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Decoupling development and energy flow during embryonic diapause in the cricket, *Allonemobius socius*

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SUMMARY

Respiration rate increases 6.3-fold during 15 days of post-oviposition development in embryos of the Southern ground cricket, *Allonemobius socius*. This ontogenetic increase in metabolism of non-diapause insects is blocked during diapause, such that metabolic rate is only 36% of the rate measured for 15 days developing embryos. Surprisingly, however, there is not an acute metabolic depression during diapause entry at the point when developmental ceases (4–5 days post-oviposition), as measured by blockage of morphological change and DNA proliferation. The results indicate a decoupling of developmental arrest from metabolism. Both non-diapause and diapause embryos have unusually high [AMP]:[ATP] ratios and low [ATP]:[ADP] ratios during early embryogenesis, which suggests embryos may have experienced hypoxia as a result of an insect chorion that limits water loss but may restrict oxygen diffusion. The similar adenylate profiles for these two developmental states indicate the atypical energy status is not a specific feature of diapause. In addition embryos at day 3 have high levels of lactate that decrease as development proceeds up to day 7. Calorimetric-respirometric (CR) ratios of –353 (day 3) to –333 (day 7) kJ mol^{-1} O_2 are consistent with embryos that are aerobically recovering from hypoxia, but are inconsistent with an ongoing anaerobic contribution to metabolism. Superfusing 3-day embryos with O_2 enriched air (40% O_2) forces these metabolic indicators toward a more aerobic poise, but only partially. Taken together these biochemical data indicate the metabolic poise of *A. socius* is only partly explained by hypoxia in early development, and that the atypical set points are also intrinsic features of this ontogenetic period in the life cycle.

Key words: embryonic diapause, hypoxia, anaerobic poise, adenylate status.

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; Denlinger, 2002). 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 against a variety of environmental stressors including extreme temperatures and oxidative stress (Denlinger, 2002; Lee et al., 2002; Clegg, 1965). 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 was to characterize the bioenergetics of embryogenesis and diapause in this species, in order to understand which metabolic features, if any, are predictors of diapause and which are consequences of developmental stage. DNA content, a surrogate for cell number, allowed us to pinpoint when developmental arrest began at the onset of diapause. Although the continuous increase in metabolism that accompanied active development was blocked during diapause, an acute depression of metabolism surprisingly did not occur upon diapause entry in *A. socius*. Tissue lactate and atypical adenylate ratios suggest that hypoxia may be a feature of early ontogeny in this insect, but artificial exposure to hyperoxia has limited impact on these biochemical indicators.

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 an obligate diapause, whereas in the southern portion of its range this species produces two or more generations per year and has a facultative diapause (Fulton, 1931; Howard and Furth, 1986; Mousseu and Roff, 1989). The first generation matures during the spring months each year, and the mature adults of this generation produce a second generation of offspring that develop directly without entering diapause. These individuals hatch as nymphs after 15–20 days 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 fall, 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 (Huestis and Marshall, 2006) and Olvido et al. (Olvido et al., 1998) have shown that females reared under long day conditions (16 h:8 h L:D) produce a high proportion of non-diapause embryos, whereas rearing females under short day conditions (12 h:12 h 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 et al., 1998; Heustis and Marshall, 2006). We are 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.

Our results indicate that diapause in *A. socius* is primarily characterized by a developmental arrest that begins 4–5 days post-oviposition. Unexpectedly, there is no significant metabolic depression linked to the entry into diapause. The AMP:ATP concentration ratio ([AMP]:[ATP]) is remarkably high in early embryos of this species and [ATP]:[ADP] low, but these do not appear to be a feature of diapause *per se*. The 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 from South Carolina, USA during 2001 and 2002. Nymphs were kept in plastic boxes with food and moistened cotton. They were maintained at room temperature with a 14 h:10 h (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^{TM} Cricket Food (Fluker's Cricket Farm, Baton Rouge, LA, USA) that was supplemented with dry cat food to supply additional protein. Adults were maintained at either 22°C with a short-day photoperiod (12 h:12 h, L:D) to encourage production of diapause embryos or 28°C with a long day photoperiod (16 h:9 h, L:D) to encourage production of non-diapause embryos (Olvido et al., 1998; Heustis and Marshall, 2006). Plastic vials containing moistened cheesecloth were provided as an oviposition site. Eggs were collected every 24 h and incubated at 29°C.

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, 1959), 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 MZ7 stereomicroscope (Leica Microsystems, Wetzlar Germany), and images were digitally recorded with a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA). 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 with those of *bona fide* diapause embryos fixed 30 days post-oviposition.

Metabolic rate

Oxygen consumption was measured at 29°C with a Gilson Differential Respirometer (Gilson Medical Electronics; Middleton, WI, USA). Approximately 100 embryos were placed in 15 ml respiration flasks with moistened filter paper. A filter paper wick with 3 mol l^{-1} 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. The change in µl O_2 per minute was adjusted for standard temperature and pressure, and data were expressed in pmol O_2 min^{-1} $embryo^{-1}$.

Heat dissipation was monitored at 29°C 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 the rate of oxygen consumption obtained in the previous section, we calculated there to be sufficient air space within the ampoule to preclude oxygen limitation during the course of the experiment. 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). After temperature equilibration for 1 h upon lowering the ampoule into the calorimeter, heat dissipation data were recorded and averaged over the 3 h collection period. Calorimetric-respirometric (CR) ratios (kJ mol^{-1} O_2) were calculated from oxygen consumption and heat dissipation data recorded independently, but in parallel, from embryos harvested at the same time from the same cohort of females (Podrabsky and Hand, 1999). Values for the theoretical oxycaloric equivalent were taken from Gnaiger (Gnaiger, 1983) and Widdows (Widdows, 1987).

Metabolite extraction

Perchloric acid extracts were prepared as described by Podrabsky and Hand (Podrabsky and Hand, 1999). Approximately 200–300 embryos were pulverized in liquid nitrogen and then homogenized in 500 µl of ice-cold 1 mol l^{-1} perchloric acid containing 5 mmol l^{-1} EDTA. The homogenate was centrifuged at 10,000 ***g*** for 10 min at 4°C. The supernatant was neutralized with 5 mol l^{-1} K_2CO_3 and centrifuged at 10,000 ***g*** for 15 min at 4°C to remove perchlorate salts. Supernatants were stored at –80°C 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 (Giles and Myers, 1965) [modified from Burton (Burton, 1956)]. Briefly, the resuspended perchloric acid pellets from the above extractions were incubated at 75°C for 30 min and centrifuged at 10,000 ***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^{-1} 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 covered, 96-well microtiter plates, color development was measured at 595 nm using a SPECTRAmax Plus microplate reader (Molecular Devices, Sunnyvale, CA, USA). Corrections were made for non-specific color development and turbidity by subtracting the absorbance at 700 nm from the absorbance at 595 nm.

Adenine nucleotides were quantified in neutralized perchloric acid extracts by reverse phase chromatography with a method similar to that described by Menze et al. (Menze et al., 2005). Before analysis,

extracts were filtered through a 0.45 μm Nanosep MF Centrifugal Device (Pall Corporation, East Hills, NY, USA) to remove any remaining perchlorate salts. AMP, ADP and ATP were separated using a 4.6 mm×250 cm reversed phase column (Synergy 4 μm Hydro RP 80A, Phenomenex, Torrance, CA, USA) with a Dionex HPLC system (Sunnyvale, CA, USA) 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 mmol l^{-1} potassium phosphate buffer (pH 5.0), 10 mmol l^{-1} tetrabutylammonium hydrogen sulfate (TBS), and 1.5% acetonitrile (v/v). A linear gradient of acetonitrile (1.5–25%) 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 3-D-field with Chromeleon™ software (Dionex, Sunnyvale, CA, USA). The concentrations of nucleotides were 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 (Bergmeyer, 1974). Neutralized perchloric acid extracts were assayed in glycine–hydrazine buffer (0.43 mol l^{-1} glycine and 0.34 mol l^{-1} hydrazine, final concentrations) that contained 2.75 mmol l^{-1} NAD. Reactions (2.9 ml volume) were initiated by the addition of 55 i.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-day embryos were exposed to one of three experimental oxygen regimes: normoxia (air, O_2 concentration 20%), hyperoxia (air supplemented with pure O_2, final concentration 40%) or mild hypoxia (air mixed with N_2, final O_2 concentration 10%). Embryos were incubated at 29°C for 24 h in small plastic weighing boats enclosed in a flow-through chamber for gas equilibration. A small amount of water was included inside the chamber to prevent dehydration of embryos. The embryos were then superfused with the desired gas mixture at a rate sufficient to replace the air volume of the chamber every 3 min. Gas mixtures and air flow were regulated with mass flow controllers and a mass flow meter (Tylan FC-260 and RO-28, respectively; Mykrolis Corporation, Billerica, MA, USA). 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.

Adenylate or lactate levels for the three treatment groups were compared with those of control embryos incubated under standard rearing conditions (see above) without perfusion. To verify sufficient oxygen levels are present under these control conditions, an air-tight syringe was used to draw air from triplicate vials containing 3-day embryos. Samples were injected into a Hewlett Packard 5890 II gas chromatograph (Palo Alto, CA, USA) equipped with a molecular sieve column (3 mm diameter, 152.4 cm long) and a thermal conductivity detector maintained at 50°C during the analysis. The percentage of oxygen was calculated from the ratio of peak areas for oxygen and nitrogen.

Normalization of data and statistical analysis

Biochemical data collected for developing embryos can be problematic to normalize because the amount of metabolically active tissue increases over time and is not necessarily proportional to the mass of the embryo because of changes in amounts of yolk present. The issue is even more complicated for orthopteran embryos because they absorb water during embryogenesis (e.g. Browning, 1965; Yoder and Denlinger, 1992). As seen in Fig. 1, the wet mass of *A. socius* embryos destined to enter diapause increases dramatically after 3 days post-oviposition. However, there is very little increase in dry mass during the same period, based on measurements of embryos dried to constant mass at 90°C. With these considerations in mind, the data were either normalized per embryo or per μg DNA.

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 days of embryonic development. Additional measurements at 15 days post-oviposition were made to further characterize the biochemical and metabolic state of diapause embryos. General linear model (GLM) was used to analyze the relationship between each feature as it changed over time for each embryo type (i.e. non-diapause or diapause), as well as to analyze the interaction between time and embryo type. The GLM is a univariate ANOVA that can be used with an unbalanced design,

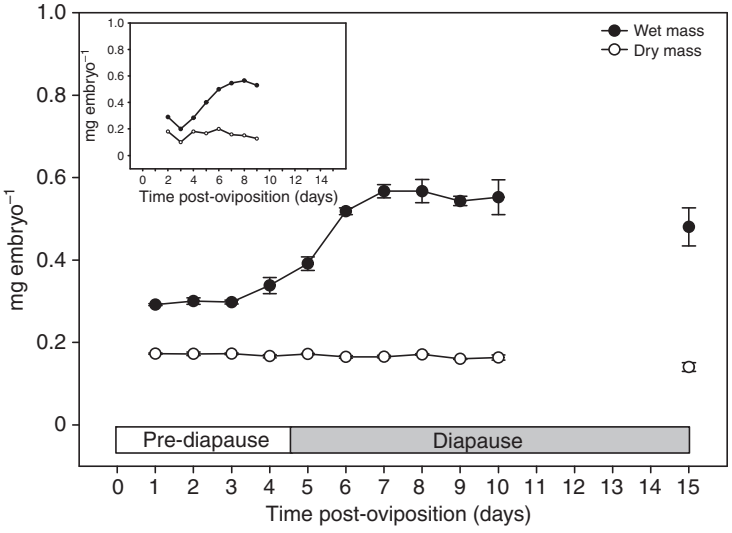

Fig. 1. Mass of embryos as a function of developmental time. Data shown in the main panel are for embryos destined to enter diapause. Values are means ± s.e.m., N=3 for each time point. Although there is little change in the dry mass (mg embryo^{-1}) during the first 15 days of development, wet mass increases dramatically beginning after day 3. The inset shows a similar pattern for non-diapause embryos (values are mean, N=1–4 for each time point).

allows multiple independent variables, and used to probe interactions between the independent variables. Tukey's method was used *a posteriori* to test for pairwise differences between means. ANOVA was used to analyze the relationship between adenylate status (or lactate) and percentage O_2 in the rearing environment; Fisher's LSD was used to test, *a posteriori*, for significant ANOVA. For all statistical tests, results were considered significant if $P \leq 0.05$. Student's *t*-test (with Bonferroni correction when appropriate) was used to test for specific pair-wise differences as discussed below. All statistical analyses were performed using MiniTab software (MiniTab, State College, PA, USA).

RESULTS

Developmental arrest

A. socius embryos enter diapause early in development at what Tanaka (Tanaka, 1984; Tanaka, 1986a) designated as 'Stage 2'. Embryos at this stage appear as elongated dumbbells with well-defined head lobes (i.e. procephalon). Rudimentary stomodea and antennae are visible, but there is no obvious segmentation. At this stage gastrulation is complete (Roonwal, 1936; Gillott, 2005), and segmentation, limbud formation, and organogenesis are beginning. 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 Fig. 2. Diapause embryos, fixed 30 days 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. 2B). By comparing embryos that were collected at 24 h intervals to Tanaka's description of diapause embryos and to our observations of *bona fide* diapause embryos based on DNA content, we estimated that embryos enter diapause approximately 4–5 days post-oviposition.

DNA content is an indirect measure of cell proliferation and can be used to help establish the time point at which development is arrested upon diapause entry as shown in Fig. 3. Analyzing µg DNA per embryo with GLM shows significant change in DNA over 15 days of development (F=12.91, d.f.=9, P=0.00) as well as a significant difference in the DNA of non-diapause embryos compared with diapause embryos (F=63.54, d.f. 1, P=0.00) and significant interaction between time and embryo type (F=5.37, d.f.=9, P=0.00). In non-diapause embryos, DNA content (µg DNA embryo^{-1}) gradually increases during the first 4 days post-oviposition (Fig. 3). Beginning on day 5 there is a steep increase from 0.203 µg DNA embryo^{-1} to 0.950 µg DNA embryo^{-1} at 10 days. For embryos programmed to enter diapause there is not a significant increase in DNA content over the first 15 days post-oviposition, as shown by comparing µg DNA per embryo on days 2 and 15 using Tukey's method for pair-wise comparison (difference of means=92.28, t=0.688, P=1.00). As described above, *bona fide* diapause embryos are morphologically the same as non-diapause embryos 4–5 days post-oviposition. We used Student's *t*-test (Bonferroni corrected) to compare µg DNA per embryo in non-diapause *versus* diapause embryos at days 5 and 6 post-oviposition to estimate the divergence point at which DNA content first differs (i.e. diapause entry). Non-diapause embryos have a greater quantity of DNA than diapause embryos as early as 6 days post-ovipositon (P=0.009, α=0.025), but not at day 5 (P=0.09, α=0.025). Thus, the morphological and biochemical data are in reasonable agreement as to when the developmental pathways of diapause and non-diapause embryos diverge.

Metabolic rate

Oxygen consumption by non-diapause embryos increases significantly over time (GLM, F=6.34, d.f.=11, P=0.00) from 19.8±0.57 pmol O_2 min^{-1} embryo^{-1} (mean ± s.e.m.) shortly after oviposition to 124±8.95 pmol O_2 min^{-1} embryo^{-1} at 15 days post-oviposition (Fig. 4). Tukey's method verifies that oxygen consumption on day 15 is significantly different from that on day zero (P=0.0035). To assess whether there is acute metabolic depression during diapause, we measured the oxygen consumption rate for diapause embryos of *A. socius* at 15 days post-oviposition. Oxygen consumption rate for these embryos is 45.3±3.9 pmol O_2 min^{-1} embryo^{-1} (mean ± s.e.m., N=22; far right bar

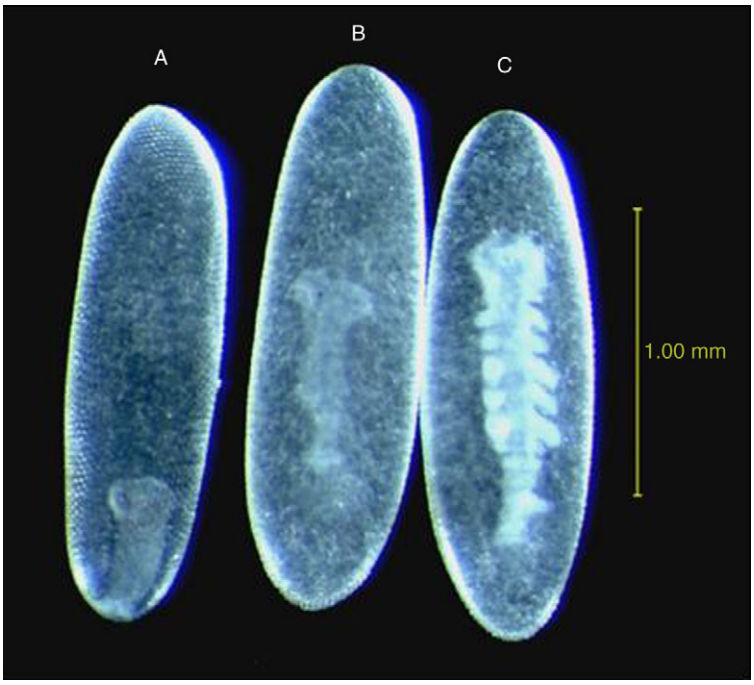

Fig. 2. Morphology of *A. socius* embryos. Diapause embryos are morphologically similar to embryo 'B', which is moderately dumbbell shaped. This embryo has migrated from the posterior end of the egg (as in egg 'A') to the center and is surrounded by yolk. In addition, embryos in diapause lack limb buds (compare with 'C').

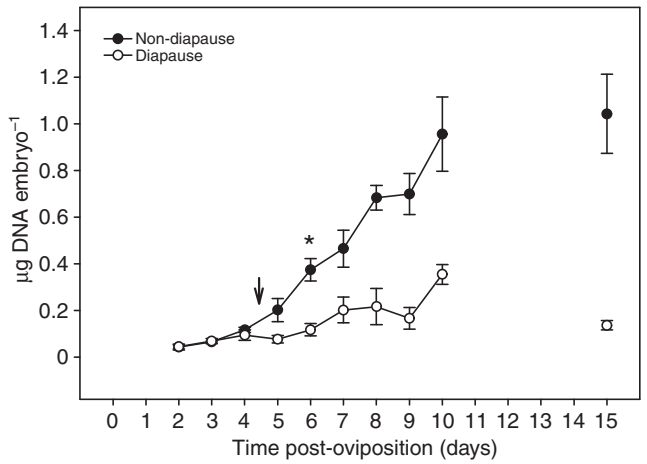

Fig. 3. Total DNA content per embryo as a function of developmental time in non-diapause and diapause embryos. Values are means ± s.e.m., *N*=3–6 samples of 200–300 embryos for each time point. The asterisk indicates the first time point when non-diapause embryos have significantly more DNA than diapause embryos of the same age (Student's *t*-test ($P \leq 0.009$; α=0.025, Bonferroni corrected). The arrow indicates the time point when morphogenesis is arrested in diapause embryos.

Fig. 4), and is the same as the metabolic rate of non-diapause embryos at 4 days and 5 days post-oviposition (*P*=0.71, and 0.13, respectively), which are a similar morphological stage. Thus, there is not a detectable metabolic depression upon diapause entry. However, the aerobic metabolism in 15 day diapause embryos is only 36% of the rate measured for 15 day non-diapause embryos (*P*=0.0032). This comparison clearly shows that the ontogenetic increase in metabolism observed in non-diapause embryos is blocked during diapause.

To estimate aerobic metabolism per embryo as a function of the amount of metabolically active cells, respiration was plotted (log–log) against total DNA per embryo. DNA content serves as an indirect indicator of cell number and increases 20-fold from day 2 to day 15. As anticipated, oxygen consumption per embryo increases linearly with increasing DNA content (Fig. 4, inset).

There is not a significant difference in the ratio of heat produced to oxygen consumed (CR ratio) by non-diapause embryos 3 and 7 days post-oviposition (Student's *t*-test, *t*=–0.40, *P*=0.7; Table 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), as considered in the Discussion section.

Adenylate status

The total quantity of adenylates in *A. socius* embryos (ATP+ADP+AMP; pmol adenylates µg^{-1} DNA) is highest soon after oviposition and decreases as a function of developmental time (Fig. 5A; *F*=13.91, d.f.=9, *P*=0.000). Significant decreases occur between days 2 and 3 and again between days 3 and 6 (Tukey's method for separation of means, *P*=0.006 and *P*=0.0322, respectively). There are significant differences between non-diapause and diapause embryos (*F*=7.64, d.f.=1, *P*=0.008), but there is not a significant difference on any particular day. There is no significant interaction between time and embryo type (*F*=0.23, d.f.=9, *P*=0.988). The same general relationship holds true for each adenylate species. AMP (Fig. 5B) shows a significant change over time (*F*=12.97, d.f.=9, *P*=0.000) and differences between embryo type (*F*=6.34, d.f.=1, *P*=0.15), but there is no interaction between time and type (*F*=0.38, d.f.=9, *P*=0.939). ADP (Fig. 5C) also decreases over time (*F*=21.60; d.f.=9, *P*=0.00) with some significant differences between non-diapause and diapause embryos (*F*=17.24, d.f.=1, *P*=0.0001) but no significant interaction between age and embryo type (*F*=0.32, d.f.=9, *P*=0.966). Similarly, for ATP (Fig. 5D) there is a significant change over time (*F*=4.40, d.f.=9, *P*=0.00) with some significant differences between non-diapause and diapause embryos (*F*=3.90, d.f.=1, *P*=0.054); there is no significant interaction between embryo age and type (*F*=0.60, d.f.=9, *P*=0.789).

The amount of AMP is remarkably high early in development in both non-diapause and diapause destined embryos (Fig. 5B), and this AMP status corresponds to an unusually high [AMP]:[ATP] ratio. At 2 days post-oviposition the [AMP]:[ATP] ratio is 3.5±0.08 (mean ± s.e.m.) for non-diapause embryos and 4.5±0.56 for diapause embryos (Fig. 6A). The [AMP]:[ATP] ratio decreases with time after oviposition (*F*=10.25, d.f.=9, *P*=0.00) and with overall differences between non-diapause and diapause embryos (*F*=4.25, d.f.=1,

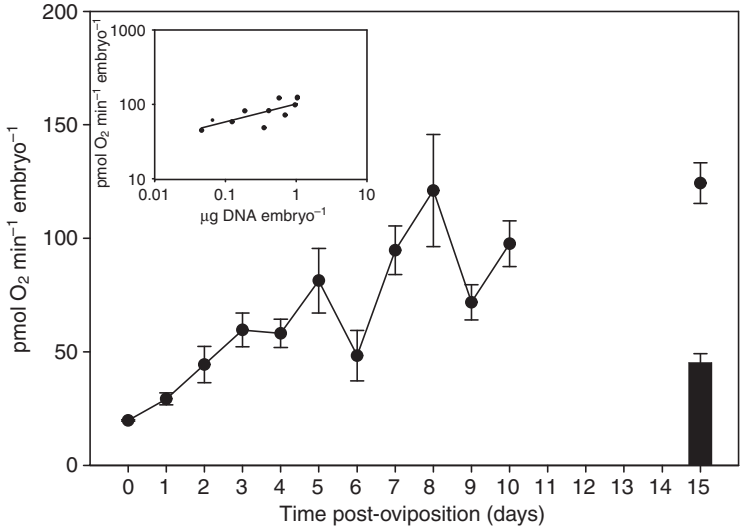

Fig. 4. Respiration rate of *A. socius* embryos as a function of time post-oviposition, presented as O_2 consumption rate per embryo of non-diapause embryos during the first 15 days of development (values are means ± s.e.m., *N*=3–12 samples of 100 embryos for each time point). The bar indicates respiration rate of diapause embryos 15 days post-oviposition (mean ± s.e.m., *N*=22). Inset shows a log–log plot of oxygen consumption *versus* total DNA content for non-diapause embryos.

Table 1. Calorimetric-respirometric (CR) ratios of non-diapause embryos measured at 3 and 7 days post-oviposition

Days post-oviposition	*N*	CR ratio (kJ mol^{-1} O_2)
3	3	353±39
7	4	333±32

Values are means ± s.e.m.

P=0.044) but no interaction between time and embryo type (*F*=1.15, d.f.=9, *P*=0.348). There is not a significant difference between non-diapause and diapause embryos on any particular day post-oviposition. The ratio reaches a steady-state of approximately 0.5 after 5 d post-oviposition for both non-diapause and diapause embryos.

Fig. 6B shows low [ATP]:[ADP] ratios in both diapause and non-diapause embryos during the first 4 days post-oviposition with a significant increase as development progresses (*F*=10.46, d.f.=9, *P*=0.00). There are significant differences between non-diapause and diapause embryos (*F*=30.20, d.f.=1, *P*=0.000) as well as significant interaction between time and embryo type (*F*=2.13, d.f.=9, *P*=0.043). In non-diapause embryos, the [ATP]:[ADP] ratio increases 85% over 15 days of embryonic development, primarily because of the large decrease in ADP. In embryos destined to enter diapause, the [ATP]:[ADP] ratio increases 73%, from 0.87 to 3.22, during the first 15 days post-oviposition. In these embryos the increase is primarily the result of 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 unusually low for tissues respiring under normoxic conditions.

The adenylate status of non-diapause (3 day) embryos of *A. socius* depends in part on the availability of oxygen in the environment in which they are incubated. As seen in Fig. 7B, 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 in samples of air collected from control, non-superfused vials containing 3-day embryos was 20±0.33% (mean ± s.e.m., *N*=3) indicating these embryos had access to sufficient oxygen. We anticipate the higher [ATP]:[ADP] ratio for embryos superfused with 20% O_2 compared with control embryos (20% O_2, embedded in moist cheesecloth) may have been due to reduced oxygen diffusion in unstirred layers surrounding controls.

Embryos exposed to hyperoxia (40% O_2) exhibit a significant 37% decrease in [AMP]:[ATP] compared with control embryos (ANOVA *F*=4.69, d.f.=3, *P*=0.024; Fisher's LSD *P*=0.05; Fig. 7A). 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 hyperoxia does not result in adenylate ratios that are typical for normoxically respiring tissues. Thus the abnormal

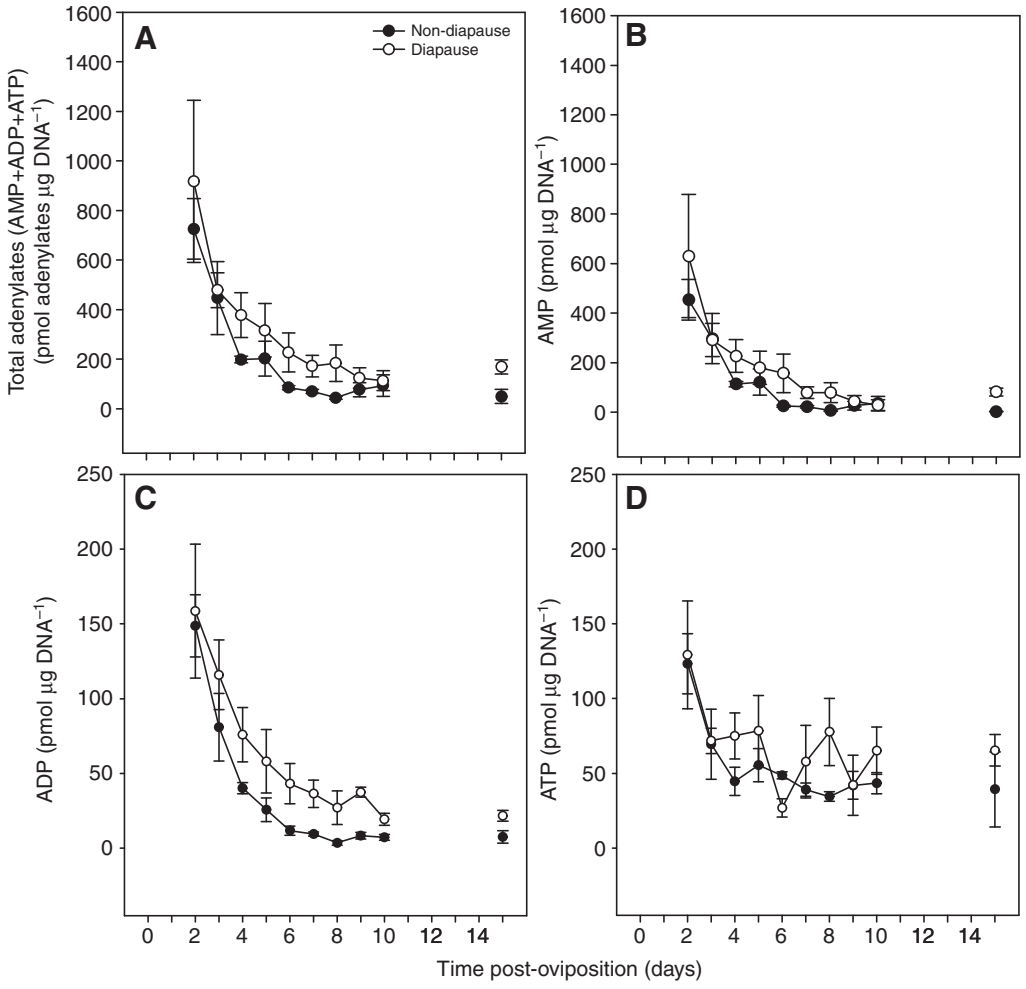

Fig. 5. Adenine nucleotides in *A. socius* embryos as a function of developmental time. (A) Total quantity of adenylates (ATP+ADP+AMP), (B) AMP, (C) ADP, and (D) ATP per µg DNA as a function of time, in non-diapause and diapause embryos. Values are means ± s.e.m., *N*=3–6 samples of 200–300 embryos for each time point.

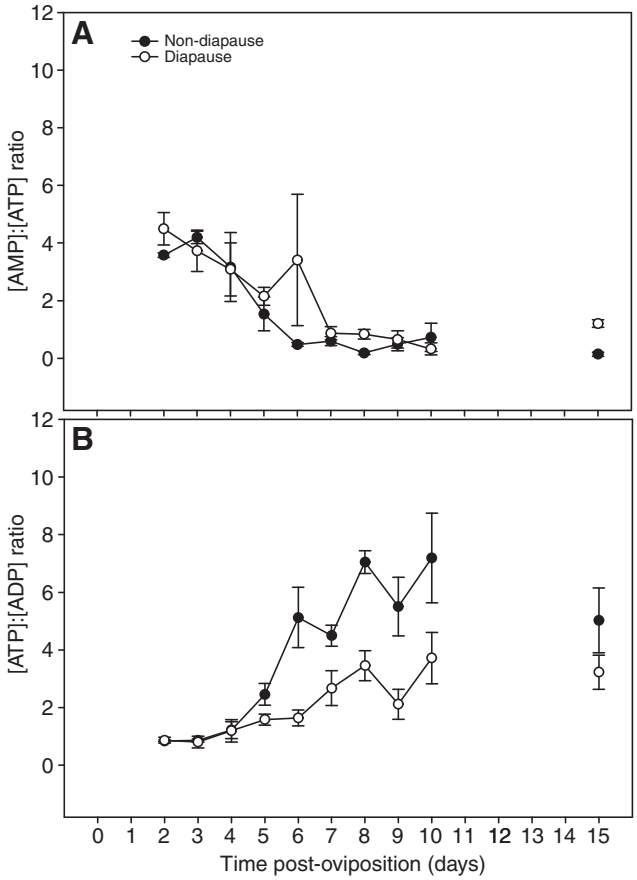

Fig. 6. Adenine nucleotide ratios in *A. socius* embryos. (A) [AMP]:[ATP] ratios and (B) [ATP]:[ADP] ratios of non-diapause and diapause embryos as a function of developmental time. Values are means ± s.e.m., *N*=3–6 samples of 200–300 embryos for each time point.

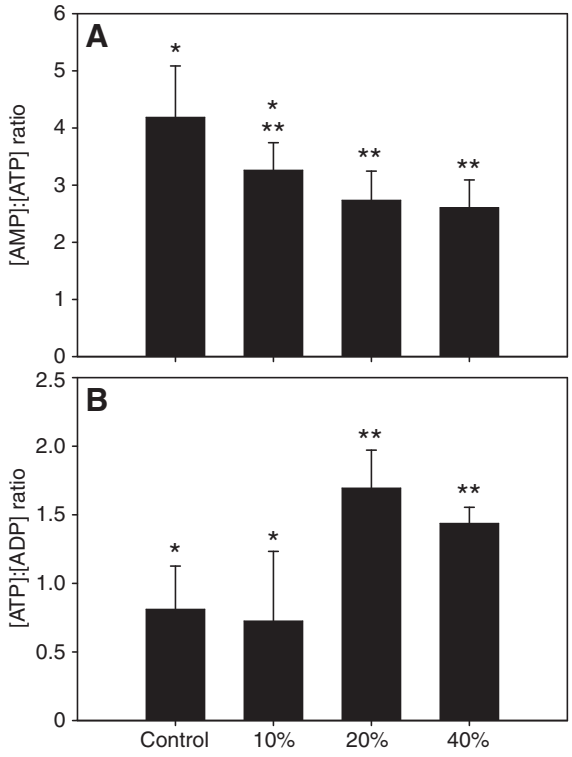

Fig. 7. Adenine nucleotide ratios under different oxygen levels. (A) [AMP]:[ATP] ratios and (B) [ATP]:[ADP] ratios of 3 days non-diapause embryos incubated for 24 h under hyperoxic (40% O_2), normoxic (20% O_2) or mildly hypoxic (10% O_2) conditions. Control embryos were placed in moist cheesecloth and incubated under normoxia but without perfusion of the incubation vials (percentage O_2=20±0.33, *N*=3). Values are means ± s.e.m., *N*=3–4 samples of 200–300 embryos. Bars marked with the same symbol are not statistically different from each other.

ratios in embryos of *A. socius* are not fully explained by oxygen limitation, but rather are in part 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 (Storey and Storey, 1990; Wegener, 1993; Hoback et al., 2000; Hoback and Stanley, 2001), was measured in non-diapause embryos. Between 3 and 7 days 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. 8A), consistent with processing of lactate stores during development. 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 with controls (20% O_2; ANOVA *F*=1.02, d.f.=3, *P*=0.421; Fig. 8B). 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 or mild hypoxia.

DISCUSSION

This study examined morphological and physiological characteristics of embryonic development and diapause for the ground cricket, *Allonemobius socius*. We show that direct developing individuals of this species typically complete the embryonic stage after 15 days, whereas development is arrested approximately 4 days post-oviposition in embryos programmed to enter diapause. Cessation of DNA proliferation can be used as a reliable marker of diapause. Unlike other species, acute depression of aerobic metabolism does not accompany the entry into diapause in this cricket and thus cannot be used as a functional indicator of this state. However, the continuing ontogenetic increase in O_2 consumption observed for non-diapause embryos is blocked in diapause. 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 exclusively explained by oxygen limitation because incubating the embryos under hyperoxia only moderately increases the [ATP]:[ADP] ratio and reduces the [AMP]:[ATP] ratio. In addition lactate, which is present in 3 day embryos, is not reduced by increasing O_2 in the incubation environment. Finally, CR ratios are not consistent with a contribution from anaerobic metabolism, but are more typical of animals recovering aerobically from hypoxia. Thus, the atypical levels of biochemical and physiological indicators of metabolic poise are at least partly an intrinsic feature of *A. socius* embryos at this stage of development.

Comparing the morphology and DNA quantity of *bona fide* diapause embryos with non-diapause embryos over a period of 10 days indicates that development is arrested 4–5 days post-oviposition. The oxygen consumption rates of non-diapause *A. socius* embryos are similar to those reported for *Gryllus veletis*, a

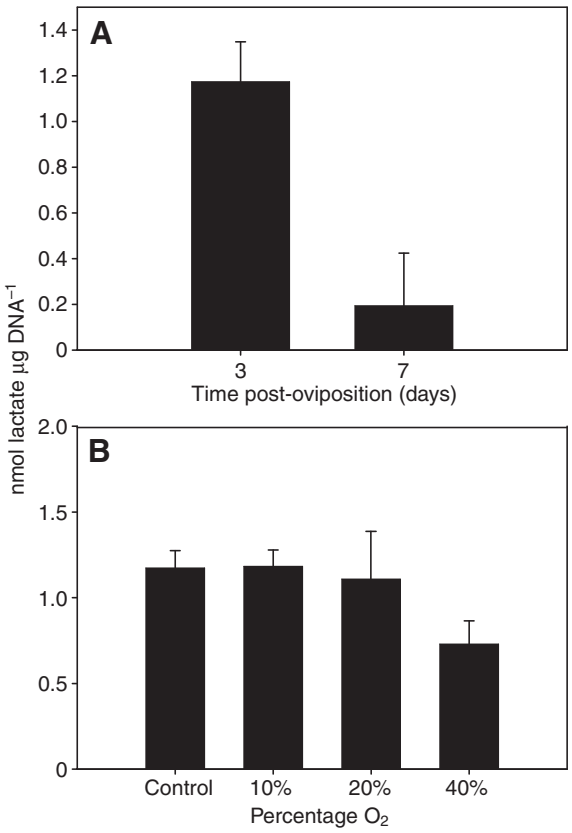

Fig. 8. Lactate levels in *A. socius* embryos. (A) Lactate (nmol µg^{-1} DNA) in non-diapause embryos at 3 and 7 days post-oviposition and (B) in 3-day embryos incubated for 24 h under hyperoxic (40% O_2), normoxic (20% O_2) or mild hypoxic (10% O_2) conditions. Control embryos were placed in moist cheesecloth and incubated under normoxia (percentage O_2=20±0.33; *N*=3) but without perfusion of the incubation vials. Values are means ± s.e.m., *N*=3–4 samples of 200–300 embryos.

cricket species that does not undergo an embryonic diapause (Rakshpal, 1962a). Aerobic metabolism is not downregulated in diapause embryos compared with 3–4 day non-diapause embryos. This result is surprising given that diapause in many species is characterized by a 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). Many species of insects remain responsive to changes in environmental conditions throughout diapause (Koštál, 2006), and consequently it is probable that if metabolic downregulation in *A. socius* were to occur in nature it would be mediated by external factors (e.g. low temperatures operating through the Q_{10} effect) rather than internal mechanisms. It is notable that 15 day 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 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 days post-oviposition in *A. socius* embryos, but decrease as development progresses to reach a steady state at 6 days in non-diapause embryos and 7 days in diapause embryos. Under normal conditions, healthy mammalian cells maintain an [AMP]:[ATP] of 0.01 (Hardie and Hawley, 2001). In this cricket species, the [AMP]:[ATP] ratio is 4:1 at 2 days 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] has been observed during embryonic diapause in a number of animals including *Artemia franciscana* (J.R., J. Covi and S.H., unpublished observations) and *Astrofundulus limneaus* (Podrabsky and Hand, 1999). The increased [AMP] observed in these species may contribute to metabolic downregulation and developmental arrest by acting through 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 processes including, but not limited to, a decrease in fatty acid synthesis and lipogenesis and inhibition of cell proliferation (Hardie and Hawley, 2001). AMPK activity is required to inhibit cell proliferation during dauer formation in *C. elegans* (Narbonne and Roy, 2005). Although high [AMP]:[ATP] ratios may activate AMPK and contribute to metabolic downregulation during diapause in some species, it is unlikely that AMPK is a major regulator of diapause in *A. socius* embryos for a number of reasons. First, [AMP]:[ATP] ratios in diapause embryos are not significantly different from those of non-diapause embryos in the first 10 days post-oviposition. Second, the highest [AMP]:[ATP] ratios were measured well before developmental arrest occurs. Third, although 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 (Izumigama and Suzuki, 1986), and adenylate levels are similar in non-diapause and diapause-destined *Bombyx mori* embryos (Suzuki and Miya, 1983). Thus, it is unlikely that activation of AMPK is a universal mechanism for regulating diapause induction. We cannot rule out the possibility that AMP may be partitioned, for example, in yolk material. There is evidence that cyclic AMP (cAMP) may be associated with yolk proteins (vitellin in particular) in the embryos of some insects (Satio et al., 1990). However, there are no data available on the distribution of AMP in insect embryos.

One plausible explanation for the unusual adenylate status of *A. socius* embryos is that early development occurs with an intracellular environment that may be hypoxic. High [AMP]:[ATP] ratios are characteristic of insects facing oxygen limitation because, unlike anoxia-tolerant vertebrates that are able to maintain high [ATP] under hypoxia (Hochachka, 1997), insects do not typically defend ATP levels under oxygen limiting conditions (Hochachka et al., 1993; Wegener, 1993; Hoback et al., 2000; 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. Although 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 functional oxygen delivery system (Gillott, 2005). The probable consequence is that insect embryos develop with an internal 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 days of embryogenesis, and Sakano et

al. (Sakano et al., 2004) suggest this is evidence of these embryos relying on anaerobic metabolism early in development. Woods and Hill (Woods and Hill, 2004) measured oxygen levels within *M. sexta* eggs and found that oxygen levels are well below air saturation throughout development and that the P_{O2} at the center may be as low as 2 kPa (Woods and Hill, 2004). Whether this P_{O2} is sufficiently low to limit oxygen availability at the mitochondrion is an open question; the only mitochondrial P_{50} (the partial pressure of oxygen at which respiration is half maximal) available for an invertebrate species is below 0.1 kPa (Gnaiger et al., 2000). If *A. socius* embryos are oxygen limited during early development, then we predict that: (1) CR ratios will be indicative of anaerobic metabolism; (2) lactate levels will be high early in development; and (3) biochemical indicators of anaerobic poise (e.g. lactate and high [AMP]:[ATP] ratios) will be reduced when embryos are incubated under hyperoxic conditions.

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 whereas higher (more negative) values suggest that anaerobic pathways contribute to the total energy flow and lower values are characteristic of animals recovering from anoxia (Hand, 1991). The interpretation of these values is based on theoretical calculations of oxycaloric equivalents, the predicted amount of heat energy expected per mol of oxygen consumed when a substrate is completely oxidized to carbon dioxide and water (Hand, 1991; Hand, 1999). CR ratios calculated for 3 days and 7 days *A. socius* embryos are well below the values predicted for aerobic metabolism on mixed substrates (Widdows, 1987; Gnaiger and Kemp, 1990) but are typical values for animals recovering aerobically from exposure to anoxic conditions (Hand, 1991). Thus the *A. socius* values are inconsistent with an ongoing anaerobic contribution to metabolism at the developmental stage for which the CR ratios were measured, but an earlier hypoxia bout during ontogeny is implicated. It is notable that CR ratios measured for these embryos are within the range of values determined for embryos of another insect, *Tribolium confusum* (Dunkel et al., 1979).

Lactate is a common anaerobic end product in many animals, including insects, and is present in 3 days *A. socius* embryos. The amount of lactate we measured for this developmental stage is comparable to lactate levels measured for 3-day *Bombyx mori* embryos (Sakano et al., 2004) and suggests anaerobic metabolic pathways are utilized early during embryogenesis. Lactate decreases by 84% during the next 4 days of development. It is notable that [AMP]:[ATP] ratios decrease and [ATP]:[ADP] ratios increase during the same period. Furthermore, the timing of these observed changes corresponds with an increase in the average mass of the embryo. This mass gain appears to result primarily from the absorption of water as there is not a corresponding increase in dry mass (see Fig. 1). Orthoptera embryos commonly absorb water during embryogenesis (e.g. Browning, 1965; Tanaka, 1986b). However, the physiological and/or biochemical relevance of this phenomenon is unknown. We hypothesize that 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 that suggests that water absorption is tied to enhanced oxygen diffusion (Seymour et al., 1991). Alternatively, water accumulation may be related to desiccation resistance in this small embryo.

That hypoxic conditions may have been experienced within embryos of *A. socius* does not fully explain the indicators of metabolic poise, because our 24 h incubation of embryos under 40% O_2 did not significantly lower the amount of lactate in 3-day embryos as we predicted it might. Superfusing 3-day embryos with 40% O_2 moderately decreased [AMP]:[ATP] ratios and moderately increased [ATP]:[ADP] ratios compared with control embryos incubated under normoxic conditions without perfusion. However, as mentioned above, 40% superfusion was not successful in moving these ratios into ranges typically observed for animal cells; and in the case of [AMP]:[ATP], there were no differences among any of the superfused treatments – hypoxia, normoxia or hyperoxia. Therefore, observed lactate and adenylate ratios are in part an intrinsic, ontogenetic feature of these embryos.

It is difficult to surmise the ontogenetic role of lactate and adenylate ratios in *A. socius* embryos because the biochemistry of embryonic development is not fully understood for many animals. The accumulation of lactate in embryos of some species including *B. mori* (Sakano et al., 2004) and the fish, *Fundulus heteroclitus* (Paynter et al., 1991) suggests there is a role for this metabolite beyond simply being an end product, for example, as a metabolic fuel during early development. Lactate may also help maintain the redox state both within and between cells (Gladden, 2004), function in cell-to-cell signaling (Brooks, 2002; Philp et al., 2005) and have a role in wound repair [Gladden (Gladden, 2004) and references therein]. Carbohydrates are known to be the primary fuel consumed during early embryogenesis for at least some Orthoptera (Hill, 1945), and a high [AMP]:[ATP] ratio may be advantageous to the developing embryo to favor the breakdown of carbohydrates and limit reactions leading to carbohydrate storage at a time of high fuel usage. The activity of glycogen phosphorylase, a key enzyme in glycogen catabolism is enhanced by AMP, and phosphofructokinase activity is stimulated by AMP and inhibited by ATP. Conversely, the activity of fructose-1,6-bisphosphatase, a control enzyme in gluconeogenesis, is strongly inhibited by AMP.

Several insights have emerged from this study about the features of embryonic diapause. In *A. socius*, diapause is not characterized by acute metabolic depression during entry, but rather by a cessation of the ontogenetic increase in metabolism evident in non-diapause embryos. There is an abrupt arrest of cell proliferation and development, which shows that diapause can serve the purpose of postponing progression through the life cycle, as part of an overwintering strategy, but without the metabolic downregulation typically associated with stoppage of direct development. If acute, stage-specific metabolic depression does occur, it must be mediated through direct environmental impact, for example by temperature change, and not through genetically programmed downregulation. The special biology of *A. socius* allowed us to compare developmental stages that were precisely matched morphologically and chronologically, so as to clearly separate ontogenetic changes from diapause-specific traits. In this regard, it is now clear that the surprising setpoints of biochemical indicators (including high AMP:ATP ratio, low ATP:ADP ratio, and high lactate) are not specific features of diapause embryos, but apply equally to the developing embryo. Our data suggest that hypoxia within the embryo may foster, to a limited degree, the unanticipated biochemical properties, because incubation under hyperoxia partially forces a more aerobic poise. Still, we are left with the conclusion that the prevailing ‘anaerobic poise’ is also a feature of this ontogenetic period of the life cycle.

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