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RESEARCH ARTICLE

Transgenic expression of late embryogenesis abundant proteins improves tolerance to water stress in *Drosophila melanogaster*

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ABSTRACT

Four lines of *Drosophila melanogaster* were created that expressed transgenes encoding selected late embryogenesis abundant (LEA) proteins originally identified in embryos of the anhydrobiote *Artemia franciscana*. The overall aim was to extend our understanding of the protective properties of LEA proteins documented with isolated cells to a desiccation-sensitive organism during exposure to drying and hyperosmotic stress. Embryos of *D. melanogaster* were dried at 57% relative humidity to promote a loss of 80% tissue water and then rehydrated. Embryos that expressed AfrLEA2 or AfrLEA3m eclosed 2 days earlier than wild-type embryos or embryos expressing green fluorescent protein (Gal4GFP control). For the third instar larval stage, all *Afrlea* lines and Gal4GFP controls experienced substantial drops in survivorship as desiccation proceeded. When results for all *Afrlea* lines were combined, Kaplan–Meier survival curves indicated a significant improvement in survivorship in fly lines expressing AfrLEA proteins compared with Gal4GFP controls. The percent water lost at the LT_{50} (lethal time for 50% mortality) for the AfrLEA lines was 78% versus 52% for Gal4GFP controls. Finally, offspring of fly lines that expressed AfrLEA2, AfrLEA3m or AfrLEA6 exhibited significantly greater success in reaching pupation, compared with wild-type flies, when adults were challenged with hyperosmotic stress (NaCl-fortified medium) and progeny forced to develop under these conditions. In conclusion, the gain of function studies reported here show that LEA proteins can improve tolerance to water stress in a desiccation-sensitive species that normally lacks these proteins, and, simultaneously, underscore the complexity of desiccation tolerance across multiple life stages in multicellular organisms.

KEY WORDS: Desiccation, LEA proteins, Salt stress, *Artemia franciscana*

INTRODUCTION

A broad suite of factors contributes to desiccation tolerance in cells and animals (Crowe and Clegg, 1978; Crowe et al., 1997, 2005; Erkut et al., 2013; Gusev et al., 2014; Hand et al., 2011; Koshland and Tapia, 2019; Somero et al., 2017; Tunnacliffe and Wise, 2007), yet mechanistically it can be informative to understand how individual molecular components impact survival during desiccation. Several studies have employed transgenic expression of late embryogenesis abundant (LEA) proteins found in desiccation-tolerant embryos of the brine shrimp *Artemia franciscana* (AfrLEA proteins) in cell lines that cannot tolerate desiccation (Czernik et al., 2020; Li et al., 2012; Marunde et al., 2013). Interestingly, sheep adult fibroblasts and human and insect cell lines that are desiccation sensitive, but accumulate transgenic AfrLEA proteins, have significantly higher survival after short-term exposure to desiccation and hyperosmotic stress than cell lines not expressing AfrLEA proteins (Czernik et al., 2020; Li et al., 2012; Marunde et al., 2013). These studies highlight the critical role that LEA proteins play in desiccation tolerance at the cellular level. To better understand the extent to which AfrLEA proteins can protect whole organisms from damage owing to desiccation and hyperosmotic stress, we have created four *Drosophila melanogaster* lines that accumulate AfrLEA proteins. The present study describes the impact of transgenic expression of AfrLEA proteins on desiccation tolerance and hyperosmotic stress in *D. melanogaster*.

Organisms generally employ one of two strategies to overcome stress caused by the loss of water: desiccation avoidance and desiccation tolerance. The vast majority of organisms, including *D. melanogaster*, are desiccation avoidant. Organisms that are desiccation avoidant attempt to minimize water loss, but when water loss exceeds the capacity to maintain water balance, desiccation-avoidant organisms eventually lose viability. Conversely, desiccation-tolerant organisms readily survive enormous decreases in tissue water and enter a state of anhydrobiosis or ‘life without water’ (Crowe et al., 1992). To enter anhydrobiosis, various nematodes (Browne et al., 2002; Solomon et al., 2000), the chironomid midge *Polypedilum vanderplanki* (Gusev et al., 2014; Kikawada et al., 2005), tardigrades (Welnicz et al., 2011; Wright, 1989) and selected rotifers (Marotta et al., 2010; Ricci et al., 2003) permit water loss in a slow, controlled manner, which provides time to accumulate various protectants and stop development in preparation for the desiccated state. Other organisms, such as diapause embryos of *A. franciscana*, are intrinsically provisioned with protectants and shut down metabolism before water stress occurs (Hand and Menze, 2015; Patil et al., 2013; Qiu and MacRae, 2010). Preceding anhydrobiosis or during early stages of entry, desiccation-tolerant organisms generally decrease metabolism through the suppression of oxidative pathways and energy usage (Erkut and Kurzchalia, 2015; Glasheen and Hand, 1988; Hand et al., 2016; Patil et al., 2013), and upon rehydration, the organism’s metabolism returns to normal levels (Clegg, 1976; Glasheen and Hand, 1988). Organic molecules such as the sugar trehalose (Clegg, 1962; Crowe et al., 1997; Dutrieu, 1960; Tapia and Koshland, 2014; Tapia et al., 2015; Watanabe et al., 2003), heat shock proteins (King and MacRae, 2012; Nesmelov et al., 2018b), antioxidants (Nesmelov et al., 2018a), DNA repair enzymes (Gusev et al., 2010) and LEA proteins (Hand et al., 2011; Tunnacliffe and Wise, 2007) are present in desiccation-tolerant life stages of anhydrobiotic organisms, and these factors contribute to reduction of damage during drying and to repair during rehydration (Crowe et al., 1998; Gusev et al., 2014; Hand et al., 2011; Welnicz et al., 2011).

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LEA proteins were initially described in the late stages of embryogenesis in cotton seeds (Dure et al., 1981) and subsequently identified in anhydrobiotic animals. As classified by Wise (2003), there are six classification groups of LEA proteins, with groups 1, 3 and 6 found in animals (Hand and Menze, 2015; Hand et al., 2011; Janis et al., 2018a,b; LeBlanc and Hand, 2020; LeBlanc et al., 2019; Wise, 2003). Most LEA proteins found in animals are hydrophilic and are composed predominantly of random coils when hydrated (Hand et al., 2011; LeBlanc et al., 2019; Tunnacliffe and Wise, 2007). In contrast to most globular proteins, many LEA proteins gain secondary structure as water is removed (Boswell et al., 2014a; Goyal et al., 2003; LeBlanc et al., 2019). It is speculated that this increase in secondary structure allows LEA proteins to take on a protective role during water-limited states. LEA proteins prevent inactivation and aggregation in globular proteins and stabilize membranes during desiccation (Boswell et al., 2014b; Goyal et al., 2005; LeBlanc et al., 2019; Moore et al., 2016; Tolleter et al., 2010). These proteins work synergistically with trehalose to stabilize macromolecules in some cases and also increase the glass transition temperature of trehalose (Buitink and Leprince, 2008; Goyal et al., 2005; Shimizu et al., 2010; Wolkers et al., 2001).

Most LEA proteins found in animals belong to group 3. *Artemia franciscana* is the only animal known to accumulate LEA proteins from groups 1, 3 and 6 (Hand and Menze, 2015; Janis et al., 2018a,b; LeBlanc et al., 2019). Based on a cDNA library created from desiccation-tolerant embryos of *A. franciscana*, we identified seven LEA genes (*Afrlea1*, *Afrlea2*, *Afrlea3m*, *Afrlea3m_47*, *Afrlea3m_43*, *Afrlea3m_29* and *Afrlea6*) that encode proteins in groups 3 and 6 (Hand et al., 2007; Hand and Menze, 2015; Janis et al., 2018b; LeBlanc et al., 2019; Menze et al., 2009). A considerable number of group 1 LEA proteins with high similarity are also expressed in *A. franciscana* (Marunde et al., 2013; Sharon et al., 2009; Toxopeus et al., 2014; Warner et al., 2016, 2010). Of the three LEA proteins chosen for the present study, AfrLEA2 and AfrLEA3m are members of group 3 and are targeted to the cytoplasm and the mitochondrion, respectively (Boswell and Hand, 2014). The third LEA protein used, AfrLEA6, shares high sequence similarity with seed maturation proteins (SMPs) found in plants such as the small legume *Medicago truncatula* (Chatelain et al., 2012). AfrLEA6 contains two SMP domains toward the N-terminus (Janis et al., 2018b) and is cytoplasmically localized in *A. franciscana* embryos (LeBlanc and Hand, 2020). In plants, SMPs (also classified as group 6 LEA proteins) have been correlated to the long-term viability of dried seeds (Chatelain et al., 2012). Long-term viability has not been achieved with desiccated mammalian cells that were genetically engineered to express group 3 LEA proteins (e.g. Li et al., 2012). Thus, the possibility to extend dry storage time by incorporating group 6 LEA proteins deserves experimental evaluation.

Drosophila melanogaster is a desiccation-avoidant organism. As such, pre-stressing *D. melanogaster* with bouts of moderate desiccation results in a reduced rate of water loss owing to changes in cuticular permeability (Bazinet et al., 2010). Attempts to artificially select *D. melanogaster* for higher desiccation tolerance has resulted in increased water retention but not an increase in the amount of water the organism can lose before dying (Gibbs et al., 1997). Studies investigating embryo and larval desiccation tolerance have shown that these developmental stages of *D. melanogaster* are also intolerant of extreme desiccation stress, although older embryos and larvae can tolerate more water loss than younger developmental stages (Kawano et al., 2010; Schreuders et al., 1996; Thorat et al., 2012). Despite this lack of substantial desiccation tolerance, *D. melanogaster* has been shown to tolerate hyperosmotic stress from NaCl after many generations of selection in media containing high NaCl (Riedl et al., 2016). While it is likely that intrinsic pathways for tolerance to extreme desiccation do not exist in *D. melanogaster*, it may be possible to enhance desiccation and hyperosmotic tolerance through transgenic expression of selected LEA proteins that are naturally accumulated by *A. franciscana* during anhydrobiosis. In the present study, we created *D. melanogaster* fly lines that accumulate AfrLEA2, AfrLEA3m and AfrLEA6 and evaluated the impact that these AfrLEA proteins have on the survival of *D. melanogaster* in embryonic, larval and adult stages of development.

MATERIALS AND METHODS

Creation of fly lines

For Gal4-UAS lines, the pUASg.attB vector (gift from the Dr Konrad Basler Lab, Zurich, Switzerland) was used to express the *Afrlea2* (GenBank accession no. EU477187) and *Afrlea3m* (GenBank accession no. FJ592175) genes when crossed with a fly line expressing Gal4 (Brand and Perrimon, 1993). The two vectors were created by employing the gateway cloning method (Invitrogen Corporation, Carlsbad, CA, USA).

For lines driven directly by the Actin 5C promoter, three additional vectors were created by inserting an Actin 5C promoter into a promoter-less vector (pC4scs plasmid; generously provided by Dr Craig Hart, Baton Rouge, LA, USA). The Actin 5C promoter used was a 461 bp region of the *D. melanogaster* Actin 5C promoter that has been shown to promote gene expression at higher levels than achieved with the entire Actin 5C promoter sequence (Chung and Keller, 1990; Qin et al., 2010). A 750 bp nucleotide string was synthesized (IDTDNA, Coralville, IA, USA) that contained this 461 bp promoter followed by a 56 bp multi cloning site (MCS) with eight restriction sites and ended with a 233 bp Simian virus 40 PolyA tail from the gfpTub-UASpBacNPF vector [Drosophila Genomics Resource Center (DGRC), Bloomington, IN, USA]. This 750 bp string was amplified by PCR using primers containing 5′ overhangs that were complementary to the plasmid region where the promoter would be inserted. The pC4scs plasmid was cut with the BamHI restriction enzyme, and then the PCR product was inserted into the cut plasmid using an exonuclease, DNA polymerase and DNA ligase via the Gibson assembly method (New England Biolabs, Ipswich, MA, USA). The new plasmid (pC4scs-A5C) was then cut again with the restriction enzyme SpeI (part of the MCS) and the *Afrlea2*, *Afrlea3m* or *Afrlea6* (GenBank accession no. MH351624) gene was inserted into the pC4scs-A5C plasmid using the Gibson assembly method.

After transgene insertion into the expression vector, the sequence fidelity and structure of all plasmids was confirmed by PCR, restriction enzyme digestion and sequencing in the region of the transgene. Sequencing was performed with BigDye terminator chemistry and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Proper transgene insertion was analyzed using the bioinformatics programs Sequence Scanner V1.0 (Applied Biosystems), Emboss:merger (Rice et al., 2000) and Biology Workbench (Subramaniam, 1998), and sequences were compared with those of bona fide *Afrlea2*, *Afrlea3m* and *Afrlea6*.

Three fly lines that express the PhiC31 integrase were employed for transgenesis: (1) y[1] w[1118]; PBac{y[+]-attP-3B}VK00031 (VK31), (2) y[1] w[1118]; PBac{y[+]-attP-3B}VK00037 (VK37) and (3) y[1] M{3xP3-RFP.attP}ZH-2A w[*]; M{RFP[3xP3.PB] GFP[E.3xP3]=vas-int.Dm}ZH-102D. These fly lines were injected with the vectors containing the *Afrlea* genes by the GenetiVision

Corporation (Houston, TX, USA), specifically: pUASg.attB-*Afrlea2* and pC4scs-A5C-*Afrlea3m* into VK31, pUASg.attB-Afrlea3m and pC4scs-A5C-*Afrlea2* into VK37, and pC4scs-A5C-*Afrlea6* into y[1] M{3xP3-RFP.attP}ZH-2A w[*]; M{RFP[3xP3.PB] GFP[E.3xP3]=vas-int.Dm}ZH-102D (Bischof et al., 2007; Venken et al., 2006). After transgenesis, selection of the injected fly lines was then performed to cross out the integrase gene used for plasmid incorporation and to create fly lines carrying homologous copies of each individual *Afrlea* transgene. Furthermore, because the VK31 and VK37 docking sites are on separate chromosomes, homologous co-expression of both AfrLEA2 and AfrLEA3m in the same fly was completed by crossing *Afrlea* fly lines containing pUASg.attB-*Afrlea2* and pUASg.attB-*Afrlea3m* (*Afrlea2*×*Afrlea3m*). The Oregon-R-C (wild type) and Act5C-GAL4/CyO-GFP (Gal4GFP) fly lines (DGRC) were employed as controls and the Gal4GFP line was also crossed to *Afrlea*-UAS lines to promote the ubiquitous expression of *Afrlea* genes.

AfrLEA accumulation during the embryo and larval stages

Expression of the *Afrlea* transgenes in pUASg.attB-*Afrlea* lines was driven by crossing the lines to flies expressing the Gal4 transcription activator. Gal4 expression was driven by the Actin 5C promoter and therefore allows ubiquitous expression of the *Afrlea* genes in progeny resulting from the transgenic pUASg.attB-*Afrlea*×Gal4 cross. To visualize AfrLEA accumulation, non-dechorionated embryos and third instar larvae were removed from the media and rinsed in water, then 70% ethanol, and then water again. After rinsing, larvae and embryos were homogenized at a ratio of 2.5 mg wet tissue to 1 µl of ice-cold LEA storage buffer, and a sample of the homogenate was frozen for total protein quantification using a modified Lowry method (Peterson, 1977). The remaining homogenate was then heated at 90°C for 20 min and spun at 20,000 ***g*** for 30 min. The resulting supernatant was mixed with the appropriate amount of 4× Laemmli sample buffer [1×=62.5 mmol l^{-1} Tris–HCl (pH 6.8), 2% SDS, 10% glycerol and 5% 2-mercaptoethanol (Laemmli, 1970)] and frozen at −20°C until SDS-PAGE was performed. Samples were then thawed and placed in a heating block for 10 min at 90°C. The protein samples were loaded onto an SDS polyacrylamide gel (4% stacking gel, 11% resolving gel) alongside a concentration range of the corresponding purified AfrLEA protein. The gels were electrophoresed at 120 V for 90 min in a Bio-Rad Mini Protean 3 cell (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were transferred at 90 V for 60 min in Towbins buffer (25 mmol l^{-1} Tris, 192 mmol l^{-1} glycine, 20% v/v methanol, 0.025% SDS) to a nitrocellulose membrane. The membrane was stained with Ponceau Red to visualize the proteins and then blocked for 1 h in 5% fat free dry milk prepared in TBS-T (20 mmol l^{-1} Tris, 500 mmol l^{-1} NaCl, 0.1% Tween 20, pH 7.6) at room temperature with rocking. The blots were incubated overnight in fresh blocking solution with primary antibody against each AfrLEA protein at the following dilutions: 1:50,000 (anti-AfrLEA2), 1:100,000 (anti-AfrLEA3m) and 1:20,000 (anti-AfrLEA6). The specificity and quality of AfrLEA polyclonal antibodies (raised in chicken eggs, Aves Labs Inc., Tigard, OR, USA) have been evaluated by Boswell et al. (2014b) (AfrLEA2 and AfrLEA3m) and LeBlanc et al. (2019) (AfrLEA6). After the primary antibody incubation, the blots were rinsed in three changes of TBS-T (15 min each). Finally, the blots were incubated with secondary antibody [horseradish peroxide labeled (HRP) goat anti-chicken (Aves Labs Inc.)] at a concentration of 1:10,000 for 1 h at room temperature with shaking. The Amersham ECL Prime Western Blotting Detection Kit (GE Healthcare, Chicago, IL, USA) was used for detection of bands, and the blots were imaged using a Bio-Rad ChemiDoc™ XRS+ Imager.

HPLC analysis of trehalose content in embryos

Drosophila melanogaster embryos (non-dechorionated) were collected and aged to obtain embryos 5–21 h old, washed with H_2O, and then immediately homogenized into ice-cold 6% perchloric acid (PCA). PCA extracts were neutralized with 0.5 mol l^{-1} K_2CO_3 and then centrifuged at 20,000 ***g*** for 5 min and the resulting supernatant was filtered (0.22 µm pore size). HPLC analyses were performed using a Dionex HPLC system (Dionex, Sunnyvale, CA, USA) that contained a PDA-100 photodiode array detector, GP-50 gradient pump, ED40 module and AS50 autosampler (Patil et al., 2013). The peaks in the PCA extract were separated using a Dionex MA-1 column (250×4 mm i.d.) operating at 25°C. The mobile phase was 600 mmol l^{-1} NaOH (degassed) and was run at a flow rate of 0.3 ml min^{-1}. Peaks of trehalose in the PCA extracts were identified by comparing them to the retention times of a trehalose standard (Ferro Pfanstiehl Laboratories, Inc., Waukegan, IL, USA). Quantification of the eluted peaks was performed using pulsed amperometric detection employing waveform-A on the Dionex ED40 module. Calibration curves of trehalose standards were linear over the assay range.

Manipulation of embryos in preparation for drying and rehydration

Egg laying chambers (Genesee Scientific, San Diego, CA, USA) were first attached to grape juice agar plates that had been coated with yeast paste. To obtain embryos expressing a given *Afrlea* transgene, *Afrlea* males×Gal4 virgin female crosses were placed in the egg laying chambers approximately 1 h before the dark phase of a 12 h:12 h light:dark cycle. It has been documented that the rate of oviposition peaks just after the dark phase begins (Allemand, 1976). Embryos were collected for approximately 16 h and then washed with deionized H_2O onto a fine mesh screen. At room temperature, embryos were then dechorionated by immersion for 3 min in a 2.5% hypochlorite solution (prepared by diluting commercial bleach) and then rinsed with deionized water for 5 min. Dechorionated embryos were returned to a grape agar plate and arranged in rows in groups of 50–100 (Fig. 2B). Double-sided, acid-free tape (Scotch Brand, Hutchinson, MN, USA) was attached to Whatman filter paper (Whatman PLC, Maidstone, Kent, UK) and used to transfer the embryos from the grape agar plate. Embryos were then dried and subsequently rehydrated while attached to this filter paper. Eclosion of embryos after rehydration was tracked daily for 5 days by observing the embryos under a dissection microscope. If embryos completed development to the larval stage and broke free from the vitelline membrane, they were scored as ‘eclosed’. Larval survival after eclosion was not determined.

Desiccation of developmental stages

Drying of larvae and embryos was performed in desiccators (Fisher Scientific, Pittsburgh, PA, USA) equilibrated with saturated solutions of phosphorus pentoxide or sodium bromide (Fisher Scientific) to maintain relative humidities (RH) of approximately 0% or 57%, respectively (Young, 1967). Larval expression of *Afrlea2* and *Afrlea6* was driven directly by the Actin 5C promoter, while expression of *Afrlea3m* and *Afrlea2*×*Afrlea3m* was driven by the Gal4-UAS system under an Actin 5C promoter. Desiccation of 18 h embryos was conducted at 57% RH, similar to conditions used in a previous study of embryo drying (Schreuders et al., 1996),

while desiccation of third instar larvae required 0% RH to quickly desiccate the larvae before pupation occurred. Embryos were dried for 4 h to a residual water content of 20%, and larvae were dried for multiple time periods, yielding a range of residual water contents. Larvae were dried separately in individual cells that had mesh bottoms to allow air flow (Fig. 4B) at 15°C or 21°C. It is important to note that if larvae are allowed to dry in contact with other larvae, they wander and form groups, which promotes heterogeneous drying. Water loss for embryos and larvae was measured gravimetrically. Total water content was determined by drying animals to constant mass overnight at 90°C. Then the percent water lost during experimental drying was subtracted from 100 to yield the percent residual water.

Rehydration was performed by placing the embryos (still attached to filter paper) or larvae on top of grape agar plates in 100 or 20 µl of deionized H_2O, respectively. Recovery was defined as the ability of embryos to eclose and larvae to move after rehydration. It is important to note that embryos and larvae expressing LEA proteins did not show differences in total water loss when compared with controls that experienced similar drying regimes.

Impact of osmotic stress on the production of pupae

Groups of adult flies (three females and three males) were placed in vials containing culture medium that was supplemented with different amounts of NaCl. The groups were composed of wild-type flies or transgenic flies that expressed Afrlea2, Afrlea3m or Afrlea6 under a direct Actin 5C promoter. The culture medium was based on a cornmeal–molasses medium and contained 150 ml H_2O, 1.15 g agar, 10.4 g yellow cornmeal, 4.3 g brewer's yeast, 10.4 ml molasses, 3.325 ml Nipagin M and 0.825 ml propionic acid. The osmolality of the culture medium without agar was 250 mosmol kg^{-1} H_2O as measured by vapor pressure osmometry (Vapro 5600 Vapor Pressure Osmometer; Wescor, Inc, Logan, UT, USA). NaCl was added to the culture medium to create a range of osmotic concentrations: 445, 605, 773, 973 and 1196 mosmol kg^{-1} H_2O. Flies were cultured on these media for 12 days, and then the number of pupae that formed were counted. Differences in the number of pupae generated by each fly line were normalized by dividing the number of pupae formed in salt-containing vials by the number of pupae formed in control vials (no salt added) for each respective fly line.

Statistical analyses

Statistics were performed using R (https://www.r-project.org/). Data processing was performed using packages in the tidyverse (Wickham et al., 2019). Base R was used to perform one-way ANOVAs. Duncan's new multiple range and Games–Howell *post hoc* tests were performed using the packages DescTools (https://cran.r-project.org/package=DescTools) and userfriendlyscience (https://cran.r-project.org/web/packages/userfriendlyscience/index.html), respectively. The sigmoid curve was generated using the sicegar package (Caglar et al., 2018). The non-parametric Kaplan–Meier method was used for analysis of survivorship curves. Statistical significance between survivorship curves was determined using a log-rank test (Mantel–Cox). The Kaplan–Meier and Mantel–Cox tests were performed using the survminer package (https://cran.r-project.org/package=survminer).

RESULTS

Expression of AfrLEA proteins in *D. melanogaster*

Three AfrLEA proteins were expressed after successful transformation in *D. melanogaster* (Fig. 1). Expression was driven either indirectly using a Gal4-UAS promoter system and an Actin 5C-Gal4 fly line (Fig. 1A,B) or directly by the Actin 5C promoter (Fig. 1C). Without crossing in the Actin 5C-GAL4 fly line, no AfrLEA expression occurred in the *Afrlea*-UAS fly lines. Each of the three AfrLEA proteins accumulated in both the embryonic and larval stages of development. To manage the possibility that AfrLEA proteins could be deleterious to *D. melanogaster*, the GAL4-UAS system was employed so that expression of AfrLEA proteins could be tightly controlled if necessary. Because negative effects were not observed as a result of AfrLEA expression, we also created fly lines that constitutively expressed *Afrlea* genes under an Actin 5C promoter alone (Fig. 1, A versus C). Overall, there was not a major difference in expression between these two strategies, although the indirect (GAL4-UAS) system qualitatively appeared to give higher expression of AfrLEA2 (Fig. 1, A versus C). Finally, the technically challenging expression of two LEA proteins simultaneously in the same fly line was successfully accomplished for AfrLEA2 and AfrLEA3m (Fig. 1B). The purified recombinant forms of AfrLEA2 and AfrLEA3m migrate slightly slower than the respective proteins expressed in *D. melanogaster*, which would be predicted owing to the 6×-His tag and associated sequence (+4.2 kDa) for recombinant AfrLEA2 and the uncleaved mitochondrial targeting sequence and 6x-His tag (+4.0 KDa) for recombinant AfrLEA3m (Boswell et al., 2014b). In contrast, the purified recombinant AfrLEA6 contains no expression tag or targeting sequence (LeBlanc et al., 2019).

HPLC analysis of trehalose in embryos

Trehalose is the major blood sugar in insects (Becker et al., 1996) and consequently could be relevant to desiccation tolerance if sufficiently high in concentration in developmental stages. Embryos aged between 5 and 21 h contained 2.60±1.34 µmol trehalose g^{-1} wet embryo (n=6, mean±s.d.), which corresponds to a concentration of ∼3.2 mmol l^{-1} trehalose. This low concentration of trehalose is unlikely to have any influence on survival during desiccation or hyperosmotic stress. Trehalose content has been thoroughly evaluated in third instar larvae of *D. melanogaster* and is approximately 25 mmol l^{-1} (reared at 25°C with normal diet; Koštál et al., 2012).

Impact of AfrLEA proteins on embryo eclosion after desiccation

Dechorionated embryos dried at 57% RH lost approximately 80% of their water after 240 min (4 h) of drying. During this period of desiccation, the morphology of the embryos compresses from a three-dimensional oblong form (Fig. 2B) to a flattened, essentially two-dimensional shape (Fig. 2C). Upon rehydration with the direct application of water on grape-juice agar plates, the flattened embryos return to their original morphology within an hour. Embryos were tracked daily for 5 days for evidence of eclosion. Eclosion of dried embryos expressing AfrLEA proteins was dramatically higher (up to 90.2±13%, mean±s.d., n=7) than wild-type and Gal4GFP (control) embryos without LEA protein at 72 h (3 days) post-rehydration (8.8±8%, n=9, Welch's ANOVA, P<0.05) (Fig. 3). Importantly, the Gal4 fly line expressing green fluorescent protein (GFP) exhibited statistically identical results to wild-type controls [n=9 (3 days) and n=6 (5 days), Welch's ANOVA, P>0.05]. These results suggest a major impact of AfrLEA proteins on the rate of development after drying and subsequent rehydration. AfrLEA2 and AfrLEA3m were equally effective in protecting developmental capacity during desiccation versus controls (n=7, Welch's ANOVA, P>0.05). After 120 h (5 days) of recovery, AfrLEA2 and AfrLEA3m embryos eclosed significantly

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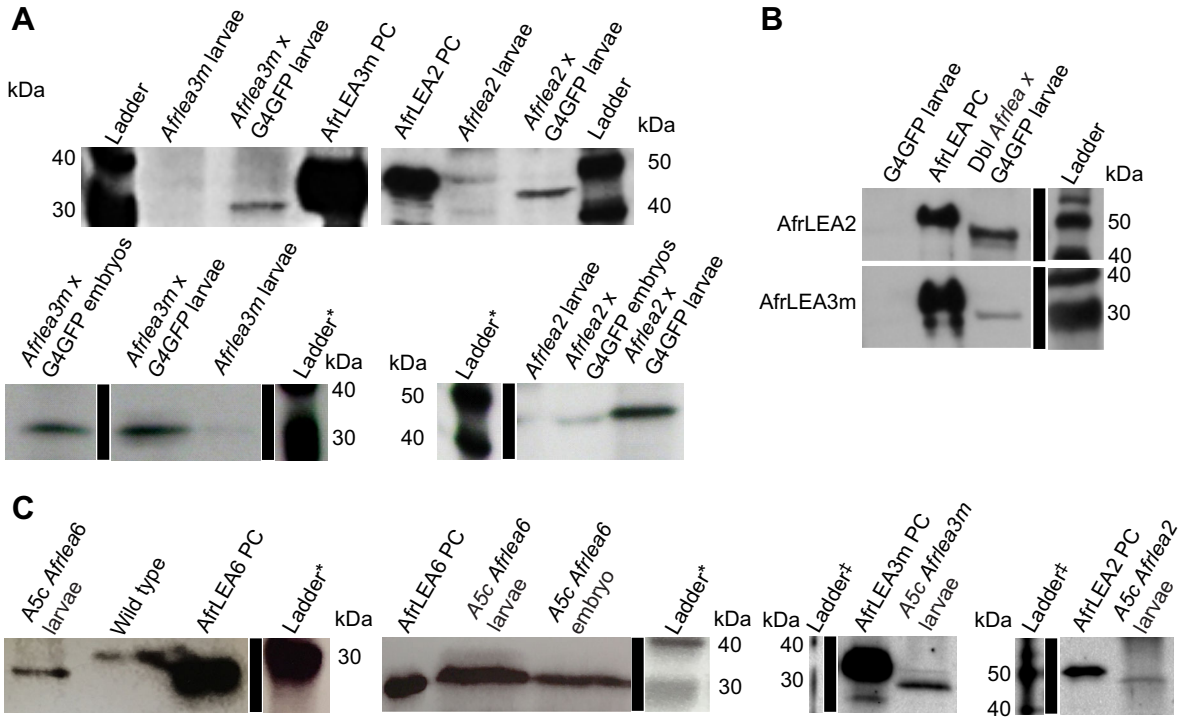

Fig. 1. Western blots of third instar larvae and embryos that express *Afrlea* transgenes. (A) In the left group of lanes, Gal4GFP (G4GFP) fly lines were crossed to the Afrlea-UAS fly lines to induce transcription of the *Afrlea* transgenes and expression of AfrLEA3m and AfrLEA2 proteins in larvae. Without the cross to Gal4, no AfrLEA protein is expressed in the larvae. The lower blots show expression of AfrLEA3m and AfrLEA2 in embryos. Larvae expressing AfrLEA protein are used as the positive control. (B) Afrlea-UAS fly line that contains both the *Afrlea2* and *Afrlea3m* transgenes and expresses both proteins simultaneously when crossed with a Gal4 fly line. As expected, larvae of the Gal4GFP line do not express either AfrLEA2 or AfrLEA3m. (C) *Afrlea* fly lines that contain an Actin 5C (A5c) promoter to drive transgene expression of AfrLEA6, AfrLEA2 or AfLEA3m in larvae and AfrLEA6 in embryos. PC, purified control protein (recombinant). Black vertical lines indicate points where lanes from the same blot and exposure time were re-positioned horizontally for easier comparisons (non-germane lanes were removed). Asterisks indicate ladders that were from the same blot, but from a different exposure, and double dagger indicates a ladder that was partially cut during trimming of the blots prior to incubation with antibodies.

better than Gal4GFP embryos (n=6 for wild type and Gal4GFP, and n=7 AfrLEA lines, Welch's ANOVA, P<0.05), but not significantly better than wild-type embryos. Eclosion of wild-type and Gal4GFP embryos was retarded dramatically after desiccation and 72 h of recovery, but eventually exhibited a non-significant trend toward improved eclosion after 120 h post-rehydration (Fig. 3).

Survival of larvae after desiccation

The drying time course at 0% RH shows that water loss was statistically identical between 15 and 21°C (one-way ANOVA, P>0.05) (Fig. 4A). In either case, approximately 6 h was required for the water content of larvae to decline to 50%. The lack of thermal dependence of water loss at 0% RH suggests that diffusive processes with low Q_{10} values likely predominate (e.g. Lange et al., 2012; Wang, 1965). It is essential that larvae remain separated during drying or else they behaviorally form clumps that alter the rate of water loss per individual (Fig. 4B). Larvae of control and AfrLEA-expressing fly lines all showed substantial declines in survival as dehydration proceeded (Fig. 5A). However, it is noteworthy that 10–25% of larvae containing AfrLEA proteins tolerated up to 13 h of drying at 0% RH, which promoted approximately 90% water loss; in contrast, Gal4GFP larvae (without AfrLEA proteins) displayed 0% survival after only 70% water loss (Fig. 5A). When all AfrLEA-expressing lines are combined, non-parametric Kaplan–Meier survival curves documented that survivorship was significantly greater compared with Gal4GFP (control) larvae at low water contents (P<0.0001, Fig. 5B). When larvae of AfrLEA-expressing lines are separately compared with Gal4GFP controls using the Kaplan–Meier survival curves, AfrLEA2, AfrLEA3m and AfrLEA6 larvae exhibited significantly higher survival compared with Gal4GFP controls (P<0.001), but the *Afrlea2*×*Afrlea3m* line was not significantly different (possibly owing to low sample size). The water loss at the LT_{50} (lethal time for 50% mortality) for larvae of the combined AfrLEA lines was 78.1% versus 52% for Gal4GFP controls (Fig. 5B).

AfrLEA proteins improve overall developmental progression when challenged with osmotic stress

Adult wild-type flies exhibited a marked depression in the ability of their offspring to reach pupation at osmolarities above 445 mosmol kg^{-1} H_2O in the culture medium (an osmotic concentration of 445 mosmol kg^{-1} H_2O actually increased pupation in all lines) (Fig. 6). At 605 mosmol kg^{-1} H_2O, pupation was less than 50% of control values (no salt supplementation; 250 mosmol kg^{-1} H_2O) and was only 20% of control values at 773 mosmol kg^{-1} H_2O (Fig. 6). In contrast, offspring of fly lines containing AfrLEA2, AfrLEA3m or AfrLEA6 fared much better. AfrLEA3m and AfrLEA6 lines produced significantly more pupae than wild type at 605, 773 and 973 mosmol kg^{-1} H_2O, while the AfrLEA2 line produced significantly more pupae than wild type at 773 mosmol kg^{-1} H_2O (one-way ANOVA, P<0.05, Duncan's *post hoc* test) (Fig. 6). An upper limit of tolerance to hyperosmotic stress in growth medium appears to be ∼1200 mosmol kg^{-1} H_2O, where all fly lines produced very few pupae.

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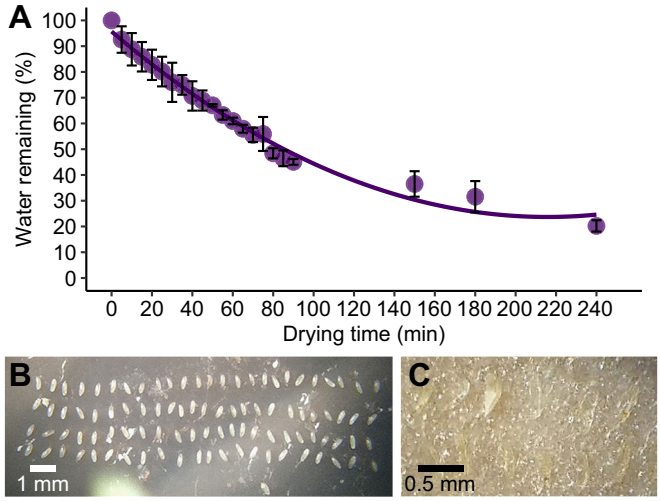

Fig. 2. Kinetics of water loss by embryos and their morphology before and after desiccation. (A) Percent of water remaining in dechorionated *D. melanogaster* embryos after dehydration at 55% relative humidity and 21°C (n=14 at 0 min; n=12 at 5, 10 and 15 min; n=10 at 20, 25 and 30 min; n=8 at 35, 40 and 45 min; n=3 at remaining points; means±s.d.) as a function of drying time. Groups of 50–100 embryos were dried per replicate. After 50% water loss, the embryos contained ~1.88 g H_2O g^{-1} dry mass. (B) Dechorionated embryos are shown that have been aligned prior to dehydration. (C) Desiccation for 90 min causes the embryos to compress (flatten) dorsoventrally on the filter paper and become semi-transparent.

DISCUSSION

In this study, we have shown that accumulation of transgenic AfrLEA proteins in embryos and third instar larvae of *D. melanogaster* under an Actin 5C promoter improves survival after exposure to desiccation and hyperosmotic stress. Embryos in the late stages of embryogenesis that expressed AfrLEA proteins tolerated the loss of up to 80% of their water and remarkably eclosed 2 days earlier than wild type. Third instar larvae that expressed AfrLEA proteins showed significant improvement in survival after water loss as compared with larvae from the Gal4GFP line without LEA proteins. Moreover, LEA proteins promoted recovery of 10–25% of rehydrated larvae that experienced up to 90% water loss, whereas recovery of Gal4GFP larvae was 0% after even 70% water loss. Finally, offspring of fly lines that expressed AfrLEA2, AfrLEA3m or AfrLEA6 exhibited significantly greater success in reaching pupation, compared with wild-type flies, when challenged with hyperosmotic stress. These findings represent a successful proof of principle test that group 3 and group 6 LEA proteins improve tolerance to water stress for a desiccation-sensitive species that normally lacks these proteins.

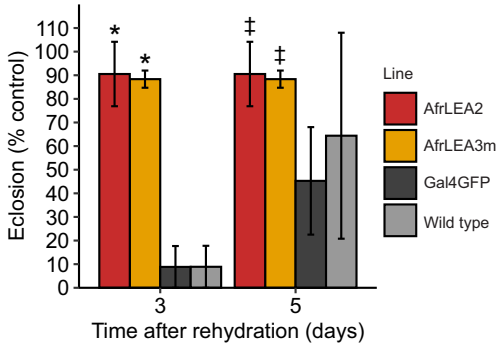

Fig. 3. Embryo eclosion after drying and subsequent rehydration/recovery. Groups of 50–100 embryos per independent replicate were dried for 240 min (20% water remaining). Eclosion is expressed as a percentage of the number of eclosed embryos from the same batch that were never dried (control). Bars with asterisks indicate significant differences from the eclosion of wild type and Gal4GFP lines after 3 days of rehydration/recovery (n=9, wild type and Gal4GFP; n=7, AfrLEA lines; means±s.d. and *P<0.05) and double daggers indicate significant differences from the eclosion of the Gal4GFP line after 5 days of rehydration/recovery (n=6, wild type and Gal4GFP; n=7, AfrLEA lines; means±s.d. and ‡P<0.05). Significance was assessed using Welch's ANOVA and the Games–Howell *post hoc* test. Gal4GFP represents a fly line expressing green fluorescent protein that serves as a negative control for the *Afrlea* transgenes.

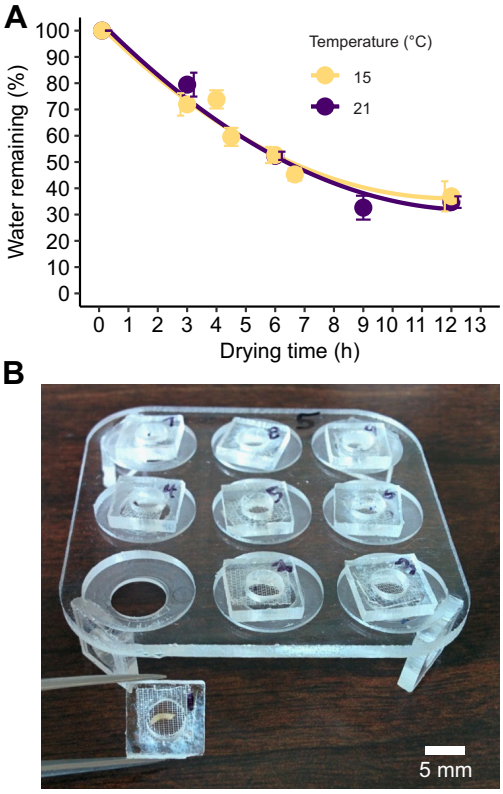

Fig. 4. Kinetics of water loss by larvae and configuration of drying chambers. (A) Percent of water remaining in third instar larvae of *D. melanogaster* after dehydration at 0% RH and either 15 or 21°C (n=3 for larvae dried for 3 h at 15°C, and n=4 for all others; means±s.d.) as a function of drying time. Larvae were dried in separate chambers and weighed individually. After 50% water loss, the larvae contained ~1.77 g H_2O g^{-1} dry mass. (B) Plastic chambers with mesh bottoms used to keep larvae separated during drying. Without this safeguard, multiple larvae behaviorally form clumps during drying, which alters the rate of dehydration.

Expression of AfrLEA proteins

Drosophila melanogaster transformed with plasmids containing *Afrlea* genes accumulate observable quantities of AfrLEA proteins in embryonic and larval life stages. As expected, lines using the GAL4-UAS system require a GAL4 line to be crossed in before expression occurs (Duffy, 2002). Lines driven directly by Actin 5C constitutively express *Afrlea* genes. The concentrations of AfrLEA2, AfrLEA3m and AfrLEA6 in desiccation-tolerant life stages of *A. franciscana* are approximately 0.8–1.8 mg ml^{-1} embryo water, 1.2 mg ml^{-1} matrix volume and 0.18 mg ml^{-1} embryo water, respectively (Boswell et al., 2014b; LeBlanc et al., 2019). The titers of these group 3 and group 6 proteins fall sharply as the embryos progress through pre-emergence development to the

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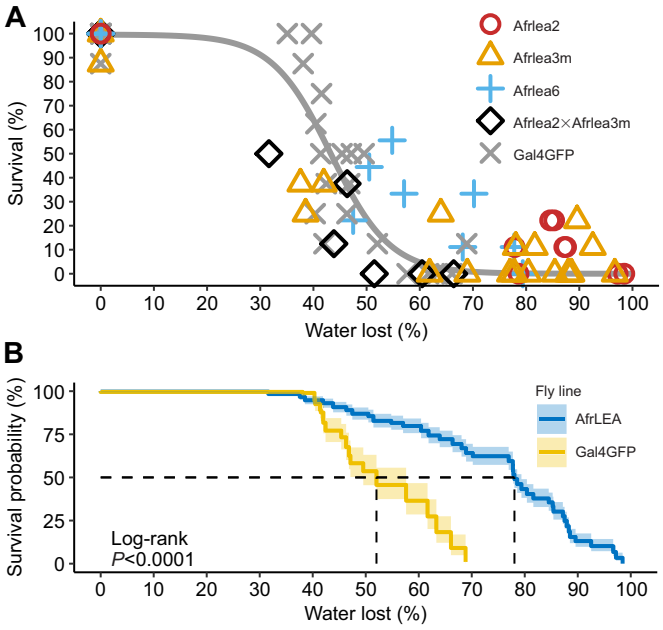

Fig. 5. Larval survival after various degrees of desiccation and subsequent rehydration/recovery. (A) Survival of larvae as a function of percent water remaining. Desiccation was accomplished by drying at 0% relative humidity and 15–21°C. Individual symbols represent groups of nine larvae that were dried and rehydrated simultaneously. A sigmoidal trend line was calculated for the Gal4GFP fly line to illustrate the sharp decline in survivorship at ∼45% water loss. (B) Kaplan–Meier survival curves plotted as a function of the percentage of water lost for AfrLEA-expressing lines combined versus Gal4 controls. Shaded areas around the curves represent 95% confidence intervals, and the dotted lines indicate the LT_{50} for each group. A statistically significant difference exists between the AfrLEA expressing lines and the Gal4GFP controls (log-rank test, P<0.0001).

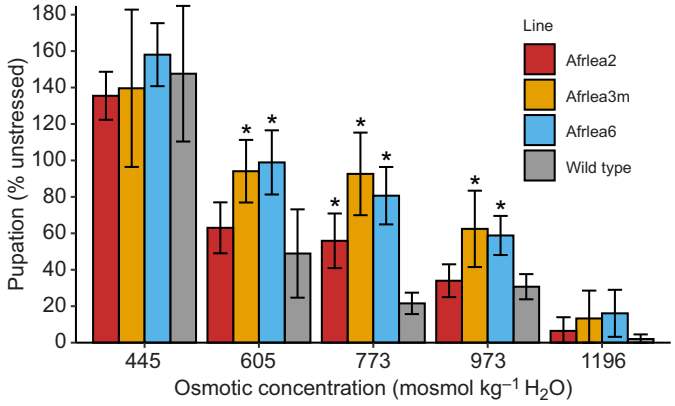

Fig. 6. Pupation of *D. melanogaster* after parental, embryonic and larval exposure to various concentrations of salt-supplemented medium. Values are expressed as a percentage of unstressed control flies. Replicates represent the pupation in individual fly vials, and a replicate was excluded if no eggs were laid [445 mosmol kg^{-1} H_2O: n=8 for wild type, AfrLEA3m and AfrLEA2 and n=7 for AfrLEA6; 605 mosmol kg^{-1} H_2O: n=8 for wild type, AfrLEA6 and AfrLEA2 and n=4 for AfrLEA3m; 773 mosmol kg^{-1} H_2O: n=4 for wild type, n=6 for AfrLEA3m, n=7 for AfrLEA2 and n=8 for AfrLEA6; 973 mosmol kg^{-1} H_2O: n=7 for wild type, n=6 for AfrLEA3m and AfrLEA2, and n=8 for AfrLEA6; 1196 mosmol kg^{-1} H_2O: n=4 for all fly lines; ±s.d.). Asterisks indicate a significant difference from the wild type at a given osmotic concentration (*P<0.05).

larval stage (Boswell et al., 2014b; LeBlanc et al., 2019), and these decreases are correlated with complete loss of desiccation tolerance in the nauplius. AfrLEA concentrations achieved in *D. melanogaster* embryos and larvae either using the GAL4-UAS system or controlled directly by the Actin 5C promoter have not been rigorously quantified but appear far lower than those naturally expressed in embryos of *A. franciscana*. Previous studies have not identified the minimum titers of AfrLEA proteins needed *in vivo* for increased tolerance to desiccation and osmotic stress, but apparently the values achieved in *D. melanogaster* are adequate to support significant amelioration of water stress.

Eclosion is enhanced after desiccation of embryos expressing LEA proteins

Up to 90% of embryos that contained AfrLEA2 or AfrLEA3m and were dried to 20% residual water eclosed after 3 days of rehydration, compared with only 9% for control embryos not expressing LEA proteins; most control embryos required 5 days to eclose and exhibited large variation in time requirements. In the absence of desiccation, full embryogenesis at 25°C requires about 24 h. The delayed eclosion owing to desiccation stress suggests to us that desiccation caused damage that impaired development. It is plausible that the presence of AfrLEA2 and AfrLEA3 reduced the damage and allowed the organism to overcome impairment and eclose more quickly. A previous study only tracked embryo eclosion for 24 h after rehydration, and as such, missed the ability of embryos to repair damage and resume development (Schreuders et al., 1996). However, survival to pupation of larvae that had eclosed from desiccated embryos was not quantified, but appeared low with or without LEA proteins.

Survival of larvae after desiccation

Survival of larvae generally declined as body water was removed but significantly improved with expression of AfrLEA proteins, which was documented by Kaplan–Meier survival curves (Fig. 5B). Indeed, some larvae containing AfrLEA proteins survived up to 90% water loss, while no control larvae survived even after 70% water loss. These results bear noteworthy resemblance to the improved eclosion of the embryos that experienced 80% water loss. LEA proteins are intrinsically disordered in aqueous solution and do not gain major amounts of secondary structure until ambient conditions reach low water content (e.g. below 20% water content, Li and He, 2009; equilibration to 75% RH, B. M. LeBlanc and S. C. Hand, unpublished). Accordingly, some authors argue that the full protective effects of LEA proteins are seen only with extensive drying when folding and acquisition of α-helix structure becomes prominent (Goyal et al., 2003; Shimizu et al., 2010; Tolleter et al., 2010). Direct experimental evidence for this important concept is lacking, and it may well be that certain functions of LEA proteins require secondary structure while other functions do not (see below). Nevertheless, in the present drying experiments, we observed greater protective effects by LEA proteins at lower water contents where these intrinsically disordered proteins would be predicted to fold.

Tolerance to osmotic stress during development is improved by LEA proteins

Our work extends a previous study that documented improved tolerance to osmotic stress when AfrLEA proteins are expressed in an insect cell line, Kc167 derived from *D. melanogaster* (Marunde et al., 2013). Here, with whole animals, we show that during osmotic stress, a significantly greater fraction of offspring reach pupation if they expressed AfrLEA proteins compared with wild-type controls without the proteins. The overall tolerance range (∼450 up to

1200 mosmol kg^{-1} H_2O) was not extended by AfrLEA expression. Interestingly, when *D. melanogaster* were selected for tolerance to osmotic stress across several hundred generations, maximum tolerance reached approximately 3700 mosmol kg^{-1} H_2O (Riedl et al., 2016). Gibbs et al. (1997) showed that improved desiccation tolerance in flies observed after selection across multiple generations was the result of greater water retention, larger body size and higher lipid content – not enhanced tolerance to water loss per se.

Conclusions and perspectives

Most anhydrobiotic animals are tolerant to desiccation only during specific life stages, and may possess behavioral adaptations that allow them to control the rate of drying (Halberg et al., 2013; Hand et al., 2018; Kikawada et al., 2005; Marotta et al., 2010). Both the stage of development and drying regime are likely important. As shown in the present study, larvae and embryos differ in their ability to recover from desiccation stress. Furthermore, because of the complex nature of the damage that occurs during desiccation, many molecular mechanisms are interwoven to promote survival. Although LEA proteins represent only one part of the puzzle (Gusev et al., 2014; Hand et al., 2011; Welnicz et al., 2011), the improvement in tolerance afforded by AfrLEA proteins in this study is nevertheless quite instructive. Our gain of function studies here with the transgenic expression of LEA proteins in a desiccation-sensitive species complement nicely the loss of function experiments published by Toxopeus et al. (2014) for a desiccation-tolerant species. In that work, RNAi technology was used to knock down group 1 LEA proteins in *A. franciscana* embryos, which resulted in compromised desiccation tolerance.

Amelioration by LEA proteins of desiccation stress versus mild hyperosmotic/ionic stress could be occurring through different mechanisms. In addition to the folding of LEA proteins that occurs at low water contents discussed above, protection by LEA proteins during osmotic/ionic stress suggests that these proteins may have protective functions in the unfolded state, because it is unlikely that gain of secondary structure occurred under these mild conditions with very limited water loss. For example, we have previously documented that trehalose concentrations up to 340 mmol l^{-1} (physiological for *A. franciscana* embryos) do not promote folding of AfrLEA proteins (LeBlanc et al., 2019); by comparison, the maximum concentration of trehalose reached naturally in larvae of *D. melanogaster* is 25 mmol l^{-1} (Koštál et al., 2012). The strong ability of trehalose to drive the folding equilibrium towards the native state of globular proteins has been well documented (Auton et al., 2011; Street et al., 2006; Xie and Timasheff, 1997). Similar to our trehalose results, model peptides based on a group 3 LEA protein could not be induced to fold in the aqueous state in the presence of salts including NaCl up to a 10-fold molar ratio per amino acid residue (Furuki et al., 2011), although divalent cations did shift the secondary structure from α-helix toward β-sheet during drying. Thus, major water removal or the addition of non-physiologically high crowding agents seem necessary to trigger secondary structure in LEA proteins. Yet, AfrLEA proteins appear to improve biological outcomes during our mild osmotic challenges with NaCl-fortified media.

Work is underway to create *D. melanogaster* lines that accumulate AfrLEA2, AfrLEA3m and AfrLEA6 simultaneously and to further optimize drying regimes for embryos and larvae. Further improvements in desiccation tolerance may require additional engineering of pathways for trehalose accumulation, DNA repair and antioxidant production, and lowering the temperature for desiccation and storage.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.M.A., S.C.H.; Methodology: J.M.A., S.C.H.; Validation: J.M.A., S.C.H.; Formal analysis: J.M.A., S.C.H.; Investigation: J.M.A.; Data curation: J.M.A.; Writing - original draft: J.M.A.; Writing - review & editing: S.C.H.; Supervision: S.C.H.; Project administration: S.C.H.; Funding acquisition: S.C.H.

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