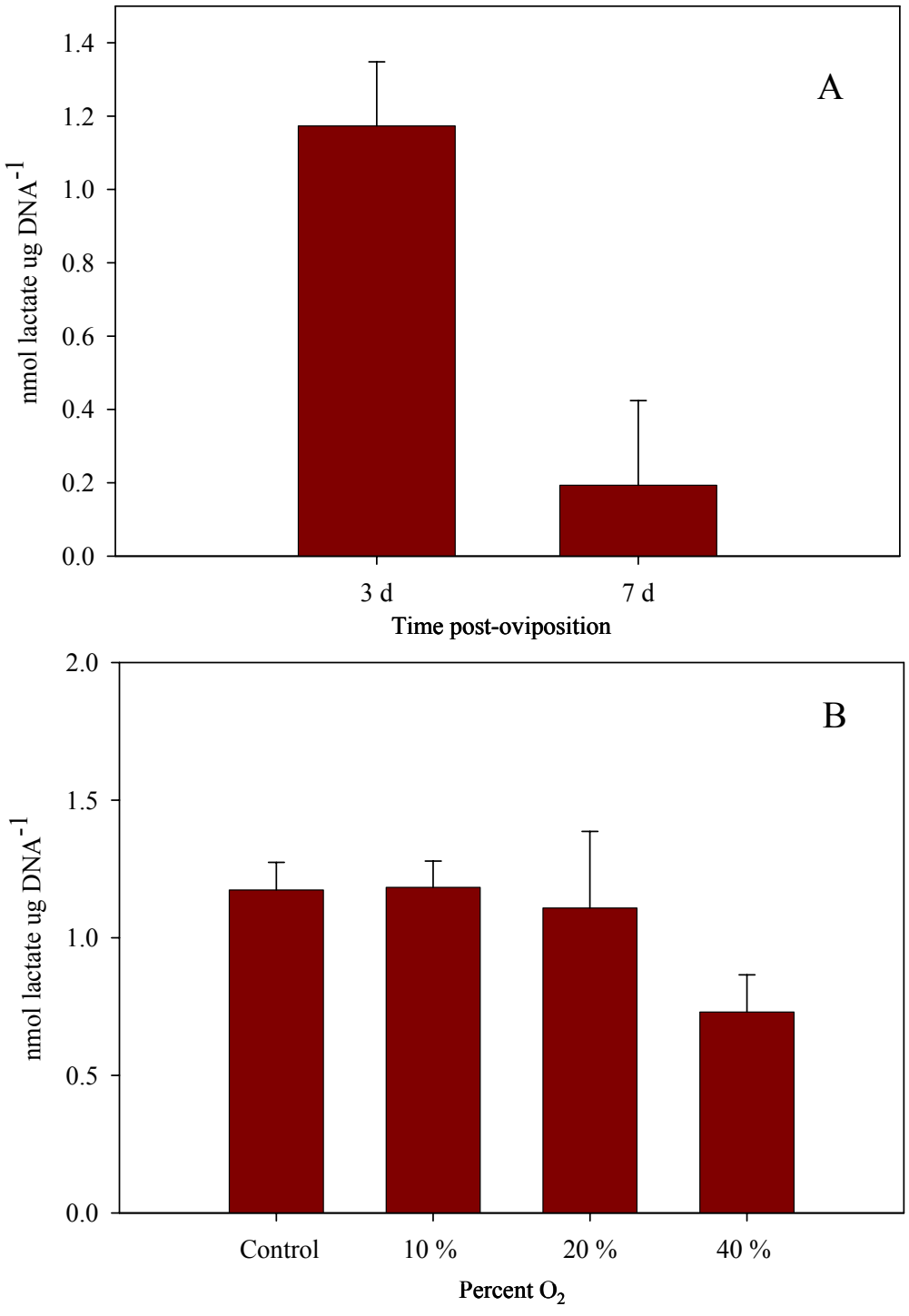

Figure 3.9. (A) Lactate (nmol DNA^{-1}) in non-diapause embryos at 3 and 7 d post-oviposition and (B) in 3 d embryos incubated for 24 h under hyperoxic (40 % O_2), normoxic (20 % O_2), or mild hypoxic (10 % O_2) conditions. Control embryos were embedded in moist cheese cloth and incubated under normoxia (percent O_2 = 20 ± 0.33; n = 3) but without perfusion of the incubation vials. Mean ± SEM; N = 3-4 samples of 200-300 embryos.

Both diapause and non-diapause embryos exhibit remarkably high [AMP] and low [ATP] early in development. The levels of these adenylates do not appear to be correlated with oxygen limitation because incubating the embryos under hyperoxia only moderately reduces [AMP]. In addition lactate, which is present in 3 d embryos, is not reduced by increasing O_2 in the incubation environment. Finally, CR ratios are not consistent with fully aerobic metabolism, but are more typical for animals recovering from hypoxia. Thus, the atypical set point for biochemical indicators of metabolic poise is an ontogenetic feature only partially explained by oxygen availability.

Comparing the morphological characteristics of *bona fide* diapause embryos with non-diapause embryos over a period of 10 d indicates that morphogenesis arrests approximately 4 d post-oviposition. The quantity of DNA in diapause and non-diapause embryos supports this conclusion. The oxygen consumption rates of non-diapause *A. socius* embryos are similar to those reported for *Gryllus veletis,* a cricket species that does not undergo an embryonic diapause (Rakshpal 1962a). Aerobic metabolism is not downregulated in diapause *A. socius* embryos compared to 3-4 d non-diapause embryos. This result is surprising given that diapause in many species is characterized by deep metabolic depression. For example, oxygen consumption is reduced by 87 % in *G. pennsylvanicus* embryos (Rakshpal 1962b) and by as much as 92 % in *Artemia franciscana* embryos (Reynolds and Hand 2004). However, the lack of significant metabolic arrest during diapause is not unique to *A. socius,* because embryos of the grasshopper, *Alocara elliot*, continue to consume oxygen at a "pre-diapause" rate even after entering diapause (Roemhild 1965), and there may be other species for which metabolism is not endogenously depressed. Many species of insects remain responsive to changes in environmental conditions

throughout diapause, and it is possible that metabolic downregulation in some species is due to low temperatures operating through a Q_{10} effect. It is notable that 15 d diapause embryos consume oxygen at a rate that is only 36 % of the rate observed for non-diapause embryos of the same chronological age. This observation suggests that the ontogenetic increase in metabolism observed for non-diapause embryos is blocked in diapause embryos.

[AMP]:[ATP] ratios are atypically high during the first 5 d post-oviposition in *A. socius* embryos, but decrease as development progresses to reach a steady state at 6 d in non-diapause embryos and 7 d in diapause embryos. Under normal conditions, healthy mammalian cells maintain an [AMP]:[ATP] ratio of 0.01 (Hardie and Hawley 2001). In this cricket species, the [AMP]:[ATP] ratio is 4:1 at 2 d post-oviposition, and the lowest [AMP]:[ATP] ratios observed in these embryos (0.5 in non-diapause embryos and 0.7 in diapause embryos) are considerably higher than the value typically measured for mammalian cells. One hypothesis for the unusual levels of adenylates is that elevated AMP is a feature of the entry into diapause. Increased AMP levels have been observed during embryonic diapause in a number of animals including *Artemia franciscana* (J. Reynolds, J. Covi, and S. Hand, unpublished observations) and *Astrofundulus limneaus* (Podrabsky and Hand 1999). Podrabsky and Hand (1999) have hypothesized that during diapause in *A. fundulus*, increased AMP activates the AMP-activated protein kinase (AMPK). AMPK is a protein that acts as a cellular fuel gauge and is part of a signaling cascade that modulates a number of metabolic responses, including, but not limited to, a decrease in fatty acid synthesis and lipogenesis, and inhibition of cell proliferation (Hardie and Hawley 2001). Although elevated [AMP]:[ATP] ratios may activate AMPK, and thus contribute to metabolic downregulation during diapause in some species, it is unlikely that AMPK is a major regulator of diapause in

A. socius embryos. First, [AMP]:[ATP] ratios of diapause embryos are not significantly different from those of non-diapause embryos during the first 10 d of development. Second, the highest [AMP]/[ATP] ratios were measured well before developmental arrest occurred and are maintained at relatively low levels after diapause is initiated. Third, while the high [AMP]:[ATP] ratios are unusual, even higher values have been measured for embryos of the field cricket, *Gryllus bimaculatus*, a species that does not enter diapause (Izumiuama and Suzuki 1986). Adenylate ratios during late diapause in sponge gemmules are apparently similar compared to those during germination (Loomis et al. 1996). Thus activation of AMPK by high AMP levels upon entry into diapause does not appear to be a universal mechanism for controlling the diapause state.

A more plausible explanation for the unusual adenylate status of *A. socius* embryos is that early development occurs in a hypoxic environment. High [AMP]:[ATP] ratios are characteristic of insects facing oxygen limitation because, unlike anoxia tolerant vertebrates that are able to maintain high ATP levels under hypoxia (Hochachaka 1997), insects do not typically defend ATP levels under oxygen limiting conditions (Hochachka et al., 1993; Wegener 1993; Hoback and Stanley 2001). During embryonic development, insects are typically enclosed within a system of membranes and other structures that protect the embryo from desiccation and environmental hazards. While the chorion, wax layer, and vitelline membrane clearly have a protective role, they may also limit oxygen diffusion, which is the only means of obtaining oxygen for embryos that do not yet have a functioning oxygen delivery system. The likely consequence is that insect embryos develop within an environment that is hypoxic, at least early in development. This conclusion is supported by two recent studies on embryonic development in *Bombyx mori* (Sakano et al. 2004) and

Manduca sexta (Woods and Hill 2004). Lactate accumulates in *B. mori* embryos during the first 3 d of embryogenesis, and Sanko et al. (2004) interpret this to mean that these embryos rely on anaerobic metabolism early in development. More direct evidence that insect embryos develop in a hypoxic environment comes from Woods and Hill (2004). These researchers measured oxygen levels within *M. sexta* eggs and found that oxygen levels are well below air saturation throughout, and the P_{O2} at the center may be as low as 2 kPa (Woods and Hill 2004). Hypoxia may be a challenge commonly faced by insects during embryogenesis because the extra embryonic structures are similar for most terrestrial insect species (Gillott 2005).

To test the hypothesis that *A. socius* embryos are oxygen limited early in development, I measured lactate levels as a function of developmental time. Lactate levels in 3 d embryos are comparable to those measured by Sakano et al. (2004) but fall during the next 4 d of development. By 7 d post-oviposition, lactate is reduced by 84 %. It is notable that [AMP]: [ATP] decreases and [ATP]:[ADP] increases during the same period. Furthermore, the timing of these observed changes in lactate and adenylate ratios corresponds with an increase in the average wet weight of the embryo. This weight gain appears to result primarily from absorption of water as there is not a corresponding increase in dry weight (see Fig. 3.1). Orthoptera embryos commonly absorb water during embryogenesis (e.g., Banks 1949; Browning 1953; Tanaka 1986b). However the physiological and/or biochemical relevance of this phenomenon is unknown. I hypothesize water absorption is tied to a change in the permeability of the chorion and may increase the amount of oxygen available to the embryo. This conclusion is indirectly supported by research on embryonic development in frogs and salamanders which suggests that water

absorption enhances oxygen diffusion in these species by increasing the effective surface area and decreasing the viscosity of the yolk surrounding the embryo (Seymour et al. 1991).

Comparing the [AMP]:[ATP] and [ATP]:[ADP] ratios of embryos incubated under normoxia, hyperoxia, and hypoxia suggests the atypical adenylate status observed early in development results from hypoxia, at least in part. Superfusing 3 d embryos under 40 % O_2 decreases [AMP]/[ATP] ratios and increases [ATP]/[ADP] ratios compared to control embryos incubated under normoxic conditions without perfusion. However, superfusing embryos alters [AMP]:[ATP] regardless of the percent oxygen. In addition, increasing O_2 does not significantly change the amount of lactate in 3 d embryos. Therefore the ontogenetic change in [AMP]/[ATP] and lactate is only partially related to oxygen availability.

Calorimetric-respirometric ratios (CR ratios; kJ produced per mole O_2 consumed) can be used to test for a contribution from anaerobic pathways to overall metabolic rate. CR ratios between -443 and -478 are thought to represent completely aerobic metabolism while higher (more negative) values suggest that anaerobic pathways contribute to the total energy flow. These conclusions are based on theoretical calculations of oxycaloric equivalents, the predicted amount of heat energy expected per mol of oxygen consumed when a given substrate is completely oxidized to carbon dioxide and water (Hand 1991, 1999). CR ratios calculated for 3 d and 7 d *A. socius* embryos are not indicative of aerobic metabolism because they are well below -450 (the value estimated for mixed substrate oxidation, see Widdows 1987; Gnaiger and Kemp 1990). While the CR ratios of *A.socius* embryos are consistent with values measured for animals recovering from anoxia (Hand 1991), they are also within the range of values determined for *Tribolium confusum* embryos (Dunkel et al.

1979). Clearly, additional research needs to be done before we will understand the bioenergetics of embryonic development in these and other insects.

In summary, this chapter characterizes bioenergetics of embryogenesis and diapause in *A. socius* embryos. Diapause in this species is not characterized by deep metabolic depression, but the ontogenetic increase evident in non-diapause embryos is blocked in diapause embryos. [AMP] is atypically high early in development in this species, which may be indicative more generally of oxygen limitation in insects at this stage. I provide biochemical evidence that *A. socius* embryos are hypoxic early in development. Further, the calculated CR ratios indicate that the embryos have indeed experienced oxygen limitation and are recovering from it. However, if the embryos were still anoxic at day 3-7, this condition should be reflected by a CR ratio much more negative that the oxycaloric equivalent (which I did not observe). Increasing the percent O_2 in the incubation environment partially reverses the biochemical indicators of hypoxia. A change in chorion permeability that begins around 3-4 d post-oviposition appears to relieve hypoxia and may explain the ontogenetic decrease in lactate and the increase in ATP/ADP after that time. Nevertheless, the atypical set points for biochemical indicators of metabolic poise appear to be an ontogenetic feature that is only partially explained by oxygen availability.

CHAPTER 4

DIFFERENTIAL GENE EXPRESSION IN PRE-DIAPAUSE AND DIAPAUSE EMBRYOS OF THE CRICKET, *ALLONEMOBIUS SOCIUS*

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, Denlinger 2002). Diapause is endogenously controlled, and entry typically begins well in advance of the advent of harsh conditions. This state is widespread among insects and can occur at any stage of the life cycle; whether diapause is initiated in the embryo, larva/nymph, pupa, or adult depends on the species. The physiological and molecular changes that occur are also species specific, and the physiological changes that occur during diapause have been characterized for a number of species (for example see Rakshpal 1962a,b; 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 et al 2002; Hong et al. 2006; Kim et al. 2006, Robich et al. 2006), 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 identify genes that may have a role in the entry into diapause in embryos of the southern ground cricket, *Allonemobius socius.* Specifically, I used PCR-based subtractive hybridization and quantitative PCR (qPCR) to identify genes that are significantly up or down regulated in embryos that are preparing to enter diapause, as well as in embryos that have been in diapause for approximately 10 days.

The only insect embryo for which gene expression during diapause has been studied extensively is the silkworm, *Bombyx mori* (e.g., Hong et. al. 2006, Suzuki et al. 1999, Dorel and Coulon 1988). 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 alters 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. While 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. This

plasticity permits 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.

Chapter 3 describes previous research that focuses on morphological, metabolic and biochemical characteristics of *A. socius* embryos that are in diapause compared with embryos that are not in diapause. Results indicate that *A. socius* embryos enter diapause 3-4 d post-oviposition. Although diapause in many species is characterized by downregulation of metabolism (Reynolds and Hand 2004 and references therein), *A. socius* appears to be unusual in that there is not an endogenously controlled metabolic depression. The oxygen consumption rate of embryos that have been in diapause for about 10 days is not significantly different from that of non-diapause embryos at the same morphological stage. However, the ontogenetic increase in respiration expressed per embryo for the non-diapause state is fully blocked during diapause. Given these data, I hypothesize that genes upregulated in prediapause embryos will encode for stress proteins that protect against cellular damage and extend protein half-life. I also predict there to be significant downregulation of genes encoding for proteins involved in DNA replication, cell cycle control, and protein synthesis. 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 are not necessary for maintaining the diapause state. Thus, I 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 were kept in 20 x 13 cm plastic boxes with moistened cotton and food, and they were maintained at ambient temperature with a 14:10 (L: D) photoperiod. Upon reaching adulthood, males and females were transferred to 38 L glass aquaria; approximately 50 adults were kept in each tank with access to food and water *ad libitum*. All crickets were fed Fluker's™ Cricket Food (Fluker's Cricket Farm, Baton Rouge, LA) that was supplemented with dry cat food for additional protein. Adults were maintained at either 22°C with a short-day photoperiod (12:12, L:D) to encourage production of diapause embryos or 28° with a long-day (16:9, L:D) photoperiod to encourage production of non-diapause embryos (Olvido et al 1998; Huetis and Marshall 2006). Plastic vials containing moistened cheese cloth were provided as an ovipositon substrate; embryos were collected every 24 h and were reared under ambient light at 29°C.

RNA Isolation and cDNA Synthesis

In order to identify genes that may have a role in the initiation of diapause in *A. socius*, total RNA was isolated from pre-diapause embryos and from non-diapause embryos that were both reared at 29 °C for 2-4 d. The RNA samples were then used in a PCR based subtraction method to generate two pools of cDNA. One pool, referred to as the "upregulated" library was expected to be enriched for transcripts that are more abundant in pre-diapause embryos and the second pool, the "downregulated" library, should be enriched for transcripts that are

more abundant in non-diapause embryos of the same chronological and morphological stage as the pre-diapause embryos.

Total RNA was isolated using RNAwiz reagent (Ambion, Austin, TX). 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 weight) were combined with 500 ml of RNAwiz in an RNase-free microtube and were homogenized with a Kontes Pellet Pestle driven by a cordless Kontes Pellet Pestle motor (Kimble/Kontes, Vineland NJ). The homogenate was drawn through an 18 gauge needle to further disrupt the tissue and to improve mixing. Following a15 min incubation at room temperature, 0.2 volumes of chloroform and 0.1 volumes of RNase-free water were added to the homogenate. After an additional 15 min incubation at room temperature they were centrifuged at 18,000 x g for 30 min at 4°C to separate the RNA from DNA and protein. Total RNA was precipitated with 0.5 volumes of RNase-free water and 1 volume of isopropanol, and the RNA pellet was washed with cold 75% ethanol. 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). Samples were stored at -80° until they were 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 and non-diapause embryos, it was necessary to verify the diapause status of the embryos before combining the RNA samples into either 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 using the method published by Hogan (1956). Briefly, embryos were incubated in water at room temperature for 30 min. Following a 45 min incubation in a mixture of chloroform, glacial acetic acid, and absolute ethanol (2:2:1) at 37 °C, they were transferred to a solution of glycerol and 70% ethanol (1:1) and incubated overnight at room temperature. Embryos were observed within the egg using a Leica dissecting microscope, and the number with the diapause morphology (see below) was counted. *A. socius* embryos that do not enter diapause typically hatch after about 15 d. Therefore after 10 d, 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, and lacked obvious limb development. An RNA sample was categorized as 'diapause' if at least 70% of the embryos from which it was prepared showed the diapause morphology, while a sample was considered to be 'non-diapause' if no more than 30% of the embryos exhibited the diapause morphology. RNA from confirmed diapause or non-diapause samples was pooled to yield 100-155 μg per batch.

Messenger RNA (mRNA) was isolated from the pooled, total RNA using Oligotex™ beads (Qiagen, Valencia, CA), and the standard manufacturer's protocol was followed. Aliquots of mRNA (0.53 μg from diapause embryos and 0.54 μg from non-diapause embryos) were then used to synthesize cDNA using a SMART cDNA synthesis kit (Clontech, Mountain View CA) following the manufacturer's protocol. The SMART cDNA system can generate large amounts of cDNA from small amounts of RNA because an

amplification step is incorporated to increase the amount of cDNA while maintaining the original ratios of mRNA. Briefly, the first strand synthesis is primed with a modified oligo dT. When the reverse transcriptase (RT) reaches the 5' end of the mRNA, additional nucleotides, primarily deoxycytidines, are added to the 3' end of the cDNA by means of the enzyme's terminal transferase activity. A SMART™ oligonucleotide anneals to the deoxycytidines that were added to the 3'end. The reverse transcriptase then switches templates and continues replicating to the end of the oligonucleotide. The resulting single-stranded (ss) cDNA contains the complete RNA sequence plus a region that is complementary to the SMART oligonucleotide. This added sequence is then used as a universal primer for the amplification step and increases the amount of cDNA for use in subtractive hybridization.

Subtractive Hybridization

Subtractive hybridization allows comparison of two populations of mRNA in order to identify genes that are expressed in one population but not the other. In this approach, one population of mRNA is designated as the "tester" and contains the potentially upregulated genes of interest, while the second population, or "driver", is the reference. In this study, I performed two subtractions. The first, or forward, subtraction was intended to isolate genes that were upregulated in pre-diapause embryos compared to non-diapause embryos, so cDNA synthesized from pre-diapause mRNA was designated the tester and cDNA from non-diapause mRNA was the driver. The second subtraction, or reverse subtraction, was intended to identify genes that were only present in non-diapause cDNA (i.e., downregulated in pre-diapause embryos). Therefore, the tester was the cDNA from non-diapause embryos and the driver cDNA came from pre-diapause embryos.

The forward and reverse subtractions were performed using a PCR-Select™ cDNA Subtraction Kit from Clontech (Mountain View CA) according to the manufacturer's protocol. Briefly, double stranded (ds) cDNA from pre-diapause and non-diapause embryos was digested with the restriction enzyme Rsa1 to remove the oligonucleotides added during the SMART cDNA synthesis and to generate shorter cDNA molecules with blunt ends. The tester cDNA was divided into two portions and each was ligated to a different cDNA adaptor. To confirm that at least 25% of the cDNA strands possessed adaptors, as recommended by the manufacturer's protocol, two PCRs were run for the forward and reverse subtractions. One PCR used two primers for Cox subunit I that were designed by aligning sequences from several Orthoptera species; the forward primer sequences was 5'-AGCTCCTGATATAGCATTCCCACG-3' and the reverse primer sequence was 5'-AGGGCTGTAATACCAACGGCTCAT-3'. The second PCR used one primer for Cox I and one primer for the ligated adaptor. Gel electrophoresis confirmed that over 25% of cDNA molecules had been ligated to the adaptors (data not shown).

In the next step, two hybridizations were performed. During the first hybridization, excess driver was added to each of the two tester populations. The samples were heat denatured and allowed to anneal, forming four types of molecules. Type "a" molecules were ss cDNA with the adaptor, and type "b" molecules were ds cDNA hybrids with an adaptor on each strand. Type "c" molecules were ds cDNA hybrids of tester and driver cDNA, and type "d" molecules were ss or ds driver cDNA. During this hybridization, the concentrations of high- and low- abundance molecules were equalized within type "a" molecules because more abundant molecules reanneal faster than less abundant ones due to the second-order kinetics of hybridization (James and Higgins 1985). In addition, the pool of type "a" molecules was

enriched for differentially expressed transcripts because sequences that were not differentially expressed formed type “c” molecules with the driver.

In the second hybridization, the two primary hybridizations were mixed together without additional denaturation, which promoted the formation of type “e” molecules, which are ds cDNA molecules with one strand ligated to adaptor 1 and one strand ligated to adaptor 2. Adding freshly denatured driver further enriched the population of “e” molecules. The differentially expressed sequences were amplified by PCR using primers complementary to the adaptors. During this step, only type “e” molecules were exponentially amplified because the other molecule types did not contain sequences that were complementary to both of the primers. A secondary PCR with nested primers further enrich differentially expressed sequences. These products were used to construct forward and reverse subtracted libraries (see below). A final PCR with degenerate primers for actin (forward primer 5’-ACAATGGMTCYGGWATGTGCAARGCT; reverse primer 5’-CCCAGTTKGTWACAATWCCRTGCT), which was originally a highly abundant gene, confirmed that the subtraction was successful because actin levels were greatly reduced relative to unsubtracted controls.

Sequencing and Bioinformatics Analysis

The forward and reverse subtracted libraries were cloned using JM109 cells using pGem vector to insert cDNA into competent cells (Promega, Madison WI). Transformed cells were grown overnight on Luria-Bertani (LB) plates containing ampicillin, X-gal, and IPTG for blue/white selection. For each library 300-400 white clones 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). Sequences were identified by homology to known genes using a 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 (qPCR) was used to quantify the relative level of expression in pre-diapause and diapause embryos compared to non-diapause embryos. Total RNA was isolated from pre-diapause and non-diapause embryos that had developed for 2-4 d post-oviposition and from diapause embryos that were incubated for 15 d post-oviposition as described above. For each sample, 4µg of total RNA was reverse transcribed in a 20µl reaction containing 500 ng of random primers (Integrated DNA Technology, Coralville, IA), 0.5 µM dNTPs, 1X first strand buffer, 5 mM DTT, 1 µl RNaseOUT and 200 U of SuperScript III™ Reverse Transcriptase (Promega, Madison WI). 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. Following a 60 min incubation at 50 °C, samples were heated to 70°C for 15 min to inactivate the enzyme.

qPCR was performed with a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA) using SYBR Green mix (Roche Indianapolis, IN), to which 10 nM fluorescein (Bio-Rad) was added to allow well-factor correction. The total reaction volume of 20 µl included 300 nM of primers and 2 µl of template. PrimerQuest software (IDT DNA, Coralville IA) was used to design primer sequences, as listed in Table 4.1. Cycling parameters were 10 min at 95°C

followed by 50 cycles of 95 °C for 15 sec, 58 °C for 30 sec, and 72 °C for 30 sec. Analysis of melt curves verified that only one product was amplified in each reaction.

mRNA expression for each developmental stage was evaluated in four to six independent experiments, each with three replicates per primer pair. Each experimental plate included three wells with primers for a reference gene, which in all cases was 18S rRNA. After subtracting the baseline value with software provided by Bio-Rad, the cycle threshold (C_t) values were set to give a Ct 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 and to define relative gene expression profiles in relation to the reference gene (Mimmack et al. 2004). Values for fold change in expression are given as mean ± SEM. Minitab software was used to assess significance with a One-Way Student's T test (H_0: mean = 1; H_a: mean ≠ 1) (Minitab, Inc., State College, PA).

Results

Of the 288 clones sequenced from the upregulated library, 78 had a significant homology with a sequences 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 Expressed Sequence Tags (ESTs) showed significant homology with sequences in GenBank, with 68% representing unique genes. Identification of all 233 genes with significant homology to known genes is found in Appendix 1. A total of 33 sequenced ESTs were selected for further analysis with qPCR to quantitatively estimate the abundance of these transcripts in prediapause 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 (see Chapter 3). Eleven of the transcripts were from the upregulated library (Table 2), while 22 were from the downregulated library (Table 3). To facilitate the evaluation, these 33 transcripts were separated into six functional groups.

Stress Proteins and Chaperones

Eight transcripts were selected that encode proteins that 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 correlated with increased cold tolerance in *Drosophila* (Misener 2001). This mRNA increases by 58 ± 16 % ($P = 0.017$) in pre-diapause and is downregulated by 69 ± 4.4 % in diapause ($P < 0.001$) when compared to non-diapause embryos (Fig. 1A). In addition, mRNA encoding Δ9-acyl CoA desaturase (desaturase), which converts saturated fatty acids to monounsaturated fatty acids (MUFAs), is upregulated by 240 % ($P = 0.021$) in pre-diapause embryos (Fig. 4.1A). There was no difference in amount of this transcript in diapause embryos ($P = 0.16$).

Glyoxalase has been reported to protect cells from oxidative stress (Sommer et. al. 2001), and its transcript is downregulated by 32 ± 6.9 % ($P = 0.02$) in pre-diapuse embryos and 80 ± 4.0 % ($P < 0.01$) 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 is no difference in the mRNA abundance of a BI-1 homolog in pre-diapause embryos compared to non-diapause embryos ($P = 0.28$), but an 86 ± 3.0 % ($P < 0.01$) reduction in the abundance of this transcript occurs during diapause (Fig. 4.1A).

The transcript abundance of six heat shock proteins were examined (Fig. 4.1B). 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 to non-diapause embryos, only CHORD is differentially expressed in pre-diapause embryos, where it is upregulated by 44 $\pm$ 8.9 % ($P = 0.016$).

Hsp20.7 ($P = 0.6$), Hsp70 ($P = 0.72$), Hsp90 ($P = 0.24$), and endoplasmin ($P = 0.42$) show comparable levels of mRNA in pre-diapause and non-diapause embryos. In diapause embryos, Hsp20 and Hsp90 mRNAs are reduced by approximately 60 % ($P = 0.005$ and $P = 0.008$, respectively); endoplasmin expression is downregulated by 86 $\pm$ 3.0 % ($P = 0.002$). The mRNA level of Hsp70 is the same in diapause and non-diapause embryos ($P = 0.08$).

Energy Production and Conversion Proteins

Cytochrome c oxidase subunit II (COX II), COX IV, COX VII, and arginine kinase (AK) are involved in the production of ATP; and capthesin B-like protease, ATP-citrate lyase (ACLY), aldo/keto-reductase (AKR), 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. As seen in Fig.4.2A, compared to non-diapause embryos, the transcript abundances of COX II and AK are downregulated in pre-diapause embryos by 31 and 36 %, respectively ($P \leq 0.01$ for both). There is no change in the expression of COX VII ($P = 0.9$), but the mRNA abundance of COX IV increases 40 $\pm$ 8.4 % ($P = 0.02$). When mRNA levels in diapause embryos are compared to values for non-diapause controls, all four of these genes are downregulated by values ranging from about 36 % for COX II to 86 % for AK (COX II $P = 0.03$, COX IV $P < 0.01$, COX VII $P = 0.01$, and AK = $P < 0.01$).

Table 4.1. Primers sequences used for quantitative PCR

Primer name (Clone ID)	Forward Sequence	Reverse Sequence
18S Ribosomal RNA	5'-TCTCAGTGCAAGCCGAAGTAAGGT	5'-TCCCTTCCATTGCTGGTCGAGATT
Hsp90 (S2P1WA8)	5'-AACTGCCCTCCTATCTTCTGGCTT	5'-TCTTCACCTTCCTGCGCATCATCT
Endoplasmin (S2P4WF12)	5'-GGACAGTTTGGCGTTGGCCTTTAT	5'-CAAGGTATTACCACGTGGGTCTTCAG
Hsp20.7 (S1P1WE3)	5'-CTCAACAGTCTTCTCAACATTCTTCTTAGG	5'-AGGAACGTCCAATTCCAATCATCC
Hsp70 (S2P3WC11)	5'-ACTCCCACACAGGAGTATGTGGTT	5-GTGAACGAACAAGAGTGCGGAGAA
ATP citrate synthase (S2P1WH7)	5'-ACTGCTGATCATGGACCTGCTGTA	5'-AGCACCATCTAGAGCACCACCAAA
CapthesinB-like protease (S2P1WG9)	5'-AGCATGATCCGCAAGATCCCTGAT	5'-CATTCATCGGCTGATGGGTGTTCA
Tranlation elongation factor-1γ (S2P2WF11)	5'-AAGGCTGGAACCTTTCCAAGAGGA	5'-AGGTGCTCATTGCTGCCTCTTACT
Cytochrome p450 (S2P2WF1)	5'-GGACTTGCGTGTTAAGTTAAGCCCAG	5'-ATCACGGGCAGCGTCATCAAAGTA
Histone 2A-pp1 (S2P4WD5)	5'-TAGACCCGGATGTTGACCCTGTTT	5'-ATTGGAGTTAGCTGGCAATGCTGC
Histone 2A pp2 (S2P4WD5)	5'-ATTACTGCAGCCAAATACACCGGC	5'-TCAGGAAGAGGCAAAGGAGGCAAA
Desaturase pp1 (S2P4WD6)	5'-ATGGCCTCTGCGACTTCTATTGGT	5'-TTGTGTGGATCGGCATCAGTCTCA
CHORD Containing Protein (S1P3WB2)	5'-TAAGGGCTGCACAAGGTCGTATCA	5'-CCTTGACAATTGCTGCAGGTGCTT
Bax Inhibitor pp1 (S2P4WB5)	5'-CTTCAGCCTCTGCGTTTACGCTTT	5'-ATCGAGAAGCGTCGAAGTGGTGAT
RACK 1 pp1 (S2P4WA4)	5'-ATCTCTACAGCTTCGAGGCACCTT	5'-CATCACGAGTGAGTTTCCACACGA
RACK1 pp5 (S2P4WA4)	5'-TCTCTTTCCAGCGTGGCCATTAGA	5'-CCTCGAAGCTGTAGAGATTCCGACAT
Nedd8 (S1P3WF8)	5'-GGAGGATCGGCTTCTTCATTTGGT	5'-AGCATAGGCAATCTGAGCACGCTA
CG1532-PA /glyoxalase (S2P2WA12)	5'-GGATGGCCCTTTACTGAAGAAGGA	5'-TGCGAGGTAGGCTGAGGTTCATTT

Table 4.1 cont.

Primer name (Clone ID)	Forward Sequence	Reverse Sequence
Mitochondrial processing peptidase (S2P1WG6)	5'-ATACAACAGGACGGGCTTCCAAGT	5'-GGCTGGCAAAGTTGGAGATGTTGA
Translation initiation factor 4 pp1 (S2P2WF8)	5'-AAAGCCTGGGTCTGTGTGATGAGA	5'-TGAGATCCTTGAGGAGCGTGGTTT
IFRD pp2 S2P4WH4	5'-ATCAGGTGGCTCTCCATCCTCAAT	5'-AGAGGACATGACTCCTCAGTTGGT
TIF pp2 S2P2WF8	5'-GGTCTGTGTGATGAGACGGATCCAAA	5'-TGAGATCCTTGAGGAGCGTGGTTT
RACK1 pp1 (S2P2WA4)	5'-ATCTCTACAGCTTCGAGGCACCTT	5'-CATCACGAGTGAGTTTCCACACGA
RACK1 pp5 (S2P2WA4)	5'-TCTCTTTCCAGCGTGGCCATTAGA	5'-CCTCGAAGCTGTAGAGATTCCGACAT
TFDp2 pp3 (S2P4WH9)	5'-CAGTCTGCTGAGTAGGAGTTAAGCGT	5'-TCCATTTCAGTCACCTGGTGGAAC
RpL45 pp2 (S1P2WD5)	5'-AGTCGGCGGAGATCTATTGCATGT	5'-GGATTCCAGTGTTTCGAGCCTCTT
eEFtB-γ (S2P2WF11)	5'-AAGGCTGGAACCTTTCCAAGAGGA	5'-AGGTGCTCATTGCTGCCTCTTACT
Spaghetti squash (S2P1WG11)	5'-GGAAATGAGGGCAGTAAGGCACTA	5'-ATCGTGAAGCTCCAATCAAGGGTG
CHORD containing protein (S1P3WB2)	5'-TAAGGGCTGCACAAGGTCGTATCA	5'-CCTTGACAATTGCTGCAGGTGCTT
COX subunit II (S2P1WG7)	5'-TTCCGTCTTCTCGATGTTGACAACCG	5'-CGTCCTGGTGTAGCATCAGATTTAACCC
COX subunit IV (S2P3WF12)	5'-TGCCCGAATCCTTTACTGATGAGC	5'- CCCACTTGGATGTCAATCCATCAAC
COX subunit VII (S1P3WA12)	5'-TTCTTGGAGTCGCTGTGGACATCA	5'-TGGCTCTGGGTTTAGTGCTTCCTT
Arginine kinase (S2P4WD11)	5'-TGCTTCTGTGCACATCAAGGTTCC	5'-AAACTCCACCCTCAGCTTCTGTGT
Lipid storage protein pp3 (S1P3WA6)	5'-TCATATGTTCCACTGTCCCAGCAA G	5'-AGCTATTTCTTCCCTGGCACTGAC

Table 4.1 cont.

Primer name (Clone ID)	Forward Sequence	Reverse Sequence
RpL35 pp1 (S1P2WA5)	5’-TCCACTTGGTTGGTGTTGAGGTCT	5’-AGATGTTGCATCTACGTCGGCCAT
Reptin (S1P2WH3)	5’-GCCATCTCATTCTCCAGAGCACGATT	5’-TGAGTGGCGTGAAGAAGGGAAAG
Aldo/keto Reductase (S2P1WB10)	5’-AGGCCCATGGAGGAACAAGTGAAT	5’-GCCACAAACTGTAATGCCCAAACG
Polymerase III (S1P2WE8)	5’-ATGGACAGAAAGGTGTTACAGGGC	5’-TGATCCACCAAATGCTGTCCCA
Pyrolline carboxylate reductase (S1P1WF2)	5’-TCTCCCAATTCTACGGAGCGCTTT	5’-TGGTATTGGAAACAGGCAAGCACC
Male sterility protein (S1P1WG7)	5’-GGTCCGGCGATACCTCAATAAAGA	5’-TATACTATCACGGGCACCCAAGCA
Lipid metabolism (S2P3WE12)	5’-AAGACGTGTCCAGGGCTTATCGTA	5’-TGTCGGCTGCCATTATGACTACCT

Table 4.2. ESTs for transcripts identified from the upregulated library with significant BLAST matches.

Clone ID	Probable Homology	Closest Genbank Match	Species	E-value[a]	Identity[b]
S1P1WE3	Hsp 20.7	ABC84494	*Locusta migratoria*	1e-52	63%
S1P3WB2	CHORD containing protein	XP_967567	*Tribolium castaneum*	1e-40	69%
S1P2WH3	RubV-like 2 (Reptin/TBP interacting protein)	XP_001122537	*Apis mellifera*	3e-43	79%
S1P2WE8	DNA dependent RNA polymerase III	XP_625037	*Apis mellifera*	2e-77	84%
S1P1WF2	Pyrroline 5-carboyxlate reductase	XP_974776	*Tribolium castaneum*	9e-30	73%
S1P1WG7	Male sterility protein(acyl Co-A reductase)	EAT41033	*Aedes aegypti*	2e-39	53%
S1P2WA5	Ribosomal protein L35Ae	CAJ17420	*Carabus granulatus*	7e-23	66%
S1P3WA6	Neimann-Pick type C-like	XP_624310	*Apis mellifera*	2e-14	52%
S1P3WA12	Cytochrome c oxidase VIIc	XP_970390	*Tribolium castaneum*	9e-13	79%
S1P2WD5	Mitochondrial ribosomal protein L45 (TIMM 44)	EAT35833	*Aedes aegypti*	1e-28	41%
S1P3WF8	Nedd8	XP_972922	*Tribolium castaneum*	2e-19	98%

[a] 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.

[b] Identity indicates the percentage of amino acids that are identical in the query and result sequences.

Table 4.3: ESTs for transcripts isolated from the downregulated library with significant BLAST matches.

Clone ID	Probable Homology	Closest Genbank Match	Species	E-value	Identity
S2P1WA8	Heat shock protein 90	AAS45246	*Locusta migratoria*	1e-53	89%
S2P4WF12	Endoplasmin (Molecular chaperone – Heat shock 90 family)	EAT34979	*Aedes aegypti*	5e-52	80%
S2P3WC11	Heat shock 70 family member	AAO21473	*Locusta migratoria*	1e-36	90%
S2P1WG9	Capthesin B-like protease	NP_572920	*Drosophila melanogaster*	1e-59	60%
S2P1WH7	ATP citrate lyase	EAT44342	*Aedes aegypti*	8e-87	95%
S2P2WF11	Translation elongation factor -1 gamma	AAL78751	*Locusta migratoria*	3e-44	85%
S2P4WD6	Fatty acid desaturase	XP_967943	*Tribolium castaneum*	2e-62	76%
S2P2WF1	Cytochrome P450	AAK57914	*Blattella germanica*	6e-10	40%
S2P4WD5	Histone 2A	XP_307083	*Anopheles gambiae*	8e-48	99%
S2P3WA4	RACK1	NP_001041703	*Bombyx mori*	7e-72	93%
S2P2WF8	Eukaryotic translation initiation factor 4 gamma	EAT40334	*Aedes aegypti*	1e-48	60%
S2P4WH4	Interferon developmental regulator	XP_392883	*Apis mellifera*	5e-39	49%
S2P1WG11	Spaghetti squash (Myosin regulatory light chain)	XP_623372	*Apis mellifera*	4e-35	92%
S2P4WB5	Bax-inhibitor 1-like protein	ABM55570	*Maconellicoccus hirsutus*	8e-26	80%
S2P1WG7	Cytochrome c oxidase subunit II	AAU11284	*Allomenobius socius*	2e-39	87%
S2P4WD11	Arginine kinase	AAT77152	*Periplaneta americana*	1e-63	96%
S2P4WH9	Transcription factor Dp-2 (E2F dimerization partner)	XP_393377	*Apis mellifera*	2e-27	54%
S2P2WA12	Glyoxalase CG1532-PA	XP_625100	*Apis mellifera*	2e-40	67%
S2P3WF12	Cytochrome c oxidase subunit IV	AAY66918	*Ixodes scapularis*	2e-05	56%
S2P1WG6	Mitochondrial processing peptidase	XP_624556	*Apis mellifera*	9e-35	66%
S2P1WB10	CG2767-PA; Aldo/keto reductase family	XP_974785	*Tribolium castaneum*	1e-07	40%

[a] 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.

[b] Identity indicates the percentage of amino acids that are identical in the query and result sequences.

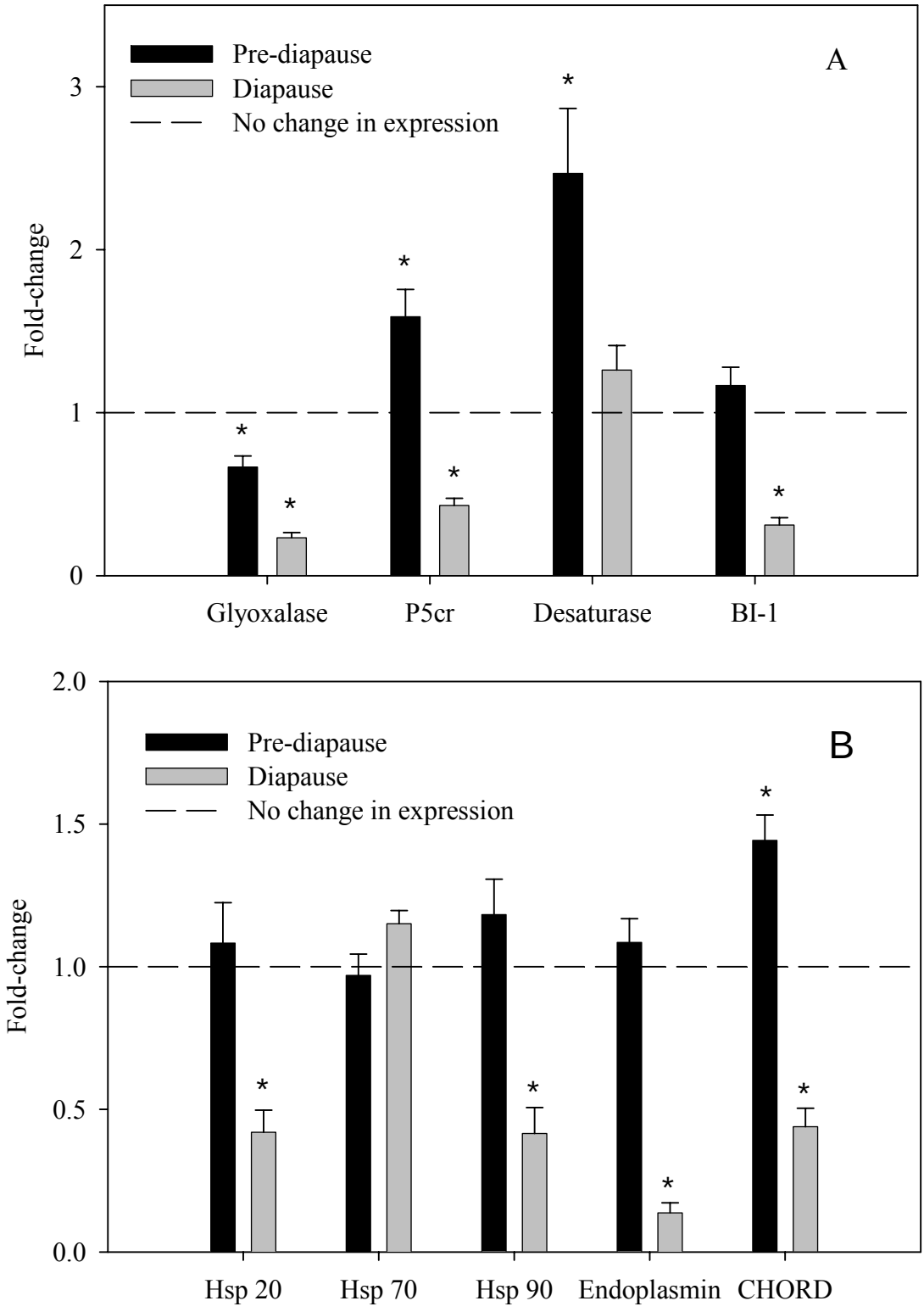

Figure 4.1. mRNA expression profiles for genes encoding (A) stress proteins and (B) chaperones in pre-diapause and diapause embryos. Mean ± SEM for 3-6 replicates; bars marked with an asterisk show significant change in transcript level compared to non-diapause embryos (One-way T-test; $P < 0.05$).

Figure 4.2B 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 capthesin-B ($P < 0.01$), ACLY ($P = 0.05$), LMP ($P = 0.01$), and MSP ($P < 0.01$) are downregulated from 24-40 % in pre-diapause embryos. Compared with non-diapause embryos AKR is upregulated 70 % ($P = 0.05$). NPC2 is not differentially expressed in pre-diapause embryos ($P = 0.6$). In diapause embryos, five out of six genes in this group are strongly downregulated by amounts ranging from 82 % (for Capthesin B; $P < 0.01$) to 98 % (for AKR; $P < 0.01$) compared to non-diapause embryos.

DNA Replication, Cell Cycle Regulation and Transcription

Developmental arrest is a hallmark feature of embryonic diapause (Chapter 3), and thus four genes were evaluated whose products have some role in cell division. Histone 2A is part of the nucleosome and is important for chromosome packaging. As seen in Fig 4.3 there is a moderate but significant 20 ± 2.0 % decrease in the amount of Histone 2A mRNA ($P = 0.02$) in pre-diapause embryos compared to non-diapause embryos, and there is an 86 ± 1.0 % decrease in this mRNA ($P < 0.01$) in embryos that have been in diapause for 10 d. *Spaghetti squash* encodes a cytoplasmic myosin that is required for cytokinesis in dividing cells of *Drosophila* (Young et al. 1993; Wheatley et al. 1995). However, this gene is not differentially expressed in pre-diapause ($P = 0.45$) or diapause embryos ($P = 0.06$)

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 (Zhen et al. 1999). There is a 99 ± 1.5 % increase in TFDP2 ($P = 0.02$) in pre-diapause embryos (Fig. 4.3), but this transcript's

abundance in diapause embryos is not significantly different from that of non-diapause embryos (P = 0.33). 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 is a 76 ± 1.8 % increase in Reptin mRNA (P = 0.03), and a 71 ± 3.0 % drop (P < 0.01) is observed in diapause embryos compared to non-diapause embryos.

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 (Hand and Hardewig 1996). RNA polymerase III (Pol III), which transcribes small ribosomal RNAs and transfer RNAs that carry amino acids to ribosomes during translation increases almost 70 % (P = 0.02) in pre-diapause embryos compared to non-diapause embryos (Fig. 4.4) and decreases 47 ± 2.0 % (P < 0.01) in diapause embryos. Only one of six transcripts encoding proteins involved in translation and post-translational modification shows a significant decrease in the abundance of their mRNA in pre-diapause embryos compared to non-diapause embryos (Fig. 4.4). Ribosomal protein L35a (RpL35a) is reduced approximately 40 % (P = 0.03). There is a small but significant increase of 19 % in mitochondrial processing peptidase (MPP) (P=0.05). There are no significant changes in the mRNA levels of translation elongation factor 1B-γ (eEF1B-γ; P = 0.86), translation initiation factor 4-γ (eIF4-γ; P = 0.26), ribosomal protein L45 (RpL45; P = 0.95), or Nedd8 (P = 0.42). However, all of the transcripts related to protein synthesis are significantly depressed by 47 – 80 % in diapause embryos compared to non-diapause embryos. eEF1B-γ is reduced 61 ± 3 % (P < 0.01), eIF4-γ by 57 ± 6.0 % (P = 0.01), RpL35a by 80 ± 1.2 % (P < 0.01), RpL45 by 56 ± 1.2 % (P < 0.01), Nedd8 by 75 ± 3.0 % (P <0.01), and MPP by 60 ± 3.0 % (P < 0.01).

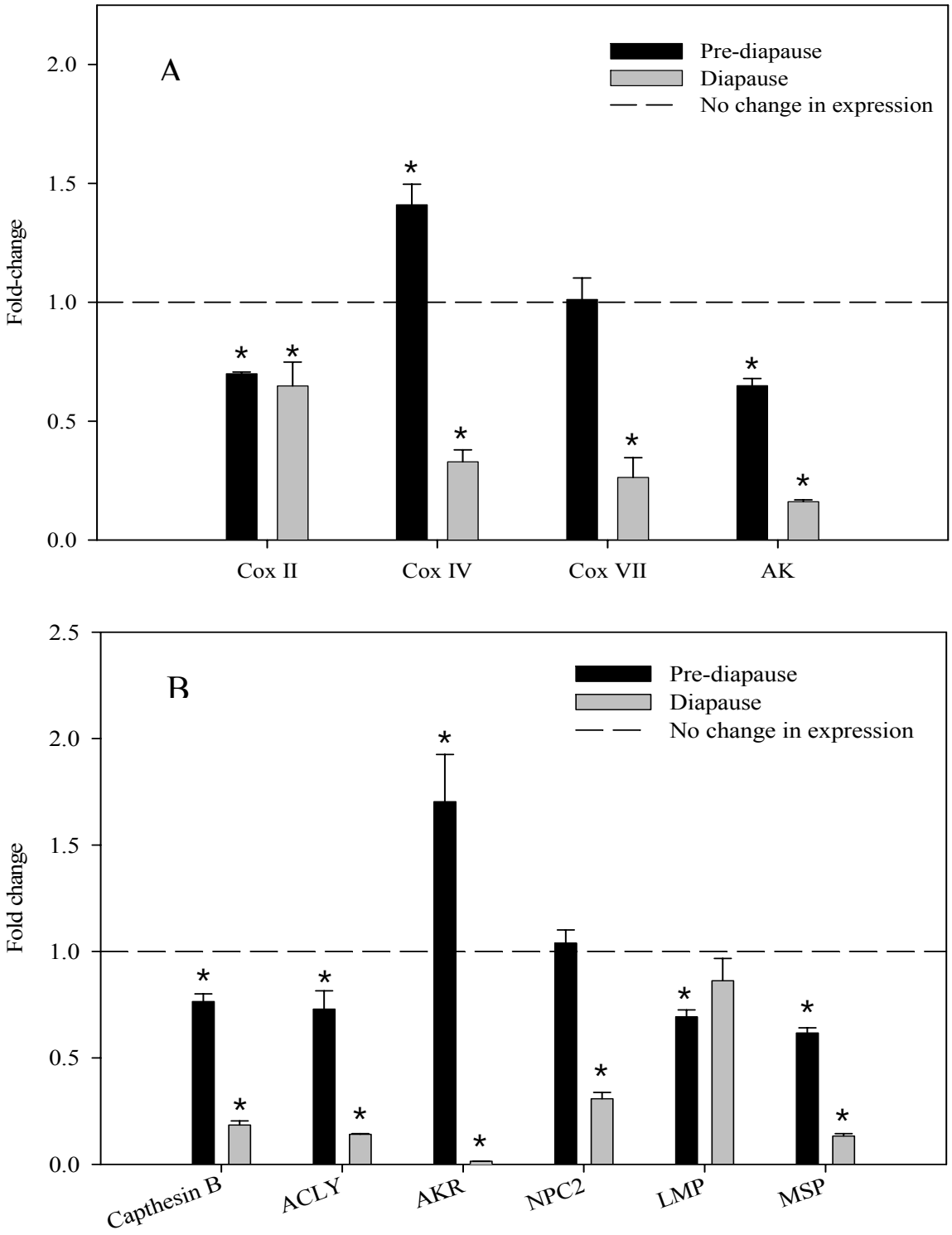

Figure 4.2. mRNA profiles for genes encoding proteins involved with energy production and conversion in pre-diapause and diapause embryos. Mean ± SEM for 3-6 replicates; bars marked with an asterisk show a significant change in transcript level compared to non-diapause controls (One-way T-test; $P \leq 0.05$). C_t values were corrected for 18S rRNA.

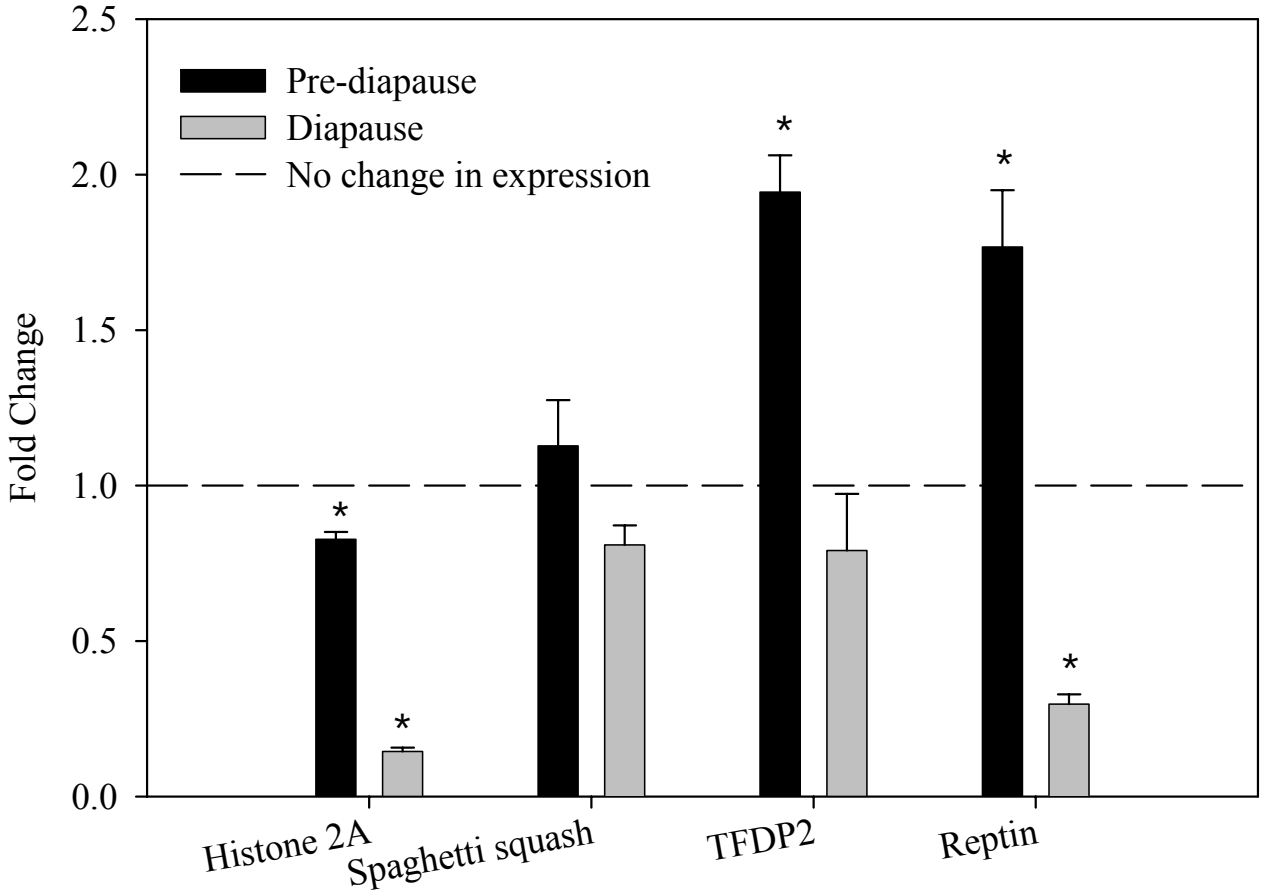

Figure 4.3. mRNA profiles for genes encoding proteins that are involved DNA replication, cell cycle regulation for pre-diapause (2-4 d post-oviposition) and diapause embryos compared to non-diapause embryos Mean ± SEM for 3-6 replicates. Bars marked with an asterisk show a significant change in transcript level compared to non-diapause embryos (One-way T-test; $P < 0.05$). Dotted line indicates no change in transcription.

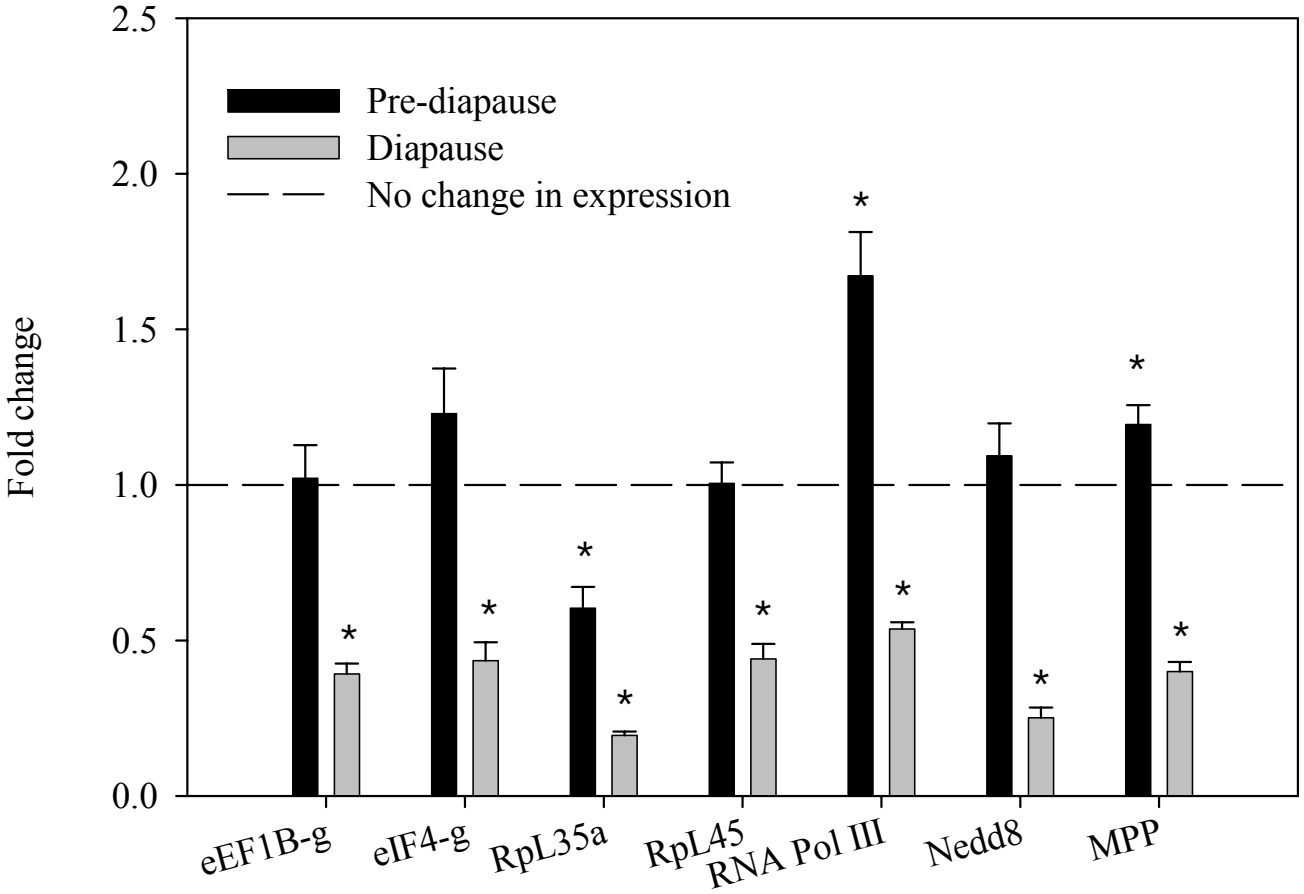

Figure 4.4. mRNA profiles of genes encoding proteins involved in transcription, translation, and protein processing in pre-diapause and diapause embryos. Mean ± SEM for 3-6 replicates. Bars marked with an asterisk show significant change in transcript abundance (One-way T-test; $P \leq 0.05$). Dashed line marks a fold change of 1, which indicates no change in expression.

Endocrine and Signal Transduction

Figure 5 illustrates the expression profiles of selected signaling molecules. All of the transcripts evaluated are significantly upregulated in pre-diapause embryos compared to non-diapause embryos. Cytochrome P450 (CYP450), which may be involved in ecdysone synthesis, is upregulated 203 % ($P = 0.03$). Interferon related developmental regulator (IFRD), which is homologous to mammalian genes Tis7 and PC4 and is required for muscle differentiation (Micheli et al. 2005), is 40 ± 13 % higher ($P = 0.03$) in pre-diapause embryos. Activated protein kinase c receptor (RACK1), which is reported to have a role in ecdysone signaling (Quan et al. 2006) is moderately, but significantly, upregulated 23 ± 2.1 % ($P =$ 0,01). In diapause embryos, the abundance of CYP450 is reduced 50 % ($P = 0.04$) compared to non-diapause embryos. IFRD is reduced 45 ± 2.2 % ($P < 0.01$), and RACK1 is downregulated by 67 ± 1.0% ($P < 0.01$).

Discussion

PCR-based subtractive hybridization and qPCR show 11 genes to be significantly upregulated in pre-diapause *A. socius* embryos and 9 genes to be downregulated compared to morphologically similar non-diapause embryos. Based on the functional arguments discussed below, four upregulated genes (CYP450, AKR, 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 ACLY and Capthesin B-like protease. Embryos that have been in diapause for approximately 10 d exhibit a very different expression profile compared to pre-diapause embryos. In these embryos, 87 % of the genes examined were downregulated and 13% were expressed at the same levels as non-diapause embryos. It is notable that upregulated genes were not found in these diapause embryos,

because analysis of other insect species shows late diapause to be characterized by both up- and down-regulated genes (Denlinger 2002; Robich et al. 2007). One interpretation 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 needed after 10 d of diapause. It could be argued that an overall depression in transcription (and therefore translation) is consistent with a physiological state like diapause where energy conservation and developmental arrest are advantageous (Hand and Hardewig 1996; Hahn and Denlinger 2007).

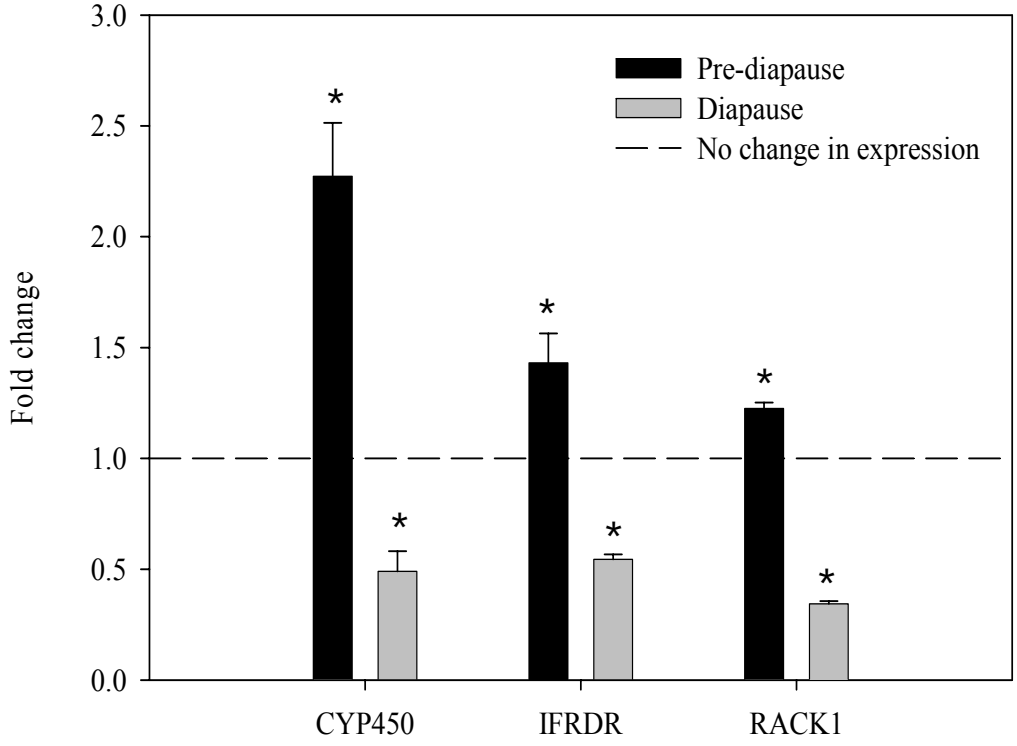

Figure 4.5. mRNA profiles of genes encoding endocrine and signaling proteins in pre-diapause and diapause relative to non-diapause embryos. Mean ± SEM, N= 3-6. Bars marked with an asterisk are show significant change transcript abundance (One-way T-test; $P < 0.05$). C_t values were corrected for 18S rRNA. Dotted line demarcates a fold change of 1 which indicates no change in expression.

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). However, upregulation of stress proteins is not a general feature of diapause in *A. socius* because only 3 of 9 genes analyzed increased in pre-diapause embryos. Two of the genes that are upregulated in pre-diapause embryos, P5cr and Δ9desaturase, are also upregulated in some overwintering insects. P5cr is involved in the final step in proline biosynthesis. 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 (Gracey 1996; Cossins 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 (Hochachka and Somero 2002). 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 ∝-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 type of 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* while Hsp90 is downregulated (Denlinger et. al 2002). In 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. Hsp20.7, Hsp70, Hsp90, and endoplasmin are not differentially expressed in pre-diapause or diapause embryos; thus upregulating these genes is not a requirement for diapause entry in this species.

Energy Conversion and Metabolism

Although metabolic depression is a defining characteristic of diapause for many species, previous research shows that *A. socius* embryos that have been in diapause for approximately 10 d consume oxygen at the same rate as morphologically similar non-diapause embryos (see Chapter 3). However, the several fold ontogenetic increase in aerobic metabolism observed in non-diapause embryos is blocked during diapause. It is appropriate to note that a decrease in protein amount is only one potential mechanism for downregulating metabolism. Additional mechanisms, including allosteric inhibition and covalent modification do not

necessarily require changes in enzyme level. Thus, it was of interest to evaluate the contribution of differential expression of metabolic enzymes during diapause.

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 a system to buffer ATP levels in insects (Tankak et al. 2007). Downregulation of this enzyme is consistent with data that show low levels of ATP in pre-diapause and diapause *A. socius* (Chapter 3). 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 of the COX complex in mammals and yeast (Aggler and Capaldi 1990; Youfen 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. These data are consistent with measurements of oxygen consumption in diapause and non-diapause embryos that suggest the ontological increase in aerobic metabolism observed for non-diapause embryos is blocked upon entry into diapause.

The majority of genes involved in interconversion of cellular energy reserves are downregulated in pre-diapause embryos. Capthesin B is involved 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* would prevent 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 are fatty acid/lipid usage enzymes that are all downregulated approximately 20 % in pre-diapause embryos. NCP2 is a lipid storage protein that does not show differential

expression in pre-diapause embryos. With this one exception, the data of this study are consistent with conservation of lipids during diapause. Previous studies on *Sarcophaga crassipalpis* suggest that pupae that enter diapause have larger lipid stores than non-diapause pupae (Adedokun and Denlinger 1985). Finally, it is notable that ACLY and NCP2 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).

AKR, a member of the aldo-keto reductase superfamily, is upregulated 70 % in pre-diapause embryos. The AKR family includes more than 120 members that have a wide range of physiological functions including steroid metabolism, xenobiotic detoxification, and potassium channel regulation (Sieglaff et al. 2005, Luccio et al. 2006). The abundance of this transcript is depressed dramatically by 98 % during diapause. Coupled with its significant upregulation during pre-diapause, AKR displays the largest differential expression of any gene in the study. Consequently, it appears that the function of this gene product is necessary for diapause entry but is not required to maintain the dormant state.

DNA Replication, Cell Cycle Regulators and Transcription Factors

Developmental arrest is a hallmark of diapause, and development is arrested 3-4 d post-oviposition when *A. socius* embryos enter diapause (Chapter 3). Histone 2A, which is downregulated in pre-diapause embryos, is a core histone protein and is essential to the structure of condensed chromatin. The amount of histone proteins, in general, are tightly regulated to ensure an equal amount of histone proteins and DNA in each cell. 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. 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 2-fold in pre-diapause embryos, 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. Specifically, the expression 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), so 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.

Endocrine and Signal Transduction.

Diapause in insects is, in part, regulated by the endocrine system (Nijhout 1984; Yamashita and Hasagawa 1985; Denlinger 2002). In many species, embryonic diapause results from a decrease in ecdysone (e.g., Gharib et al. 1981) and/or juvenile hormone, a sesquiterpenoid (e.g., Neumann-Visser 1976). Incubating diapause embryos with exogenous ecdysone and juvenile hormone terminates diapause in at least two species of locusts (Kidokoro et al. 2006). RACK1 and CYP450 are two genes that are part of the ecdysone signaling pathway and are upregulated in pre-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).

CYP450 encodes an ecdysone 20-hydroxylase and is part of the ecdysone biosynthesis pathway (Wen and Scott 2001). Specifically, CYP450 converts ecdysone to 20-hydroxyecdysone, the active form of the hormone (Chavez 2000; Horike et al. 2000; Wen and Scott 2001). It is surprising that CYP450 is upregulated 2.4-fold in pre-diapause

embryos because embryonic diapause is typically correlated with a decrease, or lack, of ecdysone. Indeed, ecdysone activity and amount are known to be reduced during diapause in embryos of several species including *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). Future studies that quantify the amount of ecdysone, 20-hydroxyecdysone, and their conjugates are required to ascertain the role of this gene in the entry into diapause in *A. socius* embryos.

In summary**,** six candidate genes have been identified that show promise as regulators of diapause entry in *A. socius* embryos and warrant additional study. Reptin and TFDp2 are upregulated almost 2-fold in pre-diapause embryos. Reptin forms a complex with HATs epigenetically represses transcription by altering chromatin structure (Qi et al. 2006) which could lead to a general reduction in transcription. TFDp2 is a transcription factor kown to interact with cell proliferation regulators such as cyclinA, cyclinE, and c-Myc, and it can repress cell division if Rb is complexed with the TFDp2-E2F heterodimer. Analyzing transcript levels of E2F, Rb, c-Myc, cyclinA, cyclinE and other genes known to complex with TFDp2 and/or be targets of TFDp2-E2F should improve our understanding of mechanisms underlying developmental arrest. CYP450 and AKR are predicted to have a role in ecdysone metabolism. For the majority of embryos that have been studied, diapause is considered to result from a lack of ecdysone – not a peak in hormone activity. Quantification of ecdysteroids would solidify the predictions emerging from the current study on of the role of hormones in diapause entry in this species.

Capthesin B-like protease and ACLY are two downregulated genes that hold promise for a significant role in diapause entry. Capthesin B-like protease is a yolk digestion protein;

directly assessing nutrient storage pools like yolk could explain the downregulation of capthesin B-like protease during diapause. ACLY may be a key gene in juvenile hormone synthesis. As JH is commonly reduced or even absent in diapause embryos, downregulation of this gene may be essential to diapause entry. Measuring the temporal titers of JH during prediapause and diapause would test this prediction. These and other future experiments should complement the present work to provide a more comprehensive picture of the molecular regulation of embryonic diapause.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

This dissertation is by no means the first study of diapause in arthropods; researchers have been interested diapause since the 1920s (Lees 1955). However, only a few studies address mechanisms that regulate diapause. While the research discussed in the previous data chapters begins to build a foundation for understanding diapause mechanistically, considerable work remains before a comprehensive picture of diapause will exist.

Chapter 2 shows that mitochondria in diapause *Artemia* embryos appear to be well differentiated and are structurally similar to those in post-diapause embryos. In addition, mitochondria isolated from diapause embryos are functionally similar to post-diapause embryos that have been developing for 8 h. RCR values for diapause embryos are greater than or equal to those calculated for post-diapause embryos, and P:O flux ratios are statistically equivalent in both states. Therefore, the acute metabolic depression that characterizes diapause in this species does not result from intrinsic changes in the mitochondria. However, the mechanisms that do regulate the reduction in O_2 consumption remain unknown. Future studies on this species will test the hypothesis that metabolic depression is the result of substrate (e.g, pyruvate) limitation. Specifically, assays will test the hypothesis that pyruvate is prevented from entering the TCA cycle because the pyruvate dehydrogenase complex (PDC) is inhibited *in vivo*. Carbohydrate is the sole metabolic fuel for *A. franciscana* embryos at this stage in development; respiration will cease if carbon compounds from glycolysis are not available to the mitochondrion. The inhibition of pyruvate utilization by PDC could be caused, for example, by a diffusible inhibitor that is

removed during mitochondria isolation or by an altered phosphorylation state of the protein complex that occurs during isolation. These mechanisms could be addressed experimentally by isolating mitochondria under conditions that preserve the phosphorylation state of PDC and by experiments where mitochondrial-free extracts prepared from diapause embryos, which could contain an inhibitor, are incubated with mitochondria from active embryos.

Taken together, Chapter 3 and 4 provide an integrative examination of diapause in *A. socius* embryos. Chapter 3 shows that diapause embryos are morphologically similar to non-diapause embryos 3-4 d post-oviposition, and the quantity of DNA measured for diapause embryos provides additional evidence that development is during this time frame. Unlike *Artemia* embryos, metabolic depression is not a feature of diapause in *Allonemobius socius* embryos. Rather, embryos that have been in diapause for approximately 10 d consume O_2 at the same rate as morphologically similar non-diapause embryos. One notable characteristic of early embryonic development in this species is a high [AMP]:[ATP] ratio. It is unlikely that the unusual adenylate status is a feature of the entry into diapause because it is observed in both diapause and non-diapause embryos. Although high levels of AMP are suggestive of oxygen limitation in insects, an increase in the percent O_2 in the incubation environment only partially reduces [AMP] and does not significantly lower the amount of lactate in these embryos. Thus, the atypical set points for metabolic poise are an ontogenetic feature of these embryos and are not completely explained by oxygen limitation.

As seen in Chapter 4, gene expression in pre-diapause embryos (2-4 d post-oviposition) is defined by a unique pattern of up- and downregulated transcripts. However, there is a very different pattern of gene expression observed for embryos that have been in diapause for 10 d. In these embryos, the majority of the 33 genes I analyzed are substantially

downregulated. Two downregulated genes, ACLY and capthesin B-like protease, are predicted to be important for the entry into and maintenance of diapause. ACLY is part of the juvenile hormone synthesis pathway. Lack of this hormone is important for diapause entry in a number of insect species. Capthesin B-like protease degrades yolk proteins; downregulation of this gene is predicted to prevent the depletion of an important fuel source during diapause. Four genes that are upregulated in pre-diapause embryos are predicted to have a role in regulating diapause entry. TFDp2 is a transcription factor with the potential to arrest DNA synthesis and cell proliferation. TFDp2 dimerizes with E2F which represses the G_1-S transition when Rb is also present. Reptin is part a complex that plays a role in epigenetic silencing in *Drosophila* (Qi et al. 2006). CYP450 and AKR are also predicted to have a significant regulatory function in pre-diapause embryos. CYP450 is part of the endocrine system in insects in that it converts ecdysone to 20-hydroxyecdysone, which is the active part of a hormone that is known to regulate diapause. AKR encodes a member of a protein superfamily whose members have been reported to have numerous functions, including ecdysone synthesis. Upregulation of CYP450 and AKR suggest ecdysone regulates the entry into diapause in *A. socius* embryos.

Future research on embryonic diapause in *A. socius* will build on the foundation established in Chapters 3 and 4. In particular, I plan to further examine metabolism in *A. socius* embryos. There is not an endogenous metabolic depression in this species, but without some way to modulate O_2 consumption these embryos will deplete nutrient reserves needed for post-diapause development. I predict that metabolism in this species slows in response to low temperatures during the winter months. A number of researchers have shown that insects in diapause remain responsive to changes to temperature. While in

diapause, both the gall fly, *Eurosta solidaginis* (Irwin and Lee 2000, 2003) and the rose galling wasp, *Diplolepis spinosa* (Williams et al. 2003), consume oxygen at lower rates when exposed to cold temperatures than when incubated at warm temperatures. In these cases, metabolic rates are reduced through the standard Q_{10} effect of temperature on reaction rates (Guppy and Whithers 1999). Q_{10} effects reduce metabolic rate two- to three-fold for every 10 ^{o}C drop in temperature (Whithers 1992). One future study on *A. socius* embryos will test the hypothesis that low temperature makes a substantial contribution of metabolic depression in these embryos.

As mentioned above, I have identified two genes that are upregulated in diapause and are predicted to regulate developmental arrest, reptin and TFDp2. Because the predicted activities of the proteins encoded by these two genes depend on the presence of members of larger complexes, future research on reptin and TFDp2 will analyze the transcript abundances of their binding partners. For example, I predict that the abundance of transcripts encoding histone acyltransferases (HATs), which are also part of the chromatin silencing complex, will also be upregulated in pre-diapause embryos. I also predict that genes encoding E2F and Rb, which bind with TFDp2 to repress the G_1-S transition in the cell cycle will be upregulated at this stage and will promote developmental arrest.

Modern molecular techniques, including microarrays, differential display, and subtractive hybridization are valuable methods for identifying differentially expressed genes in a variety of circumstances. However, while transcript abundance can predict protein abundance in many cases, it is not always an accurate indicator of protein amount. For example, posttranscriptional modifications, which may not be detected with expression analysis methods, can silence mRNA and prevent its translation into protein. Even in cases

where mRNA abundance accurately predicts the amount of a protein, the amount of protein present is not necessarily correlated with function. Allosteric inhibition and covalent modification commonly result in decreased enzyme activity for example. Taking these factors into consideration, further analysis of genes encoding CYP450 and AKR will focus on the interactions of the proteins they encode.

CYP450 and AKR are both predicted to have a role in ecdysone metabolism in insects, and because diapause typically results from a lack of ecdysone, not an increase, future studies will measure the amount and activity of the CYP40 and AKR proteins in pre-diapause and non-diapause embryos. Additional studies will quantify ecdysone, 20-hydroxyecdysone, and their respective, and inactive, cognates in diapause and non-diapause *A. socius* embryos. Information about the quantities of these molecules, as well as measurements of the ecdysone receptor (EcR) and ultraspiracle (i.e., components of a functional ecdysone receptor – see Yao et al. 1993) will add to our understanding of the role of the ecdysone signaling pathway in diapause induction in this species. In summary, while the results presented in the previous chapters of this dissertation increase what is known about embryonic diapause, there is still much that needs to be done before a comprehensive picture of regulatory mechanisms will be available.

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APPENDIX A

IDENTITY OF UP- AND DOWNREGULATED ESTS

Table A1.1 Expressed Sequence Tags for transcripts identified from the upregulated library with significant BLASTX matches.

Clone ID	Probable Homology	Closest Genbank Match	Species	E-value	Identity
S1P1WA5	FK506 binding protein-like protein	ABM55671	*Maconellicoccus hirsutus*	4e-39	84%
S1P1WA7	Hydroxyacylglutathione hydrolase	NP_956337	*Danio rerio*	2e-45	49%
S1P1WA10 S1P1WE3	Hsp 20.7	ABC84494	*Locusta migratoria*	1e-52	63%
S1P1WA11	Single strand recognition protein (Chorion factor 5)	XP_973014	*Tribolium castaneum*	3e-22	73%
S1P1WA12 S1P1WD7	Acidic ribosomal protein P0	XP_975859	*Tribolium castaneum*	4e-59	73%
S1P1WB11 S1P1WF10	Eukaryotic initation factor 5C		*Apis mellifera*	4e-60	69%
S1P1WB12	Isocitrate dehydrogenase	NP_001040134	*Bombyx moir*	6e-16	62%
S1P1WC8	Mitochondrial ATP synthase-gamma subunit	ABD98774	*Graphocephala atropunctata*	1e-29	67%
S1P1WC9	Nascent polypeptide associated complex protein	XP_623555	*Apis mellifera*	3e-47	85%
S1P1WD11	Ribosomal protein S28e	CAJ01882	*Byphillus lunatus*	1e-10	91%
S1P1WD12	Na+/K+ ATPase alpha subunit	EAT35801	*Aedes* aegypti	1e-65	86%
S1P1WE5	Ubiquinol-cytochrome c reductase	XP_972400	*Tribolium castaneum*	1e-47	84%
S1P1WE10	Alpha tubulin-1	AAM73986	*Homarus gammarus*	7e-28	81%
S1P1WF2	CG6009-PA Pyrroline 5-carboyxlate reductase	XP_974776	*Tribolium castaneum*	93-30	73%
S1P1WF3	Ribosomal protein S27A	AAT01911	*Pseudopleuronectes americanus*	2e-23	77%
S1P1WG2	Alpha tubulin-1	XP_966492	*Tribolium castaneum*	6e-87	99%

Table A1.1 cont.

Clone ID	Probable Homology	Closest Genbank Match	Species	E-value	Identity
S1P1WG7	Conserved hypothetical protein (acyl Co-A reductase/male sterility protein)	EAT41033	*Aedes aegypti*	2e-39	53%
S1P1WH10	Ribosomal protein L36e	CAJ17423	*Agriotes lineatus*	3e-37	81%
S1P2WA4	CG2774-PA isoform 2	XP_001120260	*Apis mellifera*	2e-24	43%
S1P2WA5	Ribosomal protein L35Ae	CAJ17420	*Carabus granulatus*	7e-23	66%
S1P2WA6	Ribosomal protein L18e	CAJ17292	*Cicindela campestris*	9e-18	55%
S1P2WA9	Hephaestus CG31000-PC	XP_62508	*Apis mellifera*	2e-22	54%
S1P2WB11	Cytochrome c oxidase subunit VIIC	ABF18472	*Aedes aegypti*	1e-10	76%
S1P2WC1	mitochondrial acetyl-coenzyme A acyltransferase 2	ABN11953	*Maconellicoccus hirsutus*	7e-52	71%
S1P2WC7	XTP3-transactivated protein B (Protein kinase c substrate)	XP_394479	*Apis mellifera*	6e-32	66%
S1P2WC8	Triosephosphate isomerase 1	NP_001090623	*Apis mellifera*	2e-35	74%
S1P2WC9 S1P2WG2 S1P3WG4	CG5920-PA Ribosomal protein S2	XP_975415	*Tribolium castaneum*	3e-63	94%
S1P2WD3	Proteasome subunit beta 7	NP_001040536	*Bombyx mori*	8e-14	59%
S1P2WD5	Mitochondrial ribosomal protein L45 (TIMM 44)	EAT35833	*Aedes aegypti*	1e-28	41%
S1P2WD6	Ferretin 2	AAY68366	*Anoplophora glabripennis*	7e-09	41%
S1P2WD10 S1P3WD11	Beta tubulin	ABL11314	*Cryptocercus punctulatus*	2e-66	97%
S1P2WD12	CG3869-PC (mitofusin)	XP_970147	*Tribolium castaneum*	1e-24	68%
S1P2WE2	Ribosomal protein Ubq L40e	ABD72690	*Acyrthosiphon pisum*	9e-39	92%
S1P2WE4	Adenine phosphoribosyl transferase	XP_788872	*Strongylocentrotus purpuratus*	3e-31	47%
S1P2WE5 S1P3WB11	mitochondrial F1F0-ATP synthase subunit c	ABI83790	*Anopheles funestus*	4e-20	75%
S1P2WE7	Ribosomal protein L5	O65353	*Helianthus annuus*	2e-22	67%

Table A1.1 cont.

Clone ID	Probable Homology	Closest Genbank Match	Species	E-value	Identity
S1P2WE8	DNA dependent RNA polymerase III	XP_625037	*Apis mellifera*	2e-77	84%
S1P2WF4	Mitochondrial assembly regulation factor	AAM00196	*Drosophila melanogaster*	3e-20	60%
S1P2WF6	Conserved hypothetical protein (Translocation protein Sec62)	EAT34545	*Aedes aegypti*	5e-10	46%
S1P2WF8	CG5931-PA (Dead-like helicase superfamily)	XP_970554	*Tribolium castaneum*	1e-56	87%
S1P2WG3	CG15860-PA	XP_001122160	*Apis mellifera*	7e-11	44%
S1P2WG4	CG9953-PA Serine carboxy peptidase	XP_972061	*Tribolium castaneum*	6e-31	46%
S1P2WG11	CG11200-PB (Retinol dehydrogenase 11)	XP_970723	*Tribolium castaneum*	8e-32	55%
S1P2WH1	Ribosomal protein L8e	ABM55545	*Maconellicoccus hirsutus*	2e-53	89%
S1P2WH2	Proteasome subunit alpha type 1	XP_625032	*Apis mellifera*	6e-52	88%
S1P2WH3	RubV-like 2 (Reptin/TBP interacting protein)	XP_001122537	*Apis mellifera*	3e-43	79%
S1P2WH5	N-acetyl-glucosamine-6-phosphate isomerase	NP_001040296	*Bombyx mori*	3e-33	45%
S1P2WH7	Ribosomal protein L27e	AAX62447	*Lysiphlebus testaceipes*	2e-46	83%
S1P3WA5	CG14813-PA Clathrin adaptor comples	XP_967725	*Tribolium castaneum*	3e-81	72%
S1P3WA6	Epididymal secretory protein E1 precursor	XP_624310	*Apis mellifera*	2e-14	52%
S1P3WA8	Cyclophilin A	ABJ98670	*Scophthalmus maximus*	9e-19	88%
S1P3WA9	UDP-glucose glycoprotein:glucosyltransferase	EAT48502	*Aedes aegypti*	2e-31	74%
S1P3WA10	Translationally-controlled tumor protein homolog	Q6XIN1	*Drosophila yakuba*	2e-31	75%
S1P3WA12	CG2249-PA Cytochrome c oxidase VIIc	XP_970390	*Tribolium castaneum*	9e-13	79%
S1P3WB2 S1P3WC6	CG6198-PA CHORD containing protein	XP_967567	*Tribolium castaneum*	1e-40	69%
S1P3WB5	Proteasome subunit beta type 4 precursor	XP_394993	*Apis mellifera*	7e-28	50%

Table A1.1 cont.

Clone ID	Probable Homology	Closest Genbank Match	Species	E-value	Identity
S1P3WC7	SEC13-like 1 isoform b	XP_393516	*Apis mellifera*	1e-66	86%
S1P3WC11	Ribophorin I	XP_001378255	*Monodelphis domestica*	3e-43	53%
S1P3WD2	CG9916-PA isoform 1 (PPIase)	XP_966308	*Tribolium castaneum*	1e-52	84%
S1P3WD9	CG1814-PA (5` nucleotidse family)	XP_393672	*Apis mellifera*	5e-50	76%
S1P3WD12	CG4422-PA GDP dissociation inhibitor	XP_968281	*Tribolium castaneum*	5e-91	95%
S1P3WE2	Zinc finger, CCHC domain containing 9	XP_001121478	*Apis mellifera*	2e-06	51%
S1P3WE8	CG1386-PA (short-chain alcohol dehydrogenase)	XP_970890	*Tribolium castaneum*	2e-29	45%
S1P3WE9	Tetracycline resistance CG5760-PA	NP_524429	*Drosophila melanogaster*	1e-33	61%
S1P3WE10	Translation elongation factor 2	ABN12062	*Maconellicoccus hirsutus*	9e-33	87%
S1P3WE11	Unnamed protein product	CAA41925	*Drosophila miranda*	4e-12	38%
S1P3WF5	Carnitine acetyltransferase	XP_001364014	*Monodelphis domestica*	3e-18	53%
S1P3WF7	Ribosomal protein L3	XP_624821	*Apis mellifera*	1e-46	75%
S1P3WF8	CG10679-PA Nedd8	XP_972922	*Tribolium castaneum*	2e-19	98%
S1P3WF12	Probable DNA replication factor GINS	XP_392289	*Apis mellifera*	3e-50	56%
S1P3WG5	Disulfide oxidoreductase	EAT36789	*Aedes aegypti*	5e-15	56%

Table A.1.2. ESTs for transcripts identified from the downregulated library with significant BLASTX matches

Clone ID	Probable Homology	Closest Genbank Match	Species	E-value	Identity
S2P1WA5 S2P2WB4 S2P4WB2 S2P4WD2	Cytochrome c oxidase subunit III	NP_982314	*Drosophila sechellia*	6e-31	62%
S2P1WA8 S2P3WG2	Heat shock protein 90	AAS45246	*Locusta migragtoria*	1e-53	89%
S2P1WA9	ER degradation enhancer	EDK99415	*Mus musculus*	4e-57	67%
S2P1WA10 S2P1WD7 S2P1WE5 S2P1WE7 S2P2WG3	Alpha tubulin	ABM55668	*Maconellicoccus hirsutus*	2e-46 2e-55	87% 96%
S2P1WA11 S2P1WE3 S2P3WA4	RACK1	NP_001041703	*Bombyx mori*	2e-75 7e-72	91% 93%
S2P1WB2	Phosphoribosylaminoimidazole carboxylase	ABM05496	*Bombyx mori*	1e-38	73%
S2P1WB9	CG1109-PA, isoform A (contains WD40 repeat)	XP_397060	*Apis mellifera*	7e-72	94%
S2P1WB10	CG2767-PA Aldo/keto reductase family	XP_974785	*Tribolium castaneum*	1e-07	40%
S2P1WB12 S2P2WF3	Heat shock protein 20.7	ABC84494	*Locusta migratoria*	6e-31 2e-35	61% 70%
S2P1WC4	Alcohol dehydrogenase	XP_393266	*Apis mellifera*	3e-46	72%
S2P1WC6 S2P4WC11 S2P4WD11	Arginine kinase	AAT77152	*Periplaneta americana*	1e-63	96%
S2P1WC8	Pol-like protein	ABN58714	*Biomphalaria glabrata*	2e-14	50%
S2P1WC12	CG9916-PA (Cyclophilin-like PPIase)	XP_966308	*Tribolium castaneum*	6e-50	91%
S2P1WE1 S2P4WG6	CG8444-PA (Renin receptor-like protein)	XP_973593	*Tribolium castaneum*	3e-16	37%
S2P1WE2 S2P3WE11	Proteasome subunit beta type 6 precursor	XP_973571	*Tribolium castaneum*	8e-50	81%

Table A1.2 cont.

Clone ID	Probable Homology	Closest Genbank Match	Species	E-value	Identity
S2P1WE8 S2P3WA8 S2P4WE5	CG5920-PA (Ribosomal protein S2	XP_975415	*Tribolium castaneum*	4e-44	92%
S2P1WE11	Translation elongation factor 1 alpha	ABP57611	*Tegosa etia*	1e-34	73%
S2P1WF2 S2P1WF8	Dead-box-1 CG9054-PA isoform 1	XP_392325	*Apis mellifera*	5e-57	82%
S2P1WF10 S2P3WC11 S2P4WE1	Heat shock 70 family member	AAO21473	*Locusta migratoria*	1e-36	90%
S2P1WG3 S2P2WC2	Ribosomal protein S9	XP_392726	*Apis mellifera*	8e-50	66%
S2P1WG5	ATP binding cassette	XP_001120768	*Apis mellifera*	3e-13	60%
S2P1WG6	CG8728-PA (Mitochondrial processing peptidase)	XP_624556	*Apis mellifera*	9e-35	66%
S2P1WG7	Cytochrome c oxidase subunit II	AAU11284	*Allomenobius socius*	2e-39	87%
S2P1WG9 S2P2WA4	CG10992-PA (Capthesin B-like protease)	NP_572920	*Drosophila melanogaster*	1e-59	60%
S2P1WG11	Spaghetti squash (Myosin regulatory light chain)	XP_623372	*Apis mellifera*	4e-35	92%
S2P1WG12	Alpha-tubulin isotype M-alpha-6	XP_526036	*Pan troglodytes*	2e-78	88%
S2P1WH6	Superoxide dismutase	NP_990395	*Gallus gallus*	9e-22	58%
S2P1WH7	ATP citrate synthase	EAT44342	*Aedes aegypti*	8e-87	95%
S2P1WH12 S2P3WF12	Cytochrome c oxidase subunit IV	AAY66918	*Ixodes scapularis*	2e-05	56%
S2P2WA3 S2P4WG8	CG9350-PA NADH-uibquinone oxidoreductase	NP_611538	*Drosophila melanogaster*	3e-17	46%
S2P2WA6	Ribosomal protein S27	ABM55459	*Xenopsylla cheopis*	2e-28	96%
S2P2WA12	CG1532-PA Glyoxalase	XP_625100	*Apis mellifera*	2e-40	67%
S2P2WB3	Ribosomal protein L18e	CAJ17294	*Hister sp. APV-2005*	4e-47	71%
S2P2WC8	Thioredoxin peroxidase		*Apis mellifera*	2e-55	84%
S2P2WD3	Ribosomal protein S10e	CAJ17181	*Georissus sp. APV-2005*	3e-10	65%
S2P2WD12 S2P3WG5	Small heat shock protein ArHsp21	ABD19712	*Artemia franciscana*	1e-09	42%

Table A1.2 cont

Clone ID	Probable Homology	Closest Genbank Match	Species	E-value	Identity
S2P2WE1	CG1240-PA (a chromatin associated protein)	XP_392065	*Apis mellifera*	3e-18	82%
S2P2WE7 S2P2WH12 S2P4WA9	Elongation factor-1 alpha	AAL78750	*Locusta migratoria*	1e-52	96%
S2P2WF1	Cytochrome P450	AAK57914	*Blattella germanica*	6e-10	40%
S2P2WF6	Ribosomal protein L10Ae	CAJ17257	*Cicindela littoralis*	3e-42	92%
S2P2WF7	Ayl-coa dehydrogenase	EAT43846	*Aedes aegypti*	3e-75	84%
S2P2WF8	Eukaryotic translation initiation factor 4 gamma	EAT40334	*Aedes aegypti*	1e-48	60%
S2P2WF11 S2P4WD8	Translation elongation factor -1 gamma	AAL78751	*Locusta migratoria*	3e-44	85%
S2P2WG6 S2P3WB8 S2P3WD3 S2P3WE2 S2P4WE10	Alpha tubulin	ABL61211	*Cryptocercus punctulatus*	6e-111	92%
S2P2WH4 S2P3WH11	Elongation factor-1 alpha	AAC03153	*Periplaneta americana*	3e-108	94%
S2P2WH8 S2P3WD6	CG8331-PA Receptor expression enhancing factor	XP_624070	*Apis mellifera*	2e-33	59%
S2P2WH11	Beta-actin	BAF43306	*Misgurnus anguillicaudatus*	6e-13	89%
S2P3WA2	CG10527-PA farnesoic acid O-methyl transferase-like protein	XP_623207	*Apis mellifera*	6e-30	54%
S2P3WA3	Protein ROP isoform A (Intracellular trafficing secretion)	XP_396375	*Apis mellifera*	4e-29	73%
S2P3WA5	26S proteasome non-ATPase regulatory subunit 5	XP_001120302	*Apis mellifera*	6e-16	51%
S2P3WA6	Dynactin isoform 6	EDL35430	*Mus musculus*	6e-52	64%
S2P3WA10 S2P4WC7	CG1796-PA (has WD-40 domain)	XP_968267	*Tribolium castaneum*	1e-20	67%
S2P3WB2	SUMO-like protein	ABQ41279	*Artemia franciscana*	5e-35	94%
S2P3WB3	Cystathionine beta-lyase	EAT44604	*Aedes aegypti*	8e-14	71%
S2P3WB5	Hypothetical protein (Immune related gene)	CAM36311	*Thermobia domestica*	9e-11	66%

Table A1.2 cont.

Clone ID	Probable Homology	Closest Genbank Match	Species	E-value	Identity
S2P3WB7 S2P3WB10 S2P4WA7	Aubergine CG6137-PA	XP_395884	*Apis mellifera*	6e-41	66%
S2P3WC1	CG12262-PA (Acyl-CoA dehydrogenase)	XP_970506	*Tribolium castaneum*	2e-72	73%
S2P3WC2	Catalase	AAZ50618	*Anemonia viridis*	6e-63	70%
S2P3WC3	Elongation factor-2	AAR01309	*Periplaneta americana*	1e-102	88%
S2P3WD1	GA12335-PA	XP_001360485	*Drosophila pseudoobscura*	2e-21	59%
S2P3WD2 S2P3WD4	CG4878-PB (eIF3-S9)	XP_972849	*Tribolium castaneum*	2e-06	64%
S2P3WE1 S2P4WC9	ATP/ADP translocase	ABM55522	*Maconellicoccus hirsutus*	1e-41	88%
S2P3WE7 S2P4WH3	CG40006-PC	XP_396241	*Apis mellifera*	4e-52	49%
S2P3WE8	Nuclear pore complex protein 93	EAT36220	*Aedes aegypti*	1e-12	38%
S2P3WE12	CG32645-PB	XP_969264	*Tribolium castaneum*	3e-66	79%
S2P3WF3	Gasp precursor (Chitin binding domain)	NP_001040470	*Bombyx mori*	1e-15	69%
S2P3WF4	Translocase of outer membrane 70 CG6756-PA	XP_001121853	*Apis mellifera*	1e-71	62%
S2P3WF8	Ribosomal protein S7e	CAH04318	*Carabus granulatus*	6e-51	90%
S2P3WF11	Cxpwmw03 (Electron transport flavoprotein)	ABC61672	*Periplaneta americana*	3e-57	76%
S2P3WG3	CG15439-PA	XP_970496	*Tribolium castaneum*	4e-65	79%
S2P3WG7	Ribosomal protein L5	O65353	*Helianthus annuus*	1e-50	91%
S2P3WG8	CG3662-PA (BRACHIOS domain)	XP_392881	*Apis mellifera*	4e-33	45%
S2P3WG12	CG3861-PA (Citrate synthase)	XP_970124	*Tribolium castaneum*	9e-38	91%
S2P3WH1 S2P4WF4	CG4618-PA	XP_973478	*Tribolium castaneum*	1e-15	66%
S2P3WH3	CG1518-PA (Oligosaccharyl transferase)	XP_392786	*Apis mellifera*	6e-47	85%
S2P3WH4	Ribosomal protein L27Ae	CAJ17396	*Agriotes lineatus*	4e-56	83%

Table A1.2 cont.

Clone ID	Probable Homology	Closest Genbank Match	Species	E-value	Identity
S2P3WH6 S2P4WG11	Acidic ribosomal protein P1	ABM55444	*Xenopsylla cheopis*	8e-26	78%
S2P3WH10	Zinc finger protein 595	XP_001120298	*Apis mellifera*	7e-13	39%
S2P3WH12	MGC85351 protein (Zinc metallopeptidase)	NP_001087923	*Xenopus levis*	8e-56	59%
S2P4WA4	ENSANGP00000009944	XP_317548	*Anopheles gambiae*	1e-76	74%
S2P4WA10	CG7430-PA Pyridine nucleotide-disulphide oxidoreductase	XP_623438	*Apis mellifera*	8e-101	75%
S2P4WB3	Chromosome associated protein	XP_393700	*Apis mellifera*	5e-39	67%
S2P4WB5	Bax-inhibitor 1-like protein	ABM55570	*Maconellicoccus hirsutus*	8e-26	80%
S2P4WB6	Ribosomal protein L13A	XP_623813	*Apis mellifera*	3e-50	76%
S2P4WB7	CG17569-PB	XP_971240	*Tribolium castaneum*	9e-41	65%
S2P4WB12 S2P4WC12	Actin-related protein 87C	XP_623834	*Apis mellifera*	3e-60	89%
S2P4WC3 S2P4WC4	CG31523-PA (Long chain fatty acid elongation)	XP_971544	*Tribolium castaneum*	9e-26	54%
S2P4WC6	Putative innexin 3	AAU84945	*Toxoptera citricida*	1e-26	62%
S2P4WC10	CG3001-PA (Hexokinase)	XP_966410	*Tribolium castaneum*	6e-57	69%
S2P4WD5	ENSANGP00000032064 (Histone 2A)	XP_307083	*Anopheles gambiae*	8e-48	99%
S2P4WD6 S2P4WG5	CG9743-PA (Fatty acid desaturase)	XP_967943	*Tribolium castaneum*	2e-62	76%
S2P4WE4	CG7483-PA eIF4 subunit III	XP_393356	*Apis mellifera*	7e-61	88%
S2P4WE6	Hypothetical protein	ABR27858	*Triatoma infestans*	6e-46	70%
S2P4WE8 S2P4WG3	HEL	CAD21558	*Chironomus tentans*	3e-36	89%
S2P4WE9	CG18445-PA	XP_973957	*Tribolium castaneum*	3e-63	75%
S2P4WF1	CG6084-PA (Aldo-keto reductase)	XP_969526	*Tribolium castaneum*	6e-51	75%
S2P4WF12	Endoplasmin (Molecular chaperone – Heat shock 90 family)	EAT34979	*Aedes aegypti*	5e-52	80%
S2P4WG9	CG9350-PA (SAM dependent methyltransferase)	XP_972432	*Tribolium castaneum*	1e-51	80%

Table A1.2 cont.

Clone ID	Probable Homology	Closest Genbank Match	Species	E-value	Identity
S2P4WG10	CG8498-PA (Acyl CoA binding protein)	XP_974824	*Tribolium castaneum*	3e-28	71%
S2P4WH4	CG31694-PA (Interferon developmental regulator)	XP_392883	*Apis mellifera*	5e-39	49%
S2P4WH6	C-type lectin	NP_001091747	*Bombyx mori*	7e-56	79%
S2P4WH7	CG8756-PD (Very low density lipoprotein receptor)	XP_969401	*Tribolium castaneum*	1e-68	87%
S2P4WH8	Apoliphophorin precursor	CAB51918	*Locusta migratoria*	1e-07	31%
S2P4WH9	Transcription factor Dp-2 (E2F dimerization partner)	XP_393377	*Apis mellifera*	2e-27	54%
S2P4WH11	Hypothetical protein	XP_001120369	*Apis mellifera*	3e-13	46%
S2P4WH12	Fibroinase precursor	AAR87763	*Bombyx mori*	6e-47	75%

APPENDIX B

LETTER OF PERMISION

From: **Julie Reynolds** (julierey52@hotmail.com)
Sent: Mon 9/10/07 6:08 PM
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VITA

Julie Annette Lipe Reynolds was born in Lawton, Oklahoma, and grew up in Colorado Springs, Colorado, and near Huntsville, Alabama. She first became interested in science at the age of 10, when she studied ecosystems for a 5th grade science project. Julie went on to study biology at the University of Alabama in Huntsville where she was fortunate enough to work in the labs of Drs. Marian Lewis and Richard Modlin. During her time as an undergraduate research assistant, Julie worked in the Microgravity Biotechnology Lab and was involved in the science mission support for five space-shuttle flights. Through these experiences, Julie learned not only to correctly load and assemble spaceflight hardware, but also how to remain good humored when she and everyone around her had been awake for 24 hours straight assembling mission payload experiments. Following her time as a space cadet, Julie attended Pennsylvania State University where she earned a Master of Science degree in entomology while studying the feeding behavior of the European corn borer, *Ostrinia nubilalia,* with Dr. Dennis Calvin. In 2000, she joined Dr. Steven Hand's lab at Louisiana State University where she has since studied diapause in brine shrimp and crickets.

Julie is married to Matthew Reynolds and has identical twin daughters – Alison and Kaitlin. Upon completion of her doctorate, she plans to move her family to Ohio State University where she will work with Dr. David Denlinger and study additional facets of insect diapause.