GROUP 1 LATE EMBRYOGENESIS ABUNDANT (LEA) PROTEINS CONTRIBUTE TO STRESS TOLERANCE IN *ARTEMIA FRANCISCANA*

by

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This thesis is dedicated to the spirit of curiosity.

"Life is short, and potential studies infinite. We have a much better chance of accomplishing something significant when we follow our passionate interests and work in areas of deepest personal meaning."

-Stephen Jay Gould

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ABSTRACT

The encysted embryos (cysts) of the crustacean *Artemia franciscana* have several molecular mechanisms to enable anhydrobiosis – life without water. This study examines the function of group 1 Late Embryogenesis Abundant (LEA) proteins, hydrophilic unstructured proteins which accumulate in the stress-tolerant cysts of *A. franciscana*. Group 1 LEA proteins were knocked down in cysts using RNA interference. Cysts without group 1 LEA proteins exhibited low survival following desiccation and/or freezing, suggesting a role for these proteins in tolerance of low water conditions. In contrast, cysts with or without group 1 LEA proteins responded similarly to hydrogen peroxide exposure , indicating little to no function in reducing damage due to oxidative stress. This is the first *in vivo* functional study of group 1 LEA proteins in an animal, and may have applied significance in aquaculture, where *Artemia* is an important feed source, and in the cryopreservation of cells for therapeutic applications.

LIST OF ABBREVIATIONS AND SYMBOLS USED

aa	amino acid
AfrLEA1	*Artemia franciscana* group 1 LEA protein gene
AU	arbitrary units
bp	base pairs
DPBS	Dulbecco's phosphate buffered saline
dsRNA	double-stranded RNA (ribonucleic acid)
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
g	acceleration due to gravity, 9.81 m/s^2
GFP	green fluorescent protein
GSL	Great Salt Lake
h	hour
H_2O_2	hydrogen peroxide
HRP	horse radish peroxidase
HST	high salt/Tween
IgG	immunoglobulin G
kDa	kiloDaltons
LEA	late embryogenesis abundant
min	minute
mRNA	messenger RNA (ribonucleic acid)
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species

RT-qPCR	reverse transcription quantitative PCR
SDS	sodium dodecylsulfate
siRNA	short interfering RNA
sHsp	small heat shock protein
TBE	Tris, boric acid, EDTA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline Tween
TE	Tris, EDTA
Tris	tris-(hydroxymethyl)aminomethane
Tween	polyoxyethylene sorbitan monolaurate
UV	ultraviolet

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CHAPTER 1 INTRODUCTION

1.1 Stress and Stress Tolerance

1.1.1 Desiccation Stress

Water, one of the most abundant compounds on Earth, is required for growth and reproduction by all known life forms. At the cellular level, water makes the formation of phospholipid bilayers (membranes) and the folding of proteins into well-defined three-dimensional structures energetically favourable (Caramelo and Iusem 2009, Hoekstra et al. 2001). Hydration shells, layers of water molecules, stabilize many important cellular macromolecules such as DNA and proteins, facilitating function and excluding detrimental small molecules from their immediate vicinity (Berjak 2006, Caramelo and Iusem 2009). Clearly, water is absolutely essential for the structural integrity and function of cells, as well as the macromolecules contained in these cells (Berjak 2006, Caramelo and Iusem 2009, Hoekstra et al. 2001). Loss of water from cells, therefore, is a considerable stress for most organisms. When cells dehydrate, the removal of water causes molecular crowding, protein denaturation and aggregation, and potential fusion of cell and organelle membranes (Caramelo and Iusem 2009, Hoekstra et al. 2001). Damage due to reactive oxygen species (ROS) is enhanced during molecular crowding (França et al. 2007). Membrane fusion and protein aggregation are often irreversible processes, making it unlikely that cells regain function upon rehydration (Ellis and Minton 2006, Hoekstra et al 2001).

Terrestrial environments with subfreezing temperatures, xeric climates and aquatic environments with high osmolarity cause cellular water deficits, stress that varies from mild drought to intense desiccation (Hand et al. 2011). Freezing removes free liquid

water, disrupting the ionic balance of cells, and can cause mechanical damage due to ice crystal formation (Storey and Storey 2013). Desiccation is the most severe form of water loss, with reduction of body water content to less than 10% of body mass, indicating loss of bulk cytoplasmic water and the hydration shells surrounding macromolecules (Berjak 2006, Hoekstra et al. 2001, Walters et al. 2005). Surviving freezing or desiccation involves protecting against extreme water loss and maintenance in the stressful state for prolonged periods, tasks that most organisms cannot accomplish (Berjak 2006). However, many plant taxa (Illing et al. 2005, Proctor and Pence 2002), lichens (Kranner et al. 2005), fungi (Walters et al. 2005), and bacteria (Battista et al. 2001, Berjak 2006) tolerate desiccation and cold temperatures. Several animals, including nematodes (Browne et al. 2002, Tyson et al. 2012), collembolans, chironomids, tardigrades, bdelloid rotifers (Caprioli et al. 2004, Lapinski and Tunnacliffe 2003), and crustaceans (Clegg 1986) undergo anhydrobiosis, a state of metabolic dormancy in which compounds accumulate to protect against water loss and promote survival for long periods without water, often at subzero temperatures.

1.1.2 A. franciscana Development and Diapause

A genus of aquatic crustaceans, *Artemia*, exhibits anhydrobiosis in the stress-tolerant embryonic stage of its life cycle. *Artemia spp.* inhabit hypersaline lakes and ponds around the globe, with *A. franciscana* being the dominant species in North America (Triantaphyllidis et al. 1998). *Artemia* develop by ovoviviparous and oviparous pathways (MacRae 2005). In the former, fertilized eggs develop into nauplii in the female's brood sac, being released from the mother approximately five days post-fertilization as swimming larvae (MacRae 2005). These larvae undergo several molts and

grow into adults in approximately one month if conditions are optimal. In the oviparous pathway, embryonic development is halted at the gastrula phase (approximately 4000 cells) and these embryos are coated with a rigid, chitinous shell impermeable to non-volatile compounds (de Chaffoy et al. 1978, Liang and MacRae 1999, Ma et al. 2013, MacRae 2003). The encysted embryos, termed cysts, are released from the female brood sac and enter diapause over the next few days (Clegg et al. 1996). Diapause is a form of metabolic dormancy that is terminated once the organism receives specific environmental cues, and is characterized by high stress tolerance to several biotic and abiotic insults (Clegg et al. 1996, MacRae 2005). *Artemia* cysts withstand temperature extremes (Morris 1971, Liang and MacRae 1999, MacRae 2003), high ultraviolet (UV) radiation (Tanguay et al. 2004), anoxia (Clegg 1997, Clegg et al. 2000), and desiccation (MacRae 2005, MacRae 2010). *A. franciscana* cysts also exhibit freezing avoidance, as their intracellular water does not freeze until exposed to temperatures at or below -2°C (Crowe et al. 1981). The specific cues that terminate diapause are complex, varying between species of *Artemia*, and between populations of a single species (Drinkwater and Crowe 1987, Van Stappen et al. 1998). In the laboratory, the stressful combination of a few weeks of desiccation followed by two to three months at -20°C effectively terminates diapause in *A. franciscana* from the Great Salt Lake (GSL), Utah (King and MacRae 2012), while desiccation alone is insufficient. Alternatively, a brief treatment of GSL *A. franciscana* cysts with hydrogen peroxide (H_2O_2) can terminate diapause in embryos from this and other populations of *Artemia* (Robbins et al. 2010, Van Stappen et al. 1998, Veeramani and Baskaralingam 2011). In GSL *A. franciscana*, diapause is most efficiently terminated a few months after the cysts are released from the female (Robbins, in prep). The

molecular pathways activated or suppressed in response to these termination cues are not well-understood.

Once diapause is broken, the embryos may resume development, hatching as nauplii when cyst shells rupture, and undergoing several molts to become adults. However, if conditions are unfavourable for development, cysts remain dormant after diapause is broken, entering quiescence (MacRae 2005). Quiescence is also a state of dormancy characterized by high stress tolerance, but terminates as soon as the environment favours metabolic reactivation, not requiring a specific cue to terminate the dormancy (MacRae 2005). Both diapause and quiescence permit *A. franciscana* to survive unfavourable circumstances, resuming growth, development, and reproduction once their surroundings improve (MacRae 2010).

1.1.3 Mechanisms of Stress Tolerance in A. franciscana

The stress-tolerant embryos of *A. franciscana* are protected mechanically by the cyst shell, which shields against UV irradiation and may reduce the rate of water loss upon drying (Clegg 2005, Dai et al. 2011, Tanguay et al. 2004). Metabolic dormancy is thought to reduce the production of harmful ROS during long periods without water (Berjak 2006, Hand et al. 2011b). In addition, there are several molecular mechanisms by which cysts tolerate desiccation and cold temperatures during diapause and quiescence, including the developmentally-programmed accumulation of molecular chaperones, antioxidants, glycerol, disaccharides, and hydrophilic proteins as cysts enter diapause.

A. franciscana embryos synthesize molecular chaperones such as artemin, p26, ArHsp21, and ArHsp22, which reduce protein aggregation in *in vitro* experiments (Chen et al. 2007, Qiu and MacRae 2008a,b, Warner et al. 2004, Willsie and Clegg 2001).

ArHsp22 is also stress-inducible in adults (Qiu and MacRae 2008b, King et al. 2013). Two of these chaperones function in both stress tolerance and development, as p26 and artemin affect timing of embryonic development, and p26 impacts diapause maintenance and termination (King and MacRae 2012, King et al. 2014). Cysts also accumulate high amounts of the antioxidant carotenoid, canthaxathin, which neutralizes high energy ROS and UV radiation due to its many conjugated double bonds (Clegg and Trotman 2002, Gilchrist and Green 1960, Godar et al. 1993, Huang et al. 1996, Nelis et al. 1989, Tanguay et al. 2004).

While antioxidants and molecular chaperones are important in response to several forms of stress, glycerol accumulation regulates osmotic pressure and protects against freezing. Glycerol comprises 2 to 2.5% of *A. franciscana* cyst dry weight (Clegg 1997), and likely reduces damage due to ice formation by lowering the freezing point of water as it does in other cold-tolerant organisms (Storey and Storey 2013). As cysts lose water, the freezing point of water lowers further, and dried *A. franciscana* cysts survive exposure to temperatures below -20°C (Crowe et al. 1981). Any remaining "bound water", that is water associated with the surfaces of macromolecules, does not freeze (Ramløv and Hvidt 1992).

Water replacement and glass formation (vitrification) contribute specifically to desiccation tolerance (Crowe et al. 1998). Many anhydrobiotic animals accumulate the disaccharide trehalose, and it comprises 16-17% of the dry weight of *A. franciscana* cysts (Caramelo and Iusem 2009, Clegg 1997, Goyal et al. 2005b). This sugar preferentially hydrates proteins and membranes under mild water loss, and is thought to act as a water replacement molecule during desiccation, reducing membrane fusion and protein

aggregation (Buitink and LePrince 2004, Clegg 1986, Crowe et al. 1987, Crowe et al. 1992, Hoekstra et al. 2001, Potts 1994). Intracellular glass formation occurs in dried *A. francisana* cysts at room temperature (Hengherr et al. 2011). This glass, a mix of trehalose and proteins, is a structurally rigid support that helps prevent cell collapse and membrane fusion when water availability is low, as well as reducing molecular mobility and the detrimental effects of molecular crowding (Berjak 2006, Hand et al. 2011). In many species, the glasses contain late embryogenesis abundant (LEA) proteins (Hand et al. 2011, Shimizu et al. 2010, Wolkers et al. 2001), hydrophilic polypeptides that protect against desiccation but are not as well characterized functionally as molecular chaperones and trehalose.

1.2 Late Embryogenesis Abundant (LEA) Proteins

1.2.1 LEA Protein Synthesis and Function

LEA proteins were first described late in the embryogenesis of cotton seeds (Dure et al. 1981, Galau et al. 1986). These proteins are strongly associated with the acquisition of desiccation tolerance in plant seeds (Espelund et al. 1992) and several anhydrobiotic animals, including nematodes (Browne et al. 2002), rotifers (Tunnacliffe et al. 2005), collembolans (Bahrndorff et al. 2009, Clark et al. 2007), chironomids (Cornette et al. 2010, Kikawada et al. 2006), tardigrades (Förster et al. 2009), and brine shrimp (Chen et al. 2009, Hand et al. 2007, Menze et al. 2009, Sharon et al. 2009). LEA proteins also function in protection from cold or freezing in some organisms (Bhyan et al. 2012, Gai et al. 2011, Zhao et al. 2011). In addition, LEA proteins reduce desiccation- and freezing-induced protein aggregation *in vitro* (Chakrabortee et al. 2007, Furuki et al. 2012, Goyal et al. 2005a, Reyes et al. 2005), protect protein function (Grelet et al. 2005) and stabilize

membranes (Li et al. 2012, Tolleter et al. 2010). Transgenic experiments show LEA proteins improve desiccation tolerance of mammalian and *Drosophila melanogaster* cell cultures (Chakrabortee et al. 2007, Marunde et al. 2013) and whole organisms such as maize (Amara et al. 2013) and yeast (Duan and Cai 2012). In nematodes, group 3 LEA protein knockdown reduces water stress tolerance (Gal et al. 2004).

LEA proteins range in molecular mass from 8 kDa in *Typha latifolia* to 77 kDa in *Caenorhabditis elegans*, but all are characterized by conserved regions of protein sequence (Tunnacliffe and Wise 2007). While several classification schemes are suggested for the LEA protein family (Cuming 1999, Dure et al. 1989, Galau et al. 1986), most LEA proteins fall into one of three groups: group 1, group 2 (dehydrins) and group 3 (Tunnacliffe and Wise 2007, Wise and Tunnacliffe 2004). Only groups 1 and 3 are found in organisms other than plants, and representatives of both groups occur in brine shrimp (Hand et al. 2007, Sharon et al. 2009, Tunnacliffe and Wise 2007, Warner et al. 2010). All group 1 LEA proteins contain at least one copy of a hydrophilic 20 amino acid sequence motif (GGQTRREQLGEEGYSQMGRK), and all group 3 proteins contain copies of an 11 amino acid motif (TAQAAKEKAXE) (Cuming 1999, Tunnacliffe and Wise 2007). These highly hydrophilic proteins are natively unfolded and have little secondary structure, characteristics that are hypothesized to contribute to their function in desiccation tolerance (Shih et al. 2012, Tompa 2002, Tunnacliffe and Wise 2007).

LEA proteins have been partially characterized functionally, with several mechanisms proposed to explain how the proteins confer desiccation tolerance (Hand et al. 2011). The hydrophilicity of LEA proteins is thought to enhance their water binding capacity, slowing water removal during freezing or desiccation and allowing preferential

hydration of important macromolecules (Hoekstra et al. 2001, McCubbin et al. 1985, Tompa et al. 2010), although this may not substantially contribute to stress tolerance given the relative abundance of LEA proteins in cells (Hand et al. 2011). Like trehalose, LEA proteins may act as water replacement molecules (Cuming 1999, Hoekstra et al. 2001). Some group 3 LEA proteins reduce oxidative stress (Mowla et al. 2006), perhaps by binding ions and thereby reducing the frequency of, for example, the Fenton reaction (Berjak 2006, Cuming 1999). The hydrophilic unstructured nature of LEA proteins prevents them from aggregating under stress, and allows these entropic chains to act as molecular shields between denatured proteins, thereby preventing protein aggregation (Chakrabortee et al. 2012, Goyal et al. 2005b, Wise and Tunnacliffe 2004). Some LEA proteins acquire secondary structure (amphipathic α-helices) under desiccation and low temperature (Goyal et al. 2003, Hundertmark et al. 2012, Soulages et al. 2002), which allows them to interact with and protect membranes (Hincha and Thalhammer 2012, Tolleter et al. 2010). The coiled-coil structure of group 1 and 3 LEA proteins facilitates glass formation *in vitro* and contributes to the structural rigidity of glasses (Buitink and LePrince 2004, Shimizu et al. 2010, Wolkers et al. 2001).

1.2.2 A. franciscana and LEA Proteins

The upregulation of six group 1 LEA proteins or their mRNAs (Table 1), transcripts of eight group 3 LEA proteins (Appendix A, Table A1), and mRNAs of four LEA-like proteins has been confirmed in *A. franciscana* cysts (Boswell et al. 2013, Chen et al. 2009, Hand et al. 2007, Menze et al. 2009, Sharon et al. 2009, Warner et al. 2010, Warner et al. 2012, Wang et al. 2007, Wu et al. 2011). Proteomic analysis suggests up to 9 more group 3 LEA proteins (Warner et al. 2012), although sequence data are not

Table 1. Group 1 LEA proteins in *A. franciscana*.

LEA Protein[a]	Repeats	NCBI[b]	Size, Mass	Expression/Function	Ref.[d]
AfrLEA1-1m[c], Mit	8	ACX81198, GQ406334	217 aa, 21 kDa		[1]
AfrLEA1-1[c] (LEA-1a), Cyt	8	ABR67402, EF656614	182 aa, 21 kDa	↑ in diapause (mRNA and protein) ↓ protein aggregation	[1,2]
AfrLEA1-2 (LEA-1b), Cyt/mit	7		150 aa, 19 kDa	↑ in diapause (protein)	[1,3]
AfrLEA1-3 (LEA-1c), Cyt	6	ADE45145, GU568033	142 aa, 15.5 kDa	↑ in diapause (protein)	[1]
AfrLEA1-4 (LEA-1d)	5	ADE45146, GU568034	122 aa, 13 kDa	↑ in diapause (protein)	[1]
AfrLEA1-5	4	ABX89317, ES500255	102 aa, 10 kDa	↑ in diapause (mRNA)	[1,3]
AfrLEA1-6 (LEA-1g)	2	ADE45147, GU568035	62 aa, 5 kDa		[1]

[a] Nomenclature based on Wu et al. 2011. Original author nomenclature in parentheses. Cellular location when known, cyt, cytosol; mit, mitochondria; aa, amino acid. ↑, increased abundance; ↓, decrease

[b] NCBI (National Centre for Biotechnology Information) accession number of the protein and the cDNA sequence accession numbers.

[c] Both sequences are highly similar and differ nearly exclusively in the presence (ACX81198)/ absence (ABR67402) of a mitochondrial localization sequence.

[d] References: [1] Warner et al. 2010, [2] Sharon et al. 2009, [3] Wu et al. 2011

available (Appendix A, Table A1). The LEA proteins accumulate through transcription and translation as part of a developmentally-programmed entrance into diapause, much like that which occurs in production of rotifer resting eggs (Denekamp et al. 2010). Group 1 LEA proteins (AfrLEA1s) and their mRNA are detected in *A. franciscana* cysts, and disappear from the embryos shortly after diapause termination and hatching (Warner et al. 2010). Most group 3 LEA proteins are found only in cysts, with at least three detectable in gravid females and larvae (Warner et al. 2012). Two group 1 (AfrLEA1-1m, AfrLEA1-2) and several group 3 LEA proteins localize to the mitochondria of *A. franciscana* cysts, and small amounts of AfrLEA1-1 are found in the nucleus (Boswell et al. 2013, Menze et al. 2009, Warner et al. 2010). The *A. franciscana* group 3 LEA proteins have distinct sequences, while the *A franciscana* group 1 LEA proteins, AfrLEA1-1 to -6, differ only in the number of repeats of the conserved 20 amino acid motif (Table 1) (Warner et al. 2010, Wu et al. 2011). Of the group 1 proteins, AfrLEA1-1 and AfrLEA1-2 are the most abundant, estimated to comprise approximately 5% of total soluble cyst protein (Warner et al. 2010).

In addition to a small number of localization and expression studies, *A. franciscana* LEA proteins have been examined in cell culture. When expressed in transfected human cells, the group 3 LEA proteins AfrLEA3-2 and AfrLEA3-4 improved membrane integrity under dehydrating conditions, and the mitochondrially targeted group 1 LEA protein, AfrLEA1-1m, improved tolerance of *D. melanogaster* mitochondria to osmotic challenges (Li et al. 2012, Marunde et al. 2013). However, there is a lack of *in vivo* evidence for LEA protein function in *A. franciscana*, and further study is required to better understand the function of LEA proteins in this stress tolerant crustacean.

1.3 LEA Protein Function in *A. franciscana*

This study examines the function of group 1 LEA proteins in *A. franciscana* through knockdown by RNA interference (RNAi) of the expression of *AfrLEA1* genes. Cysts with and without group 1 LEA proteins were tested for their ability to withstand desiccation stress and subfreezing temperatures that remove cellular water, providing insight into the role of these proteins in desiccation and freezing tolerance of *A. franciscana* cysts. As some LEA proteins are known to protect against oxidative stress, survival of cysts following exposure to H_2O_2, a ROS, was measured. This test also allowed assessment of whether group 1 LEA proteins function in the response to diapause termination signals. Developmental parameters were observed in *A. franciscana* with and without group 1 LEA proteins because other stress tolerance proteins such as p26 and artemin affect development.

This study provides a better understanding of molecular mechanisms for desiccation and freezing tolerance in *A. franciscana*, an economically important species in aquaculture, and contributes to the growing body of knowledge surrounding LEA proteins in general. In addition, further elucidating how animal cells survive water loss may have biomedical applications through increasing storage longevity and thereby enhancing their therapeutic value (Kanias and Acker 2006, Loi et al. 2013).

CHAPTER 2 MATERIALS AND METHODS

2.1 Culture of *A. franciscana*

A. franciscana cysts from the GSL, Utah (INVE Aquaculture, Inc., Ogden, UT, USA) were rehydrated with aeration at room temperature in filtered (22 μm) and autoclaved sea water from Halifax Harbour, Nova Scotia, Canada, hereafter called sea water. Hatched nauplii were harvested 24 to 48 h later, and grown to adults in aerated sea water at room temperature. Cultures were fed daily with *Isochrysis sp.* (clone synonym T-Iso) from The Provasoli-Guillard National Center for Culture of Marine Phytoplankton, West Boothbay Harbor, Maine, USA. During experiments, adults were maintained in sea water in 6-well culture plates at room temperature (24°C) unless otherwise specified.

2.2 Knockdown of Group 1 LEA Proteins in *A. franciscana* Cysts

2.2.1 Synthesis of Double-stranded RNA

Platinum *Taq* DNA Polymerase (Invitrogen, Burlington, ON, Canada) and primers containing the T7 promoter sequence (Table 2) at 0.2 mM were used to amplify *AfrLEA1-1* by PCR from full length *AfrLEA1-1* cDNA (Fig. 1) cloned in the pCR 2.1 vector (Invitrogen) provided by Dr. A. H. Warner (University of Windsor, Windsor, ON, Canada) using the following reaction: 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, with a final 10 min at 72°C. In addition, the *Green fluorescent protein* (*GFP*) cDNA sequence was amplified from the commercial vector pEGFP-N1 (Clontech, Mountain View, CA, USA) using the above reaction cycle with Platinum *Taq* DNA Polymerase (Invitrogen) and primers (Table 2) at 0.2 mM (Zhao et al. 2012, King and MacRae 2012). *AfrLEA1-1* and *GFP* PCR products and GeneRuler 100 bp DNA

Table 2. Primers used for the production of dsRNA templates and for RT-qPCR.

Primer function	Primer sequence (5' to 3')	
Gene	Forward	Reverse
dsRNA template production[a]		
AfrLEA1	TAATACGACTCACTATAGGGAAAGCTAAGCCGCCAAG	TAATACGACTCACTATAGGGAGTCTGCTCTTGACATTTGCT
GFP [b]	TAATACGACTCACTATAGGGAGACACATGAAGCAGCACGACTT	TAATACGACTCACTATAGGGAGAAGTTCACCTTGATGCCGTTTC
RT-qPCR		
AfrLEA1	GAAGTTCAAGCGTTCTCC	TAGCTTGACCACCTTTCC
α-tubulin [c]	CGACCATAAAAGCGCAGTCA	CTACCCAGCACCACAGGTCTCT

All primers were synthesized by Integrated DNA Technologies (IDT), Coralville, IA, USA.

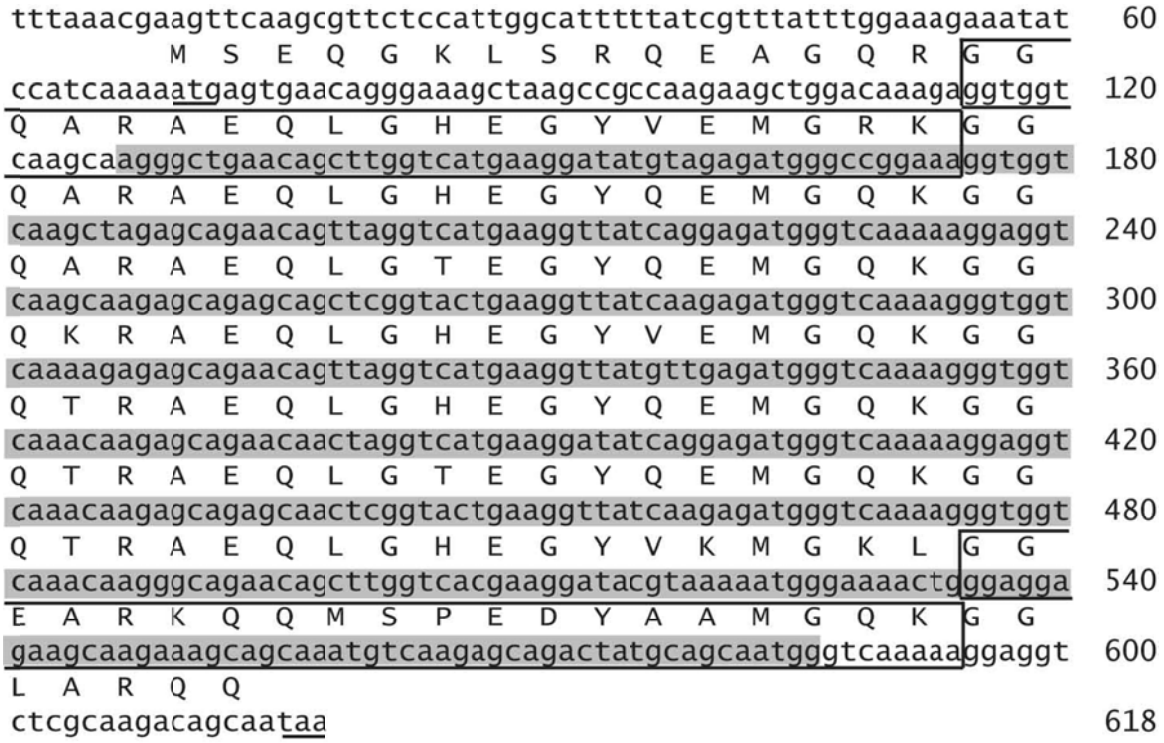
[a] Highlighted sequences indicate the T7 promoter region

[b] Zhao et al. 2012

[c] King et al. 2013

Figure 1. Full length *AfrLEA1-1* cDNA containing 8 repeated sequences.

Nucleotide and amino acid sequences for the group 1 LEA protein, AfrLEA1-1, with 8 repeats, are shown (Accession # EF656614, Warner et al. 2010). The *AfrLEA1* dsRNA is complementary to the shaded region of *AfrLEA1-1*. Start and stop codons are underlined. The first and eighth repeated sequences are boxed.


tttaaacgaagttcaagcgttctccattggcatttttatcgtttatttggaaagaaatat 60
M S E Q G K L S R Q E A G Q R G G
ccatcaaaaatgagtgaacagggaaagctaagccgccaagaagctggacaaagaggtggt 120
Q A R A E Q L G H E G Y V E M G R K G G
caagcaagggctgaacagcttggtcatgaaggatatgtagagatgggccggaaaggtggt 180
Q A R A E Q L G H E G Y Q E M G Q K G G
caagctagagcagaacagttaggtcatgaaggttatcaggagatgggtcaaaaaggaggt 240
Q A R A E Q L G T E G Y Q E M G Q K G G
caagcaagagcagagcagctcggtactgaaggttatcaagagatgggtcaaaagggtggt 300
Q K R A E Q L G H E G Y V E M G Q K G G
caaaagagagcagaacagttaggtcatgaaggttatgttgagatgggtcaaaagggtggt 360
Q T R A E Q L G H E G Y Q E M G Q K G G
caaacaagagcagaacaactaggtcatgaaggatatcaggagatgggtcaaaaaggaggt 420
Q T R A E Q L G T E G Y Q E M G Q K G G
caaacaagagcagagcaactcggtactgaaggttatcaagagatgggtcaaaagggtggt 480
Q T R A E Q L G H E G Y V K M G K L G G
caaacaagggcagaacagcttggtcacgaaggatacgtaaaaatgggaaaactgggagga 540
E A R K Q Q M S P E D Y A A M G Q K G G
gaagcaagaaagcagcaaatgtcaagagcagactatgcagcaatgggtcaaaaaggaggt 600
L A R Q Q
ctcgcaagacagcaataa 618

Ladder (Thermo Scientific) were resolved in 1.5% agarose gels in 0.5 x TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.5) at 90 V and stained with SYBR Safe® (Invitrogen). Stained gels were examined with a DNR Bio-Imaging Systems MF-ChemiBIS 3.2 gel documentation system (Montreal Biotech, Montreal, QC, Canada). PCR products were sequenced at The Centre for Applied Genomics DNA Sequencing Facility, Toronto Sick Kids Hospital, Toronto, ON, Canada. Five µg of each PCR product was used as template for the generation of *AfrLEA1* and *GFP* dsRNA with the MEGAscript® RNAi kit (Ambion Applied Biosystems, Austin, TX, USA) according to manufacturer's instructions. The purified dsRNAs were resolved by electrophoresis in agarose gels as documented above, and the dsRNA concentration was determined by measuring absorbance at 260 nm. Prior to injection of females, 3 volumes each of *AfrLEA1* and *GFP* dsRNA were separately diluted with 1 volume of 0.5% phenol red in Dulbecco's phosphate buffered saline (DPBS) (King and MacRae 2012).

2.2.2 Microinjection of A. franciscana Females with dsRNA

Mature females carrying diapause-destined unfertilized eggs were injected in the egg sac with either *AfrLEA1* or *GFP* dsRNA mixed with 0.5% phenol red in DPBS (Sigma-Aldrich, Oakville, ON, Canada) (King and MacRae 2012). Females destined to produce cysts were identified by the presence of a shell gland (Liang and MacRae 1999). Injections were performed under an Olympus SZ61 stereomicroscope (Olympus Canada, Inc., Markham, ON, Canada) with a borosilicate micropipette pulled with a custom programed P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Co., Novato, CA, USA) and broken at 45° using a clean razor blade (Copf et al. 2004). Each female was placed on cold 5% agar and lightly blotted with Kimwipes (Sigma-Aldrich) prior to

injection with 60 to 80 ng of dsRNA in approximately 250 nl using the Nanoject II Microinjector (Drummond Scientific, Co., Broomall, PA, USA). Females were returned to sea water and observed for 2 h to ensure retention of injected dsRNA (King and MacRae 2012). Each female was mated with a male 24 h post-injection in a 6-well culture plate containing sea water.

2.2.3 AfrLEA1 mRNA Knockdown

A. franciscana cysts were harvested ten days post-release from females injected with *AfrLEA1* or *GFP* dsRNA. RNA was extracted from 25 to 30 cysts by homogenization with a micropestle (Fisher Scientific, Ottawa, ON, Canada) in a 1.5 ml microtube with 100 µl TRIzol® (Invitrogen), and following manufacturer's recommendations. Six RNA preparations of cysts released from females injected with *AfrLEA1* dsRNA were generated, 3 each of first and second brood cysts. Four RNA preparations of cysts released from females injected with *GFP* dsRNA were generated, 2 each of first and second brood cysts. RNA concentration was quantified as described above, and 0.1 µg mRNA was used as template for generating cDNA with SuperScript® III First-Strand Synthesis Kit for RT-PCR (Invitrogen) and oligo dT_{20} primers according to manufacturer's instructions. For all RNA preparations, negative controls were incubated in the absence of reverse transcriptase with the SuperScript® III First Strand Synthesis Kit for RT-PCR (Invitrogen) to ensure the absence of genomic DNA.

qPCR was conducted with a QuantiTect® SYBR® Green PCR Kit (Qiagen, Mississauga, ON, Canada) in a Rotor-Gene RG-3000 system (Corbett Research, Sydney, NSW, Australia) using 0.5 µl cDNA as template (King et al. 2013). Primers (Table 2) for *α-tubulin* and *AfrLEA1* were used at 1 mM. The *AfrLEA1* primers amplified the 5'UTR

region (-62) to the second repeat of the 20 amino acid motif (+119) of *AfrLEA1-1m*, *-1*, *-3*, *-4* sequences (Table 1). cDNA copy numbers for *α-tubulin* and *AfrLEA1* were determined from standard curves, $R^2 > 0.99$ (Appendix A, Fig. A1) (King et al. 2013). Samples for the standard curves were generated by PCR using 0.5 µl cDNA with Platinum PCR SuperMix (Qiagen) and 0.4 mM primers for *α-tubulin* and *AfrLEA1* (Table 2). The concentrations of PCR products were determined by measuring absorbance at 260 nm, and copy number calculated based on the length of PCR product and an assumed base pair mass of 650 Da (http://cels.uri.edu/gsc/cndna.html). The DNA was diluted in a 10-fold series with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and 0.5 µl of each dilution was used as template with the QuantiTect® SYBR® Green PCR Kit (Qiagen). All samples were assayed in duplicate. The standard curve of resulting Ct values was fitted with a linear regression for each of *α-tubulin* and *AfrLEA1*, and the copy numbers of cDNA for both genes in each sample were determined using Rotor-Gene 6 software (Corbett Research). The copy numbers of *AfrLEA1* were normalized against the cDNA copy numbers representing *α-tubulin*. Melting curve analysis was conducted to assess primer fidelity.

2.2.4 Group 1 LEA Protein Knockdown

Fifty to 75 cysts released from females injected with either *AfrLEA1* or *GFP* dsRNA were harvested 10 days post-release and homogenized on ice with a micropestle (Fisher Scientific) in a 1.5 ml microtube containing 30 µl treatment buffer for SDS polyacrylamide gel electrophoresis (62.5 mM Tris, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.05% (w/v) bromophenol blue, pH 6.8). Protein preparations of cysts released from females injected with either *AfrLEA1* or *GFP* dsRNA

were generated from first and second brood cysts. Tubes containing protein samples were placed in a boiling water bath for 5 min prior to centrifugation for 10 min at 10,000 x g at 4°C. The supernatants and PiNK Plus Prestained Protein Ladder (FroggaBio Inc., Toronto, ON, Canada) were resolved in 12.5% SDS polyacrylamide gels at 30 mA for 60 min, and then transferred to Protran nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA) in transfer buffer (25 mM Tris, 200 mM glycine in 20% (v/v) methanol) at 100 mA overnight at room temperature.

Each membrane was blocked for 1 h in 5% (w/v) Carnation low fat milk in TBS (10 mM Tris, 140 mM NaCl, pH 7.4) at room temperature, incubated for 20 min with rabbit anti-group 1 LEA antibody (Warner et al. 2010) diluted 1:5,000 in TBS, and washed three times with TBS-T (10 mM Tris, 140 mM NaCl, 0.1% Tween-20, pH 7.4) for five min each and three times with HST (10 mM Tris, 1 M NaCl, 0.5% Tween-20, pH 7.4) for five min each. The membranes were then incubated for 20 min at room temperature with HRP-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich) diluted 1:10,000 in TBS, and washed three times with TBS-T and HST as above. Following a final 3 min wash in TBS, the secondary antibody was localized with ECL Plus Western Blotting Detection Reagents (GE Healthcare, Baie d'Urfé, QC, Canada) and a DNR Bio-Imaging Systems MF-ChemiBIS 3.2 gel documentation system. The experiment was conducted in triplicate.

2.3 Female and Cyst Development Following Knockdown of Group 1 LEA Proteins

The number of females surviving successive brood releases was documented in females injected with either *GFP* or *AfrLEA1* dsRNA. Seventy-six females were incubated at 24°C for the experiments. Brood size for the first three brood releases was

noted. The time from fertilization, defined by the fusion of egg sacs, to release of cysts was monitored for these brood releases, as well as the time required for complete release of the brood. An additional 76 females were incubated at 18°C, which slows development, to assess above parameters that might differ in a more subtle time frame.

2.4 Stress Tolerance of *A. franciscana* Cysts Lacking Group 1 LEA Proteins

2.4.1 Viability Following Diapause Termination via Desiccation and Freezing

After 10 days in sea water following release from females injected with either *AfrLEA1* or *GFP* dsRNA, cysts were collected in small containers and excess sea water was removed with a Pasteur pipette. These cysts were either blotted dry with a Kimwipe and placed in 1.5 ml microtubes in Styrofoam containers and incubated at -20°C for 12 weeks, or they were placed in aluminum foil-covered weigh boats in a desiccator containing Indicating Drierite (Sigma-Aldrich) for four weeks at room temperature, and then frozen at -20°C for 8 weeks. All cysts were then incubated in sea water at room temperature in 6-well plates to quantify hatching, a measure of viability. Cysts remained in sea water until 5 days after hatching no longer occurred. The experiments were done in triplicate with 38 to 113 cysts in each trial.

2.4.2 Viability Following Diapause Termination via H_2O_2

Following release from females injected with either *AfrLEA1* or *GFP* dsRNA, diapause was terminated by exposure of cysts to H_2O_2 after incubation at room temperature in sea water for 13 to 14 weeks. Cysts were collected by filtration and then immersed in 3% H_2O_2 for 20 min followed by filtration and washing twice with sea water at room temperature. Washed cysts were then transferred to sea water in 6-well plates and hatching observed as above. Due to high amount of abnormal hatching with this

treatment, subsequent cysts were washed 5 times to remove more residual H_2O_2. Images of emergence and hatching were obtained using Infinity Capture image software (Lumenera, Ottawa, ON, Canada) with an Olympus SZ61 stereomicroscope. The experiments were done in triplicate with 30 to 70 cysts in each replicate.

2.5 Statistical Analysis

A one-tailed student's t-test ($\alpha = 0.05$) was used to assess whether *AfrLEA1* mRNA was more abundant in cysts from females injected with *GFP* dsRNA as compared to *AfrLEA1* dsRNA. One-way ANOVA was used to assess differences in brood size from females injected with *AfrLEA1* and *GFP* dsRNA. Viability of cysts from females injected with either *AfrLEA1* or *GFP* dsRNA after exposure to stressors to terminate diapause were compared using χ^2 independence of factors tests.

CHAPTER 3 RESULTS

3.1 Generation of *AfrLEA1* and *GFP* dsRNA

dsRNAs generated from *GFP* and *AfrLEA1-1* cDNA were 309 bp and 505 bp, respectively, and they migrated to the expected position in agarose gels (Fig. 2). The *AfrLEA1* dsRNA is complementary to 463 bp (75%) of the full length *AfrLEA1-1* mRNA and it is comprised predominantly of a repeating 20 amino acid motif (Fig. 1). *GFP* dsRNA is not complementary to *AfrLEA1* mRNAs, nor to any other mRNA known to be produced in *A. franciscana* (Zhao et al. 2012).

3.2 *AfrLEA1* mRNA and Group 1 LEA Proteins Were Significantly Reduced in Cysts from *A. franciscana* Females Injected with *AfrLEA1* dsRNA

Females injected with *AfrLEA1* dsRNA released cysts with decreased abundance of *AfrLEA1* mRNA transcripts when compared to cysts from females injected with *GFP* dsRNA, $p = 0.0004$ (Fig. 3). Group 1 LEA proteins were not detected via Western blotting and chemiluminescence in protein extracts of cysts produced by females injected with *AfrLEA1* dsRNA, whereas group 1 LEA proteins of 21 kDa and 19 kDa were visible in protein extracts from the same number of cysts from females injected with *GFP* dsRNA (Fig. 3, inset). Group 1 LEA proteins with molecular masses lower than 19 kDa were not detected.

Figure 2. Synthesis of *AfrLEA1* and *GFP* dsRNA.

AfrLEA1 and *GFP* sequences were amplified by PCR using cDNA templates and primers containing the T7 promoter. *AfrLEA1* and *GFP* dsRNA were synthesized using PCR templates of the respective genes as described in Materials and Methods. *AfrLEA1* and *GFP* PCR products, dsRNA, and GeneRuler 100 bp DNA Ladder (Thermo Scientific) were resolved in 1.5% agarose, stained with SYBR®Safe (Invitrogen) and visualized under UV light with the DNR Bio-Imaging Systems MF-ChemiBIS 3.2 gel documentation system. M, marker; bp, base pairs.

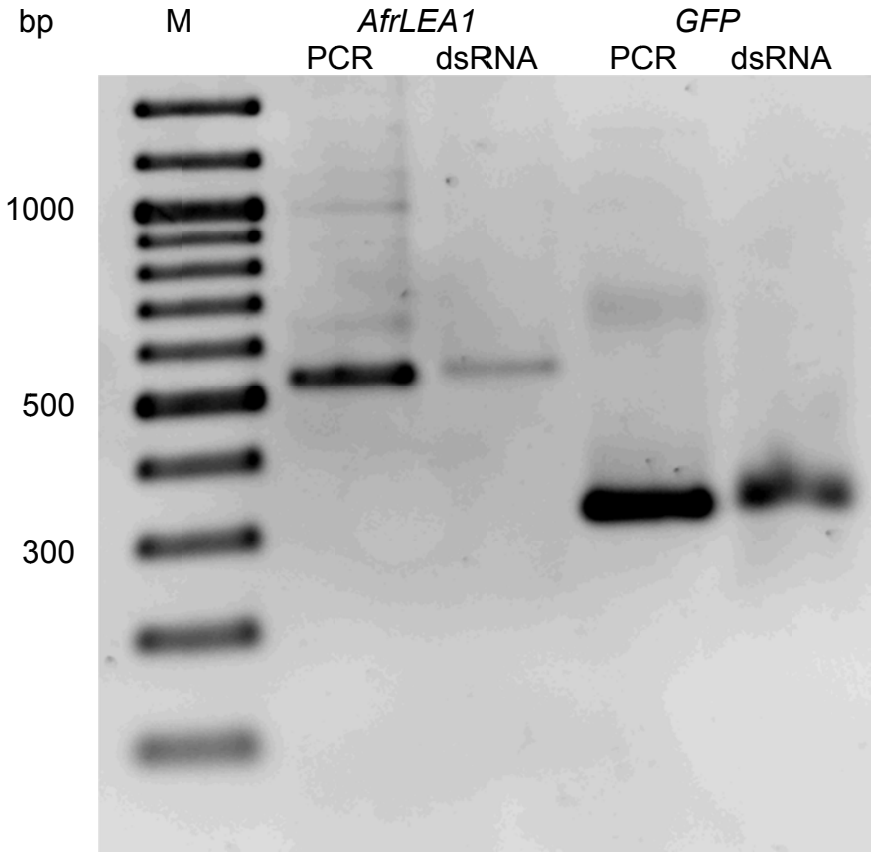
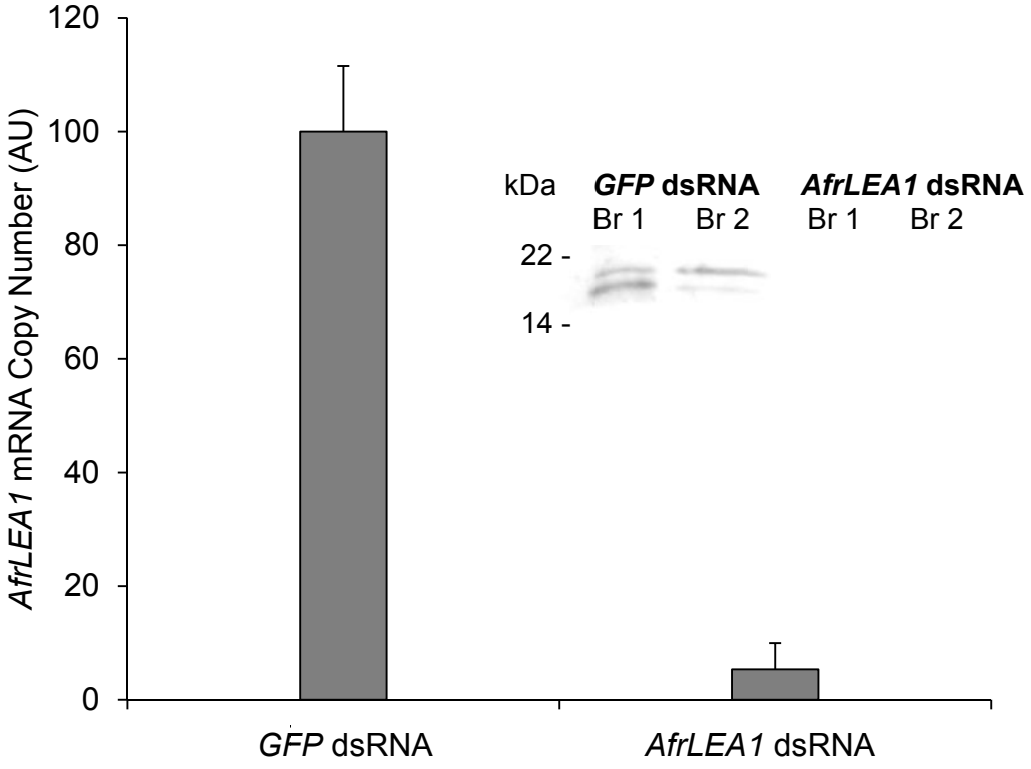

bp
M
AfrLEA1
PCR
dsRNA
GFP
PCR
dsRNA
1000
500
300

Figure 3. *AfrLEA1* mRNAs and group 1 LEA proteins were reduced in cysts from females injected with *AfrLEA1* rather than *GFP* dsRNA.

RNA and proteins were extracted from cysts produced in females injected with either *AfrLEA1* or *GFP* dsRNA. RNA was reverse transcribed and qPCR was performed in duplicate with SYBR® Green to determine *AfrLEA1* and *α-tubulin* transcript copy number in each sample. *AfrLEA1* copy numbers were normalized against *α-tubulin* copy numbers and averaged for samples of 25 to 30 cysts obtained from females injected with either *GFP* dsRNA (n = 4) or *AfrLEA1* dsRNA (n = 6). Values were scaled to set *AfrLEA1* cDNA copy number in cysts from females injected with *GFP* dsRNA to 100. Error bars represent standard deviation of mRNA copy numbers. Inset, protein extracts from 50 to 75 cysts and PiNK Prestained Protein Ladder (FroggaBio Inc.) were resolved in SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with rabbit anti-group 1 LEA protein antibody followed by HRP-conjugated goat anti-rabbit IgG antibody and chemiluminescence. The experiment was done in triplicate for the first two broods released from females. The inset shows a representative western blot. AU, arbitrary units; kDa, kiloDaltons; Br, brood.

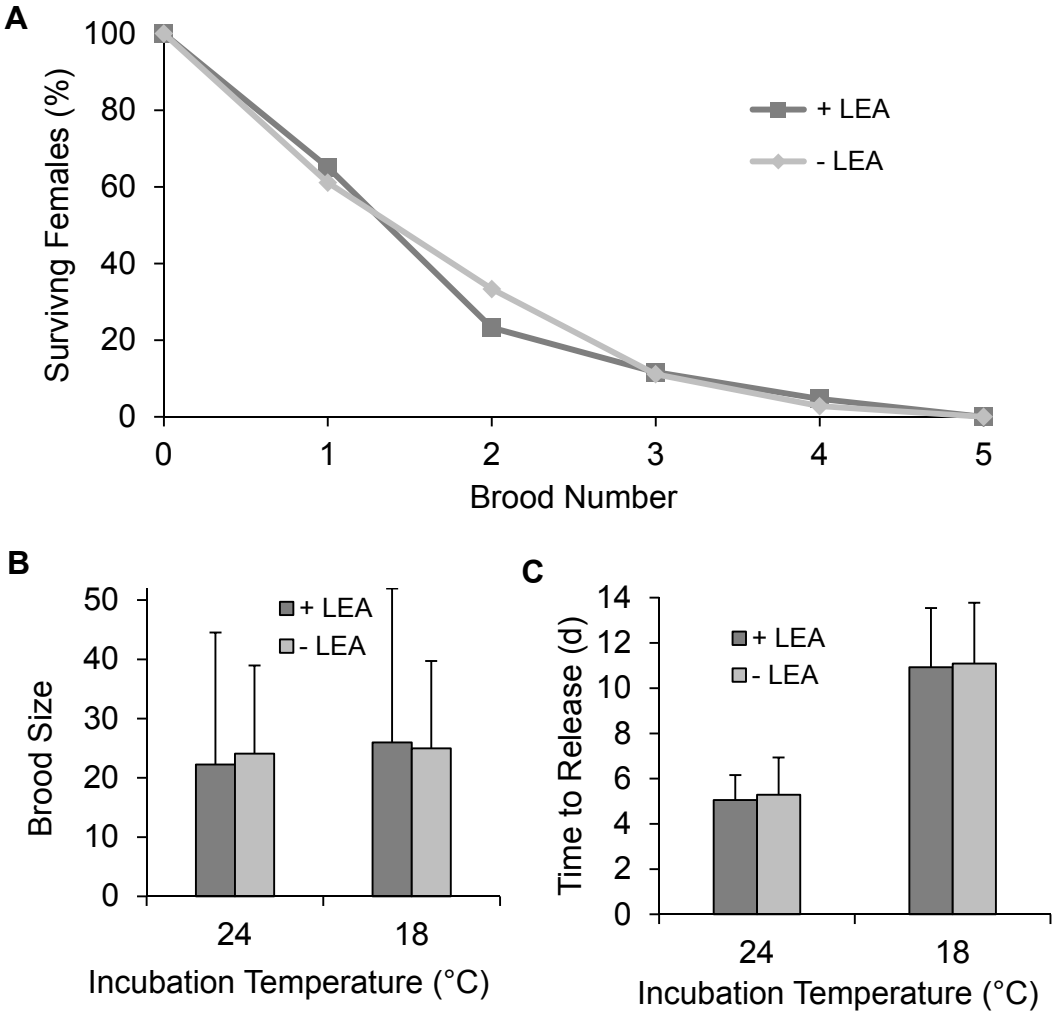

120
100
80
60
40
20
0
AfrLEA1 mRNA Copy Number (AU)
GFP dsRNA
AfrLEA1 dsRNA
kDa
GFP dsRNA
AfrLEA1 dsRNA
Br 1
Br 2
Br 1
Br 2
22 -
14 -

3.3 Group 1 LEA Protein Knockdown Did Not Affect *A. franciscana* Females or the Development of Cysts

Females injected with either *AfrLEA1* or *GFP* dsRNA survived equally well at 18°C (Fig. 4A), as did females monitored in experiments at room temperature (data not shown). Sixty to 65% of females released at least one brood of cysts, while fewer females released two (23-33%), three (11%) or four (2-4%) broods prior to death. No females produced and released a fifth brood under the conditions employed in this study (Fig. 4A). Based on data from first, second, and third brood releases, females injected with *AfrLEA1* dsRNA had the same average brood sizes (24-25 cysts) compared to females injected with *GFP* dsRNA (22-26 cysts), regardless of whether females were incubated at room temperature (24°C) or 18°C, $p = 0.57$ (Fig. 4B). Brood sizes from females injected with either *AfrLEA1* or *GFP* dsRNA were highly variable, ranging from 3 to 60 cysts per release. Embryos in females injected with either *AfrLEA1* or *GFP* dsRNA developed at a similar rate, all being released from the brood sac 5 days following fertilization when females were incubated at 24°C, $p = 0.32$ (Fig. 4C). Release time at 18°C was longer (11 days) than at 24°C, with no difference between females injected with either *AfrLEA1* or *GFP* dsRNA, $p = 0.79$ (Fig. 4C). In addition, all cysts within a brood were released from the brood sac in less than a day regardless of the type of dsRNA injected (data not shown).

Figure 4. Female survival and development of cysts were similar following injection of females with either *AfrLEA1* dsRNA or *GFP* dsRNA.

A. Representative survival of 43 females injected with *GFP* dsRNA (+ LEA) and 36 females injected with *AfrLEA1* dsRNA (- LEA) following successive brood releases at 18°C. **B**. Average brood sizes produced by + LEA and – LEA females were calculated from first, second, and third brood releases when females were incubated at 24°C (+ LEA, 53 broods; - LEA, 54 broods) or 18°C (+ LEA, 39 broods; - LEA, 32 broods). **C**. The average time from fertilization to cyst release from + LEA and – LEA females was determined from first, second, and third brood releases when females were incubated at 24°C (+ LEA, 70 broods; - LEA, 76 broods) or 18°C (+ LEA, 43 broods; - LEA, 34 broods). Females were fed daily and maintained in 6 well culture plates. Error bars represent standard deviation. d, days


A
100
80
60
40
20
0
Survivng Females (%)
+ LEA
- LEA
0
1
2
3
4
5
Brood Number
B
50
40
30
20
10
0
Brood Size
+ LEA
- LEA
24
18
Incubation Temperature (°C)
C
14
12
10
8
6
4
2
0
Time to Release (d)
+ LEA
- LEA
24
18
Incubation Temperature (°C)

3.4 Group 1 LEA Protein Knockdown Altered Cyst Viability in Response to Stress

3.4.1 Cysts Lacking Group 1 LEA Proteins Exhibited Low Survival in Response to Desiccation and Freezing

When diapause was terminated via removal of surface water by blotting with a Kimwipe and incubation at -20°C for 12 weeks, cysts lacking group 1 LEA proteins were less stress tolerant (22% survival) than cysts with group 1 LEA proteins (51% survival), as indicated by hatching, $p = 0.02$ (Fig 5). Cysts lacking group 1 LEA proteins exhibited 11-fold lower viability (2.3%) than cysts containing LEA proteins (26%) when diapause was terminated by 4 weeks desiccation and 8 weeks freezing, $p = 0.03$ (Fig. 5).

Regardless of the presence of group 1 LEA proteins, the viability of cysts exposed to 4 weeks desiccation and 8 weeks freezing was lower than cysts that had surface water removed with a Kimwipe prior to freezing for 12 weeks, $p < 0.001$ (Fig. 5). Cysts with LEA proteins were 2-fold less viable when desiccated and frozen compared to cysts that were blotted and frozen. Cysts without LEA proteins were 9 times less viable when desiccated rather than blotted dry prior to freezing.

3.4.2 Group 1 LEA Proteins Had No Effect on Cyst Diapause Termination via H_2O_2

When diapause was terminated by H_2O_2 after incubation for 13 to 14 weeks in sea water at room temperature, the proportion of cysts washed twice that hatched completely was similar with (25%) or without (10%) group 1 LEA proteins, $p = 0.63$ (Fig. 6A). A large fraction of cysts did not complete emergence, and hatching was disrupted equally in twice-washed cysts from females injected with either *GFP* or *AfrLEA1* dsRNA was disrupted equally (32% and 20% incomplete hatching, respectively), $p = 0.51$ (Fig. 6A).

Figure 5. Viability following desiccation and freezing decreased in cysts lacking group 1 LEA proteins.

Hatching (%) of cysts from females injected with either *GFP* dsRNA (+ LEA) or *AfrLEA1* dsRNA (- LEA) following removal of surface water with a Kimwipe and 12 weeks incubation at -20°C (Freezing), or 4 weeks desiccation at room temperature and 8 weeks incubation at -20°C (Desiccation & Freezing). Cysts were rehydrated in sea water at room temperature and hatched nauplii were enumerated. The experiment was done in triplicate for batches of first brood cysts; Freezing: + LEA: n = 65, 60, 62; - LEA: n = 38, 53, 60, Desiccation & Freezing: + LEA: n = 56, 113, 92; - LEA: n = 65, 87, 82. Error bars represent standard deviation. Lowercase letters indicate statistical difference.

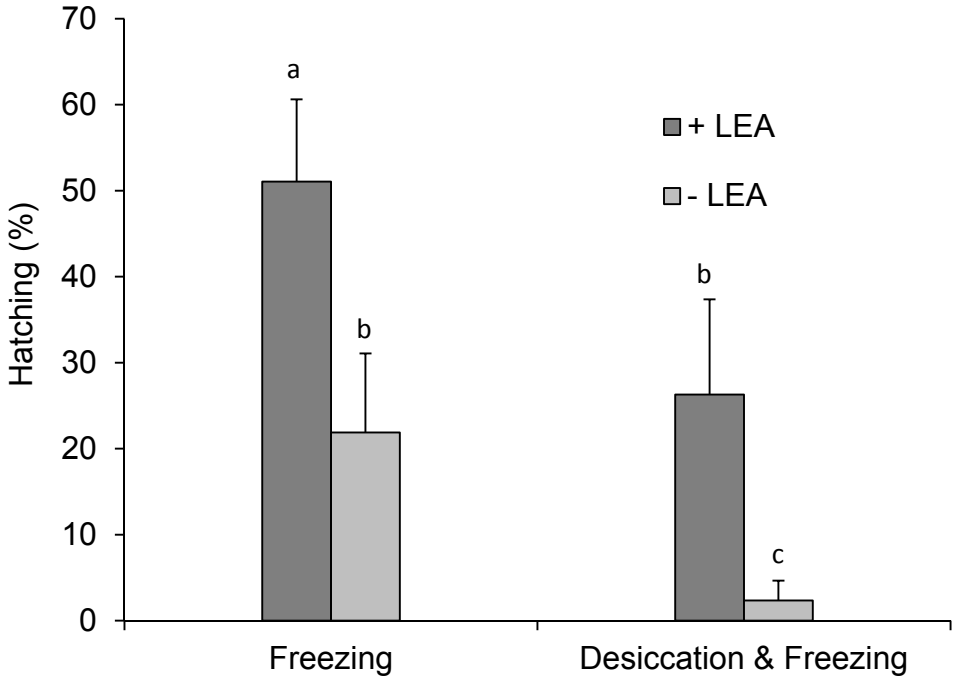
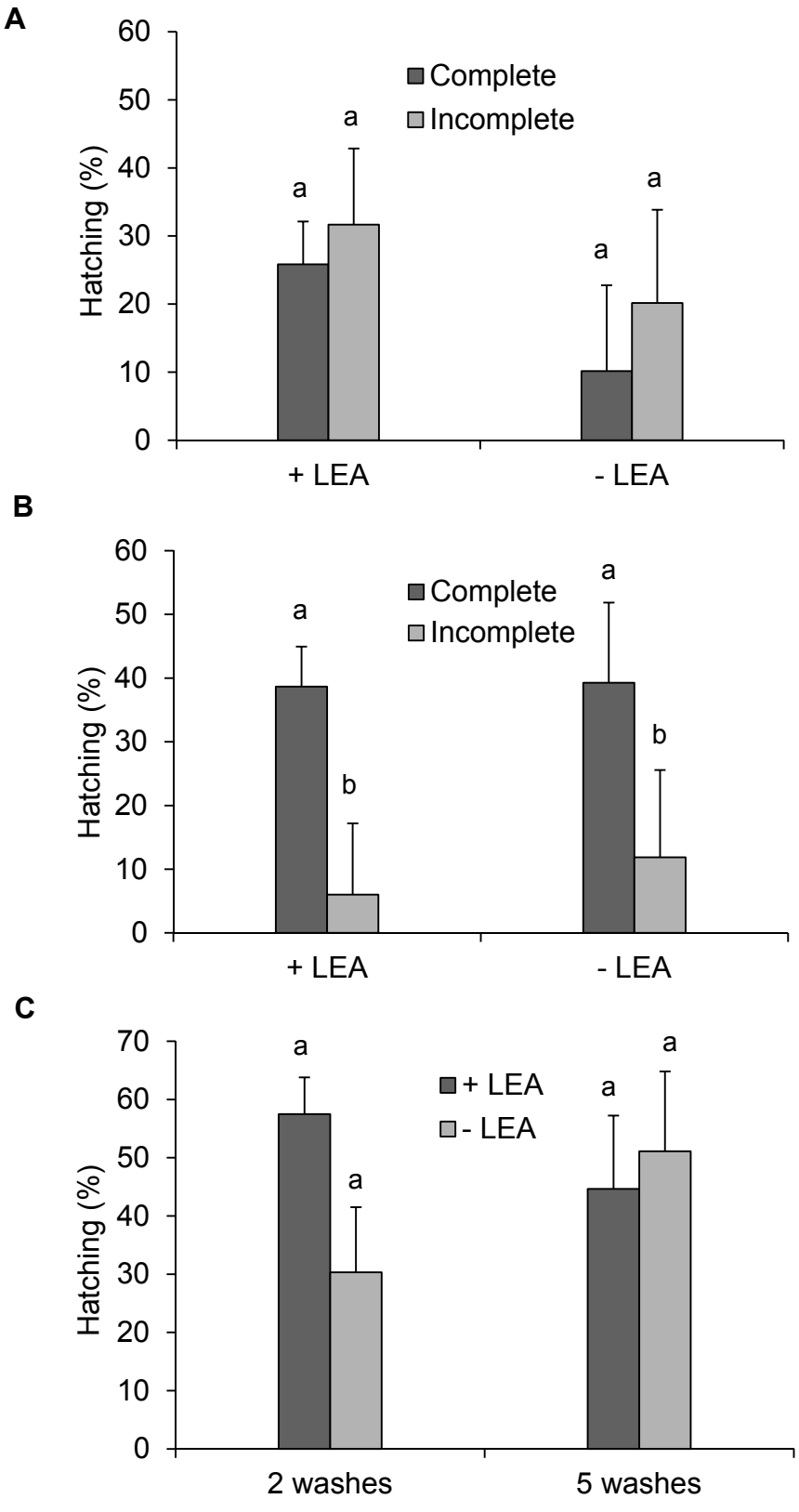

70
60
50
40
30
20
10
0
Hatching (%)
a
b
b
c
+ LEA
- LEA
Freezing
Desiccation & Freezing

Figure 6. Hatching of cysts following diapause termination with H_2O_2 was similar in cysts from females injected with either *AfrLEA1* or *GFP* dsRNA.

Cysts either containing (+ LEA) or lacking (- LEA) group 1 LEA proteins were incubated in sea water at room temperature for 13 to 14 weeks, exposed to H_2O_2, washed with sea water, and then incubated in sea water for 5 days after the last nauplius emerged. Hatching (Complete) and partial emergence (Incomplete) were enumerated **A**. Cysts were washed 2 times with sea water after H_2O_2 treatment. The experiment was done in triplicate for batches of between 30 and 70 first brood cysts (+ LEA: n = 40, 40, 30; - LEA: n = 40, 70, 40). **B**. Cysts were washed 5 times with sea water after H_2O_2 treatment. The experiment was done in duplicate for batches of between 24 and 61 first brood cysts (+ LEA: n = 61, 25; - LEA: n = 24, 26). **C**. Total viability (Complete and Incomplete hatching) of + LEA and – LEA cysts washed either 2 or 5 times post-H_2O_2 treatment was determined for the samples in **A** and **B**. Error bars represent standard deviation. Lowercase letters indicate statistical difference.

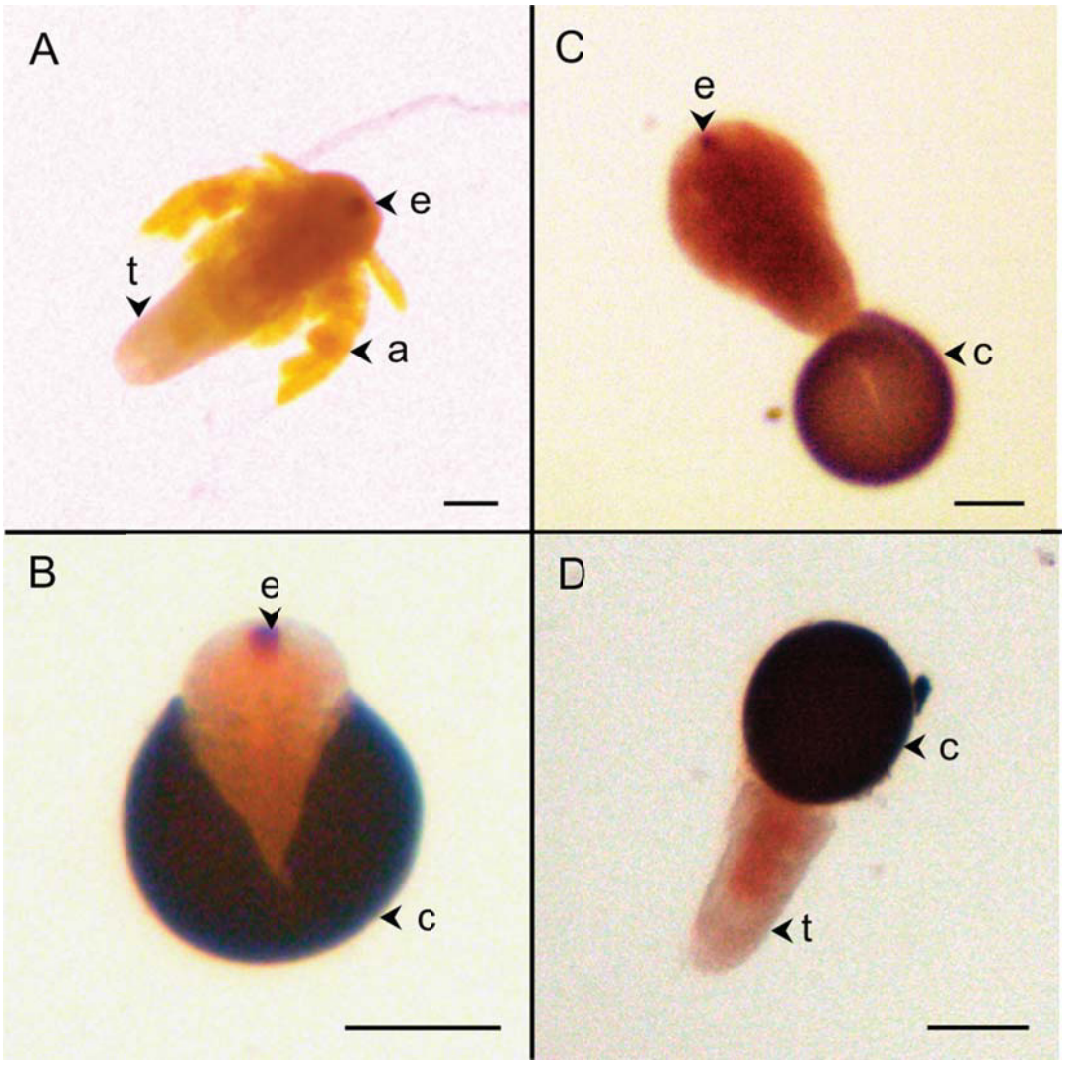

A
60
50
40
30
20
10
0
Hatching (%)
Complete
Incomplete
a
a
a
a
+ LEA
- LEA
B
60
50
40
30
20
10
0
Hatching (%)
Complete
Incomplete
a
b
a
b
+ LEA
- LEA
C
70
60
50
40
30
20
10
0
Hatching (%)
+ LEA
- LEA
a
a
a
a
2 washes
5 washes

A large portion (39%) of cysts washed 5 times completed hatching regardless of the presence of group 1 LEA proteins, $p = 0.39$ (Fig. 6B). Disruption of hatching was low after 5 washes, and was not significantly different in cysts with and without group 1 LEA proteins (6% and 12% incomplete hatching, respectively), $p = 0.42$ (Fig. 6B). There was no significant difference in viability (the sum of complete and incomplete hatching) of cysts with or without group 1 LEA proteins (Fig. 6C). When cysts were washed 5 times after H_2O_2 treatment, similar proportions of cysts containing group 1 LEA proteins (45%) and cysts lacking LEA proteins (51%) exhibited partial or complete hatching, $p = 0.11$ (Fig. 6C). When washed twice following H_2O_2 exposure, 30% of cysts lacking group 1 LEA proteins and 58% of cysts containing LEA proteins hatched or partially emerged, values that were not statistically different, $p = 0.35$ (Fig. 6C).

Complete hatching yielded a swimming nauplius (Fig. 7A), whereas for incomplete hatching, emergence halted just after the shell cracked (E1) (Fig. 7B) or with the membrane-enclosed nauplius attached to the shell (E2) (Fig. 7C). In extreme cases of abnormal exit from the cyst shell the membrane-enclosed nauplii emerged backwards, with the nauplius tail rather than the head protruding from the cyst shell (Fig. 7D). All three forms of abnormal emergence were documented in cysts either containing or lacking group 1 LEA proteins.

Figure 7. Emergence of nauplii from cysts following diapause termination with H_2O_2

Diapause was terminated using H_2O_2 and washing cysts from females injected with either *GFP* dsRNA or *AfrLEA1* dsRNA as described in Materials and Methods. Images of emergence and hatching were obtained 3 days post-H_2O_2 under bright field microscopy using Infinity Capture imaging software with an Olympus SZ61 stereomicroscope. **A**, hatched nauplius; **B**, arrested in E1; **C**, arrested in E2; **D**, reverse emergence. a, antenna; c, cyst shell; e, eyespot; t, tail. Scale bars represent 1 mm.


A
e
t
a
C
e
c
B
e
c
D
c
t

CHAPTER 4 DISCUSSION

4.1 *AfrLEA1* mRNAs and Group 1 LEA Proteins Are Knocked Down in Cysts by RNAi

The intrinsically unstructured LEA proteins are not well characterized functionally, particularly in animals. Expression in human and *D. melanogaster* cells supports a general role for LEA proteins in desiccation and osmotic stress tolerance of animals (Li et al. 2012, Marunde et al. 2013). *In vitro* studies suggest LEA proteins prevent protein aggregation (Chakrabortee et al. 2012, Goyal et al. 2005b), stabilize membranes (Hincha and Thalhammer 2012, Tolleter et al. 2010), and contribute to the stability of sugar glasses (Shimizu et al. 2010, Wolkers et al. 2001), conferring desiccation tolerance on cells. Plant LEA proteins expressed in yeast play a role in oxidative stress tolerance (Mowla et al. 2006). However, studies examining animal LEA protein function *in vivo* are lacking.

Six group 1 LEA proteins and/or their mRNA transcripts accumulate as part of a developmentally-regulated program in which *A. franciscana* embryos become encysted and enter a stress-tolerant state of dormancy known as diapause (Sharon et al. 2009, Warner et al. 2010, Wu et al. 2011). RNAi of *AfrLEA1* expression in *A. franciscana* was performed to study group 1 LEA protein function during diapause, a procedure employed previously to examine sHsp function in *A. franciscana* embryos and cysts (King and MacRae 2012, King et al. 2013, 2014). Gravid females were injected with dsRNA complementary to either *AfrLEA1-1* mRNA or *GFP* mRNA, the latter not anticipated to alter gene expression in *A. franciscana* (Zhao et al. 2012), as the animal does not encode GFP, and *GFP* shows low sequence similarity to *AfrLEA1* genes.

Exposure of females to *AfrLEA1* dsRNA reduced both group 1 LEA proteins and their transcripts in cysts. qPCR, which amplified regions of *AfrLEA1-1m*, *-1*, *-3*, and *-4* cDNA, illustrated a reduction in the mRNA encoded by group 1 LEA protein genes in cysts released from females injected with *AfrLEA1* dsRNA. It is not certain whether mRNA reduction of *AfrLEA1-2* was detected through qPCR because the cDNA sequence, although confirmed (Chen et al. 2009), is not available. The two largest group 1 LEA proteins, with molecular masses of approximately 21 and 19 kDa were not detected via immunoprobing of Western blots in cysts following RNAi with *AfrLEA1* dsRNA, while they were present in cysts from females injected with *GFP* dsRNA. Based on mass, these two LEA proteins are AfrLEA1-1 and AfrLEA1-2 (Sharon et al. 2009, Warner et al. 2010). Given that AfrLEA1-2 protein levels were lowered by RNAi, it is probable that the transcript for *AfrLEA1-2* was reduced. No smaller LEA proteins were detected in cyst extracts, contrary to previous work in which AfrLEA1-3 and AfrLEA1-4 were observed (Warner et al. 2010). AfrLEA1-3 and -4 are less abundant than AfrLEA1-1 and -2 (Warner et al. 2010), and thus unlikely to be detected in the small quantities of cyst extract used in the present study. AfrLEA1-5 and -6 proteins have not been detected previously in *A. franciscana* cysts, although their transcripts are present (Warner et al. 2010, Wu et al. 2011), and neither was detected herein.

The results in this report represent the first time more than one protein was knocked down in *A. franciscana* cysts by injecting females with a single dsRNA sequence (King and MacRae 2012, King et al. 2013, 2014). The *AfrLEA1* dsRNA is largely comprised of repeating sequences encoding a 20 amino acid motif present in all group 1 LEA proteins (Fig. 1). Due to high sequence similarity between the six group 1

LEA proteins and their mRNAs, short interfering RNAs (siRNAs) generated from *AfrLEA1* dsRNA *in vivo* likely targeted all *AfrLEA1* mRNA transcripts for degradation within developing embryos. Moreover, no group 1 LEA proteins were detected in cysts from females injected with the *AfrLEA1* dsRNA by immunoprobing of Western blots.

4.2 *A. franciscana* Female Survival and Embryo Development Occurred Independently of Group 1 LEA Proteins

Many intrinsically unstructured proteins such as the LEA proteins (Hincha and Thalhammer 2012) "moonlight", their flexible structure allowing them to perform multiple roles (Tompa et al. 2005). Therefore, in addition to observing the effects of LEA knockdown on stress tolerance, *A. franciscana* cysts were monitored while developing in the brood sacs of females injected with either *AfrLEA1* or *GFP* dsRNA. Group 1 LEA protein knockdown by RNAi did not impact injected female survival or cyst brood size. This is in contrast to results observed for ArHsp22, a protein that is stress-inducible in adults (Qiu and MacRae 2008b), where injection of ArHsp22 dsRNA is fatal to *A. franciscana* adults (King et al. 2013). The negligible effect of group 1 LEA protein absence on adults is likely because they are not expressed in adults (Warner et al. 2010).

Cysts with and without group 1 LEA proteins developed at similar rates and were released from brood sacs in comparable time frames at room temperature. Even when development was slowed by incubation at 18°C, no difference in developmental timing was observed between cysts containing or lacking group 1 LEA proteins. These proteins in *A. franciscana* do not appear to regulate developmental processes as shown for the molecular chaperones p26 and artemin, the former altering the residence time of cysts in

the egg sac and the latter affecting the duration of brood release from the female (King and MacRae 2012, King et al. 2014).

4.3 Group 1 LEA Proteins Protect *A. franciscana* Cysts Against Desiccation and Freezing

4.3.1 Group 1 LEA Proteins Contribute to Tolerance of Subzero Temperatures

Members of the group 1 and 3 LEA protein families are predicted to enhance tolerance of desiccation, and cold or freezing (Bhyan et al. 2012, Gai et al. 2011, Tunnacliffe and Wise 2007, Zhao et al. 2011). In the present study, group 1 LEA proteins contributed to protection of *A. franciscana* cysts exposed to subzero temperatures, as indicated by hatching following freezing stress. Absence of group 1 LEA proteins resulted in a two-fold decrease in hatching of cysts following removal of surface water by blotting and prolonged incubation at -20°C. Hatching was employed as a measure of viability, as only cysts that survived the stress could terminate diapause and subsequently emerge from the cyst shells. It is also possible for viable cysts to remain in diapause or quiescence rather than hatch, so viability was potentially underestimated.

Bulk cytoplasmic water in fully hydrated *Artemia* cysts freezes at approximately -2°C (Crowe et al. 1981), but "bound water", that is water associated with the surfaces of macromolecules and organelles, in *Artemia* cysts remains liquid at much colder temperatures (Ramløv and Hvidt 1992). Thus cysts incubated at -20°C for 12 weeks likely had some ice formation, but maintained substantial liquid water with high solute concentrations (Crowe et al. 1981), a cold-induced desiccation. The group 1 LEA proteins may contribute to this freeze avoidance because, much like glycerol, they have a strong binding affinity for water (McCubbin et al. 1985, Tompa et al. 2010).

A. franciscana cysts have a greater proportion of bound water as compared to life stages in other animals, consisting of approximately 0.6 g H_2O per g of dry weight cysts (Crowe et al. 1981). The absence of group 1 LEA proteins may have decreased bound water, resulting in more intracellular ice formation that negatively impacted survival of cysts. Ice crystal formation causes mechanical damage and augments cellular desiccation (Storey and Storey 2013).

Hand and colleagues (2011) argue that LEA proteins do not bind large amounts of water, therefore group 1 LEA proteins most likely protected *A. franciscana* cysts from freezing by mediating damage to proteins and/or membranes. Other LEA proteins are known to prevent protein aggregation by acting as molecular shields between denatured proteins (Chakrabortee et al. 2012) and some interact with and protect phospholipid bilayers (Tolleter et al. 2010). The mitochondrially-targeted AfrLEA1-1 and AfrLEA1-2 may preserve mitochondrial membrane and protein integrity, allowing cysts to resume respiration and development upon termination of diapause (Menze et al. 2009). It should be noted, however, that while membrane protection has been demonstrated for group 3 LEA proteins (Shimizu et al. 2010, Tolleter et al 2010), this function has yet to be demonstrated for group 1 LEA proteins.

4.3.2 Group 1 LEA Proteins Are Required for Desiccation Tolerance of A. franciscana Cysts

When cysts were desiccated prior to freezing, the reduction in survival was greater than by freezing alone. Cysts lacking group 1 LEA proteins rarely hatched after desiccation and freezing, with an eleven-fold reduction in hatching compared to cysts with group 1 LEA proteins. This strongly suggests that *A. franciscana* group 1 LEA

proteins protect against desiccation. At low water content, such as occurs upon desiccation, remaining intracellular water is not expected to freeze (Crowe et al. 1981, Ramløv and Hvidt 1992). Therefore, while cold stimulus is required for diapause termination, incubation at -20°C for several weeks probably contributed minimally to freezing stress in desiccated cysts.

The most probable role for *A. franciscana* LEA proteins under intense desiccation is intracellular glass formation, a protective state that occurs in desiccated cysts (Hengherr et al. 2011). Representatives of group 1 and 3 LEA proteins partially fold under low water conditions, protecting membranes and enhancing the formation and strength of sugar glasses (Buitink and LePrince 2004, Goyal et al. 2003, Hincha and Thalhammer 2012, Hundertmark et al. 2012, Soulages et al. 2002, Wolkers et al. 2001). Intracellular glass formation is vital to the survival of larvae in at least one animal, the chironomid, *Polypedilum vanderplanki* (Sakurai et al. 2008). If group 1 LEA proteins are required for *A. franciscana* glass formation, absence of the proteins is potentially fatal, accounting for minimal hatching when group 1 LEA proteins are not present.

Cysts from females injected with *GFP* dsRNA showed lower hatching (16-38%) compared to cysts in previous studies (50-60%, King and MacRae 2012, King et al. 2013) where diapause was terminated by desiccation and freezing. While similar methods were employed, including removal of surface water and desiccation at room temperature for 4 weeks, King (personal communication, 2013) often incubated cysts at -20°C for longer than 8 weeks. It is unclear why longer freezing periods increase hatching, because how this treatment terminates diapause is unknown. However, higher hatching after longer freezing periods has been observed with *A. franciscana* cysts other than those used in

these experiments (Appendix A, Fig. A2), and this trait may have evolved to improve survival of overwintering GSL cysts. Salt Lake City, Utah, experiences temperature below 0°C from November to February (National Oceanic and Atmospheric Administration, 2011). Cysts remaining dormant for at least the four months when below-freezing temperatures are commonly observed will have higher survival than those that break diapause and hatch during a mid-winter thaw.

4.3.3 Hatching of Cysts Is High Following Prolonged Periods of Freezing

Desiccation and subzero temperatures both pose significant stress to *A. franciscana* cysts, and when they lack molecular chaperones such as p26 (King and MacRae 2012) or the group 1 LEA proteins, many cysts fail to survive. Freezing may be the milder of the two stresses, as cysts that were only frozen showed higher hatching than cysts that were desiccated prior to freezing regardless of the presence or absence of group 1 LEA proteins. Usually if cysts are hydrated, their stress tolerance is reduced in comparison to dried cysts (Clegg and Trotman 2002, Crowe et al. 1981), and freezing hydrated cysts is expected to be more detrimental than freezing desiccated cysts. However, slow freezing (-0.5°C/min) of hydrated *Artemia* cysts results in high hatching (Yoshida et al. 2011). Freezing rate was not measured in this study, but cysts were placed in Styrofoam containers to slow the rate of freezing when transferred from room temperature to -20°C, which may have contributed to survival.

Conversely, high survival in cysts that were only frozen may be related to the overwintering strategy mentioned above. Cysts that were desiccated and then frozen spent 8 weeks at -20°C, while cysts that were only frozen were incubated at -20°C for 12 weeks. As evident in Fig. A2 (Appendix A), this difference in 4 weeks may explain some

of the variation in survival. Increased hatching after longer freezing periods may have evolved because cysts that break diapause before winter is over are less likely to survive.

4.4 Group 1 LEA Proteins Do Not Influence the Response of *A. franciscana* to H_2O_2

4.4.1 Group 1 LEA Proteins Are Not Required for Diapause Termination

Intrinsically unstructured proteins often function in cell signalling (Tompa 2002, Tompa et al. 2005), and knockdown of the molecular chaperone p26 potentially alters signalling in diapause termination (King and MacRae 2012). Therefore cysts with and without group 1 LEA proteins were examined for differences in their ability to respond to a relatively benign diapause termination cue, H_2O_2 (Robbins et al. 2010, Van Stappen et al. 1998). In the present study, there was no significant difference in diapause termination due to H_2O_2 exposure of cysts with or without group 1 LEA proteins, as demonstrated by similar viability in both conditions. This suggests that group 1 LEA proteins are not involved in signalling pathways that prolong diapause maintenance in cysts nor begin diapause termination in response to external H_2O_2.

H_2O_2 is unlikely to enter the cyst when in diapause because the shell is impermeable to non-volatile compounds (de Chaffoy et al. 1978). However, external H_2O_2 causes changes in shell-bound iron that subsequently increase nitric oxide synthesis within cysts of *A. parthenogenetica* (Veeramani and Baskaralingam, 2011). That is, H_2O_2 stimulates internal changes in encysted *Artemia* embryos, leading to diapause termination through an unknown mechanism. The results of the present study support that H_2O_2 does not penetrate the cyst shell. Cysts were either washed 2 or 5 times following H_2O_2 exposure, the latter of which resulted in lower levels of residual ROS in the sea water, as supported by Fig. A3 (Appendix A). Despite different environmental H_2O_2

concentrations, statistically similar proportions of cysts washed either twice or 5 times terminated diapause, a process that occurs while the cyst shell is intact (MacRae 2005).

4.4.2 Group 1 LEA Proteins Do Not Protect A. franciscana Against Oxidative Stress

Once diapause termination has occurred and environmental conditions are adequate, embryos resume development and generate an osmotic potential that rapidly and completely breaks all membranes and components of the cyst shell (Trotman et al. 1989). Once the cyst has cracked, the embryo is no longer physically protected from small molecules in its environment. For example, failure to complete emergence has been observed when *Artemia* cysts are hatched in medium with either heavy metals such as mercury, zinc, and cadmium, or potassium cyanide (KCN), an inhibitor of cellular respiration (Bagshaw et al. 1986, Go et al. 1990, Pandey and MacRae 1991, Rafiee et al. 1986, Trotman et al. 1980). These molecules typically didn't enter the cyst, but likely damaged the embryo once the cyst shell cracked (de Chaffoy et al. 1978, Go et al. 1990). In some cases the prenauplii arising from cysts treated with KCN emerge backwards; the posterior end of the prenauplius protruding while the anterior end of the prenauplius is trapped within the shell (Trotman et al. 1980).

In the present study, cysts were negatively impacted by environmental H_2O_2 following diapause termination. When washed only twice with sea water after H_2O_2 treatment, a high proportion (20-25%) of cysts exhibited incomplete or abnormal hatching. Cysts washed 5 times with sea water after H_2O_2 exposure typically completed hatching, while a small proportion (6-12%) exhibited incomplete or abnormal hatching. H_2O_2 remaining in sea water was not measured following the washes, but there was a clear inverse relationship between the number of washes and the proportion of abnormal

hatching in commercially-obtained quiescent cysts treated with H_2O_2 and washed between 2 and 5 times (Appendix A, Fig. A3), implicating that residual H_2O_2 impeded post-diapause development when cysts were not washed thoroughly. Robbins and colleagues (2010) observed that concentrations of H_2O_2 as low as 0.003% (w/v, 1060 μM) inhibited or delayed emergence and hatching. Environmental H_2O_2 may inhibit complete hatching by entering cracked cysts and disrupting cellular respiration machinery such as mitochondrial proteins, or transport proteins involved in generating osmotic potential (Trotman et al. 1980, 1989).

While residual H_2O_2 impeded post-diapause development, this affect was not moderated by group 1 LEA proteins. When washed twice, the proportion of incomplete hatching was similar regardless of the presence of LEA proteins. When cysts were washed 5 times and less residual H_2O_2 was present, emergence was not disrupted as severely, but there was no difference between cysts with or without group 1 LEA proteins. Several group 3 LEA proteins are thought to protect cells from oxidative stress due to ROS (Cuming 1999, Mowla et al. 2006). Under the conditions tested, group 1 LEA proteins in *A. franciscana* cysts did not protect against damage from H_2O_2 during post-diapause development. It would be valuable to test a greater range of H_2O_2 concentrations on cysts to determine if LEA proteins confer oxidative stress tolerance at other levels of this ROS.

4.5 Conclusions

Group 1 LEA proteins are part of a suite of molecular defenses known to protect *A. franciscana* diapause cysts during stress. Using RNAi to knock down all or most of the group 1 LEA proteins had a profound effect on tolerance of both desiccation and

freezing, but not oxidative stress under the conditions tested. Protection from freezing is not typically associated with group 1 LEA proteins, suggesting their function is more broad than previously anticipated. As group 1 LEA protein absence is most detrimental when cysts are desiccated, it is likely that these proteins offer the strongest protection in low water environments, perhaps contributing to intracellular glass formation, although prevention of protein aggregation is also likely.

It is of interest to examine the effect of group 1 LEA protein knockdown on desiccation stress in the absence of freezing. This was not possible with GSL *A. franciscana* because cold is required to break diapause. However, diapause is terminated with desiccation alone in San Francisco Bay *A. franciscana*, providing an appropriate model for this study. Knowing whether group 1 LEA proteins protect against heat shock in *A. franciscana* would contribute to our understanding of protein function, because heat stress, much like desiccation, causes protein denaturation and aggregation. Future studies should examine if the functions of group 1 and group 3 LEA proteins overlap in diapause cysts, or whether they are distinct.

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APPENDIX A SUPPLEMENTAL DATA

Table A1 Group 3 LEA proteins in *A. franciscana*

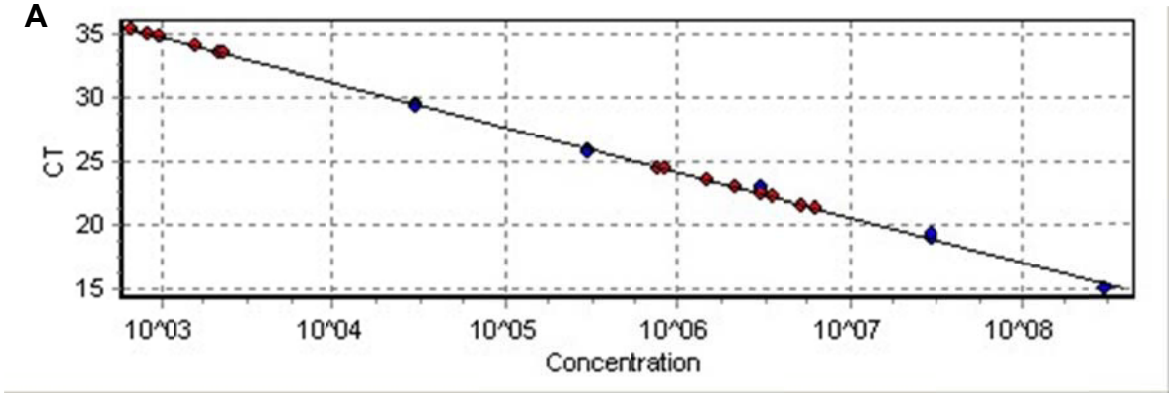
LEA Protein[a]	NCBI[b]	Size/Mass	Expression/Function	Ref.[c]
AfrLEA3-1 Mit	ACA47267	357 aa, 38 kDa	↑ in diapause (mRNA, protein)	[1,2]
AfrLEA3-2, Mit	ACA47268	364 aa, 38 kDa	↑ in diapause (mRNA, protein)	[1,2]
AfrLEA3-3	ES492663	<227 aa, 26 kDa	↑ in diapause (mRNA, protein)	[2,3,4]
AfrLEA3-4, Cyt, mit	ACM16586	307 aa, 36 kDa	↑ in diapause ↑mitochondrial function in water stress (freezing)	[5,2]
AfrLEA3-5	AAA85367, ES504155	11 kDa	↑ in diapause (mRNA)	[6]
Cyt, mit	-	125 kDa	↑ in diapause (protein)	[2]
Cyt, mit, nuc	-	98 kDa	↑ in diapause (protein)	[2]
Mit, nuc	-	89 kDa	↑ in diapause (protein)	[2]
Mit, nuc	-	80 kDa	↑ in diapause (protein)	[2]
	-	73 kDa	↑ in diapause (protein)	[2]
Yolk	-	66 kDa	↑ in diapause (protein)	[2]
	-	54 kDa	↑ in diapause (protein)	[2]
Cyt, mit	-	50 kDa	↑ in diapause (protein)	[2]
	-	20 kDa	↑ in diapause (protein)	[2]

[a] Nomenclature based on Wu et al. 2011. Cellular location based on Warner et al. 2012, cyt, cytosol; mit, mitochondria; nuc, nucleus; yolk, yolk platelets. ↑ = increase.

[b] NCBI accession number of the proteins and the cDNA sequence accession numbers

[c] References: [1] Hand et al. 2007, [2] Warner et al. 2012 ,[3] Wang et al. 2007, [4] Chen et al. 2009, [5] Menze et al. 2009, [6] Wu et al. 2011

Figure A1 Standard curves for quantifying copy numbers of group 1 LEA protein and α-tubulin transcripts. qPCR was performed in duplicate on templates of known concentration (copy number per µl) with SYBR® Green to generate standard curves of Ct (cycle threshold) values for *AfrLEA1* (**A**, R^2 =0.9963) and *α-tubulin* (**B**, R^2 =0.99615) as described in Materials and Methods.


A
35
30
25
20
15
CT
10^03
10^04
10^05
10^06
10^07
10^08
Concentration

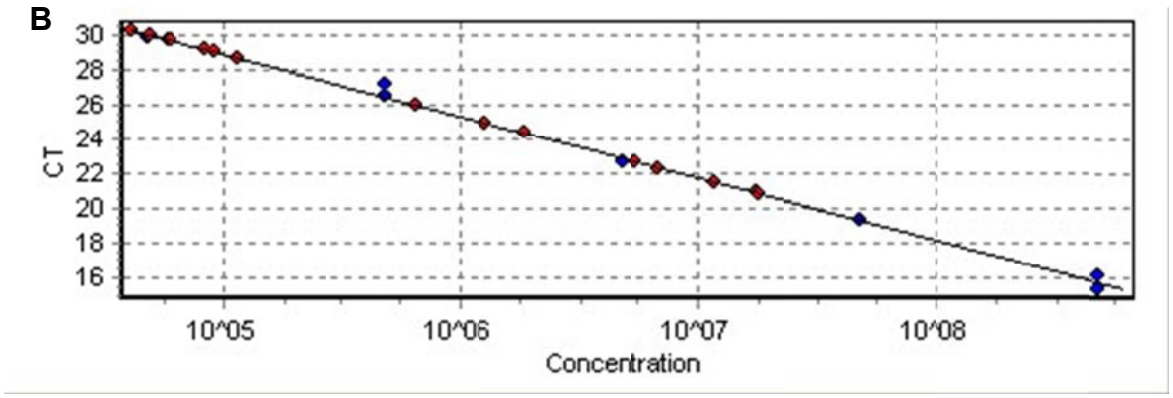

B
30
28
26
24
22
20
18
16
CT
10^05
10^06
10^07
10^08
Concentration

Figure A2 Cyst hatching improves as length of incubation at -20°C increases.

Hatching (%) of cysts released from uninjected females that were incubated in sea water at room temperature for 10 days prior to removal of surface sea water and incubation at -20°C for between 6 and 12 weeks. Cysts were returned to sea water at room temperature to enumerate hatching until 5 days after the last hatching was observed. Each treatment included 65 cysts.

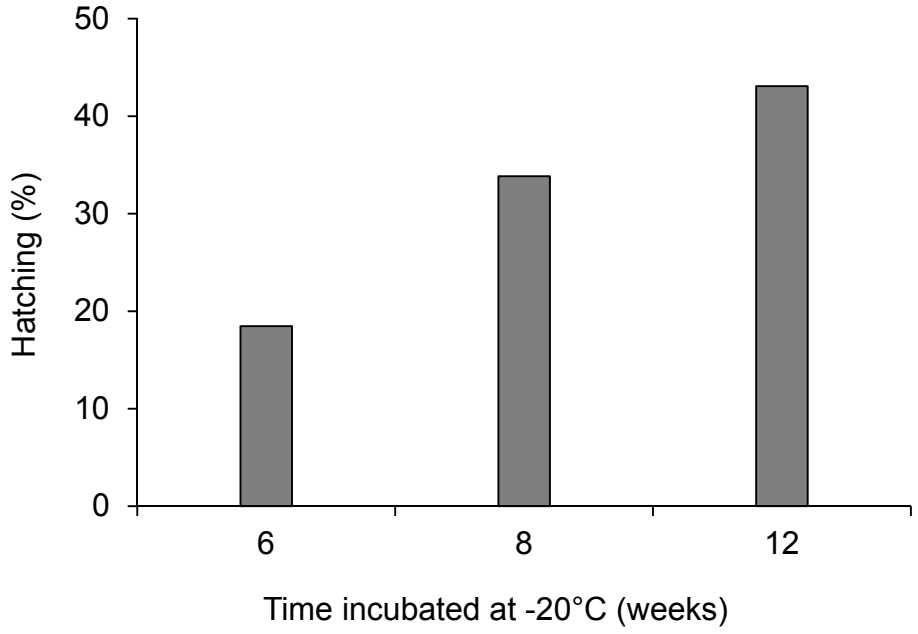
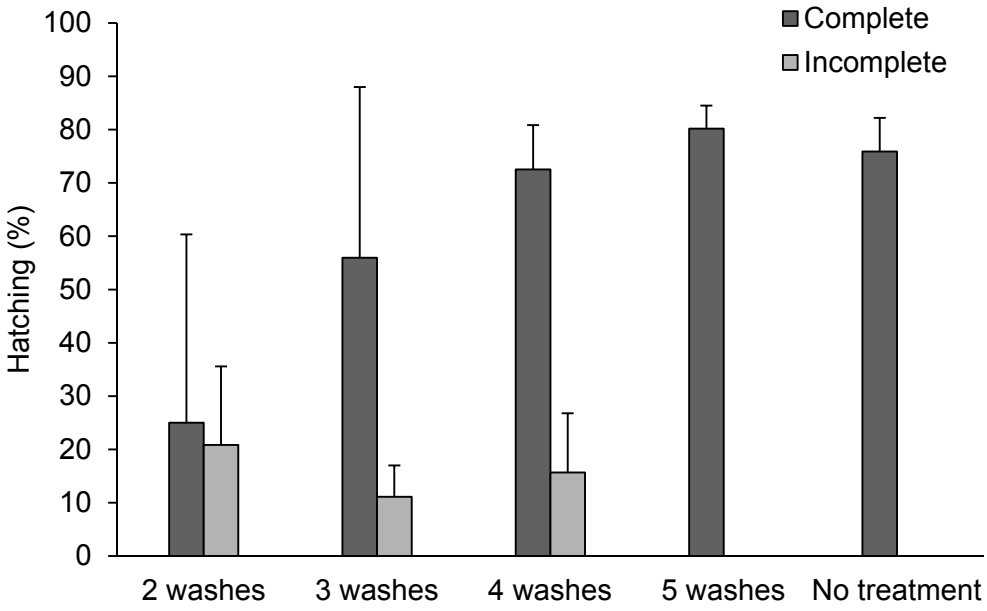

50
40
30
20
10
0
Hatching (%)
6
8
12
Time incubated at -20°C (weeks)

Figure A3 Thorough washing of cysts prior to H_2O_2 exposure reduces incomplete/abnormal hatching. Hatching (%) of commercially obtained cysts following rehydration in sea water at 4°C overnight, incubation in 3% H_2O_2 for 20 minutes at room temperature, filtration and between 2 and 5 washes with sea water at room temperature. The "No treatment" cysts were transferred to sea water without exposure to H_2O_2. The proportions of cysts that hatched fully (Complete) and those that only partially emerged (Incomplete) were documented until 5 days after the last complete hatching was observed. Each treatment included between 50 and 100 cysts. The experiment was performed in duplicate. Error bars represent standard deviation.


Complete
Incomplete
Hatching (%)
100
90
80
70
60
50
40
30
20
10
0
2 washes
3 washes
4 washes
5 washes
No treatment

APPENDIX B MICROPIPETTE PREPARATION

Setting for pulling micropipettes used in microinjection of *A. francsicana* females.

Heat:	560
Pull:	150
Velocity:	100
Time:	150
Pressure:	300

APPENDIX C SOLUTIONS AND RECIPES

Arranged by the order in which they appear in the Materials and Methods.

Solutions for Agarose Gel Electrophoresis

5 X TBE (diluted 10-fold for electrophoresis)

54 g	Tris
27.5 g	boric acid
20 ml	0.5 M EDTA, pH 8.0
dH_2O to 1 litre	

Solutions for SDS-Polyacrylamide Gel Electrophoresis

1A: acylamide/bis-acrylamide, 37.5:1 in dH_2O

1B: 1.5 M Tris (pH 8.8) with 0.5% (w/v) SDS

18.3 g	Tris
2.5 ml	20% (w/v) SDS
pH 8.8	
dH_2O to 100 ml	

1C: 0.2% (v/v) TEMED

100 µl	TEMED
dH_2O to 50 ml	

1D: 5% (w/v) ammonium persulfate

50 mg	ammonium persulfate
dH_2O to 10 ml	

2B: 0.5 M Tris (pH 6.8) with 0.5% (w/v) SDS

6.0 g	Tris
2.5 ml	20% (w/v) SDS
pH 6.8	
dH_2O to 100 ml	

2C: 2% (v/v) TEMED

100 µl	TEMED
dH_2O to 5 ml	

12.5% SDS Polyacrylamide Running Gel

1A (acrylamide/bis-acrylamide, 37.5:1 in deionized H_2O) 5.0 ml, 1B 4.0 ml, 1C 2.0 ml, dH_2O 3.0 ml, 1D 2.0 ml

SDS Polyacrylamide Stacking Gel

1A 1.0 ml, 2B 2.5 ml, 2C 1.25 ml, dH_2O 4.0 ml, 1D 1.25 ml

4 X Treatment Buffer (diluted four-fold for electrophoresis): 250 mM Tris, 280 mM SDS, 40% (v/v) glycerol, 20% (v/v) β-mercaptoethanol,0.2% (w/v) bromophenol blue, pH 6.8

1.2 g	Tris
3.2 g	SDS
16 ml	glycerol
8 ml	β-mercaptoethanol
0.08 g	bromophenol blue
pH 6.8	
dH_2O to 40 ml	

Running Buffer: 25 mM Tris, 200 mM glycine with 0.04% (w/v) SDS

12.0 g	Tris
57.6 g	glycine
8.0 ml	20% (w/v) SDS
dH_2O to 4 L	

Solutions for Western Blotting

Transfer Buffer: 25 mM Tris, 200 mM glycine in 20% (v/v) methanol)

800 mL	methanol
12.0 g	Tris
57.6 g	glycine
dH_2O to 4 L	

TBS: 10 mM Tris, 140 mM NaCl, pH 7.4

1.21 g	Tris
8.18 g	NaCl
pH 7.4	
dH_2O to 1 L	

TBS-T: 10 mM Tris, 140 mM NaCl and 0.1% Tween-20, pH 7.4

1.21 g	Tris
8.18 g	NaCl
1 mL	Tween-20
pH 7.4	
dH_2O to 1 L	

HST: 10 mM Tris, 1 M NaCl, 0.5% Tween-20, pH 7.4

1.21 g	Tris
58.4 g	NaCl
5 ml	Tween-20
pH 7.4	
dH_2O to 1 L	