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Diapause and Anhydrobiosis in Embryos of *Artemia franciscana*: Metabolic Depression, LEA Proteins and Water Stress

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Metabolic depression is typically correlated with extended survival of environmental challenge and energy-limitation in early life stages of various invertebrates and vertebrates. Diapause is an ontogenetically-programmed reduction of development and often metabolism seen in many invertebrates. When embryos of *Artemia franciscana* enter the state of diapause, the overall metabolic depression is estimated to be greater than 99%. These embryos also contain trehalose and express multiple isoforms of Late Embryogenesis Abundant (LEA) proteins, constituents often present in a number of such anhydrobiotic animals. The mRNA levels for LEA proteins are highest in diapause and post-diapause embryos that possess desiccation tolerance, but are very low in desiccation-intolerant nauplius larvae. Stable transfection of human HepG2 cells with AfrLEA2 and AfrLEA3m was performed to evaluate the possibility of improved survivorship during drying. A trehalose transporter was used for intracellular loading of this disaccharide. LEA proteins improved desiccation tolerance in mammalian cells during acute drying and immediate rehydration.

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INTRODUCTION

In embryonic stages, metabolic depression and cell stasis are often prerequisites to survival for animals whose evolutionary history has provided natural adaptations to desiccation, freezing temperatures and anoxia[1-7]. For example, Podrabsky et al.[8] attribute the extreme anoxia tolerance of embryos of the annual killifish *Austrofundulus limnaeus* to various metabolic traits exhibited during diapause: depressed rates of metabolism, protein synthesis and ion transport, and large stores of glycogen and amino acids like glutamate and glutamine. These latter amino acids can be metabolized to γ-aminobutyrate (GABA) for protection of neural tissues[8]. Anoxia tolerance peaks

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during diapause II with an LT50 of about 65 days at 25°C[8,9]. Thus, the diapause state predisposes these fish embryos to survival under anoxia. The metabolic depression exhibited by embryos of *A. franciscana* is perhaps the deepest ever reported for an animal in diapause[10,11]. The proximal mechanisms for this arrest have recently been elicidated[11] and will be briefly reviewed below. Diapause embryos of *A. franciscana* exhibit extreme tolerance to desiccation and anoxia[12].

Other important features of *A. franciscana* as related to desiccation tolerance are differential expression of LEA proteins and high intracellular levels of trehalose, specifically during the embryonic stages of the life cycle[13-15]. Multiple LEA isoforms are present, which are targeted to various cellular compartments. Finally, stable transfection of two of these LEA genes from *A. franciscana* into human HepG2 cells improve membrane integrity and survival during rapid drying in the presence of trehalose[16].

Metabolic arrest during diapause

Encysted embryos of *Artemia franciscana* undergo a dramatic respiratory depression upon release from the adult female as they enter diapause (Fig. 1). We have recently shown that strategic enzymes involved in trehalose catabolism are inhibited during diapause, namely trehalase, hexokinase, pyruvate kinase and pyruvate dehydrogenase[11]. Thus restriction of oxidative substrate to the mitochondrion involves an orchestrated inter-play at multiple enzymatic sites (Fig. 2). Trehalose is the sole source of fuel in the embryos of *A. franciscana*, and hence downregulation of trehalose catabolism results in severe limitation of metabolic fuel available to the embryo during diapause[11]. Western blot data demonstrated that pyruvate dehydrogenase (PDH) was phosphorylated during entrance into diapause, and as a consequence, a strong inhibition of PDH would be expected[11,17]. Restriction of glycolytic flux will lead to metabolic 'starvation' of the mitochondrion, and in turn will reduce mitochondrial oxidative phosphorylation during diapause.

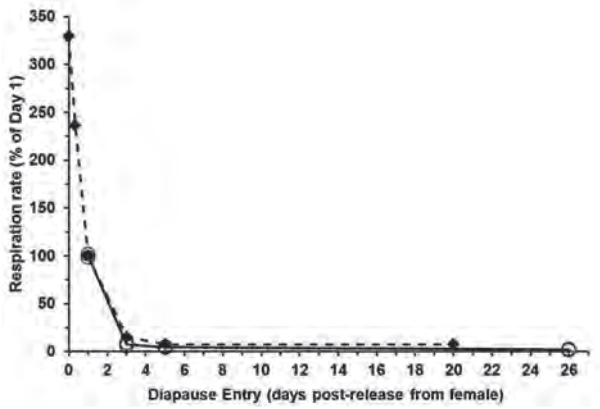

Fig. 1. Depression of respiration rate in diapause embryos of *A. franciscana* from the Great Salt Lake (solid line) during entry into diapause. Embryos were synchronized to within 4 h of their release from ovigerous females. Independent replicates for two batches are displayed by superimposed open circles. Data are also provided for San Francisco Bay embryos [dashed line, solid diamonds; Clegg et al.[10]] expressed relative to day 1 values to allow comparison. Modified from Patil et al.[11].

Measurements of ATP, ADP and AMP showed that substantial decreases occur in ATP:ADP ratio during diapause (Table 1). The concentration of ATP in diapause embryos was about 5 fold lower compared to post-diapause embryos, and there was a corresponding increase in AMP. While the elevated AMP and lowered ATP in diapause are consistent with an energy-limited state, the changes in adenylates are not nearly as dramatic as those measured under anoxia in post-diapause embryos, as reviewed previously[7].

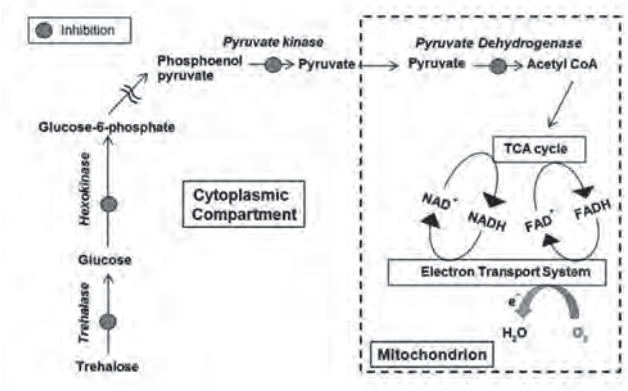

Fig. 2. Schematic representation of metabolic arrest in *A. franciscana* during diapause. Carbon flux is depressed at the trehalase reaction, two sites in the glycolytic pathway (hexokinase, pyruvate kinase), and at least one site in the mitochondrion (pyruvate dehydrogenase). The restriction of carbohydrate metabolism leads to substrate limitation at the mitochondrion and promotes downregulation of oxygen consumption. Modified after Patil et al.[11].

Table 1. Adenylate amounts in diapause and post-diapause embryos. Modified from Patil et al.[11].

Adenylates	Diapause (μmoles/g wet embryo)	Post-diapause (μmoles/g wet embryo)
AMP	0.622 ± 0.036	0.031 ± 0.005
ADP	0.126 ± 0.010	0.112 ± 0.006
ATP	0.168 ± 0.008	0.812 ± 0.010
ATP/ADP	1.306 ± 0.036	7.300 ± 0.276

Concentrations are expressed as mean ± SE, n = 10 (diapause), n = 8 (post-diapause); $p<0.0001$ (diapause versus post-diapause for AMP and ATP). ADP concentration is not statistically different between diapause and post-diapause embryos.

Expression of lea genes in *A. franciscana*

Expression of LEA proteins is tightly correlated with desiccation tolerance in anhydrobiotic animals. We have identified and sequenced multiple LEA genes in embryos of the brine shrimp *Artemia franciscana*[13-15]. Developmental stages with the capacity for anhydrobiosis (diapause and post-diapause embryos) expressed high LEA mRNA levels compared with the nauplius larva, which is desiccation-intolerant. For example, mRNA levels for *Afrlea1* and *Afrlea2* (which encode two cytoplasmic LEA proteins) were 7-fold and 14-fold higher in the desiccation-tolerant stages compared with the nauplius larva. The mRNA for the mitochondrial *Afrlea3m* was elevated 9-fold and 11-fold. These expression patterns are consistent with a role for these gene products in survivorship during dehydration [for extended review, see Hand et al.[15]].

Two of the six genes (*Afrlea2* and *Afrlea3m*) have been cloned into a bacterial expression system, and the proteins over-expressed, and purified. Multiple molecular weight variants of AfrLEA2 and AfrLEA3m have been detected in *A. franciscana* embryos with antibodies raised against the two recombinant proteins (Boswell, Moore and Hand, unpublished). Similar to mRNA expression summarized above, amounts of AfrLEA2 and AfrLEA3m protein are highest in diapause and post-diapause embryos, but are much lower in the desiccation-intolerant nauplius larvae. Preliminary data suggest that the endogenous protein concentration of the 75 kDa variant of AfrLEA2 is above 1 mg per g embryo water during diapause and early post-diapause development (Boswell, Moore and Hand, unpublished).

Transfection of human HepG2 cells with LEA genes and improved tolerance to drying

When one considers the growing literature on the protective roles of LEA proteins during desiccation[15,18], a logical extrapolation is that genetically equipping mammalian cells with LEA proteins targeted to different subcompartments should improve cellular outcomes during drying, particularly in the presence of the protective sugar trehalose[15,16]. Recently, we described the stable transfection of human HepG2 cells with AfrLEA2 and AfrLEA3m with a tetracycline-inducible expression system in order to evaluate the possibility of improved survivorship during drying[16]. Trehalose was loaded into these cells using a trehalose transporter [TRET1 from *Polypedilum vanderplanki*[19]],which was stably transfected into the HepG2 cells.

HepG2 cells suspended in a trehalose solution were rapidly and uniformly desiccated to a low moisture content (<0.12 g H_2O/g dw) using a recently described spin-drying technique[20]. Spin-drying has several important advantages over the more generally practiced sessile droplet evaporative drying. Spin-drying is orders-of-magnitude faster because the bulk of extracellular fluid is removed by forced convection during the spinning process followed by drying of a very thin film by natural convection under dry nitrogen, whereas, droplet drying removes water solely by natural convection and thus is a slow process. Thus, biological stresses associated with removal of water from cells by spin-drying should be significantly less deleterious than with droplet drying[20].

Upon immediate rehydration, membrane integrity of cells was assessed by SYTO 13/propidium iodide staining (Fig. 3). Control HepG2 cells (without LEA protein or intracellular trehalose) exhibited 0 % membrane integrity (n = 9)[16]. HepG2-TRET1 cells preloaded with trehalose showed 44.5 ± 22.2 % integrity (mean ± SD, n = 3); HepG2-AfrLEA2 cells without trehalose, 57.2 ± 13.0 % (n = 9); HepG2-AfrLEA2 cells preloaded with trehalose, 98.3 ± 2.2 % (n = 9); HepG2-AfrLEA3 cells without trehalose, 93.6 ± 4.6 % (n = 9); and HepG2-AfrLEA3 preloaded with trehalose, 97.7 ± 3.8 % (n = 9). The high membrane integrity of HepG2 cells with AfrLEA3m in the absence of trehalose loading was remarkable. Work with model synthetic peptides composed of several tandem motifs of group 3 LEA proteins vitrify at a high T_g, which suggests that dried LEA proteins may vitrify and contribute as protectants in this manner[21]. While AfrLEA3m is indeed mitochondrial-targeted, synthesis occurs in the cytosol and import into mitochondria is time dependent, so presumably, some amount of the protein is continuously resident in the cytoplasm[16]. Growth studies across subsequent days after rehydration revealed higher proliferation for cells containing LEA protein and trehalose than those without.

It is important to note that HepG2 cells provisioned with intracellular trehalose and LEA proteins are not stable at room temperature in the dried state and begin losing viability if not rehydrated within minutes[16]. As discussed by Li et al, the spin-dried samples gave a T_g of approximately 4.5 °C, which suggested that the cells were not in a vitrified state at room temperature. Stable, long-term storage of cells will require, for example, increasing the intracellular concentration of trehalose to elevate the T_g and thereby hopefully improve the storage outcomes at room temperature. Indeed, the loss of viability can be retarded by immediate storage of the dried cells at liquid nitrogen temperatures[20]. Our ultimate goal is to identify conditions that

support survival of cells during extended storage in the dried state at room temperature. However, these current data support the ability of LEA proteins to enhance tolerance to water stress in mammalian cells.

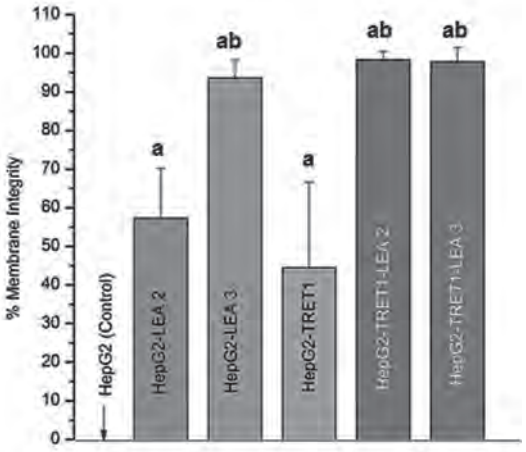

Fig. 3. Membrane integrity of HepG2 cells after spin-drying and immediate rehydration. HepG2 cells with TRET1 transporters were incubated in 50 mM trehalose containing medium for 18 h prior to spin-drying. Membrane integrity was determined using Syto-13 and ethidium bromide viability dyes. Values represent means ± SD (n = 3-9 independent determinations). The letter 'a' indicates statistically significant differences ($p<0.05$) versus the control (no intracellular trehalose or LEA protein), and 'b' indicates significance ($p<0.05$) versus cells with intracellular trehalose alone (HepG2-TRET1). Modified from Li et al.[16].

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