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Diapause Termination and Hatching of Artemia franciscana: Impacts on Expression of a Late Embryogenesis Abundant Protein

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Diapause Termination and Hatching of *Artemia franciscana*: Impacts on Expression of a Late Embryogenesis Abundant Protein

by

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Abstract

Embryonic stages of organisms like the brine shrimp, *Artemia franciscana,* that survive anhydrobiosis, enter a developmental and metabolic arrest that is termed diapause prior to the onset of drying. Late Embryogenesis Abundant (LEA) proteins contribute to desiccation tolerance during water stress. Expression of AfrLEA6, a group 6 LEA protein found in *A. franciscana*, is high in encysted diapause embryos and decreases during post-diapause development, eventually disappearing by the larval stage. However, it is unclear what effects various treatments used to terminate diapause may have on AfrLEA6 expression. In this project, I evaluated drying and exposure to H_2O_2 as diapause terminators. H_2O_2 treatment is far more effective at terminating diapause than drying (75.9 ± 4.1% hatching versus 1.7 ± 0.2%, respectively; means ± SEM; n = 3) for embryos from the Great Salt Lake, Utah. Extending the drying time from 24 h to 1 week resulted in no statistical change. When both treatments were combined, no significant improvement occurred compared to using H_2O_2 alone. H_2O_2 treatment was far more effective when administered to hydrated versus dehydrated embryos, at least when 24 h bouts of drying were applied. AfrLEA6 was quantified in diapause embryos as well as in those given treatments that could potentially terminate diapause. Treatment with H_2O_2, which is shown above to be highly effective at diapause breakage, resulted in a 30.4% decrease in AfrLEA6 concentration (0.47 ± 0.01 µg of AfrLEA6/mg total protein; mean ± SEM; n = 3), which is similar to the AfrLEA6 content in commercial post-diapause embryos. These findings provide an improved understanding of the mechanisms regulating diapause. Information on the quantities of AfrLEA6 expressed may help clarify the role of this protein during development and may aid future studies designed to transfect LEA proteins into mammalian cells, with the ultimate goal of bioengineering tissues for better desiccation storage.

Introduction

Diapause is a genetically-programmed form of developmental and often metabolic arrest seen in the life cycle of many species that occurs prior to the onset of harsh environmental conditions (Danks, 1987; Denlinger, 2002; Denlinger et al., 2012; Drinkwater and Clegg, 1990; Hahn and Denlinger, 2011; Hairston and Kearns, 2002, Hand, 1991; Hand et al., 2016; Hand et al., 2018; Hand and Hardewig, 1996; Lees, 1955; MacRae, 2010; Podrabsky and Hand, 2015). Controlled by internal factors within the organism, diapause is often displayed by organisms that inhabit environments prone to changing drastically (Hand et al., 2016). For example, the brine shrimp *Artemia franciscana* found in the Great Salt Lakes (GSL) enters diapause in the fall of the year at the gastrula stage of embryonic development (Clegg and Conte, 1980; Hand and Menze, 2015). Because the lake is shaped as a flat basin with shallow depth, it undergoes substantial changes in salinity due to evaporation as well as seasonal temperature fluctuations (Baxter, 2018). Diapause serves as an overwintering strategy vital for the survival of the *A. franciscana* embryos until they are released from the diapause state in the spring and resume development. Transitions in water content occur during its life cycle, and protective agents including sugars and Late Embryogenesis Abundant (LEA) proteins (Tunnacliffe and Wise, 2007; Hand et al., 2011) protect the animal from damage during desiccation. This thesis examines incubation conditions that promote termination (breakage) of diapause in *A. franciscana* embryos and reports changes in concentrations of a LEA protein before and after diapause termination.

The adult female of *A. franciscana* can alternate from producing embryos that develop within its ovisac and are released into the water column as free-swimming nauplius larvae (ovoviviparous reproduction) to producing encysted embryos released from the ovisac in

diapause that overwinter in the lake or on the shoreline (oviparous reproduction) (Patil et al., 2013). The transition in mode of reproduction typically occurs in response to mainly photoperiod cues and (to a lesser extent) colder temperature sensed by the adult female in the environment (Nambu et al., 2004; Podrabsky and Hand, 2015). In addition to the developmental arrest at the gastrula stage, the metabolism of the diapause embryos can decrease by as much as 97% based on oxygen consumption (Clegg et al., 1996; Patel et al., 2013). However, the arrest in both metabolism and development do not occur at the same time. When the diapause embryos are released into the water, it typically takes 4-5 days for the embryos to reach maximal depression of metabolic rate (Clegg et al. 1996). This metabolic and developmental arrest preserves internal energy stores across the winter so that sufficient energy is available in the spring when development resumes (Patil et al., 2013). High concentrations of the sugar trehalose (Clegg and Filosa, 1961; Crowe et al., 1987a; Crowe et al., 1997; Xie and Timasheff, 1997; Tapia and Koshland, 2014) and LEA proteins (Hand et al., 2007, Warner et al., 2010; Hand et al., 2011; Warner et al., 2012; Toxopeus et al., 2014) are found in diapause and post-diapause embryos of *A. franciscana*. Trehalose (a non-reducing disaccharide of glycose) is well documented to protect lipid membranes and proteins during desiccation through mechanisms that include vitrification and water replacement (Webb, 1965; Clegg and Conte, 1980; Clegg and Jackson, 1992; Yancey et al., 1982; Crowe et al., 1997; Yancey, 2005).

LEA proteins are intrinsically disordered proteins found in the multiple cellular compartments of bacteria, slime molds, fungi, plants and four phyla of animals (Nematoda, Rotifera, Tardigrada, Arthropoda) (Hand et al., 2011). Reports of LEA-like protein in animals have steadily increased and have linked their functions to improvement of desiccation tolerance (Goyal et al., 2005; Toxopeus et al., 2014; Boswell et al., 2014). LEA proteins assist in

desiccation tolerance by preventing other proteins from aggregating in addition to stabilizing the membrane of organelles such as the chloroplast and mitochondria. (Goyal et al., 2005; Steponkus et al., 1998; Tolleter et al., 2010; Hand and Menze, 2015). There are also synergistic effects between LEA proteins and trehalose, with LEA proteins stabilizing the sugar glasses formed by trehalose to resist physical stress caused by desiccation (Wolkers et al., 2001; Goyal et al., 2005). As water content decreases, LEA proteins gain secondary structure (predominantly alpha helix) and lose random coil, indicating a gain in function during desiccation (Goyal et al., 2003; Mouillon et al., 2006; Li and He, 2009). There are three groups of LEA proteins found in *A. franciscana*, with Group 1, 3, and 6 reported to be present in diapause embryos (Hand and Menze, 2015).

LEA proteins are classified by homologous amino acid motifs shared within a specific group (Wise, 2003). Group 1 LEA proteins in *A. franciscana* contain a 20 amino acid repeating hydrophilic motif and are associated with desiccation and cold temperatures tolerance (Toxopeus et al., 2014). Group 3 LEA proteins contain a repeating 11 amino acid motif (Tunnacliffe and Wise, 2007), and the four known proteins in the group found in *A. franciscana* (AfrLEA2, AfrLEA3_m, AfrLEA3_m29, and AfrLEA3_m47) are localized in specific parts of the cell with AfrLEA2 localizing in the cytoplasm (Hand et al., 2007) and AfrLEA3_m, AfrLEA3_m29, and AfrLEA3_m47 localizing in the mitochondria (Menze et al., 2009). All four of these AfrLEA3 proteins in *A. franciscana* are reported to also contribute to desiccation tolerance in diapause embryos, but they vary in size with AfrLEA2 being slightly larger (38.9 kDa as a monomer and ~70 kDa in its native dimeric state) and AfrLEA3_m being smaller (34.1 kDa) even with its mitochondrial targeting sequence included (Boswell et al. 2014). The roles of Group 1 and Group 3 LEA proteins in *A. franciscana* have both been evaluated, but not much is known about

the role of the Group 6 LEA protein AfrLEA6 (27.1 kDa) plays in *A. franciscana* embryos with ongoing studies currently assessing its localization and protective capabilities (LeBlanc and Hand, unpublished). However, the sequence of AfrLEA6 is reported to be homologous to a subset of Group 6 LEA proteins called seed maturation proteins (SMP) in plants (Hand and Menze, 2015; Janis et al., 2017), which were associated with dissociation of protein aggregates during rehydration (Boucher et al., 2010;) and long-term desiccation tolerance (Chatelain et al., 2012). Thus, AfrLEA6 is predicted to show similar capabilities to those found in SMPs. Additionally, the involvement of multiple LEA proteins within one organism calls into question the biological significance of such apparent redundancy (Hand and Menze, 2015). Understanding the role AfrLEA6 plays in concert with the other AfrLEA proteins present in diapause embryos will provide a clearer overall picture of their capabilities.

The distribution of LEA proteins across plants and animals raises the evolutionary question about their origins. It is very challenging to explain such distribution by vertical gene transfer through either sexual or asexual reproduction. For example, among arthropods, LEA proteins have been found in one insect species (the African chironomid, *Polypedilum vanderplanki*) but not in its congener, *P. nubifer* (Gusev et al., 2014). Acquisition of LEA proteins in selected animals by horizontal gene transfer (the uptake of genetic material from another organism) may explain the link between LEA proteins in animals and plants, with common ancestors likely being bacteria or fungi. The anhydrobiotic bdelloid rotifer *Adineta ricciae* has 9.7% of identifiable genes that are foreign, obtained through horizontal gene transfer (Boschetti et al., 2011, 2012; Eyres et al., 2015; Szydlowski et al., 2015). At least three of these genes obtained through horizontal gene transfer were upregulated during desiccation. It is quite

possible that *A. franciscana* may have acquired genes for LEA proteins through horizontal gene transfer. Comparative genomic studies with *A. franciscana* would be useful.

While in diapause, the embryos are more resistant to environmental stresses like desiccation and anoxia. Eventually, termination cues release the embryos from diapause. Termination cues occurring naturally in the environment include drying and cold temperatures (Drinkwater and Crowe, 1987; Lavens and Sorgeloos, 1987). Diapause embryos of *A. franciscana* in GSL often are washed ashore, where they may undergo multiple cycles of dehydration and rehydration before eventually being deposited back into the lake (Patil et al., 2013). For some diapause embryos, they remain on in the lake surface during the winter when the cold temperature eventually terminates their diapause. Additionally, diapause breakage can also occur through artificial means such as incubation in H_2O_2 (Lavens and Sorgeloos, 1987). It is important to note that the exact mechanisms for how diapause is broken because of these termination cues are not known. There are various theories on possible reasons for diapause termination such as the splitting of carotenoid proteins to free carotenoids and free proteins that disrupts the diapause mechanism (Dutrieu 1960; Lavens and Sorgeloos, 1987). Various strains of *A. franciscana* such as ones from the San Francisco Bay and the GSL have been reported to give different hatch percentages in response to diapause termination cues in the laboratory (Sorgeloos and Persoone, 1975). After diapause termination, the embryo continues preemergence development for several hours, then emerges from the cyst [emergence stage 1 (E1) and emergence stage 2 (E2)], and finally is released from the hatching membrane (termed hatching) as a free-swimming nauplius larva (Covi and Neumeyer, 2016). The emergence stages (E1 and E2) denotes when the embryo protrudes from its chorion (proteinaceous outer coat) but still is contained within the outer cuticular (‘hatching’) membrane in the shape of a parachute

(Neumeyer et al., 2015). *A. franciscana* from the GSL was chosen in this study due to the ease of obtaining large quantities of diapause cysts (commercial companies exist on the GSL with which our lab often collaborates). The purposes of this study are (1) to investigate the effects of combining H_2O_2 and drying treatments on diapause termination in *A. franciscana* to optimize the hatching percentage of post-diapause embryos and (2) to quantify the concentrations of AfrLA6 during diapause, after termination treatments, and during post-diapause.

Materials and Methods

Collection and handling of diapause embryos

Diapause embryos were collected from the surface of the GSL in the hydrated state by Dr. Brad Marden (Research Division, Great Salt Lake Artemia, Ogden, Utah). Embryos were maintained at ambient temperature in 1.25 M NaCl containing 200 units/ml nystatin, 50 mg/ml kanamycin, and 50 mg/ml penicillin-streptomycin and were protected from light (Patil et al., 2013). Hatching of recently collected embryos was very low (about 2.9%), indicative of a strong diapause condition.

Treatments for diapause termination and hatching assays

Diapause embryos were incubated for four days in artificial seawater (Instant Ocean; 35 practical salinity units) at room temperature in the dark on a shaker set at 110 rpm. Exposure to light has been associated with increased diapause breakage in the laboratory (Lavens and Sorgeloos, 1987). After the four-day incubation, diapause (unhatched) embryos were separated from any non-diapause embryos (hatched and developed into free-swimming nauplii) using a separatory funnel and subsequent filtration. The diapause embryos were then blotted using Whatman Filter Paper #2 as described in Clegg (1974) and used for various treatments below.

Treatments used for terminating diapause included air drying diapause embryos on a benchtop at room temperature and in ambient humidity (50-55%) for either 24 h or 1 week. Another treatment involved air drying of embryos for 24 h followed by rehydration at 0^{o}C in 0.25 M NaCl solution for 6 h, termed 1X dehydration/rehydration cycles (1X D/H). Drying hydrated diapause embryos for 24 h, rehydrating for 6 h, and then drying them again for 24 h was designated as 1.5X dehydration/rehydration cycles (1.5X D/H). Next, 2.0 dehydration/rehydration cycles (2X D/H) involved drying hydrated embryos for 24 h,

rehydrating for 6 h, drying again for 24 h, and then rehydrating for 6 h for a second time. Chemical treatments were performed by incubating the diapause cysts in 3% H_2O_2 prepared in 0.25M NaCl solution for either 15 or 30 min. Other experiments incorporated combinations of the above treatments to test for additive or sequence (order) effects of the various treatments.

After the above treatments, the embryos were incubated in artificial seawater in ambient light for 4 days. After the incubation, hatching assays were performed by first diluting aliquots of the treated samples with water in a plastic petri dish (bottom marked off into squares) and adding 12 N HCl to kill any free swimming nauplii. Then hatch counts were performed under a dissecting microscope. Typically, 200-400 organisms were counted for each trial. Hatching percentages were determined by counting the total nauplii and dividing by the total count (free-swimming nauplii + unhatched cysts + E1 and E2 stages).

Homogenization and extraction of diapause and post-diapause cysts

After blotting, 100 mg of embryos were weighed and homogenized in 1.9 ml of Laemmli buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, pH 6.8) for 5 min in a 5 ml ground-glass, hand-held homogenizer. In addition to treatments described above, some diapause embryos were dried in a dry-box with Drierite for 1 week and 5 weeks before homogenization. Other embryos were treated with 3% H_2O_2 prepared in 0.25M NaCl solution for 15 min at room temperature, rinsed with excess amounts of 0.25M NaCl to remove the H_2O_2, and then homogenized. Commercially-processed post-diapause embryos (dried and shipped in vacuum-packed cans) were obtained from the Great Salt Lake and homogenized directly in 1.9 mL of Laemmli buffer in a similar fashion. Hydration of these commercial post-diapause embryos was also performed prior to homogenization by incubating embryos in 0.25 M NaCl at 0^{o}C for 6 h to determine the effects of hydration on the amount of AfrLEA6 in these commercial

embryos. These preparations of commercial post-diapause embryos permitted direct comparisons to the AfrLEA6 content of post-diapause embryos prepared in our laboratory as described earlier. Homogenates were then incubated in a heating block at 95^{o}C for 5 min and subsequently centrifuged at 10000 X *g* for 10 min (25^{o}C). Afterwards, the supernatant was collected and stored at -20^{o}C until used for Western blotting. Protein concentrations of the supernatants were determined by performing Lowry assays, using bovine serum albumin (BSA) as the standard (Peterson, 1977).

Quantitative assessment of AfrLEA6 by Western blotting

In order to compare AfrLEA6 concentrations across the various treatments, samples (30-40 µg total protein per lane) were loaded onto SDS polyacrylamide gels (4% stacking and 11% resolving). Aliquots of a biotinylated protein ladder (Cell Signaling Technology, Product #81851) were also loaded onto the gels for estimates of molecular mass. The gel was then electrophoresed for 60-70 mins at 125 V in running buffer containing 25 mM Tris-base, 191 mM Glycine, 1% SDS, pH 8.3. Afterwards, proteins from the gel were electro-transferred onto a nitrocellulose membrane for 60 mins at 80 V in Towbin's buffer (192 mM Glycine, 20% methanol, 0.025% SDS, and 25 mM Tris). Successful transfer was checked using Ponceau S staining, and then the membrane was washed gently with distilled H_2O to remove the stain. The membrane was blocked for 1 h in 5% dry milk solution prepared in TBS-T (0.1% Tween 20, 20 mM Tris-HCl, 500 mM NaCl, pH 7.6). Primary antibodies against recombinant AfrLEA6m (raised in chicken, Aves Labs Inc.) and alpha-tubulin (raised in rabbit, Cell Signaling Technology) was then added at 1:50,000 and 1:1000 dilutions, respectively, in fresh 5% dry milk prepared in TBS-T, and the blots were incubated overnight in the milk solution at 4^{o}C. A portion of the blot containing the biotinylated protein ladder was incubated in milk solution containing

no antibodies. The blots were washed 3 times in TBS-T the next day, and then the blots were incubated in horse radish peroxidase-linked (HRP) secondary antibody (goat anti-chicken, 1:10,000 dilution; Aves Labs Inc.) for AfrLEA6 and HRP-linked secondary antibody for alpha-tubulin (anti-rabbit, 1:1000 dilution; Cell Signaling Technology) for 1 h. The biotinylated protein ladder blot was incubated in anti-biotin, HRP-linked antibody at a 1:1000 dilution. All blots were then washed again with TBS-T as described above and incubated in Chemilumiscence substrate (Amersham ECL Prime- RPN2236) for 5 min before imaging with a Bio-RAD ChemiDoc XRS+, using Image Lab Software 6.0 (Bio-Rad, Inc, Hercules, California). Purified recombinant AfrLEA6 (Genbank accession no. MH351624) was used to prepare a standard curve (0 ng to 40 ng) for quantification purposes. The AfrLEA6 concentration in hydrated embryos were also reported as mg of AfrLEA6/ml embryo H_2O using the water content factor reported in Glasheen and Hand (1987).

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, New York, USA). Levene's test was performed to check for equal variance among data sets. To determine significant differences among treatments, one-way ANOVA was applied for data sets with equal variance, and Welch's ANOVA was used for data sets with unequal variance. For post-hoc tests used in pair-wise comparisons, Tukey Honestly Significant Difference (HSD) was chosen for data sets with equal variance, and the Games-Howell test was applied for data sets with unequal variances (Games and Howell, 1986; McDonald, 2014).

Results

Diapause termination and hatching percentages

Hydrogen peroxide and drying were first assessed independently to determine the more effective treatment for diapause termination. Treatment of hydrated diapause embryos with the 3% H_2O_2 solution for 15 min and 30 min resulted in 75.9 ± 4.1% and 72.6 ± 2.9% hatching (means ± SEM; n = 3), respectively **(Fig. 1)**. There was no significant difference between treating diapause cysts with 3% H_2O_2 solution for 15 min versus 30 min ($p = 0.998$), but both were very effective at terminating diapause. Drying the diapause embryos for 24 h versus 1.5X D/H cycles promoted 1.7 ± 0.2% and 7.7 ± 0.7% hatching (means ± SEM; n = 3), respectively (**Fig. 1**); the hatching percentage promoted for 1.5X D/H cycles was significantly higher than for 24 h drying ($p = 0.048$), but the difference was modest. Consequently, H_2O_2 is more effective at breaking the diapause state than is either drying regime (both p values < 0.05).

To assess the impact of order for combined treatments on hatch percentages, the treatments that gave the highest hatch percentages for the H_2O_2 and drying experiments were selected for combination testing. This series also allowed the effectiveness of H_2O_2 treatment to be compared when applied to dry versus hydrated embryos. Treating hydrated diapause embryos with 3% H_2O_2 solution for 15 min followed by 1.5X D/H cycles gave an 80.7 ± 1.7 % hatch (mean ± SEM; n = 3) (**Fig. 2**). Reversing the order of these two treatments on the diapause embryos yielded a 63. 9 ± 1.6 % hatch (mean ± SEM; n = 3), which was significantly lower ($p = 0.02$) than the original order. Completion of two full dehydration/hydration cycles (2X D/H) followed by a 3% H_2O_2 solution for 15 min gave a hatch % of 87.1 ± 1.7 (mean ± SEM; n = 3), which was significantly higher compared to applying 1.5 D/H cycles before 3% H_2O_2 treatment ($p < 0.05$); however, 2X D/H followed by 3% H_2O_2 was not significantly different from 3%

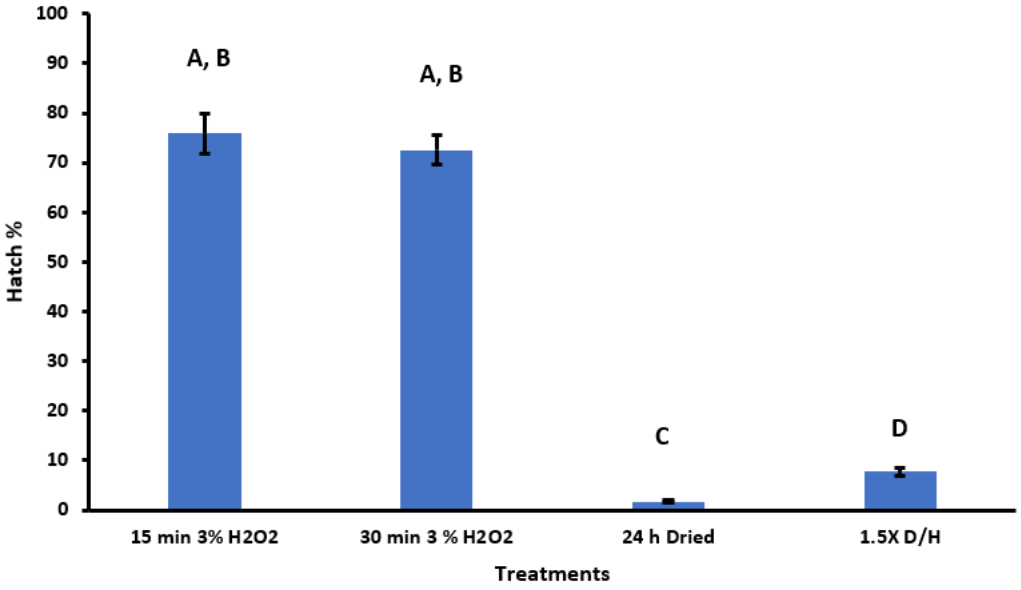

Figure 1. Hatch percentages of diapause embryos from *A. franciscana* after H_2O_2 and drying treatments. All treatments were performed at ambient humidity and at room temperature. 1.5X D/H is defined as drying hydrated diapause embryos for 24 h, rehydrating for 6 h, and drying again (24 h) prior to the 4-day incubation to promote hatching. Different letters indicate a statistically significant difference in hatching percentage. Bars represent means ± SEM, n = 3; Levene's F (9, 20) = 5.742, p = 0.001). Welch's ANOVA: F (9, 7.88) = 998.71, p = 0.000.

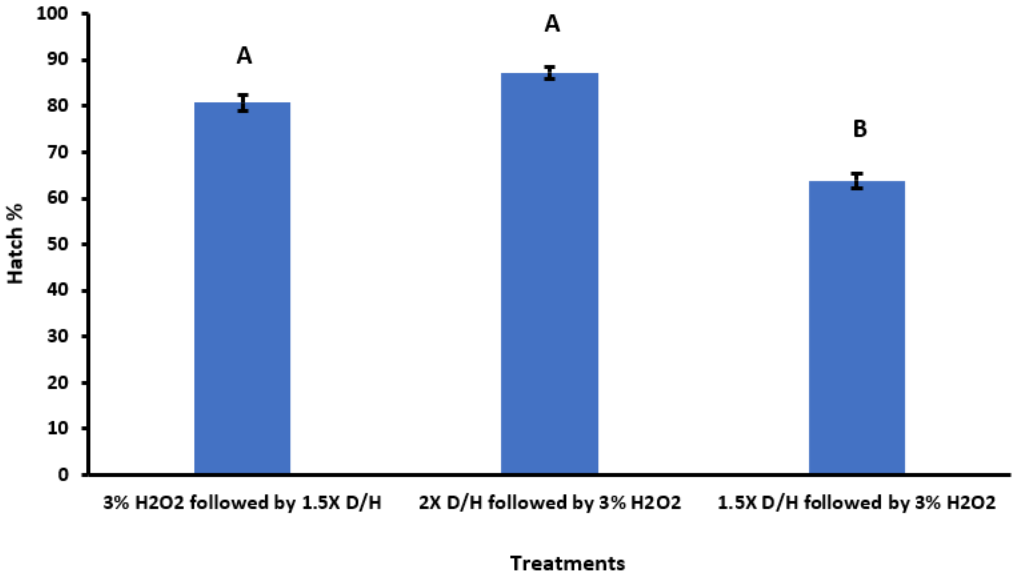

Figure 2. Hatch percentages of diapause embryos from *A. franciscana* after combined treatments with H_2O_2 and drying. All treatments were performed at ambient humidity and at room temperature. 1.5X D/H is defined as drying hydrated diapause embryos for 24 h, rehydrating for 6 h, and drying again (24 h) prior to the 4-day incubation to promote hatching. 2X D/H is defined as drying hydrated embryos for 24 h, rehydrating for 6 h, drying again for 24 h, and then rehydrating for 6 h for a second time. Different letters indicate a statistically significant difference in hatching percentage. Bars represent means ± SEM, n = 3; Levene's $F(9, 20) = 5.742$, $p = 0.001$). Welch's ANOVA: $F(9, 7.88) = 998.71$, $p = 0.000$.

H_2O_2 followed by 1.5X D/H ($p = .295$). Results suggest that applying H_2O_2 is more effective when the embryos are in the hydrated state and that treatment order per se is less important (**Fig. 2**).

One week drying was also assessed to determine whether longer term drying would impact hatch percentages. Drying diapause embryos for 1 week resulted in a 1.8 ± 0.3 % hatch (mean ± SEM; n = 3) **(Fig. 3)**. Thus, one week drying is very ineffective at terminating diapause and is equivalent to drying for only 24 h ($p = 0.019$; **Fig. 1**). Drying diapause embryos for 1 week followed by 3% H_2O_2 solution for 15 min promoted 69.08 ± 4.23 % hatching (mean ± SEM; n = 3). Switching the order with 3% H_2O_2 for 15 min first and then drying the embryos for 1 week gave a hatching percentage of 50.89 ± 0.58 (mean ± SEM; n = 3); however, there was no significant difference between the two orders ($p = 0.228$). Drying for 1 week alone was much less effective at terminating diapause than combining the 1 week drying with H_2O_2 in either order (both $p < 0.05$).

Finally, the effects of combining treatments on hatch % was also assessed by performing pair-wise comparisons between treatments of H_2O_2 by itself to any of the treatments that combined drying and H_2O_2 together. Comparing 3% H_2O_2 by itself, either 15 minutes or 30 minutes, (**Fig. 1**) to any of the combined treatments that included H_2O_2 (**Fig. 2, 3**) showed no significant differences (all p values > 0.05). Thus, the combination of drying and 3% H_2O_2 was not more effective at terminating diapause than H_2O_2.

AfrLEA6 quantification

Diapause embryos in the hydrated state prior to any additional treatments contained 0.75 ± 0.05 µg of AfrLEA6/mg total protein (mean ± SEM, n = 8) **(Fig. 4)** or 0.23 ± 0.05 mg AfrLEA6/ml embryo H_2O. Diapause embryos treated with 3% H_2O_2 for 15 min, which as shown

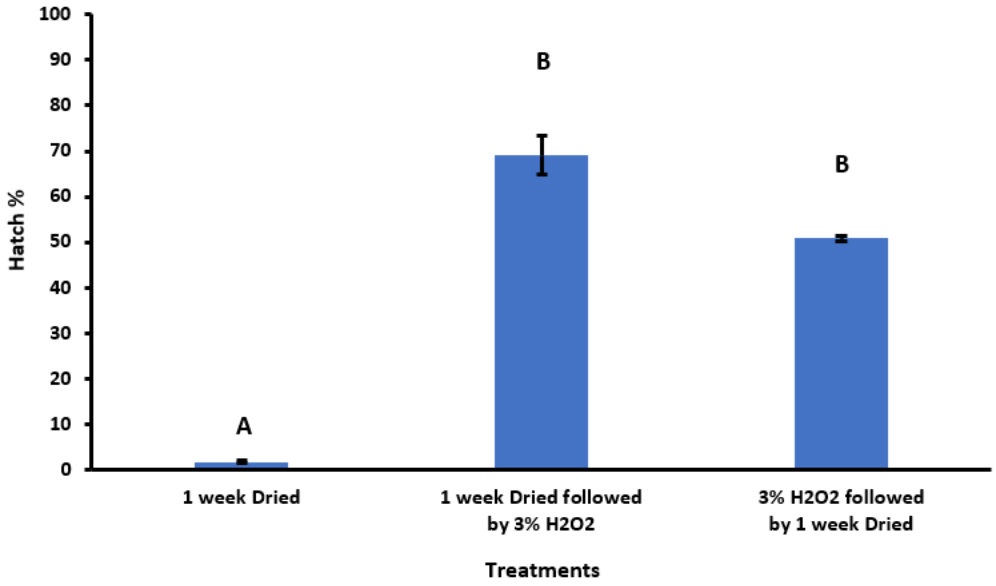

Figure 3. Hatch percentages of diapause embryos from *A. franciscana* after combined treatments with H_2O_2 and 1 week drying. All treatments were performed at ambient humidity and at room temperature. Different letters indicate a statistically significant difference in hatching percentage. Bars represent means ± SEM, n = 3; Levene's F (9, 20) = 5.742, p = 0.001). Welch's ANOVA: F (9, 7.88) = 998.71, p = 0.000.

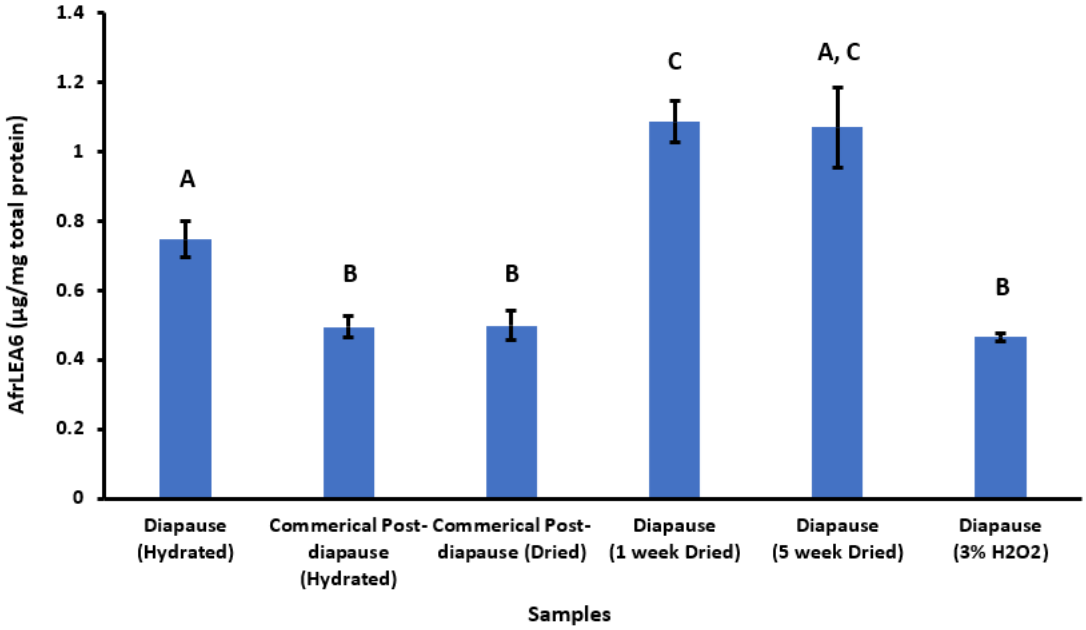

Figure 4. AfrLEA6 concentrations for various diapause and post-diapause embryos of *A. franciscana* not treated and treated with diapause-terminating protocols. AfrLEA6 was quantified by Western blotting. Different letters indicate a statistically significant difference in content. Bars represent means ± SEM. From left to right, n = 8, n = 6, n = 3, n = 6, n = 3, n =3. Levene's $F(5, 23) = 1.992$, $p = 0.118$). One-way ANOVA: $F(5, 23) = 21.1$, $p < 0.000$.

above is an effective treatment for diapause termination, contained 0.47 ± 0.01 µg AfrLEA6/mg total protein (mean ± SEM; n = 3) or 0.16 ± 0.01 mg AfrLEA6/ml embryo H_2O. This value represents a 30.4% decrease in AfrLEA6 compared to the hydrated diapause embryos (p = 0.026). Similarly, commercially-processed post-diapause embryos in the hydrated versus dried state contain 0.50 ± 0.03 µg and 0.50 ± 0.04 µg of AfrLEA6/mg total protein (means ± SEM; n = 6 and 3 respectively), which are not significantly different from each other (p = 0.999) **(Fig. 4)**. The AfrLEA6 titer of hydrated post-diapause embryos can be also be expressed as 0.15 ± 0.03 mg AfrLEA6/ml embryo H_2O. AfrLEA6 concentrations in both hydrated and dehydrated post-diapause embryos (commercially obtained) are significantly lower than the concentration of AfrLEA6 measured for the hydrated diapause embryos (p = 0.01, p = 0.028, respectively). Importantly, there are no statistical differences between AfrLEA6 concentrations in embryos released from diapause with H_2O_2 versus commercial post-diapause embryos, either hydrated or dried (p = 0.999, p = 1.0, respectively).

Diapause embryos dried for 1 week and 5 weeks contain 1.09 ± 0.06 and 1.07 ± 0.11 µg AfrLEA6 per mg total protein (means ± SEM; n = 6 and 3 respectively) **(Fig. 4)**, which are not statistically different from each other (p = 0.994). Thus, the length of drying does not influence the content of AfrLA6. Drying diapause embryos for 1 week results in a 45.3% increase in AfrLEA6 compared to hydrated diapause embryos (p = 0.002), although the change in AfrLEA6 content after 5 weeks drying is not significant (p = 0.052) due to the lower n value. Both the 1 and 5 week drying data for ArfLEA6 content are statistically different from commercial post-diapause embryos (both hydrated and dried) and the H_2O_2-treated diapause embryos (all p values < 0.05).

Discussion

In this study, I examined the effectiveness of H_2O_2 and drying on terminating diapause in embryos of *A. franciscana* from the Great Salt Lake, Utah, as well as its impact on AfrLEA6 concentrations during diapause and post-diapause. H_2O_2 was much more effective in breaking diapause than was drying, but in cases where both treatments were combined, no significant improvement was observed compared to using H_2O_2 alone. H_2O_2 treatment was also more effective when applied to hydrated embryos versus ones that were dry. Additionally, diapause termination with H_2O_2 resulted in a sharp decrease in AfrLEA6 concentration similar to the AfrLEA6 content in commercial post-diapause samples. These findings provide greater insights into efficient methods for diapause termination in *A. franciscana*, which is potentially important for understanding regulation of the diapause state, as well as improving commercial processing of *A. franciscana* embryos to yield higher hatch rates for this food source used by the aquaculture industry. Also, the impacts of these treatments on AfrLEA6 concentration are important in evaluating possible functions of this protein during development of *A. franciscana*.

Effectiveness of hydrogen peroxide and drying on diapause termination

H_2O_2 treatment for 15 min was equally effective as 30 min in breaking diapause in *A. franciscana*, and this strong oxidant was far more beneficial than drying. Drying was ineffective in releasing embryos from the GSL from diapause, even though other studies with *A. franciscana* embryos from San Francisco Bay have shown that drying under the same conditions (ambient humidity, room temperature, 24 h) was sufficient to break diapause with a maximal hatching of 80% (Clegg et al., 1996). These contrasting results underscore clear population differences in triggering the release of embryos from diapause and also perhaps in the depth of diapause among populations. The modest increase in hatch percentage observed with repeated dehydration in the

present study (1.5X D/H, 2X D/H) is comparable to the small increase in hatch percentage after multiple dehydration/rehydration cycles reported by Lavens and Sorgeloos (1987) for several populations of *A. franciscana*, including the GSL. Hydrogen peroxide has been documented previously to be effective with embryos from the GSL and San Francisco Bay populations (Clegg et al., 1996; Lavens and Sorgeloos, 1987). Unfortunately, the mechanistic basis for such differences are unknown, as is the ultimate underlying mechanism for diapause termination (Hand et al., 2016). The fact that distinctly different cues (low temperature, oxidative agents, desiccation) can be equally effective at breaking diapause depending on the population is perplexing.

Influence of order on the effectiveness of treatments

When combining treatments to determine the effects of order, treating diapause embryos with H_2O_2 first and then with 1.5X D/H gave a higher hatch percentage than treating embryos with 1.5X D/H first. A similar impact of order was seen with the 2X D/H experiments. These results suggest that H_2O_2 is more effective for diapause termination with embryos that are in the hydrated state versus the dried state, at least when 24 h bouts of drying are used. This finding is novel and has never been previously reported to my knowledge. It is puzzling, however, that when drying was extended to 1 week, the order of H_2O_2 application did not result in a statistically significant effect (**Fig. 3**). It is possible that the 1 week drying event may be somehow interfering with the effectiveness of the H_2O_2 treatment. Bogatova and Shmakova (1980) reported that drying diapause embryos of *A. salina* after H_2O_2 treatment for long term storage resulted in a loss of the H_2O_2 effects after a minimum of 7 days of storage. However, Lavens and Sorgeloos (1987) report a constant hatching rate when H_2O_2 treated *A. franciscana* embryos were dried and stored for 4 months in air or vacuum. An experiment that could address

the effectiveness of H_2O_2 in this context would be to rehydrate embryos after 1 week drying, *prior* to H_2O_2 treatment. Again, because the exact mechanism for how dehydration breaks diapause is not known, it is difficult to explain this interaction between drying and H_2O_2 for diapause termination.

Finally, the effectiveness of H_2O_2 treatment alone was not statistically improved by following this treatment with drying. The lack improvement by adding drying is most likely explained simply by the fact that drying had no impact on diapause termination alone.

AfrLEA6 quantification

Termination of diapause with H_2O_2 treatment is associated with a significant drop of AfrLEA6 content in embryos compared to the untreated diapause embryos, which is a novel finding (**Fig. 4**). The resulting concentration of AfrLEA6 in embryos released from diapause in this manner (H_2O_2) was statistically identical to that measured for the commercial post-diapause embryos (**Fig. 4**). Thus, the method for diapause termination used commercially (which is proprietary) results in the same content of AfrLEA6. The question still remains as to why there is a 30.4% drop in AfrLEA6 content upon diapause breakage. The observation could suggest that H_2O_2 triggers an immediate degradation of selected proteins, which could include AfrLEA6. Does disruption of key macromolecules initiate physiological signaling events necessary for resumption of development and metabolism (Hand et al., 2016)? For example, it has been speculated that the small heat shock protein p26 in *Artemia* embryos might bind proteins critical to the maintenance of diapause or perhaps sequester a signaling protein important for diapause termination (King and MacRae, 2012). The answers to these issues are unknown presently and are important for future progress in the field of diapause research.

Surprisingly, drying of the diapause embryos for 1 week resulted in an *increase* in AfrLEA6 concentration compared to hydrated diapause embryos; the pattern for 5 week-dried embryos was similar but not statistically significant. This drying did not result in any substantial diapause breakage. The increase in AfrLEA6 content after 1 week drying is perplexing. First, the drying of embryos in the dry box (with Drierite) was rapid, and desiccation blocks proteins synthesis (cf. Hand et al., 2016), so *de novo* synthesis of AfrLEA6 is highly unlikely. The results could suggest that initial drying of diapause embryos in some way fosters a conformational change in AfrLEA6 that promotes either a higher extraction efficiency of the protein from the embryo, or else an enhanced interaction of AfrLEA6 with primary antibody on Western blots. Certain features of these speculations could be tested via drying studies with purified recombinant AfrLEA6 *in vitro*.

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