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Structural Properties and Cellular Expression of AfrLEA6, a Group 6 Late Embryogenesis Abundant Protein from Embryos of *Artemia franciscana*

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Cell Stress and Chaperones

Structural Properties and Cellular Expression of AfrLEA6, a Group 6 Late Embryogenesis Abundant Protein from Embryos of *Artemia franciscana* --Manuscript Draft--

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Abstract:	<p>Late embryogenesis abundant (LEA) proteins are intrinsically disordered proteins (IDPs) commonly found in anhydrobiotic organisms and are frequently correlated with desiccation tolerance. Herein we report new findings on AfrLEA6, a novel Group 6 LEA protein from embryos of <i>Artemia franciscana</i>. Assessment of secondary structure in aqueous and dried states with circular dichroism (CD) reveals 89% random coil in the aqueous state, thus supporting classification of AfrLEA6 as an IDP. Removal of water from the protein by drying or exposure to trifluoroethanol (a chemical de-solvating agent) promotes a large gain in secondary structure of AfrLEA6, predominated by α-helix and exhibiting minimal β-sheet structure. We evaluated the impact of physiological concentrations (up to 400 mM) of the disaccharide trehalose on the folding of LEA proteins in solution. CD spectra for AfrLEA2, AfrLEA3m, and AfrLEA6 are unaffected by this organic solute noted for its ability to drive protein folding. AfrLEA6 exhibits its highest concentration in vivo during embryonic diapause, drops acutely at diapause termination, and then declines during development to undetectable values at the larval stage. Maximum cellular titer of AfrLEA6 was 10-fold lower than for AfrLEA2 or AfrLEA3, both Group 3 LEA proteins. Acute termination of diapause with H₂O₂ (a far more effective terminator than desiccation in this Great Salt Lake, Utah population), fostered a rapid 38% decrease in AfrLEA6 content of embryos. While the ultimate mechanism of diapause termination is unknown, disruption of key macromolecules could initiate physiological signaling events necessary for resumption of development and metabolism.</p>	

Additional Information:	
Question	Response
Are you submitting to a Special Issue?	No

Response to Reviewer's Comments

We thank Reviewer #2 for the cogent comments provided, and we have addressed them carefully as detailed below. The revisions to the manuscript have improved the clarity of the presentation significantly.

1. The definition of a 'SMP domain' has been added on page 3 of the revised manuscript.
2. A reference for 'our bioinformatics analyses' has been provided on page 5.
3. As suggested by the reviewer, we have eliminated the original Fig. 1B, and as further requested, we have added a Western blot comparing purified AfrLEA6 to the native protein in an embryo extract (new Fig. 1B). We have retained the gel lanes on the left side in Fig. 1A that show the ineffectiveness of heat treatment during purification, which we feel is important.
4. As recommended by the reviewer to improve the clarity for the non-specialist, we have replotted original Figs. 3 and 4 as histograms, which directly show how the percentages of secondary structure change with treatment, and we have eliminated the CD spectral scans in the new figures. We agree these changes serve to make the presentation more easily interpretable. Further we have removed the data for BSA in Fig. 4, as the reviewer suggested. The reviewer is correct that normally CD spectra are presented without statistics.
5. We are pleased that the referee finds the absence of a trehalose effect on protein structure in the hydrated state interesting, as do we. Our goal was to evaluate whether physiological concentrations of this sugar in *A. franciscana* embryos possessed the capacity to promote folding of LEA proteins when fully hydrated in aqueous solution. Clearly drying of the LEA proteins promoted folding and gain of secondary structure (e.g., Fig. 2). Thus, adding trehalose was not evaluated further in the dried state. It is possible that trehalose could promote the onset of folding at intermediate hydration levels, which is something we plan to test in the future.
6. Regarding H₂O₂, our time-dependent treatments were to confirm maximum effect on diapause termination, which is shown by the lack of any additional change between 15 and 30 min. We have added a sentence (page 16 of Results) that cites other studies providing expanded time courses, additional concentrations, and results for other *Artemia* species and populations.
7. Original Figs. 6 and 7 have been combined as suggested by the referee (now Fig. 6).
8. Diapause and post-diapause embryos in this study were incubated under identical osmotic conditions, so water content is predictably the same, since prior to hatching, these embryos behave as osmometers (water is permeable across the embryo; solutes are not). Further, expressing LEA protein content per mg total embryo protein yields the same developmental pattern (data not shown).
9. We have removed the second paragraph in the Discussion on AfrLEA6 purification as recommended by the reviewer.
10. As suggested by the referee, we have added comments on page 20 (Discussion) addressing the possibility that AfrLEA6 may contribute to a glassy state (vitrification) during desiccation, and the interesting observation of population-specific differences in diapause termination.

[Click here to view linked References](#)

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4 **Structural Properties and Cellular Expression of AfrLEA6, a Group 6 Late**
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7 **Embryogenesis Abundant Protein from Embryos of *Artemia franciscana***
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17 Blase M. LeBlanc¹, Mike T. Le¹, Brett Janis², Michael A. Menze² and Steven C. Hand^{1a}
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49 Key words: Late embryogenesis abundant proteins, anhydrobiosis, diapause termination,
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51 desiccation tolerance, secondary structure, circular dichroism
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4 **Abstract**
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7 Late embryogenesis abundant (LEA) proteins are intrinsically disordered proteins (IDPs)
8 commonly found in anhydrobiotic organisms and are frequently correlated with desiccation
9 tolerance. Herein we report new findings on AfrLEA6, a novel Group 6 LEA protein from
10 embryos of *Artemia franciscana*. Assessment of secondary structure in aqueous and dried states
11 with circular dichroism (CD) reveals 89% random coil in the aqueous state, thus supporting
12 classification of AfrLEA6 as an IDP. Removal of water from the protein by drying or exposure
13 to trifluoroethanol (a chemical de-solvating agent) promotes a large gain in secondary structure
14 of AfrLEA6, predominated by α -helix and exhibiting minimal β -sheet structure. We evaluated
15 the impact of physiological concentrations (up to 400 mM) of the disaccharide trehalose on the
16 folding of LEA proteins in solution. CD spectra for AfrLEA2, AfrLEA3m, and AfrLEA6 are
17 unaffected by this organic solute noted for its ability to drive protein folding. AfrLEA6 exhibits
18 its highest concentration in vivo during embryonic diapause, drops acutely at diapause
19 termination, and then declines during development to undetectable values at the larval stage.
20 Maximum cellular titer of AfrLEA6 was 10-fold lower than for AfrLEA2 or AfrLEA3, both
21 Group 3 LEA proteins. Acute termination of diapause with H₂O₂ (a far more effective
22 terminator than desiccation in this Great Salt Lake, Utah population), fostered a rapid 38%
23 decrease in AfrLEA6 content of embryos. While the ultimate mechanism of diapause termination
24 is unknown, disruption of key macromolecules could initiate physiological signaling events
25 necessary for resumption of development and metabolism.
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Introduction

Anhydrobiotic animals like the brine shrimp *Artemia franciscana* tolerate major transitions in water content at specific points during their life cycles. During anhydrobiosis tissue water can decrease to less than 2% (Crowe and Madin 1974), which imposes substantial challenges for defending the functionality of biological structures (Crowe et al. 1997; Crowe and Clegg 1973, 1978). One key strategy for desiccation tolerance in many anhydrobiotes is the accumulation of protective molecules such as late embryogenesis abundant (LEA) proteins, heat-shock proteins, and stabilizing organic solutes (Clegg 2005, 2011; Crowe et al. 1998, 2005; Hand et al. 2011, 2018; Tan and MacRae 2018; Tapia and Koshland 2014; Tunnacliffe and Wise 2007). The present study focuses on AfrLEA6, a Group 6 LEA protein also referred to as a seed maturation protein (SMP). SMP's have previously been found in plant seeds associated with desiccation tolerance (Boucher et al. 2010; Chatelain et al. 2012). In *Medicago truncatula*, the SMP D34.3, accumulates in the mature seed and is correlated with long-term viability of the seed in the dried state (Chatelain et al. 2012). We have recently identified, cloned, and expressed a Group 6 LEA protein from embryos of *A. franciscana* that exhibits strong sequence homologies to D34.3 (Hand and Menze 2015; Janis et al. 2018b; Wu et al. 2011). AfrLEA6 exhibits the lowest hydrophilicity of the LEA proteins thus far characterized in *A. franciscana* and more closely resembles the Group 6 LEA protein *Mt*PM25 (Boucher et al. 2010; Janis et al. 2018b). Other defining features of AfrLEA6 include two SMP domains (pfam family PF04927 with the consensus sequence kpVtpeDAaavqaAEaraageartap**gGv**AaaaqaAAdaNer, which contains the 'pgGvA' motif specified by Jaspard et al. 2012) and a proline rich region, which are predicted to be sites of self interaction (Janis et al. 2018b). In this study, we investigate selected structural properties of AfrLEA6 and provide evidence for its expression in the diapause state and

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4 throughout post-diapause development of the embryo to the larval stage. The state of diapause is
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6 a programmed arrest of development that is controlled by endogenous physiological factors and
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8 may or may not involve a substantial depression of metabolism (Hand et al. 2016 and references
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10 therein). Entering diapause is a common mechanism for overwintering, because life cycle delays
11
12 are beneficial for species encountering suboptimal environments.
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16 LEA proteins are a family of intrinsically disordered proteins that are expressed in
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18 developmental and/or adult life stages of anhydrobiotic organisms and have been strongly
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20 associated with survival during water stress. Originally discovered in plants, LEA proteins have
21
22 now been identified in animals such as nematodes (Browne et al. 2002; Gal et al. 2004; Solomon
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24 et al. 2000), brine shrimp (Hand et al. 2007), African chironomid insect larvae (Gusev et al.
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26 2014; Kikawada et al. 2006), rotifers (Denekamp et al. 2010; Tunnacliffe et al. 2005), as well as
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28 bacteria (Battista et al. 2001; Rodriguez-Salazar et al. 2017; Stacy and Aalen 1998). Similar
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30 tardigrade-specific intrinsically disordered proteins have also been described (Boothby and
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32 Pielak 2017; Boothby et al. 2017). Within these organisms, LEA proteins are present across a
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34 range of subcellular compartments including the nucleus, endoplasmic reticulum, Golgi
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36 apparatus, mitochondrion and chloroplast (Avelange-Macherel et al. 2018; Boswell and Hand
37
38 2014; Hand et al. 2011; Tripathi et al. 2012; Tunnacliffe et al. 2010; Tunnacliffe and Wise 2007).
39
40 Classification schemes places them into six groups, of which *A. franciscana* uniquely expresses
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42 LEA proteins from three different groups (1, 3, and 6; Hand and Menze 2015; Tunnacliffe and
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44 Wise 2007; Warner et al. 2010, 2012).
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53 While many questions still exist in terms of the varied functions of LEA proteins, in vitro
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55 and in vivo studies have contributed insights into their roles (for reviews, see Hand et al. 2011;
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57 Hinch and Thalhammer 2012; Janis et al. 2018a; Tunnacliffe and Wise 2007). For example,
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4 LEA proteins have been shown to protect lipid bilayers of various compositions during drying
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6 and freezing (Hundertmark et al. 2011; Moore and Hand 2016; Moore et al. 2016; Navarro-
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8 Retamal et al. 2018; Steponkus et al. 1998; Thalhammer et al. 2014; Tolleter et al. 2007, 2010),
9
10 preserve the activity of target enzymes (Boswell et al. 2014a; Goyal et al. 2005; Grelet et al.
11
12 2005; Popova et al. 2015), and prevent protein aggregation ('molecular shielding'; (Boucher et al.
13
14 2010; Goyal et al. 2005; Yuen et al. 2019). The gain of secondary structure (α -helix, β -sheet,
15
16 turns) that accompanies the dehydration of some LEA proteins may allow them to perform
17
18 separate/additional roles in the dried state (Goyal et al. 2003). Finally, synergistic effects of
19
20 combining trehalose, an established stabilizer of cellular components during drying and freezing
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22 (Crowe et al. 1987, 1998; Tapia and Koshland 2014), with LEA proteins have been documented
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24 (Boswell et al. 2014a; Goyal et al. 2005).

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31 AfrLEA6 is the only Group 6 LEA protein reported thus far from an animal, and our
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33 bioinformatics analyses (Janis et al (2018b) predict distinctly different structural features
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35 compared to other LEA proteins in *A. franciscana*. Consequently, we evaluated secondary
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37 structural features of AfrLEA6 with circular dichroism as a function of hydration state (with and
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39 without trehalose), along with the tissue content of the protein during diapause, immediately
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41 after diapause termination, and during post-diapause development.
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Methods

Collection and incubation of diapause embryos, post-diapause embryos, and larvae

Diapause embryos were collected in the hydrated state from the surface of the Great Salt Lake (Utah). These embryos were verified to be in diapause by performing hatching assays as outlined in Reynolds and Hand (2004). The hatch rate of diapause embryos used in these experiments were 2.9%, indicative of a strong diapause state. Diapause embryos were rinsed in lake water and stored protected from light at ambient temperature in 1.25 M NaCl containing 200 units/ml nystatin, 50 µg/ml kanamycin, 50 µg/ml penicillin-streptomycin. Prior to use, diapause embryos were rinsed and incubated with shaking at 110 rpm in artificial seawater (35 practical salinity units; Instant Ocean ®, Blacksburg, VA) for 4 d at room temperature in the dark to allow any embryos that had terminated diapause to hatch. Exposure to light has been associated with increased diapause breakage in the laboratory (Lavens and Sorgeloos 1987). The hatched larvae were discarded, and the remaining diapause embryos were used for experimentation.

Commercial post-diapause embryos were obtained in the dried state from Great Salt Lake *Artemia*, LLC (Ogden, UT; grade: laboratory reference standard) and were stored frozen at -20°C. Prior to use, commercial post-diapause embryos were hydrated overnight in ice-cold 0.25 M NaCl. To promote development, hydrated post-diapause embryos were incubated in 0.25 M NaCl at 23°C with shaking, and embryos were sampled at 2, 4, 6, 8 h. Incubation was extended through 24 h to generate free-swimming nauplius larvae. Hatched larvae were separated from embryos by phototaxis and processed separately.

Expression and purification of recombinant LEA proteins from *Artemia franciscana* and antibody production

The original nucleic acid sequence for AfrLEA6 (GenBank accession no. MH351624) from *A. franciscana* embryos was amplified from our existing *A. franciscana* cDNA library.

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4 Primer sequences used were

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6 5'ggcggcggccatgATGTCTGAGAATATTGGTCATATTAACATAAATGC and

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8 5'attagtaactagtAGTGCATCTCCCGTGATGCAGTCCATGCGGACATTCCCAAT. For the

9
10 polymerase chain reaction, Q5 DNA polymerase (New England Biolabs, Ipswich, MA) was used

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12 for 25 cycles following the instructions of the manufacturer, and the PCR product obtained was

13
14 cloned into pTXB1 using standard techniques and the two restriction enzymes Nde1 and Spe1

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16 (New England Biolabs, Ipswich, MA). Recombinant AfrLEA6 was purified from *E. coli* cells

17
18 (BL21) designed to work with the IMPACT protein expression system (Intein-Mediated

19
20 Purification with an Affinity Chitin-binding Tag; New England Biolabs Inc., Ipswich, MA). The

21
22 vector (pTXB1) was used to add a chitin binding domain and self-cleavage protein splicing

23
24 elements to the expressed fusion protein. These elements permitted binding to the chitin moiety

25
26 of an affinity column, and then dithiothreitol (DTT) was used to release AfrLEA6 free of any

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28 amino acid tag (see below). Bacterial cells were grown in the presence of 0.1 mM ampicillin at

29
30 37°C with shaking until the optical density at 595 nm reached 0.6. Then protein expression was

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32 induced with 0.4 mM IPTG for 2 h at 37°C while shaking. Cells were sedimented by

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34 centrifugation at 5,000 x g (4°C) for 15 min. The cell pellets were resuspended in 40 ml of chitin

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36 column buffer (20 mM Tris, 0.5 M NaCl, pH 8.5) containing either 1 mM phenylmethylsulfonyl

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38 fluoride (Sigma-Aldrich, St Louis, MO) or one cOmpleteTM protease inhibitor cocktail tablet

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40 (Roche Applied Science; Penzberg, Germany). Cells were lysed by sonication, and cellular

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42 debris was removed by centrifugation at 20,000 x g (4°C) for 30 min. The supernatant was then

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44 loaded onto a chitin resin column (S6651S; New England BioLabs Inc.; Ipswich, MA) by gravity

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46 flow (~1.0 ml/min). On-column cleavage was induced by incubating the column for 48 h with

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48 chitin column buffer containing 50 mM DTT at 4°C. AfrLEA6 was eluted using chitin column

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4 buffer and dialyzed overnight against anion exchange column buffer (15 mM triethanolamine, 10
5 mM NaCl, pH 7.0). For further purification, the protein sample was applied to a 5 ml anion
6 exchange column (HiTrap Q FF; GE Healthcare; Chicago, IL) using an AKTAprime plus FPLC
7 system at a flow rate of 1.0 ml/min. After washing with 30 volumes of anion exchange column
8 buffer, bound protein was eluted using a 50 ml linear gradient of 0-25% elution buffer (15 mM
9 triethanolamine, 1 M NaCl, pH 7.0). The eluted protein peak was detected by absorbance at 280
10 nm and collected in 1 ml fractions. Pooled samples were exchanged into 10 mM potassium
11 phosphate buffer (pH 7.5) and concentrated using Amicon Ultra-15 10K centrifugal filters
12 (MilliporeSigma; Burlington, MA). The techniques for expressing and purifying recombinant
13 AfrLEA2 and AfrLEA3m followed the procedures in Boswell et al. (2014b). Polyclonal
14 antibody against the purified recombinant AfrLEA6 was raised in chicken eggs by Aves Labs,
15 Inc. (Tigard, OR, USA).

33 **Circular dichroism spectroscopy**

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36 Spectra were obtained with a Jasco J-815 spectropolarimeter and analyzed using Spectra
37 Manager™ Suite (Jasco Analytical Instruments; Easton, OH). Samples were prepared in 10 mM
38 potassium phosphate buffer, pH 7.5, at a protein concentration of 0.15 mg/ml for AfrLEA2,
39 AfrLEA3m, AfrLEA6, and bovine serum albumin (BSA; Sigma; A6003). For aqueous protein
40 samples, measurements were recorded between 190-250 nm using a quartz cuvette with a 0.1 cm
41 pathlength. Dried samples were prepared by air drying a 50 µl droplet of sample containing 0.15
42 mg/ml protein onto one side of a demountable quartz cuvette overnight in a dry box containing
43 desiccant (Drierite; W. A. Hammond Drierite, Xenia, OH). For conversion of spectra for dried
44 samples to mean residue ellipticity, a pathlength of 0.01 cm was used. Spectra for blank samples
45 that lacked protein were collected for each experimental condition and subtracted from the
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4 respective protein spectra. Each spectrum was then converted to mean residue ellipticity and
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6 smoothed with a convolution width of 9 (Savitzky and Golay 1964). Spectra Manager™ Suite
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8 was used to subtract blank spectra, convert values to mean residue ellipticity, and smooth the
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10 curves. Analysis of secondary structure was achieved using DICHROWEB online analysis
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12 software (Whitmore and Wallace 2004, 2008) to apply the CONTINLL algorithm (Provencher
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14 and Glockner 1981; van Stokkum et al. 1990) and SELCON3 algorithm (Sreerama et al. 1999;
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16 Sreerama and Woody 1993). Reference dataset 7 was utilized for spectra analyses as it applies
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18 to denatured proteins with measurements between 190-250 nm wavelengths (Sreerama et al.
19
20 1999).

26 **Evaluation of diapause termination in the laboratory**

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29 Treatments to promote diapause termination included exposure of embryos to
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31 dehydration/rehydration cycles and to a chemical oxidant. Diapause embryos were air dried on
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33 the benchtop at room temperature and ambient humidity (50-55%) for either 24 h or 1 week.
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35 Diapause embryos were also dried for 24 h as above, rehydrated for 6 h (0°C), and then dried
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37 again for 24 h (designated as 1.5X dehydration/rehydration cycles, 1.5X D/H). Chemical
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39 treatment involved incubating the diapause cysts in 3% H₂O₂ prepared in 0.25 M NaCl solution
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41 for either 15 or 30 min. When embryos were to be used for analysis of AfrLEA6 by Western
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43 blotting, embryos were rinsed with excess 0.25 M NaCl to remove any residual H₂O₂ prior to
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45 preparation of extracts (see below). Other experiments incorporated combinations of
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47 dehydration and chemical treatments to test for additive or sequence (order) effects.
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53 To quantify the effectiveness of the above treatments for terminating diapause, embryos
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55 subsequently were incubated in artificial seawater for 4 days in ambient light. After the
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57 incubation, hatching assays were performed by first diluting aliquots of the treated embryos with
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4 water in a plastic petri dish (bottom marked off into squares) and adding 12 N HCl to kill any
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6 free swimming nauplii. Then hatch counts were performed under a dissecting scope. Typically,
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8 200-400 organisms were counted for each trial. Hatching percentages were determined by
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10 counting the total nauplii and dividing by the total count (free-swimming nauplii + unhatched
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12 cysts + E1 and E2 stages). Emergence stage E1 denotes the point at which the embryo first
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14 protrudes from its chorion (proteinaceous outer coat) but is still contained within the outer
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16 cuticular ('hatching') membrane, while emergence stage 2 signifies that the embryo mass has
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18 protruded even further to form the shape of a parachute (cf. Neumeyer et al. 2015). All diapause
19
20 and post-diapause embryos were gently blotted between sheets of filter paper to remove
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22 interstitial water as previously described (Boswell et al. 2014b; Clegg 1974; Glasheen and Hand
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24 1989) prior to extraction for quantification of AfrLEA6 expression (see below).
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31 **Quantification of AfrLEA6 expression by Western Blot analysis**

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33 Diapause embryos, post-diapause embryos (0, 2, 4, 6, and 8 h), and 24 h free-swimming
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35 nauplii were used to analyze AfrLEA6 across development. Extracts were prepared by
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37 homogenizing 100 mg of embryos or larvae in 1.9 ml of 1X Laemmli buffer with a ground-glass
38
39 homogenizer. Samples were heat treated at 95°C for 10 min after homogenization and then
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41 centrifuged at 20,000 x g for 20 min to remove insoluble cell debris. The supernatant was
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43 collected and frozen at -20°C prior to analysis. Total protein was measured with a modified
44
45 Lowry assay as described by Peterson (1977). Equal protein (40 µg) was loaded for each time
46
47 point onto an SDS polyacrylamide gel (4% stacking gel, 11% resolving gel). A standard curve
48
49 was prepared with 0-40 ng of purified, recombinant AfrLEA6 to quantify AfrLEA6 across the
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51 developmental stages. Electrophoresis was performed at 125 V for 80 min in a Bio-Rad Mini
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53 Protean 3 cell. Following electrophoresis, samples were transferred to a nitrocellulose
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4 membrane at 80 V for 60 min in Towbin's buffer (25 mM tris, 192 mM glycine, 20% v/v
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6 methanol, 0.025% SDS). The nitrocellulose membranes were then blocked for one hour in 5%
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8 fat-free dry milk prepared in TBS-T (20 mM Tris, 500 mM NaCl, 0.1 % Tween 20, pH 7.6) at
9
10 room temperature with rocking. The blots were then incubated overnight at 4°C with AfrLEA6
11
12 polyclonal antibody (raised in chicken eggs, Aves Labs Inc.) diluted 1:50,000 in 5% dry milk
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14 prepared in TBS-T. Because α -tubulin served as a loading control, blots were incubated
15
16 simultaneously with α -tubulin primary antibody raised in rabbits (Cell Signaling Technology,
17
18 Danvers, MA) at a 1:1,000 dilution. Afterwards, membranes were given three 15-min rinses in
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20 TBS-T with shaking. The blots were incubated at room temperature for 1 h with two horseradish
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22 peroxide-labeled (HRP) secondary antibodies: goat anti-chicken (AvesLabs Inc., Tigard OR) at a
23
24 1:10,000 dilution and mouse anti-rabbit (Cell Signaling Technologies; Danvers, MA) at a 1:1000
25
26 dilution. The blots were developed using an Amersham ECL Prime Western Blotting Detection
27
28 Kit (GE Healthcare; Chicago, IL) and imaged with a Bio-Rad ChemiDocTM XRS+ Imager. Bio-
29
30 Rad Image LabTM Software was used for quantifying bands. Values for AfrLEA6 expression
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32 were converted to mg AfrLEA6 protein per ml embryo water by calculating the total mg of
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34 AfrLEA6 present per gram embryo (hydrated and blotted as above) and then dividing by the
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36 corrected water content of metabolically-active embryos (0.573, i.e., 57.3% embryo water) as
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38 published in Glasheen and Hand (1989). Because preliminary bioinformatic analysis and
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40 histochemical localization (B. LeBlanc and S. Hand, unpublished) indicate AfrLEA6 is a
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42 cytoplasmic protein, this concentration unit is appropriate for estimating its effective titer in the
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44 cell.
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4 **Statistical analyses**
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6 Analyses were performed using IBM SPSS Statistics for Windows, version 22.0 (IBM
7 Corp., Armonk, New York, USA). Levene’s test was performed to check for equal variance
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9 among data sets. To determine significant differences among treatments, one-way ANOVA was
10 applied for data sets with equal variance, and Welch’s ANOVA was used for data sets with
11 unequal variance. For post-hoc tests used in pair-wise comparisons, Tukey Honestly Significant
12 Difference (HSD) was chosen for data sets with equal variance, and the Games-Howell test was
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14 applied for data sets with unequal variances (Games and Howell 1976; McDonald 2014).
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Results

Purification of recombinant AfrLEA6

Fractions containing protein eluted from the chitin column after treatment with DTT were analyzed via SDS-PAGE to estimate purity of recombinant AfrLEA6, which has a calculated molecular mass of 27.0 kDa. Several contaminating proteins were present in the eluent with estimated molecular masses of ~50 kDa, ~60 kDa, and ~20 kDa. Heat treatment and anion exchange chromatography were investigated as techniques to remove the contaminating proteins and further enrich AfrLEA6. Heat treatment followed by centrifugation was ineffective at precipitating these contaminating proteins (Fig. 1A). Anion exchange chromatography with a 0-250 mM NaCl gradient eluted a protein peak between 130-180 mM NaCl. The leading edge of the peak (130-150 mM NaCl) contained AfrLEA6 without visible contaminating bands; the purity of the pooled fractions was sufficient for production of AfrLEA6 primary antibody (Fig. 1A). The trailing edge of the peak at about 160-180 mM NaCl (Fig. 1A) showed contamination. The removed contaminants likely included the intact fusion protein composed of AfrLEA6 bound to the intein (22 kDa) and the chitin binding domain protein (CBD, 6 kDa), along with free intein and intein fused with the CBD. A Western blot of purified AfrLEA6 versus the native protein in an embryo extract documented equivalent migration at approximately 27 kDa (Fig. 1B).

Secondary structure of LEA proteins

The secondary structure of AfrLEA6 was investigated under various solution conditions and in the dried state. AfrLEA6 exists as an intrinsically disordered protein in solution with a minimum ellipticity of 200 nm that is characteristic of disordered, random-coil proteins (Fig. 2A). When interactions with water are decreased by drying or by the addition of 70%

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4 trifluoroethanol (TFE; a chemical de-solvating agent), AfrLEA6 gains significant secondary
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6 structure (α -helix, β -sheet, turns). A similar outcome was promoted by adding 2% SDS. A
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8 substantial increase in the proportion of α -helix was indicated in the CD spectra by double
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10 minima at 208 and 222 nm and a maximum at 191 nm (Fig. 2A). The proportion of α -helices
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12 increased from 3.9% in the aqueous state to 47.1% in the dried state (Fig. 2B). Similarly, the
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14 proportion of α -helix structure increased from 3.9% to 34% and 44.3% after the additions of 2%
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16 SDS and 70% TFE, respectively (Fig. 2B). These data were substantiated by comparisons to
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18 bovine serum albumin (BSA), which is a typical globular protein known to exhibit secondary
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20 structure in solution and to AfrLEA2, which is a positive control previously shown to behave as
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22 an intrinsically disordered protein (Boswell et al. 2014a). As expected, the structure of BSA was
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24 predominantly α -helix in aqueous solution and did not substantially change after treatment with
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26 2% SDS or 70% TFE (Fig. 3) (also see Boswell et al. 2014a; Takeda et al. 1987). In aqueous
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28 solution, there were noteworthy differences in secondary structure between AfrLEA6 and
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30 AfrLEA2 (Fig. 3). Overall, AfrLEA6 exhibited a lower percentage of secondary structure (11%)
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32 when compared to AfrLEA2 (26.7%), the major difference being the reduced content of β -sheet
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34 for AfrLEA6 (0.5%) versus AfrLEA2 (15.6%). AfrLEA6 gained more total secondary structure
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36 in 70% TFE (71.1%) than in 2% SDS (61.1%), whereas the percentages for AfrLEA2 were the
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38 same across the two treatments (70 versus 71%).
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48 Secondary structures of AfrLEA2, AfrLEA3m, and AfrLEA6 were also investigated in
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50 the aqueous state in the presence of trehalose. In embryos of *A. franciscana*, trehalose
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52 concentrations can reach at least 340 mM (Glasheen and Hand 1989) or higher, so it was of
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54 interest to evaluate whether this stabilizing organic solute might promote gain of structure in
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56 LEA proteins. In all cases, the structure of LEA proteins was unaffected by the presence of
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4 trehalose (Fig. 4). The CD spectra of LEA proteins remained indicative of a disordered, random-
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6 coil structure. The α -helix structure of BSA remained unchanged as well.
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9 **Protein expression of AfrLEA6 during development**

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11 The quantity of AfrLEA6 present in each developmental sample was determined by
12 comparing band intensities to a standard curve of purified, recombinant AfrLEA6 (Fig. 5A-C).
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14 These quantities were converted to units of mg LEA protein per ml embryo water. The in vivo
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16 titer of AfrLEA6 was highest during diapause and decreased throughout pre-emergence
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18 development (Fig. 5D) until it was no longer quantifiable in 24 h nauplii (data not shown).
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21 AfrLEA6 was present in diapause embryos at a concentration of 0.173 ± 0.016 (mean \pm SD; $n=3$)
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23 mg per ml embryo water and decreased significantly to a concentration of 0.113 ± 0.03 (mean \pm
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25 SD; $n=3$) mg per ml embryo water by 0 h of pre-emergence development – a reduction of 35%
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27 (Fig 5D). By 8 h of pre-emergence development, the titer of AfrLEA6 had been reduced to 8%
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29 of the diapause values, corresponding to 0.014 ± 0.0025 (mean \pm SD; $n=3$) mg per ml embryo
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31 water.
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38 **Diapause termination under defined conditions**

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40 We were surprised by the drop in AfrLEA6 between diapause and post-diapause (0 hour)
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42 embryos (Fig. 5D), an observation based in part on measurements for post-diapause embryos
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44 obtained from commercial sources. Consequently, trials were conducted to terminate diapause
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46 directly under controlled conditions to more rigorously evaluate this state-specific change in
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48 AfrLEA6. Two termination cues were used to break the diapause state in the laboratory –
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50 desiccation and incubation in hydrogen peroxide. As shown in Fig. 6A, H_2O_2 was by far the
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52 more effective treatment. Treatment of hydrated diapause embryos with the 3% H_2O_2 solution
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54 for 15 min and 30 min resulted in $75.9 \pm 7.0\%$ and $72.6 \pm 4.9\%$ hatching (means \pm SD; $n = 3$),
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4 respectively; the two results were statistically identical ($p = 0.998$). Further studies with H_2O_2
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6 involving time courses, additional concentrations, and other *Artemia* species and populations are
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8 available (Lavens et al. 1986; Lavens and Soorgeloos 1987; Van Stappen et al. 1998). Drying
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10 the diapause embryos for 24 h versus applying 1.5X D/H cycles promoted $1.7 \pm 0.39\%$ and $7.7 \pm$
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12 1.2% hatching (means \pm SD; $n = 3$), respectively (Fig. 6A). The hatching percentage promoted
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14 by 1.5X D/H cycles was significantly higher than for one 24 h drying bout ($p = 0.048$), but the
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16 difference was modest. Drying for one week was no more effective for diapause termination
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18 than drying for 24 h ($p = 1.000$; Fig. 6B). Combining drying with 3% H_2O_2 (in either order, Fig.
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20 6B) did not improve diapause termination compared to H_2O_2 alone (all p values > 0.05).
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27 Consequently, the 15 min treatment with H_2O_2 was used to terminate diapause and
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29 quantify the impact on the AfrLEA6 content of embryos. There was a significant 38% drop in
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31 AfrLEA6 content in embryos released from diapause with H_2O_2 compared to the untreated
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33 diapause embryos (Fig. 7). Further, the AfrLEA6 content in these H_2O_2 -treated embryos was
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35 statistically identical to that measured for the commercial post-diapause embryos (Fig. 7). Thus,
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37 the method for diapause termination used commercially (proprietary) promoted the same content
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39 of AfrLEA6.
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Discussion

Despite sequence and bioinformatic evidence for ArfLEA6 indicating substantially less hydrophilicity than other LEA proteins from *A. franciscana*, results presented here demonstrate that AfrLEA6 is indeed intrinsically disordered in aqueous solutions and gains structure as water is removed. Further, we show for the first time that the very high physiological concentration of trehalose known to exist in embryos of *A. franciscana* is insufficient to promote the gain of secondary structure for any of the three LEA proteins (AfrLEA2, AfrLEA3m, AfrLEA6) evaluated in aqueous solution. One might predict that the reduced hydrophilic poise of AfrLEA6 would render it more likely to initiate folding, particularly considering the strong ability of trehalose to force protein assembly/folding through preferential exclusion from the peptide backbone (Street et al. 2006; Xie and Timasheff 1997). AfrLEA6 exhibits its highest concentration in vivo during the diapause state, drops acutely at diapause termination, and then the content declines progressively during development to the larval stage. After confirming the capacity of H₂O₂ to quickly terminate diapause, we document that the state-dependent, step change in AfrLEA6 at diapause breakage can be produced in the laboratory under controlled conditions.

Our CD studies indicate that AfrLEA6 is clearly intrinsically disordered in solution based on an estimated 89% content of random coil. Further the α -helix content of AfrLEA6 increased from 4% in solution to 47% when dried, 44% in TFE, and 34% in SDS. Solutions containing 2% SDS and 70% TFE have been documented to induce α -helix conformation in LEA proteins (Ismail et al. 1999; Kentsis and Sosnick 1998; Shih et al. 2004, 2012; Tolleter et al. 2007). In the presence of either solution, AfrLEA6 adopts a majority of α -helix conformation. By comparison to Group 3 LEA proteins, Boswell et al. (2014a) estimated the total α -helix content of

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4 AfrLEA3m in the aqueous state was 2%, which increased to 41% and 36% after treatment with
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6 2% SDS or 70% TFE, respectively. In the dried state, however, α -helix content only increased to
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8 18%. Gains of structure were also seen for AfrLEA2 under identical conditions. However, note
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10 that recombinant AfrLEA2 and AfrLEA3m in that study contained up to 10% sequence that was
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12 atypical of the mature protein (hexa-histidine tag, amphipathic mitochondrial targeting sequence,
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14 etc.), so precise comparisons of percentages to AfrLEA6 (all native sequence due to purification
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16 with the IMPACT expression system/chitin binding column) should be limited. Recent
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18 bioinformatics predictions place AfrLEA6 at the top of the most hydrophobic LEA proteins
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20 found in *A. franciscana*, with AfrLEA2 being second. Despite its high hydrophobicity, the
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22 DisEMBL prediction of overall disorder for AfrLEA6 was 80.9% (Janis et al. 2018b). In
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24 summary both experimental data and bioinformatics strongly support a classification of
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26 AfrLEA6 as an intrinsically disordered protein (IDP).
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33 As mentioned earlier, trehalose is a stabilizing organic solute that is present in embryonic
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35 stages of *A. franciscana*. At concentrations that are physiologically relevant to these embryos in
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37 the hydrated state (≥ 340 mM), trehalose has been shown to drive folding equilibrium towards the
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39 native state of globular proteins (Auton et al. 2011; Xie and Timasheff 1997). LEA protein
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41 folding in the presence of molecular crowding agents at high concentration (≥ 2 M) has been
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43 successfully demonstrated (Bremer et al. 2017; Navarro-Retamal et al. 2018). However, our CD
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45 spectra gathered for AfrLEA2, AfrLEA3m, and AfrLEA6 in solutions of trehalose across
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47 physiological concentrations indicate no impact of trehalose on the structure of LEA proteins or
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49 BSA. If trehalose were to play a role in promoting a gain of secondary structure in LEA
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51 proteins, it likely would be seen at higher concentrations that are approached as cellular water is
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4 removed during desiccation. Further investigations into the secondary structure of LEA proteins
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7 at intermediate hydration values, with and without trehalose, could be warranted.
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9 Based on data presented, AfrLEA6 protein content is highest during the diapause state
10 and undetectable in free-swimming nauplii. This pattern parallels the loss of desiccation
11 tolerance seen at the larval stages through adults, which is consistent with a protective role of
12 LEA proteins during times of desiccation. However, there is an acute 38% decrease in AfrLEA6
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14 during the termination of diapause with H₂O₂ that was unanticipated, and the reason for this drop
15 remains an open question. This observation suggests that H₂O₂ triggers an immediate
16 degradation of selected proteins, which apparently includes AfrLEA6 [H₂O₂ treatment of
17 commercial *post*-diapause embryos has less impact (29% decrease); M. Le, B. LeBlanc, S Hand,
18 unpublished]. Disruption of key macromolecules could initiate physiological signaling events
19 necessary for resumption of development and metabolism. For example, it has been speculated
20 that the small heat shock protein p26 in *A. franciscana* embryos might bind proteins critical to
21 the maintenance of diapause, or perhaps sequester a signaling protein important for diapause
22 termination (King and MacRae 2012). Ultimately, the mechanism explaining termination of
23 diapause with hydrogen peroxide, or any other release cues (e.g., cold temperature, desiccation),
24 are currently unknown (cf. Hand et al. 2016). Population-specific differences exist in the
25 efficacy of termination cues (Lavens and Sorgeloos 1987). As documented here (Fig. 6A, B),
26 desiccation for 24 h up to one week is largely ineffective at diapause release for Great Salt Lake
27 embryos, whereas drying works well for embryos from the San Francisco Bay (cf. Clegg et al.
28 1996). Until definitive mechanisms of diapause termination in *A. franciscana* become available,
29 these interesting population differences will remain unexplained (cf. Hand et al., 2016)
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4 Finally, considering that the cellular titer of AfrLEA6 in diapause and post-diapause
5 embryos is approximately ten-fold lower than for AfrLEA2 and AfrLEA3m (cf. Boswell et al.
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9 2014b), AfrLEA6 apparently promotes its functions in *A. franciscana* at a much lower content
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11 than these Group 3 LEA proteins. Based on sequence homologies between AfrLEA6 and SMPs
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13 that are associated with desiccation tolerance in plants (Boucher et al. 2010; Chatelain et al.
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16 2012), potential functions of AfrLEA6 may involve the prevention and dispersion of protein
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18 aggregates during desiccation and/or their rapid dissolution during rehydration similar to
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20 *MtPM25*. Contribution to a glassy state during desiccation as with pollen D-7 LEA protein
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22 (Wolkers et al, 2001) is also possible. The amino acid sequence of AfrLEA6 contains features
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24 associated with proteins known to undergo liquid-liquid phase separations in the hydrated state
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26 under certain conditions (Janis et al. 2018a, b). An attractive hypothesis may be that the protein
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28 is involved in the partitioning of signaling peptides or other biomolecules that regulate diapause
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30 in the cyst. Due to the increasing concentration of solutes in the drying cell, AfrLEA6 may
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32 precipitate from solution into another liquid phase that partitions such biomolecules at moderate
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34 to low water content and releases them as it dissolves upon rehydration. Experiments to test this
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36 hypothesis are currently underway. In any case, our results underscore the concept that multiple
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38 LEA proteins exist at different amounts and within different cellular compartments within a
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40 single organism and may act together or separately to protect the organism during events of
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42 water stress.
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Figure Legends

Fig. 1 Purification of recombinant AfrLEA6. **(A)** *Left panel:* SDS-PAGE analysis of purified protein after IMPACT column chromatography combined with anion exchange chromatography. Evidence that heat treatment (HT) is ineffective for removing contaminants present after IMPACT chromatography alone. Total protein loaded per lane was 40 μ g. MW STD = ladder of molecular weight standards. *Right panel:* SDS-PAGE analysis of 1-ml fractions eluted from the anion exchange column (HiTrap Q FF resin) that contained AfrLEA6. Contamination is present in the trailing edge of the peak, which was not pooled. Each lane was loaded with 10 μ l of eluant. **(B)** Western blot of purified, recombinant AfrLEA6 versus the native protein in a diapause embryo extract shows equivalent migration. Approximately 40 ng of purified AfrLEA6 and 40 μ g total protein from an embryo extract prepared in Laemmli buffer were loaded in respective lanes. Ladder of MW standards shown at left.

Fig. 2 **(A)** Circular dichroism (CD) spectra of 0.15 mg/ml AfrLEA6 in the aqueous and dried state as well as in the presence of 2% SDS and 70% TFE. Lines smoothed via the Savitzky-Golay method with convolution width 9. **(B)** Dichroweb analysis of secondary structures in CD spectra of AfrLEA6 using the Contin-LL method (see text).

Fig. 3 Comparison of secondary structure content for AfrLEA6, AfrLEA2, and bovine serum albumin (BSA) based on CD spectra recorded in the **(A)** aqueous state, **(B)** presence of 2% SDS and **(C)** presence of 70% TFE. All proteins were measured at a concentration of 0.15 mg/ml. Analysis of secondary structure was performed using the Dichroweb server with the Contin-LL method (see text).

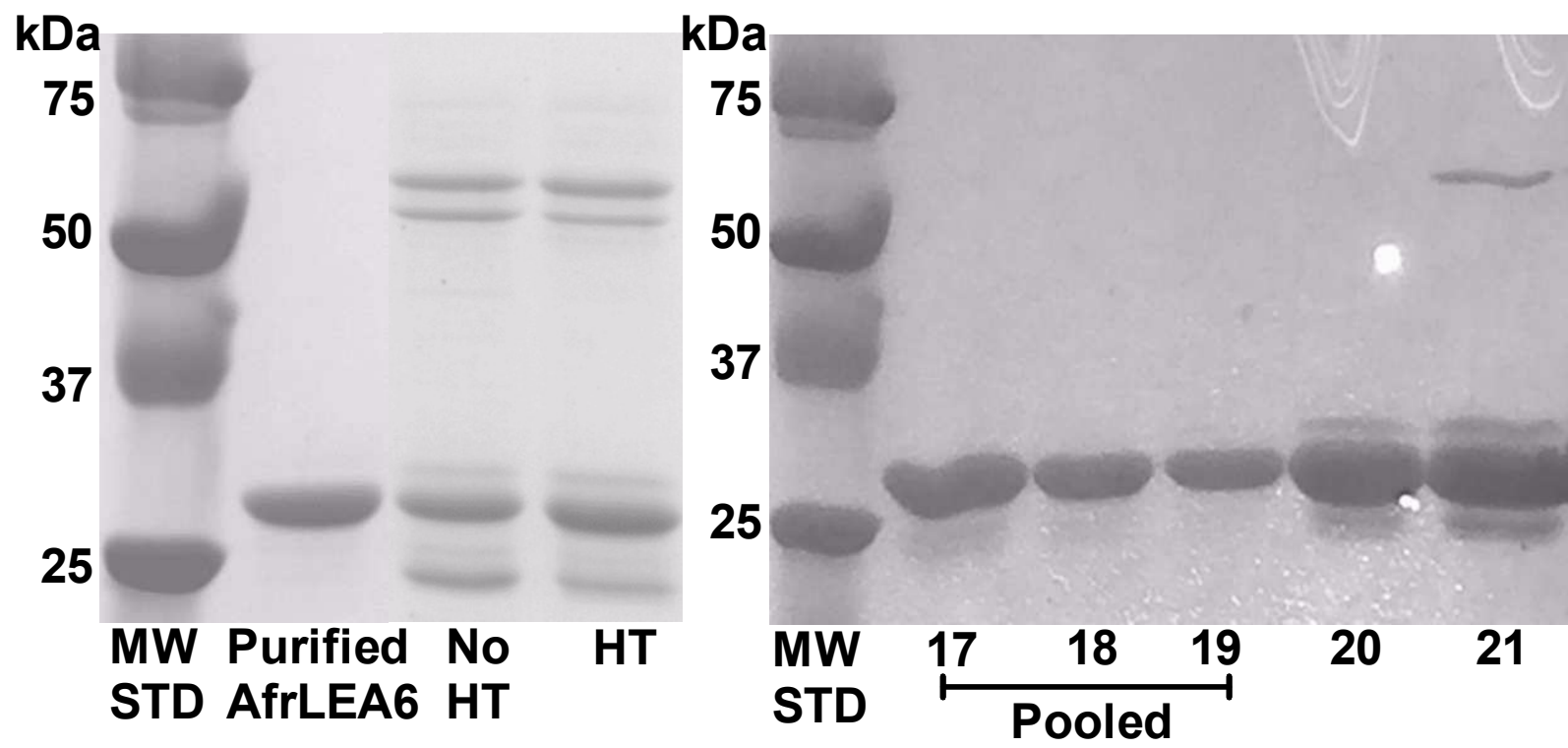
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4 **Fig. 4** Comparison of secondary structure content for AfrLEA2, AfrLEA3m and AfrLEA6
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6 based on CD spectra recorded in the presence of various concentrations of trehalose in the
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8 aqueous state. All proteins were measured at a concentration of 0.15 mg/ml. Analysis of
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10 secondary structure was performed using the Dichroweb server with the Contin-LL method (see
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12 text).
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17 **Fig. 5** Quantification of AfrLEA6 in extracts of *A. franciscana* by Western blot analysis using an
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19 AfrLEA6 polyclonal antibody. **(a)** AfrLEA6 was quantified in extracts from diapause embryos
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21 and post-diapause embryos through 8 h of pre-emergence development. Total protein loaded per
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23 lane was 40 µg. α -tubulin is included as a loading control for each time point. AfrLEA6 was too
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25 low in 24 h free-swimming nauplii to reliably quantify (data not shown). **(b, c)** Quantification of
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27 AfrLEA6 in extracts was based on a standard curve with 5-40 ng of purified recombinant
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29 AfrLEA6. Bio-Rad Image LabTM software was used to compare band intensities. **(d)** AfrLEA6
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31 concentrations from diapause throughout 8 h of pre-emergence development. All values were
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33 normalized to α -tubulin and then expressed per ml embryo water as described in Methods. Data
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35 are presented as means \pm SD. Different letters above means indicate statistically significant
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37 differences (one-way ANOVA, Tukey, $P < 0.01$, $n = 3$).
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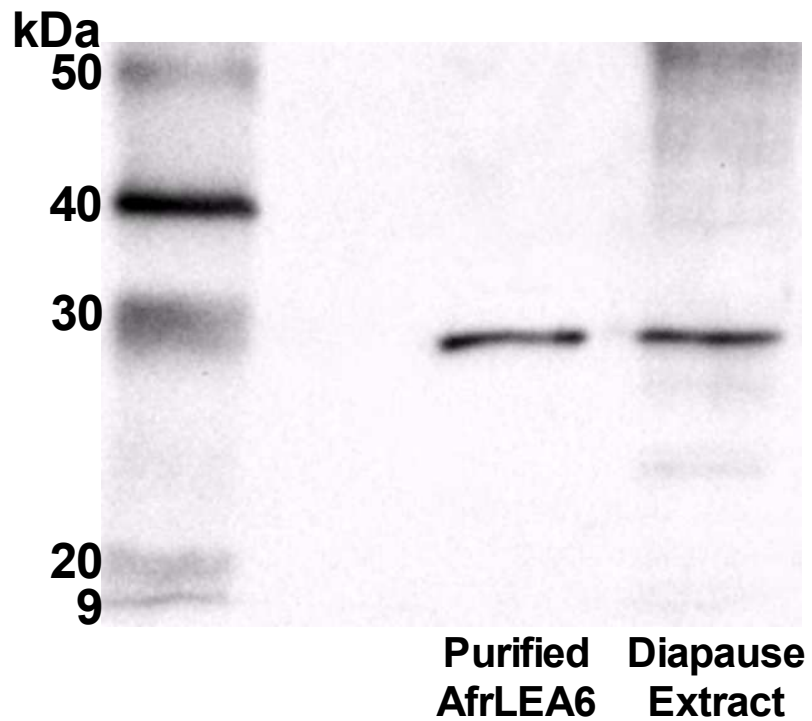
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45 **Fig. 6** Hatching percentages of diapause embryos from *A. franciscana* after H₂O₂ and drying
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47 treatments. All treatments were performed at ambient humidity and at room temperature. **(a)**
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49 1.5X D/H is defined as drying hydrated diapause embryos for 24 h, rehydrating for 6 h, and
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51 drying again (24 h) prior to the 4-day incubation to promote hatching. Bars represent means \pm
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53 SD. Different letters above bars indicate statistically significant differences among means
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55 (Levene's $F(9, 20) = 5.742$, $P < 0.05$. Welch's ANOVA, Games-Howell, $P < 0.05$, $n = 3$). **(b)**
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57 Hatch percentages after combined treatments with H₂O₂ and 1 week drying. Bars represent
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4 means \pm SD; Levene's F (9, 20) = 5.742, P<0.05. Welch's ANOVA, Games-Howell, P<0.05, n
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6 = 3). (c) AfrLEA6 contents for diapause embryos not treated and treated with H₂O₂ and for
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8 commercially obtained post-diapause embryos of *A. franciscana*. AfrLEA6 was quantified by
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10 Western blotting. Bars represent means \pm SD. Different letters above bars indicate statistically
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12 significant differences among means (Levene's F (3, 16) = 3.635, P<0.05. Welch's ANOVA,
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14 Games-Howell, P<0.05. From left to right, $n = 8, n = 6, n = 3, n = 3$).
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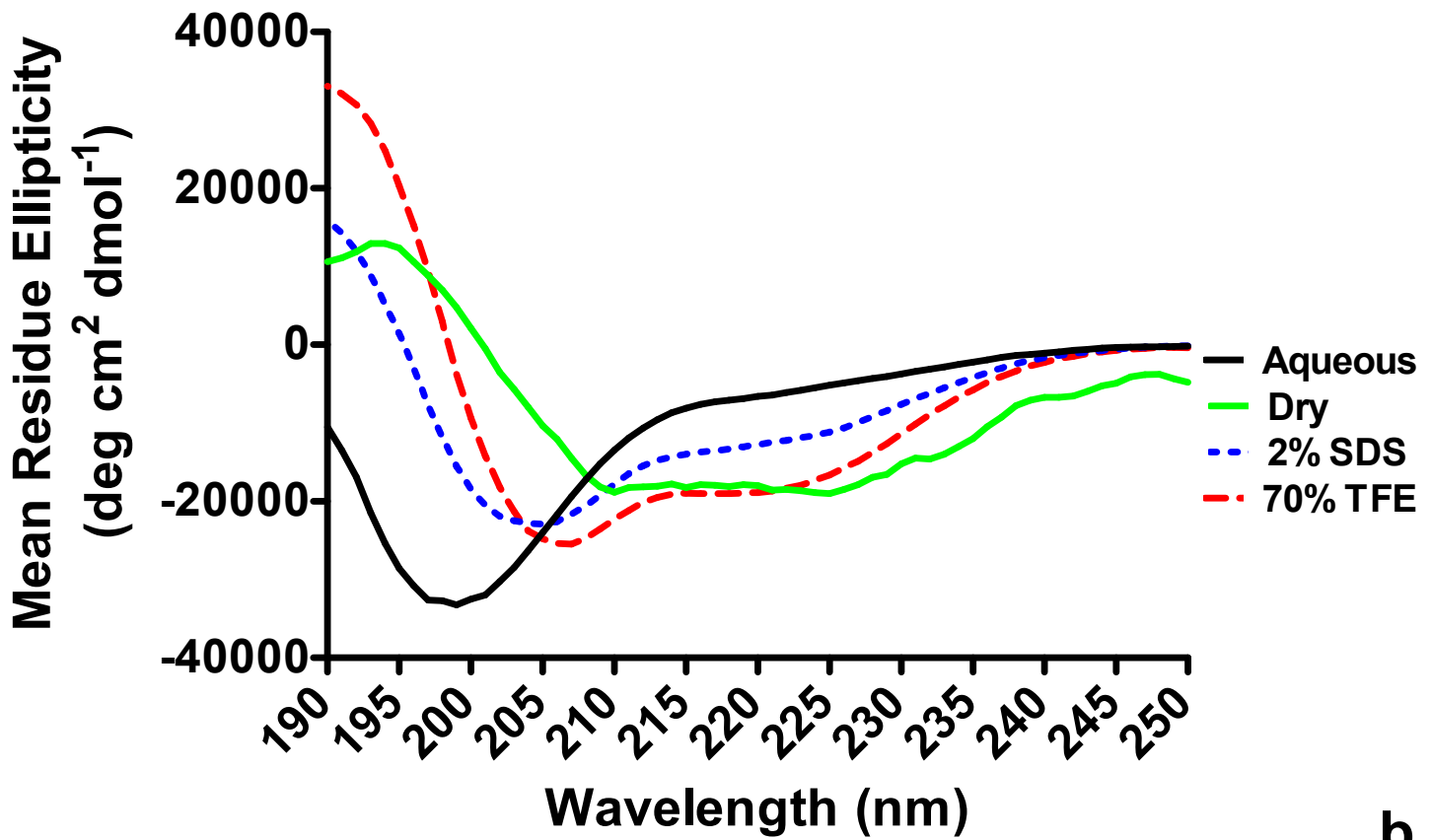
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