*p*26 KNOCKDOWN MODIFIES THE DIAPAUSE PROTEOME OF *ARTEMIA FRANCISCANA*

by

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This thesis is dedicated to my mother, Salma, for her unmeasurable support and prayers to pursue my study and for my husband, Salem and for my two kids, Hasan and Ahmed for being patient with me and giving me the courage to get my degree despite all obstacles.

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ABSTRACT

The brine shrimp, *Artemia franciscana*, produces diapausing embryos (cysts), in which biological processes are arrested. Cysts are an excellent model for studying the regulation of diapause because they remain in this state for prolonged periods showing remarkable resistant to harsh environmental conditions and they terminate diapause in response to specific stimuli. p26 is the most abundant diapause protein in the cyst proteome, playing a critical role in embryo development, diapause maintenance, and cyst stress tolerance, the latter presumably by preventing irreversible protein denaturation during stress. p26 is therefore likely to influence many proteins in the cyst diapause proteome. However, the identity of p26 substrates remained largely unknown as did the effect of p26 on protein synthesis. This study represents the first global characterization of the *A. franciscana* diapause proteome, identifies putative p26 substrates and shows that p26 regulates the synthesis of specific proteins. Advantage was taken of modern proteomic techniques, including 2D-LC MS/MS to analyze and quantify the proteome of the cyst, and RNAi was used to evaluate the affects of knocking down *p26* on the diapause proteome. 3212 proteins were detected by MS/MS. Those proteins that varied significantly upon *p26* knockdown either have the potential to be p26 substrates or possibly have their synthesis affected by p26.The functional categorization of the proteins was considered using the PANTHER classification system yielding catalysts, binding proteins, structural proteins, transporters, translation factors, receptors, antioxidants, signal transducers, and a channel regulator. This proteomics approach has added valuable information to the global picture of the diapause proteome, making a contribution to understanding p26 influence on diapause and the molecular mechanisms of diapause in *A. franciscana*.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ACN	acetonitrile
NH_4HCO_2	ammonium formate
bp	base pairs
BLAST	basic local alignment search tool
cDNA	complementary DNA
DTT	dithiothreitol
D_2O	heavy water (deuterium oxide)
2D-HPLC	two dimensional high performance liquid chromatography
DPBS	Dulbecco's phosphate buffered saline
dsRNA	double-stranded RNA (ribonucleic acid)
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ExPASy	expert protein analysis system
FA	formic acid
GFP	green fluorescent protein
GO	gene ontology
GSL	Great Salt Lake
HRP	horse radish peroxidase
HSP	Heat shock protein
HST	high salt/Tween
IAcNH2	iodoacetamide
IgG	immunoglobulin G
IDT	integrated DNA technologies
kDa	kiloDaltons

LC-MS/MS	liquid chromatography tandem mass spectrometry
mRNA	messenger RNA (ribonucleic acid)
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NCBI	national center for biotechnology information
ORCAE	online resource for community annotation of eukaryotes
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAi	RNA interference
SDS	sodium dodecylsulfate
$NaCNBH_3$	sodium cyanoborohydride
sHsp	small heat shock protein
Taq	thermus aquaticus
TBE	tris/borate/ethylenediaminetetraacetic acid
TBS	tris-buffered saline
TBS-T	tris-buffered saline tween
TE	tris, EDTA
TEAB	triethylammonium bicarbonate buffer
TEMED	tetramethylethylenediamine
TFA	trifluoroacetic acid
Tris	tris-(hydroxymethyl)aminomethane
Tween	polyoxyethylene sorbitan monolaurate
UniProt	universal protein resource
UniProtKB	uniProt knowledgebase
UV	ultraviolet

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CHAPTER 1: INTRODUCTION

1.1 Stress tolerance

1.1.1 Diapause

Diapause, a widespread state of dormancy where development stalls, cell division ceases, and morphological changes occur, is divided into several phases including initiation, maintenance and termination (Koštál, 2006; MacRae, 2010). Diapause initiation occurs in response to external signals such as photoperiod and temperature, or independently of external signals as an obligatory part of an organism's life history (Podrabsky & Hand, 1999; MacRae, 2010; Clark *et al*., 2012). During the maintenance phase of diapause many organisms remain in metabolic depression with metabolism virtually turned off, even upon exposure to environmental conditions that normally support growth and metabolism, yet many of these organisms show remarkable stress tolerance (Tauber *et al*., 1986; Koštál, 2006; Podrabsky & Hand, 2015). Diapause improves the survival of organisms under harsh circumstances. The requirements for organisms to survive during diapause include suppression of energy consumption as required for synthesis of macromolecules and the operation of transmembrane transport, as well as the suppression of protein degradation and aggregation, which results in the extension of protein half-life (Clegg, 2007). Protected proteins are required for growth when diapause terminates, which is a signal mediated phase (Clegg, 2007). Understanding diapause mechanisms and how diapause affects the survival of organisms are fundamental issues because they are related to physiological characteristics such as stress tolerance and longevity. This is especially true in habitats that experience environmental extremes where diapause is an essential feature in the life history of resident organisms (Hand, 1998). That is, in order to survive during diapause organisms must have the appropriate

macromolecular composition, an important component being the possession of molecular chaperones which are required for stress tolerance.

1.1.2 Molecular chaperones/small heat shock proteins

Cells have physiological and developmental strategies to survive environmental stress. One of the most remarkable strategies is the involvement of cell proteins called molecular chaperones, also known as stress proteins or heat shock proteins (HSPs), which have crucial roles in folding, storing, and rescuing other proteins during stress. With the exception of the small heat shock proteins (sHSPs), molecular chaperones require ATP in order to function. Molecular chaperones are classified according to molecular mass, sequence and function, yielding six major families termed HSP100, HSP90, HSP70, HSP60, HSP40, and the sHSPs (Morimoto & Santoro, 1998). These chaperones often function in networks interacting with one another and with substrate proteins. Molecular chaperone synthesis differs from one species to another and within the same species under different environmental, physiological, and developmental states. As an example, under stress the abundance of molecular chaperones often increases (Basha *et al*., 2004; Teigen *et al*., 2015; MacRae, 2016). By comparison, HSP synthesis may increase, decrease or remain stable during diapause (King *et al*., 2013; King & MacRae, 2015). The differential syntheses of molecular chaperones indicate an essential need for these proteins in the maintenance of homeostasis under stress and in surviving hostile environments during diapause.

Under stress conditions sHSPs protect substrate proteins from irreversible denaturation and they enhance protein quality control and cell protection. sHSP monomers consist of a conserved α-crystallin domain and non-conserved amino and

carboxyl-terminal regions and they tend to form oligomers that are important to their chaperone function (Sun & MacRae, 2005; Basha *et al*., 2012 & Haslbeck & Vierling, 2015). sHSP monomers differ in mass, ranging from 12 to 42 kDa, substrate interactions, and roles within cells. The oligomers differ in their overall structure from one species to another (Basha *et al*., 2012; Haslbeck & Vierling, 2015). The synthesis of sHSPs is usually induced by stress and they bind to denaturing substrates thereby preventing their irreversible denaturation and aggregation. To confer protection on proteins sHSPs must be present during the time substrates are unfolding because they cannot refold already unfolded and aggregated substrates (Haslbeck & Vierling, 2015). As an example, upon heat shock treatment *in vivo*, the amount of the sHSP, HSP25 is augmented in concert with a significant increase in the types of bound substrates when compared with the HSP25 substrates obtained under normal physiological conditions. This result demonstrates that under stress there is a greater need for HSP25 to bind specifically with and protect unfolding substrates from irreversible denaturation.

sHSPs act independently of ATP, but substrate proteins are recovered from sHSPs and reactivated by ATP-dependent chaperones, such as HSP70 (Horwitz, 1992; Liang & MacRae, 1999; Narberhaus, 2002; Sun *et al.,* 2006; Basha *et al., 2*012; King & MacRae, 2015; Haslbeck & Vierling, 2015). As an example, under heat shock treatment *in vitro* the murine sHSP, HSP25 functions as a molecular chaperone by forming stable complexes with unfolding proteins (Ehrnsperger *et al., 1*997). HSP25 efficiently traps denaturing citrate synthase in a folding-competent state which protects the enzyme from irreversible aggregation. HSP70 then releases citrate synthase from HSP25 and refolds the protein. Thus, substrate binding to HSP25 creates a reservoir of proteins that may exist for an extended period of time and these

proteins are refolded upon restoration of permissive conditions (Ehrnsperger *et al*., 1997). Molecular chaperones therefore function as networks of complementary proteins which co-operate with each other to promote cellular homeostasis, processes that are influenced by ATP availability.

sHSPs are crucial for proteostasis in normal and stressed cells, an observation supported by their presence in most organisms (Kappé *et al*., 2003) and their dramatic increase under stress conditions, making them among the most abundant of cellular proteins (Malmström, *et al*., 2009). Moreover, sHSPs are implicated in a range of diseases where their elimination enhances the aggregation of proteins (Clark, 2000). A notable case of their importance is that the knockout of *sHSPs* in the lens of mice increases the abundance of aggregated proteins and induces cataract formation. This finding indicates that sHSPs have a protective function in the lens, most likely through the binding of substrate proteins and prevention of their aggregation (Andley *et al.,* 2013). Under stress the sHSPs promote substrate protection in a background of protein synthesis inhibition.

An important consequence of the functions of sHSPs is maintaining cell homeostasis after perturbation through securing and storing protein substrates. However, recent evidence obtained by RNA interference (RNAi) indicates other consequences for sHSPs. An unfortunate result of sHSP action is the promotion of cancer progression, but this also provides clues as to other functions of sHSPs. As an example, HSP27 is involved in cancer progression by promoting resistance to chemotherapy in tumor cells (Kamada *et al*., 2007; Wettstein *et al*., 2013). Knocking down *HSP27* in human cancerous cells eliminates three polypeptides, namely histone deacetylase HDAC6, transcription factor STAT2, and procaspase-3 (Gibert *et al.,* 2012), suggesting that HSP27 regulates the levels of these polypeptides through

substrate-chaperone interactions (Gibert *et al., 2*012). The binding of HSP27 with these putative substrates and other unidentified proteins is thought to promote the survival of cancerous cells and the identification of substrate proteins through work such as this may be crucial in understanding the role of sHSPs in cancer, ultimately leading to therapeutic applications.

Molecular chaperones, including sHSPs, are modified during diapause where the physiological status of an organism changes, metabolism is reduced resulting in a substantial decrease in ATP, and stress tolerance increases (King & MacRae, 2012, 2013; Podrabsky & Hand, 2015). Identifying sHSP substrates during diapause may reveal how sHSPs promote cell survival during metabolic suppression. The differential regulation of sHSP synthesis under diapause indicates the importance of this group of molecular chaperones in adaptation to stress and maintenance of proteostasis. *Artemia franciscana* is a good example of an organism which employs a sHSP to cope with extreme environmental conditions and thereby promote survival during physiological stress.

1.1.3 *Artemia franciscana* and stress tolerance

The brine shrimp, *A. franciscana*, is an aquatic crustacean that occurs worldwide in waters of extreme salinity. During embryogenesis *Artemia* produce either motile nauplii by ovoviviparous development or gastrula stage embryos enclosed in a shell (cysts) by oviparous development (Jackson & Clegg, 1996). *Artemia* cysts enter diapause, remaining in a state of profound metabolic depression and showing extreme stress tolerance for years (Clegg & Jackson, 1998). Diapause is, in itself, a type of stress because ATP is not available in appreciable amounts. In their normal habitats *Artemia* cysts terminate diapause once they receive an external signal

such as desiccation and/or freezing (Robbins *et al*. 2010; Zhou *et al*., 2013). Under laboratory conditions cysts terminate diapause by exposure to H_2O_2 (Van Stappen *et al*., 1998; Robbins *et al*., 2010). Upon diapause termination under favourable conditions, including availability of food, suitable temperature, hydration and aeration, development resumes and cysts hatch to yield nauplii. However, if cysts encounter unfavourable conditions upon termination of diapause, they enter quiescence and remain dormant until environmental conditions permit growth. The remarkable resistance to harsh environmental and physiological stressors persists as long as diapause and quiescence continue, disappearing from both types of dormant embryos after development resumes (MacRae, 2016). Because *Artemia* embryos survive diapause and even depend on diapause for survival, cells must be protected.

The cellular components of diapause and quiescent *Artemia* embryos are unusually stable, indicating that stress tolerance involves robust adaptive mechanisms that either prevent or repair damage and promote stress tolerance. The following adaptive mechanisms possessed by cysts are thought to be responsible for high stress tolerance of *Artemia*, one of the most stress-resistant metazoans known (MacRae, 2016). Cysts are surrounded by a rigid, semi-permeable cell wall resistant to chemical compounds and which shields the embryo from ultraviolet radiation (MacRae, 2016). In addition, cysts possess high amounts of a sugar called trehalose, an energy source and a shield against desiccation and freezing (Clegg & Jackson, 1998). Equally important, cysts have late embryogenesis abundant (LEA) proteins thought to protect against desiccation (Hand *et al*., 2011; Toxopeus *et al*., 2014). Moreover, cysts exhibit high amounts of molecular chaperones including sHSPs that are thought to be crucial elements in their remarkable stress tolerance (Liang & MacRae, 1999; Clegg *et al*., 1999, 2000; MacRae, 2003; King & MacRae, 2012; MacRae, 2016). Diapause

and quiescent cysts of *A. franciscana* contain at least three sHSPs called p26, ArHsp21, and ArHsp22 which are produced during the development of diapause-destined embryos (King *et al*., 2013). All of these sHSPs have similar chaperone activity *in vitro*, and they are developmentally regulated, appearing in only those embryos that are diapause-destined (Qiu *et al*., 2007; Qiu & MacRae, 2008; MacRae, 2016). Of these sHSPs, p26, as well as other ATP-dependent molecular chaperones, are thought to promote stress tolerance by preventing irreversible denaturation of substrate proteins needed for the resumption of development following diapause termination and access to conditions favorable for growth (Qiu *et al., 2*007; Qiu & MacRae, 2008; King & MacRae, 2012). p26 in *A. franciscana* embryos extends its function beyond a role in stress tolerance, which makes it a novel member of sHSPs (King & MacRae, 2012).

1.2 *A. franciscana* and p26

1.2.1 p26 synthesis and localization

p26 was first reported in encysted *Artemia* embryos by Clegg and colleagues (1994). The synthesis of p26 is developmentally regulated but it is not stress inducible (Jackson & Clegg, 1996; Liang & MacRae, 1999; Qui & MacRae, 2008). p26 mRNA appears at 2-days post-fertilization in diapause-destined embryos, whereas p26 is first observed at 3-days post-fertilization (Clegg *et al*., 1994; Liang & MacRae, 1999). p26 mRNA and protein disappear when development resumes post-diapause, although residual p26 is observed in emerged nauplii (Clegg *et al*., 1994; Liang *et al*., 1997, 1999), p26 is not detected in embryos that are developing directly into nauplii (Liang & MacRae, 1999; King & MacRae, 2012). These findings demonstrate that p26 is a diapause-specific protein.

p26 translocates from the cytoplasm into the nucleus upon exposure of cysts to stress, suggesting that it is a multifunctional protein. Under normal conditions and when entering diapause, p26 is located in the cytoplasm, but upon exposure to stresses such as anoxia, heat shock, low pH, or diapause p26 migrates into nuclei (Clegg *et al*., 1994; Liang & MacRae, 1997; Willsie & Clegg, 2001; Clegg, 2007; Basha *et al*., 2012). This finding indicates an association of p26 with specific nuclear components under stressful conditions, perhaps including protein assemblies required for DNA replication and other molecular activities (Liang & MacRae 1999). Lamins, which are nuclear matrix proteins, are very sensitive to environmental stress (Roti *et al*., 1998) and p26 may move into nuclei to protect these proteins and assist in maintaining protein homeostasis under stress (Willsie & Clegg, 2001, 2002). During stress, p26 and HSP70 associate with one another in the nuclei and potentially stabilize nuclear matrix proteins, thereby protecting the nuclear matrix from the consequences of protein unfolding and aggregation (Willsie & Clegg, 2002). Although p26 may have important protective functions in nuclei, most sHSPs, including p26, are cytoprotective chaperones that ameliorate cell stress by preventing the irreversible denaturation and aggregation of substrates that reside in the cytoplasm. p26 substrates are, for the most part, unknown making their identification of importance in understanding how this protein functions in diapause and in understanding how sHSPs affect proteostasis.

1.2.2 p26 function

p26 is the most abundant sHSP in diapause-destined *A. franciscana* embryos, representing approximately 7% of total soluble protein which suggests that it is an important component of the high stress resistance of cysts (King *et al., 2*013;

MacRae, 2003). *In vitro* studies, whereby p26 prevents the aggregation of citrate synthase, demonstrate the chaperoning activity of p26 purified from *A. franciscana* and transformed *E. coli* (Liang *et al*., 1997; Sun *et al*., 2006; Sun & MacRae, 2005). In the same way, transformed *E. coli* synthesizing p26 are more thermotolerant than bacteria lacking this protein (Liang & MacRae 1999; Sun & MacRae, 2005; Sun *et al*., 2006). Likewise, p26 expressed in transfected mammalian cells, confers thermotolerance and inhibits apoptosis induction upon exposure to stressors such as desiccation, rehydration, oxidation, and heat shock (Villeneuve *et al*., 2006; Wu & MacRae, 2010). These findings have direct relevance to *Artemia* life history characteristics because *Artemia* is frequently exposed to similar stressors in their natural habitat and diapause is terminated by exposure to stresses such as desiccation and cold.

p26 is the best studied sHSP in *A. franciscana* and its functions have been examined by RNAi. These *in vivo* studies confirmed that p26 is required for stress tolerance and that it has other roles in diapausing embryos. For example, diapause-destined embryos where *p26* has been knocked down by RNAi develop more slowly than those with normal amounts of the protein (King & MacRae, 2012). Cysts lacking p26 terminate diapause more readily than p26-containing cysts and exhibit reduced survival upon exposure to stressors such as heat, cold, and desiccation (King & MacRae, 2012). Knocking down *p26* by RNAi reduces the stress tolerance of *A. franciscana* cysts, such that upon termination of diapause by desiccation and freezing, only 6% of cysts lacking p26 survive as opposed to 58% of cysts containing p26. Additionally, upon exposure to heat shock only 30% of cysts lacking p26 survive as opposed to 52% containing p26 (King & MacRae, 2012). These results demonstrate

clearly that p26 contributes to diverse activities including those other than the stress tolerance of encysted *A. franciscana* embryos.

Injection of *p26 dsRNA* into females knocks down *p26* in cysts but does not affect the other two sHSPs known to exist in this organism. That is, the loss of p26 had no effect on either ArHsp21 or ArHsp22 and such reciprocal effects are also lacking when *ArspP21* and *ArHsp22* are knocked down (King & MacRae, 2012). Thus, even though all three diapause-specific sHSPs in *A. franciscana* cysts are similar to one another as previously described, it appears that ArHsp21 and ArHsp22 do not assist p26 in boosting stress tolerance, nor do they affect embryo development or diapause maintenance (King & MacRae, 2012). These differing results demonstrate that among the three known *A. franciscana* sHSPs, p26 has the most important effect in embryo development, diapause maintenance, and stress tolerance. To engage in these functions, and especially in stress tolerance, p26 presumably binds to diverse substrate proteins. It will be highly informative to identify p26 substrates because they may be involved in essential physiological activities of diapausing *Artemia* cysts.

1.3 The diapause proteome in *A. franciscana* embryos

No previous attempt has been made to characterize the diapause proteome in *A. franciscana* cysts, although studies using proteomic approaches in other organisms under various stress conditions identified a significant number of sHSP substrates (Basha *et al*., 2004). To identify putative p26 substrates, a combination of RNAi and mass spectrometry (MS) was used. The experimental approach was to knock down *p26* by RNAi and then use MS-based proteomics in concert with differential isotopic labeling to compare cell free protein extracts from diapause cysts containing and lacking p26. The premise was that proteins which disappeared or were reduced in

amount upon *p26* knock down are most likely p26 substrates, whereas it is hypothesized that those that appeared or increased in amount normally have their synthesis suppressed in diapausing embryos by p26. Other scenarios could also be argued which will be mentioned later in the Discussion section.

Two-dimensional liquid chromatography tandem mass spectrometry (2D-LC MS/MS) provides high sensitivity and accuracy in protein identification. This approach has the analytical capacity to identify and quantify not only commonly found proteins, but also those proteins that have high molecular masses, extreme isoelectric points, very low-abundance and membrane associations and which cannot be easily identified using other techniques (Fournier *et al*., 2007; Slebos *et al*., 2008). RNAi is an effective research tool for the knock down of proteins for investigation of their function *in vivo* (Siomi & Siomi, 2009). Therefore, the use of RNAi is potentially an effective method to identify and quantify proteins that are influenced by p26, particularly when combined with 2D-LC MS/MS profiling.

Comparative proteomics is a powerful approach to obtain a global view of changes in protein profiles and identify functionally adaptive variation (Tomanek, 2014). To this end, the protein profiles of cysts with and without p26 were compared in this study to identify any differences in protein composition that occur when the highly abundant and a diapause specific protein, *p26*, is knocked down. Proteins down-regulated in the absence of p26 may contribute to some of the differences seen between cysts containing and lacking p26 such as reduced stress tolerance, the slowing of embryo development and facilitated diapause termination. Possibly there is a critical need for complex formation between p26 and its substrates, so that *A. franciscana* cysts survive diapause. On the other hand, should a protein increase in *p26* knock down cysts, then p26 may normally suppress the synthesis of this up-

regulated protein and its synthesis may also affect cyst development and stress tolerance of diapausing cysts upon the loss of p26. Consequently, a major objective of this study is to identify proteins modified by the elimination of p26 potentially leading to determination of their roles during diapause in processes such as the stress tolerance of *A. franciscana* cysts. This research may therefore extend our knowledge of how sHSPs function during diapause and give a better understanding of how cells cope with stress.

CHAPTER 2: MATERIALS AND METHODS

2.1 Culture of *A. franciscana*

A. franciscana cysts, purchased from INVE Aquaculture, Inc., Ogden, UT, USA, were hydrated at 4°C for 3 h in distilled water after which they were incubated at room temperature in aerated, filtered, autoclaved seawater from Halifax Harbor, hereafter called sea water. Beginning 2-days after the initiation of incubation animals were fed every 1-2 days with the microalgae *Isochrysis galbana* from The Provasoli-Guillard National Center for Culture of Marine Phytoplankton, West Boothbay Harbor, Maine, USA. Adult females, identified by the presence of ovisacs visible under a Wild M3 dissecting microscope (Wild Leitz Canada Ltd., Ottawa, Canada) were separated from males, identified by the presence of graspers, to avoid fertilization. Unfertilized females possessing a shell gland between their lateral pouches, that is those females containing oocytes that will undergo oviparous development, were maintained in separate wells of 6-well plates and used for injection of *dsRNA* and cyst production (Fig. 1).

2.2 Knockdown of *p26* in *A. franciscana* cysts

2.2.1 Preparation of *p26* and *GFP* cDNA

Plasmid DNA was obtained from *Escherichia coli* DH5α transformed with the recombinant expression vector pRSET C (Invitrogen, Burlington, Ont., Canada) containing *p26* cDNA (Liang *et al*., 1999) by using The GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, Oakville, Ont., Canada) and following the manufacturer's instructions. Plasmid DNA was harvested from *E. coli* grown overnight at 37°C in 1.0 ml LB Broth (Bio Shop Canada Inc., Burlington, ON). The plasmid DNA was used as template for amplification of *p26* cDNA using previously

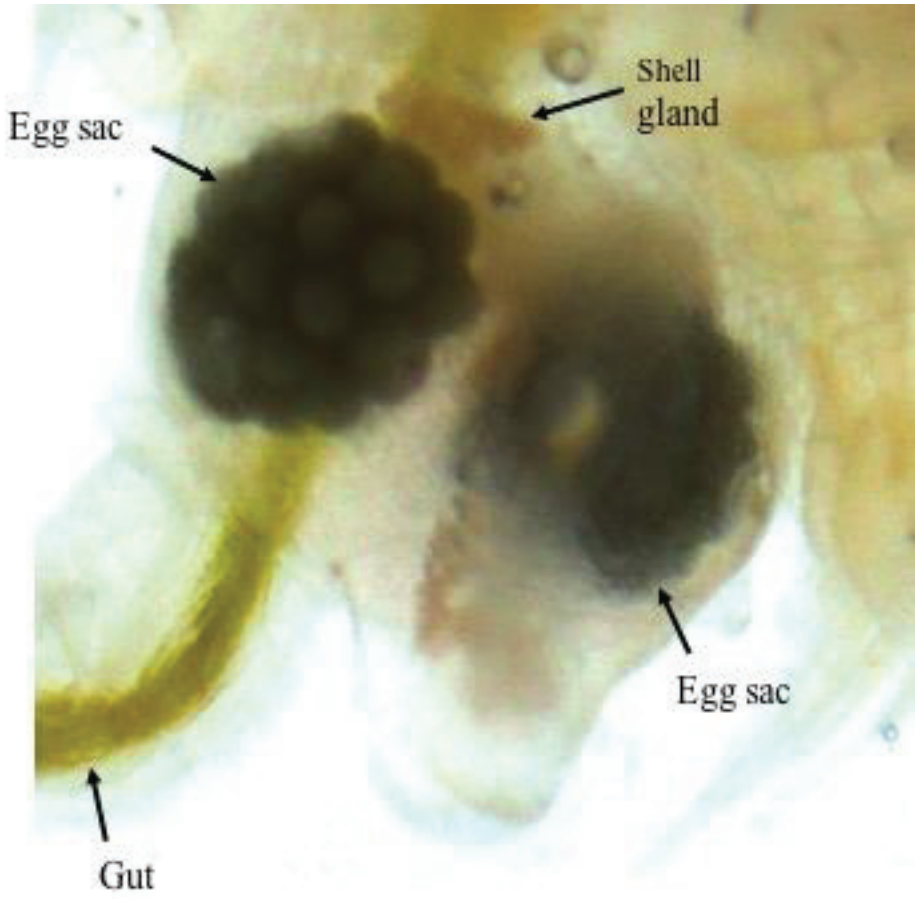

Figure 1. Ovisac of an *A. franciscana* female producing cysts. Stereomicroscopic image showing the ovisac of an unfertilized female of the type used for injection of *p26* and *GFP* dsRNA, with two distinct lateral egg sacs and a shell gland.

designed primers (King & MacRae, 2012) (Table 1). As control for RNAi experiments, green fluorescent protein (*GFP*) cDNA in the commercial expression vector pEGFP-N1 (Clontech, Mountain View, CA, USA) was used as template for amplification of *GFP* cDNA using previously designed primers (Zhao *et al*., 2012) . (Table 1). The T7 promoter sequence (TAATACGACTCACTATAGGGA) was added to the 5' end of all p26 and GFP primers. PCR was carried out with 0.2 mM Platinum® Taq DNA polymerase (Invitrogen) in accordance with the manufacturer's instructions and under the following conditions: initial denaturation for 5 min at 94°C followed by 35 cycles at 94°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 1 min, then a final incubation at 72°C for 7 min. The PCR products and GeneRuler 100 bp DNA Ladder (Invitrogen) were resolved by electrophoresis in 1.2% agarose gels in 0.5 x TBE buffer (Appendix A) at 90-95 V, and stained with SYBR Safe ® (Invitrogen). Stained gels were visualized in a DNR Bio-imagining Systems MF-Chem/BIS 3.2 Gel Documentation System (Montreal Biotech, Montreal, Quebec, Canada) to ensure that amplification products were of the appropriate size.

2.2.2 Synthesis of *p26* and *GFP* dsRNAs

dsRNAs specific to *p26* and *GFP* were synthesized using the PCR products described above as templates (King & MacRae, 2012; King *et al., 2*013) in a 3 h incubation with the MEGAscript® RNAi Kit (Ambion Applied Biosystems, Austin, TX, USA) in accordance with the manufacturer's instructions. The *p26* and *GFP* dsRNAs were resolved by electrophoresis in 1.2% agarose gels in 0.5 x TBE buffer at 90-95 V, and stained with SYBR Safe® GeneRule (Invitrogen). GeneRuler 100 bp DNA Ladder (Invitrogen) was used as size marker. Stained gels were visualized in a DNR Bio-imagining Systems MF-ChemiBIS 3.2 Gel Documentation system (Montreal Biotech) to ensure that DNA fragments of the correct size were obtained.

Table 1. Primers used for the production of *p26* and *GFP* cDNA and dsRNA.

	Primer sequence (5' to 3')	
Protein	Forward	Reverse
*p26**	TAATACGACTCACTATAGGGAGACCACTCCCAGAACATGTCAAACCA	TAATACGACTCACTATAGGGAGACCACTGCACCTCCTGATCTTGTTG
*GFP***	TAATACGACTCACTATAGGGAGACACATGAAGCAGCACGACTT	TAATACGACTCACTATAGGGAGAAGTTCACCTTGATGCCGTTTC

Underlined sequences in the primers indicate the T7 promoter region. All primers were synthesized by Integrated DNA Technologies (IDT), Coralville, IA, USA.

*King & MacRae, 2012

**Zhao *et al*., 2012

dsRNA concentration was determined by measuring absorbance at 260 nm and using a conversion factor of 50.

2.2.3 Microinjection of *A. franciscana* females with *p26* and *GFP* dsRNA

Unfertilized females, which had two separate ovisacs and were producing cysts were identified by the presence of shell glands (Liang& MacRae, 1999) (Fig. 2A). These individuals were injected under an Olympus SZ61 stereomicroscope with a glass needle pulled with a custom programmed P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Co., Novato, CA, USA) and broken with microscissors at 45° (King & MacRae, 2012). The mature females were injected in the egg sac (Fig. 2B) with 80 ng of *dsRNA* for either *p26* or *GFP* in elution buffer from the *dsRNA* kit. Prior to injection, dsRNAs were mixed separately in 1:1 ratio (v/v) with 0.5% phenol red in Dulbecco's phosphate buffered saline (DPBS). Injection was with the Nanoject II Microinjector (Drummond Scientific Co., Broomall, PA, USA) and the final volume injected was 250 nl. Females immobilized on a pre-cooled 3% agar plate and gently blotted with Kim wipes were used for injections.

To obtain enough cysts for SDS polyacrylamide gel electrophoresis (SDS-PAGE), western blotting and mass spectrometry experiments, 68 females were injected with *p26 dsRNA* and 83 with *GFP dsRNA*. After injection, females were incubated in covered six well plates containing seawater and monitored for about 2 h to ensure retention of phenol red, an indication that the *dsRNA* was retained by the animals (Fig. 2C) (King & MacRae, 2012). Females which lost the dye within 2 h and/or showed morphological or behavior abnormalities were discarded (King & MacRae, 2012). Each healthy female was placed with a male for fertilization 24 h post-injection (King & MacRae, 2012) and these females were used for cyst

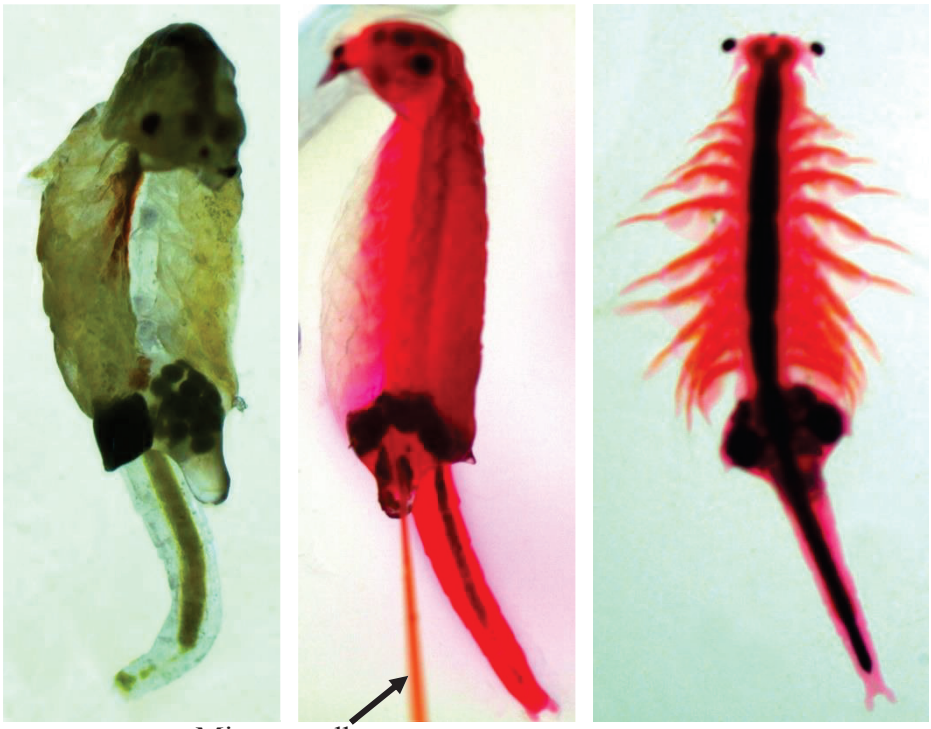

Figure 2. Injection of *A. franciscana* females with dsRNA. Stereomicroscopic images showing the injection of female *A. franciscana*. **A.** Female with shell gland producing cysts prior to fertilization. The female was immobilized on her back in an injection position. **B.** Injection of female in the egg sac with a micro needle containing dsRNA. **C.** Female of normal morphology which has retained phenol red for 2 h post injection.

production until their death. On average females that received *p26 dsRNA* produced two broods whereas those receiving GFP dsRNA produced three broods (Appendix B). Females mixed with males were observed daily under a dissecting microscope to determine the time of fertilization, as marked by the fusion of lateral egg sacs into a single sac, and to monitor embryo release. Cysts were collected from sea water 8-days post-release, washed 3 times with ice cold double distilled water and collected by centrifugation for 30 sec. at 3000 x g using a micro centrifuge in order to remove excess water. Cysts were dried in the fume hood for approximately 1 min and then kept a-80^{o} C until used.

2.3 Protein extraction for SDS-PAGE and western blotting

At least 33 cysts collected from females injected with either *p26* or *GFP dsRNA* were used to prepare protein extracts for each lane of SDS polyacrylamide gels. Cysts were homogenized on ice in the presence of protease inhibitors (Thermo Scientific^{TM} 78430, USA) and 10 µl of loading buffer (Appendix A). Homogenization was with a microfuge pestle (Fisher) in a 1 ml glass tube (Radnoti LLC 440613, USA). The homogenate was placed in a boiling water bath for 5 min and then all tubes were centrifuged for 10 min at 8600 x g in a microcentrifuge at 4°C. Fifteen µl of each protein sample was resolved in 12.5% SDS polyacrylamide gels (Appendix A) using a constant current of 45 mA for approximately 1 h. PiNK Plus Prestained Protein Ladder (Frogga Bio Inc., Toronto, ON, Canada) was used as molecular mass marker. Proteins were blotted to nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA) in transfer buffer (Appendix A) at 100 mA overnight at room temperature. Protein transfer was verified by the staining of membranes for 5 min with 2% Ponceau (Appendix A). After staining, membranes were rinsed three times with distilled water, then washed for 5 min with TBS (Appendix A).

For immunoprobing, membranes were incubated at room temperature for 1 h in 8% (w/v) Carnation low fat milk in TBS (Appendix A), followed by exposure for 15 min to a polyclonal antibody raised in rabbit against p26 and diluted 1:10000 in TBS (Liang & MacRae, 1999). After incubation in primary antibody, membranes were washed three times for 5 min with TBS-Tween (Appendix A), and then three times for 5 min each with HST (Appendix A). Membranes were incubated for 20 min at room temperature with HRP-conjugated goat anti-rabbit IgG (Sigma-Aldrich) diluted 1:10000 in TBS then washed as above three times in TBS-Tween, HST and TBS. The secondary antibody was then detected 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. In order to confirm knockdown of *p26*. The immunoblotting experiments were conducted numerous times throughout the RNAi experiments.

2.4 Sample preparation for mass spectrometric analysis

2.4.1 Protein extraction from *A. franciscana* cysts

To ensure that protein extraction was maximal from knockdown cysts, commercially obtained hydrated cysts were homogenized in increasing amounts of lysis buffer and the protein concentration of the extracts was determined (Appendix C). Subsequently, 2090 and 2226 cysts obtained from females injected respectively with *dsRNA* for either *p26* or *GFP* were used to extract protein for mass spectrometry. Cysts were homogenized on ice in 5 ml capped glass grinders (Radnoti LLC 440614, USA) under a laminar flow hood with denaturing buffer (Appendix A). Supernatants were collected after centrifugation at 3500 x g for 5 min and the protein was quantified using the Bradford Protein Assay according to manufacturer's instructions

(Bio-Rad, Hercules, CA, USA). The protein measurements were performed four times for each sample lacking and containing p26 and the average value of the protein concentration was determined. Twenty-five µg of protein extract from cysts obtained from females injected with *dsRNA* for *p26* and *GFP* were used for SDS-PAGE and western blotting as described previously to confirm the absence and presence of p26 respectively. Two hundred µg of protein sample was processed in the Proteomics and Mass Spectrometry Core Facility (PCF, Dalhousie University, Faculty of Medicine, Halifax, NS, Canada).

2.4.2 Protein digestion

Two hundred µg of cyst protein in 0.5 M dithiothreitol (DTT, Sigma D9163) (Appendix A) obtained as described above was heated at 60°C for 30 min followed by cooling for 5 min at room temperature. The samples were then water bath sonicated (Branson 2800) at room temperature for 15 min, alkylated in 0.7 M iodoacetamide (IAcNH2, Sigma I6125) (Appendix A) for 30 min at room temperature, diluted 6 X with H_2O (1:5 ratio of sample to water) and digested overnight at 37°C with 100 µl trypsin solution (Appendix A) at a 100:1 protein to trypsin ratio. Subsequently, 1 µl trifluoroacetic acid (TFA, Sigma T6508) was added to a final concentration of 0.1%, and the pH of the resulting peptides was reduced to less than 3 with 5 µl formic acid prior to centrifugation for 1 min at 3000 x g. The acidified peptides were desalted using Qasis desalting columns (PN WAT094226) which were first conditioned, once with 1 ml of 50% acetonitrile (ACN) in 0.1% TFA and twice with 1 ml 0.1% TFA. For desalting, samples were loaded into the conditioned Qasis columns followed by washing five times with 1 ml of 0.1% TFA. Elution was done twice with 0.5 ml of 50% ACN in 0.1% TFA and once with 0.5 ml

of 80% ACN in 0.1% TFA. The eluted samples were dried with a SpeedVac (Thermo Electron Corporation SPD111V) at 20°C and reconstituted in 100 µl of 50 mM TEAB before bath sonication at room temperature for 15 min to solubilize peptides.

2.4.3 Protein isotopic labeling

Two hundred µg of the digested peptides from cysts containing and lacking p26 were differently tagged by reductive methylation using light and heavy labels. For light labeling 8 µl of 37% (w/w) formaldehyde solution (Sigma F8775) was added to the peptide sample prepared from cysts containing p26 whereas for heavy labeling 15 µl of 20% (w/w) deuterated formaldehyde in D_2O (Cambridge Isotope Laboratories. DLM-805-20) was added to the peptide sample lacking p26. Each sample was mixed and incubated for 5 min at room temperature. Seventeen µl of 6 M sodium cyanoborohydride (NaCNBH3) was then added to each sample followed by incubation for 1 h at room temperature. Light and heavy samples, were combined in one tube at 1:1 ratio. The pH of the labeled sample was reduced to less than 3 with 1 µl TFA and samples were desalted as described previously using Qasis desalting columns and dried with a Speed Vac.

2.5 Mass spectrometric analysis by 2D-LC separation

2.5.1 High pH reversed-phase liquid chromatography (high pH RP-HPLC)

In the first dimension separation, high pH RP-HPLC, 200 µg of the dried, pooled and desalted sample was suspended in 100 µl of suspension buffer (Appendix A), water bath sonicated at room temperature for 15 min and transferred to a 4.6 x 100 mm monolithic C18 column (Merck, Germany). High pH RP-HPLC separation followed by fraction concatenation was performed with an Agilent 1100 HPLCsystem

consisting of a binary pump (G1312A), auto-sampler (G1367A), fluorescence detector (G1321A, excitation 280 nm, emission 348 nm) and fraction collector (G1364C, Agilent Technologies, Santa Clara, California, USA). One hundred µl of labeled peptide was injected onto the C18 column at a constant flow rate of 400 µl/min. Chromatographic separation was carried out over 86 min using a gradient of two buffers (referred to as 1st dimension buffer A and B respectively in Table 2). In this first-dimension, the column initially received buffer A for 3 min. Next, a gradient of 100 to 64% buffer A was run over 57 min, then switched to 100% buffer B for 5 min. Subsequently, the column was rinsed with 100% buffer B for 14 min followed by 100% buffer A over 11 min. Thirty-seven fractions were collected at 2 min intervals and lyophilized using a Speed Vac. As drying occurred fractions at early, middle and late stages were combined into 12 fractions (Table 3) which were re-suspended in 50 µl of 3% ACN containing 0.1% formic acid. The samples were centrifuged for 5 min at 3000 x g and water bath sonicated at room temperature for 15 min. The first-dimension, RP-HPLC separation, was conducted at the National Research Council of Canada Laboratory (NRC-Halifax).

2.5.2 Liquid chromatography tandem mass spectrometry (LC-MS/MS)

In preparation for the second dimension separation, LC-MS/MS, C18 columns were custom made by the stage tip procedure (Rappsilber *et al*., 2007). Briefly, the C18 columns were made by placing 10 small 3M Empore C18 extraction disks, shaped with a blunt-end syringe needle containing a CTFE hub (Hamilton 90516, USA), in a pipette tip (Rappsilber *et al*., 2007). Then the C18 columns were conditioned by the addition of 50 µl methanol followed by centrifugation at 3000 x g for 5 min and then100 µl 50% ACN in 0.1% TFA followed by centrifugation at 3000 x g for 5 min. The C18 columns were next washed twice with100 µl 0.1% TFA and

Table 2. First dimension of liquid chromatography separation

Time (min)	%A*	%B*
0	100	0
3	100	0
60	64	36
61	0	100
75	0	100
86	100	0
99	100	0

***1st dimension buffer A**: 5% acetonitrile, 10 mM ammonium formate, pH8

***1st dimension buffer B:** 95% acetonitrile, 10 mM ammonium formate, pH8

Table 3. Fraction combination

Sample	Fractions Combined
1	2, 14, 26
2	3, 15, 27
3	4, 16, 28
4	5, 17, 29
5	6, 18, 30
6	7, 19, 31
7	8, 20, 32
8	9, 21, 33
9	10, 22, 34
10	11, 23, 35
11	12, 24, 36
12	13, 25, 37

In order to reduce the number of fractions while increasing sample complexity, three fractions encompassing early, middle and late elution times were combined to generate 12 samples. As an example, the fractions numbered 2, 14 and 26, respectively early, middle and late were combined into sample 1. Fraction 1 was excluded because it contained concentrated buffer A that was initially introduced to the column.

then centrifuged at 3000 x g for 5 min. Each of the 12 samples was loaded on a separate C18 column which was then centrifuged at 2000 x g for 10 min and washed twice with 200 µl 0.1 % TFA by centrifugation for 5 min at 3000 x g. Peptides were eluted into fresh tubes by adding 50 µl of 50% ACN in 0.1% TFA to each C18 column followed by centrifugation for 5 min at 3000 x g. Fifty µl of 80% ACN in 0.1% TFA was added to each of the 12 freshly collected samples which were then centrifuged for 5 min at 3000 x g. The eluted samples were dried overnight in the SpeedVac, re-suspended in 20 µl of suspension buffer (Appendix A), water bath sonicated at room temperature for 15 min and transferred to micro HPLC vials for LC-MS/MS analysis.

LC-MS/MS was performed using a nano flow liquid chromatography system (Ultimate3000, Thermo Scientific) interfaced to a hybrid ion trap-orbitrap high resolution tandem mass spectrometer (VelosPRO, Thermo Scientific) operated in data dependent acquisition (DDA) mode. One µl of each sample was injected into a C18 packed capillary column (4 µm C18 Jupiter packing material, 0.0750 x 500 mm capillary column) at a flow rate of 300 nl/min. Samples were electro-sprayed at 1.2 kV using a dynamic nanospray probe at 5^{o}C. Chromatographic separation was carried out over 119 min using a linear gradient (referred as 2nd dimension buffer A and B respectively in Table 4).

In this second-dimension, the column initially received a mobile phase consisting of 97% buffer A for 3 min followed by linear gradient from 95% buffer A to 5% buffer A over 104 min. Next, the column received a second wash with the mobile phase two consisting of 97% buffer A over 15 min (Table 4).

MS/MS spectra were acquired using both collision induced dissociation (CID) and higher-energy collisional dissociation (HCD) for the top 15 peaks in the survey to

Table 4. Second dimension of liquid chromatography separation

Time (min)	Flow rate (μl/min)	*A%	*B%
0	0.3	97	3
3	0.3	97	3
5	0.2	95	5
95	0.2	70	30
102	0.3	5	95
107	0.3	5	95
110	0.3	97	3
125	0.3	97	3

***2nd dimension buffer A**: 0.1% formic acid in MS-grade H_2O, pH 2

***2nd dimension buffer B**: 0.1% formic acid in MS-grade ACN, pH 2

provide high mass accuracy and resolution. Peptide quantification was achieved by using “the peak intensity of the area” under the chromatographic peak to measure the abundance of the peptides. Second-dimension separation, LC-MS/MS, was conducted at the Proteomics and Mass Spectrometry Core Facility, Dalhousie University.

2.6 Bioinformatics

Mass spectrometry data files were acquired (Xcalibur, Thermo Fisher) and exported to Proteome Discoverer software (version 2.0, Thermo Fisher) for peptide and protein identification using SequestHT search algorithm. The search was based on a full trypsin digestion with 2 maximum missed cleavage sites, 10 ppm precursor mass tolerance and 0.8 Da fragment mass tolerance. Database searching was carried out against the *Artemia* genome database (ORCAE, a restricted genome from Ghent University, Gent, Belgium) and with the protein sequences downloaded from UniProt database including arthropods and crustaceans. The Common Repository of Adventitious Proteins (cRAP) database was used to identify common contaminants in proteomics experiments. Databases were formatted to FASTA prior to data analysis.

Parameters for the database search were that only one missed cleavage site per peptide was allowed and only tryptic peptide sequences were permitted. Modifications included in the search were phosphorylation of Thr, Tyr and Ser, oxidation of Met, carbamidomethylation of Cys, and mass shifts of +28, light labeling, and +32, heavy labeling, for N-terminus and Lys from the isotopic labeling, which was used to estimate peptide levels. The results from the search spectra were filtered based on Xcorr vs charge state, 2.2, +2; 3.75, 3+ or greater. Peptide and fragment tolerances were set at 10 ppm and 0.6 m/z, respectively. Acceptance threshold for peptides was $e \leq 0.1$. The complex LC-MS/MS resulted file which

contained identified proteins hits was transformed into clear and biologically meaningful dataset in user friendly Excel file which can be easily interpreted. Proteins that could not be identified by the procedures just described were identified manually by blasting on different web servers and the closest protein match with threshold ≤ 0.1 was chosen.

The resulting complex data were simplified by using the filtration tool in Proteomic Discover software and then checked manually to confirm the identity of proteins present in each of the profiles. In order to determine potential activities of proteins in the diapause proteome and thus to ascertain metabolic and other characteristics of *A. franciscana* cysts, GO enrichment was utilized wherein the MS/MS detected proteins (*Artemia* database protein accession numbers) were mapped to the RefSeq of the equivalent *D. melanogaster* proteins by blasting the protein sequences in the NCBI database and using the e- value of $\leq 10^{-5}$. To elaborate, the NCBI RefSeqs of *D. melanogaster* protein IDs were mapped to UniProt KB using the UniProt Mapping tool. The output " gene names" were then inserted into PANTHER. The GO analysis of the resulting candidate proteins was executed and viewed in a pie chart with PANTHER functional classification system.

To increase confidence in the data, proteins were identified on the basis of matches to two or more unique peptides. However, some identities as indicated in the tables were based on a single match. Proteins were assigned putative functions by the use of the UniProt database. Based on these results, proteins were further organized into sub-groups for the purpose of biological interpretation, indicating functional pathways in which proteins are involved, by use of KEGG Pathway Maps, http://www. genome. jp/kegg/pathway.html. KEGG pathway includes proteins involved in genetic information processing such as in transcription, translation,

ubiquitin and chaperoning based function. Proteins involved in environmental information processing were those involved in membrane transport and signal transduction. Proteins involved in cellular processes were those involved in cell growth and death.

CHAPTER 3: RESULTS

3.1 Culture of *A. franciscana*

When experiments were initiated most of the *A. franciscana* in culture were males and there were differences in body length and color when adult males were compared to one another, as was true for the smaller number of females. Specifically, some adults were bigger and darker than others. As an illustration, the color of adult females varied from pale (Fig. 3A), pink-orange (Fig. 3B) to dark yellow-brown (Fig. 3C). Moreover, it appeared that some females produced only females and they did so in the absence of males, suggestive of a parthenogenetic population. Females and males of normal morphology, swimming ability and feeding behavior were mated with one another and they produced broods of mixed offspring, which were used for RNAi experiments.

3.2 Knockdown of *p26* in *A. franciscana* cysts

Electrophoresis in agarose gels of *p26* and *GFP* cDNAs generated by PCR yielded only DNA fragments of the expected size (Fig. 4A). Similarly, *p26* and *GFP* dsRNA used for injection of *A. franciscana* females migrated to the expected positions in agarose gels and they were the only PCR products seen in these gels (Fig. 4B). Upon immunoprobing of western blots p26 was absent from protein extracts of cysts obtained from *A. franciscana* females injected with *dsRNA* for *p26*, but was easily detected in protein extracts of cysts obtained from females injected with *GFP* dsRNA (Fig. 4C). Similarly, quantification by mass spectrometry 7-days post-release from females revealed that p26 was reduced by approximately 200-fold in cysts from females receiving *p26* dsRNA as compared to cysts from females receiving *GFP* dsRNA (Fig. 5).

A **B** **C**

Figure 3. Color variation of *A. franciscana* females. Representative stereomicroscopic images showing the color of *A. franciscana* females. **A**, pale; **B**, pink-orange (female has an empty ovisac); **C**, dark yellow-brown.

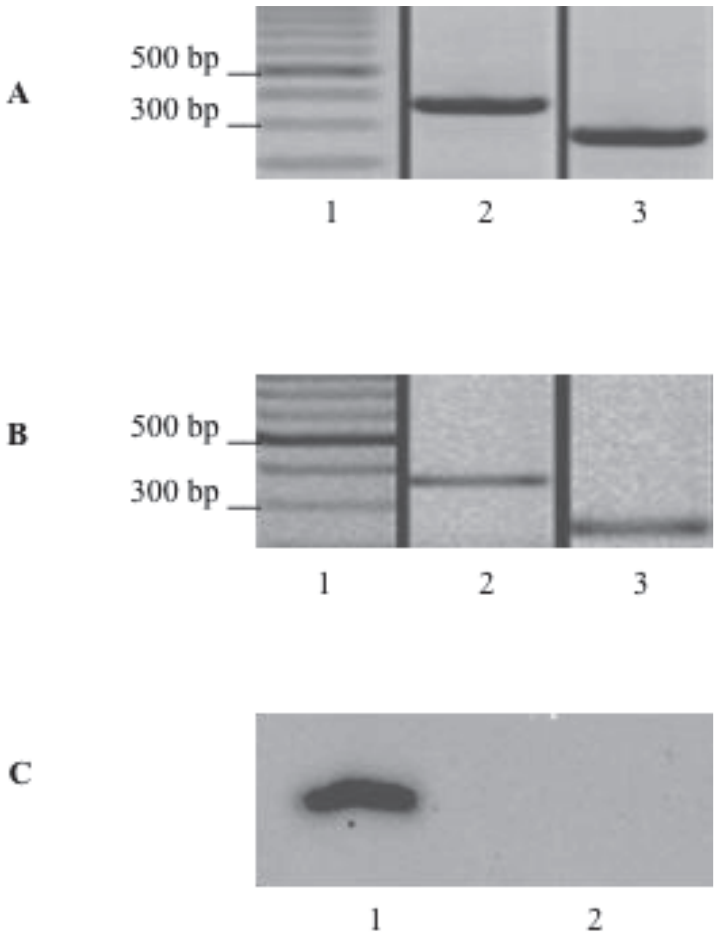

Figure 4. Knockdown of *p26* in *A. franciscana* cysts. A. *GFP* and *p26* cDNA obtained by PCR were resolved by electrophoresis in 1.2% agarose gels and stained with SYBR Safe®. Lane 1, Gene ruler 1-Kb size marker in bp; 2, *GFP* cDNA; 3, *p26* cDNA. **B.** dsRNA obtained by using *GFP* and *p26* cDNA as templates and primers containing the T7 promoter, were resolved by electrophoresis in 1. 2% agarose gels and stained with SYBR Safe®. Lane 1, Generuler 1-Kb size marker in bp; 2, *GFP* dsRNA; 3, *p26* dsRNA. **C.** Twenty-five µg of protein extract from 33 cysts obtained from females injected with either *GFP* dsRNA (lane 1) or *p26* (lane 2) was resolved in 12.5 % SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with a polyclonal antibody raised in rabbit to p26, followed by HRP-conjugated goat anti-rabbit IgG antibody and chemiluminescence.

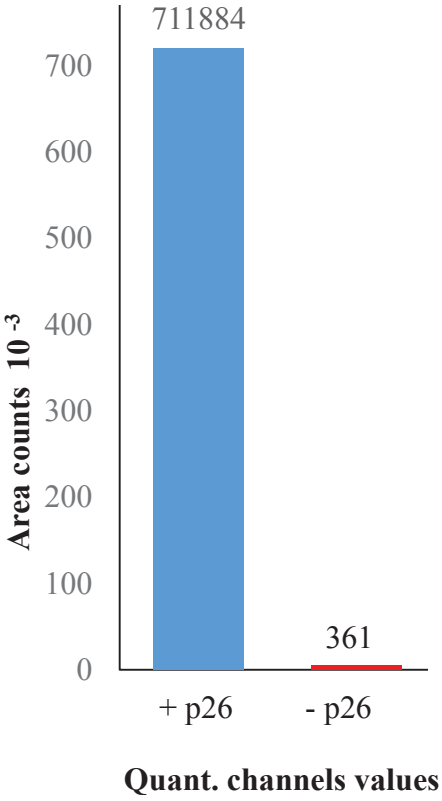

Figure 5. Mass spectrometry revealed the loss of p26 in cysts obtained from *A. franciscana* females receiving *p26* dsRNA. Spectral counting yielded the number of spectra for p26 peptides in protein samples of cysts obtained from females injected with *GFP* dsRNA (blue) and *p26* dsRNA (red) and these data were then compared to yield the relative amounts of p26 peptides in each sample.

3.3 The diapause proteome of *A. franciscana*

The proteins in cysts released from females injected with *p26*/*GFP* dsRNA and were detected by MS/MS represent a partial *A. franciscana* diapause proteome and it is archived here.[1] This is one of the few diapause proteomes available for any organism. A total of 3212 proteins were detected by MS/MS of which 1548 (48.2%) were annotated and 1664 (51.8%) were unannotated. Of the identified proteins 639 (29%) were identified with high confidence, based on 2 or more unique peptides, and the remaining 924 (71%) were identified based on a single peptide match to an archived protein**.** The raw MS file containing 3387 proteins was simplified by removing duplicate proteins as well as those proteins such as keratin and the digestive enzyme trypsin which were likely to be contaminants. For those proteins that were annotated the closest matches were generally to proteins from the Arthropods. GO enrichment analysis was not possible based on the information obtained in this manner. Subsequently, the proteins that were identified in the *A. franciscana* diapause proteome were annotated by searching for equivalent proteins in the NCBI *D. melanogaster* database, due to the fact that *D. melanogaster* is the closest annotated dataset and core module for comparison.

Of the *A. franciscana* proteins, 2515 RefSeqs matched genes associated with *D. melanogaster* proteins and of these, 1416 were successfully mapped into 1675 UniProtKB IDs using the UniProt Mapping tool, the outputs of some protein inputs were duplicates. Subsequently, the 1675 UniProtKB IDs were inserted into the PANTHER classification system. PANTHER recognized 1406 proteins and of these,

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[1] https://dalu-my.sharepoint.com/personal/hj269321_dal_ca/_layouts/15/WopiFrame.aspx?docid=03f586a0e9ee54a5ca0afc6690f0be0fb&authkey=AdwcUiu6AKtSweU6NLSaIz0&action=view

functional classification was achieved for 1204 proteins, indicating a partial classification of the diapause proteome (Fig. 6). The largest protein group within the cyst proteome contained 532 catalysts/enzymes. The next largest group was composed of 416 binding proteins followed by a set of 130 structural proteins. The other group were composed of 64 transporters, 33 translation factors, 17 receptors, 8 antioxidants, 3 signal transducers, and 1 channel regulator, the latter two categories being too small to be visible in Fig. 6. For more detailed information about the protein in each category see figure 7 A-F. Of the 532 catalytic enzymes, 604 appeared in subgroups; some enzymes may have appeared in more than one group or there may have been duplicates. Of those 604 enzymes, hydrolases were the most prominent subgroup, followed by transferases and oxidoreductases then by ligases, isomerases, lyases, enzyme regulators, helicases, and deaminases (Fig. 7A). The second largest group within the cyst proteome was composed of binding proteins, forming 35 % of the total number of the diapause proteome proteins, and mainly divided into 4 sub-groups. Of those, low numbers of proteins were identified as follows: 14 involved in nucleic acid binding, 9 involved in protein binding, 2 engaged in calcium ion binding and one participating in calcium dependent phospholipid binding (Fig. 7B).

The next largest group within the cyst proteome was composed of structural proteins, forming 11% of the total number of the diapause proteome proteins, and mainly divided into ribosomal proteins and cytoskeletal proteins (Fig. 7C). Of the total number of the diapause proteome proteins, 5% were recognized as transport proteins of which one protein was a carbohydrate transporter and another was a lipid transporter. The remaining 41 proteins, which form the most prominent subgroup of transporters, were

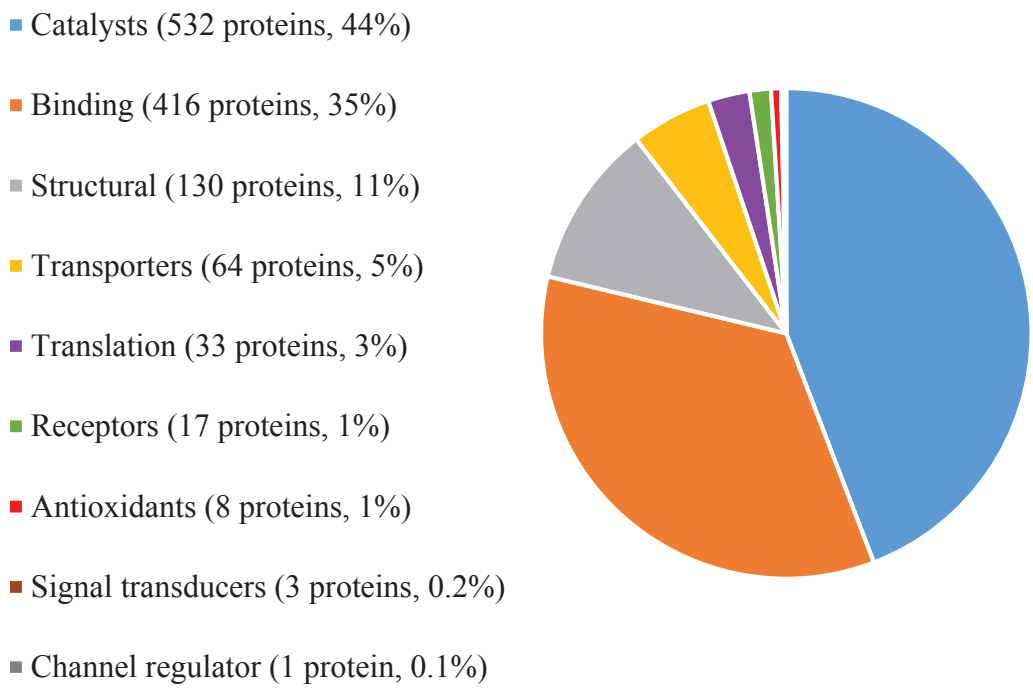
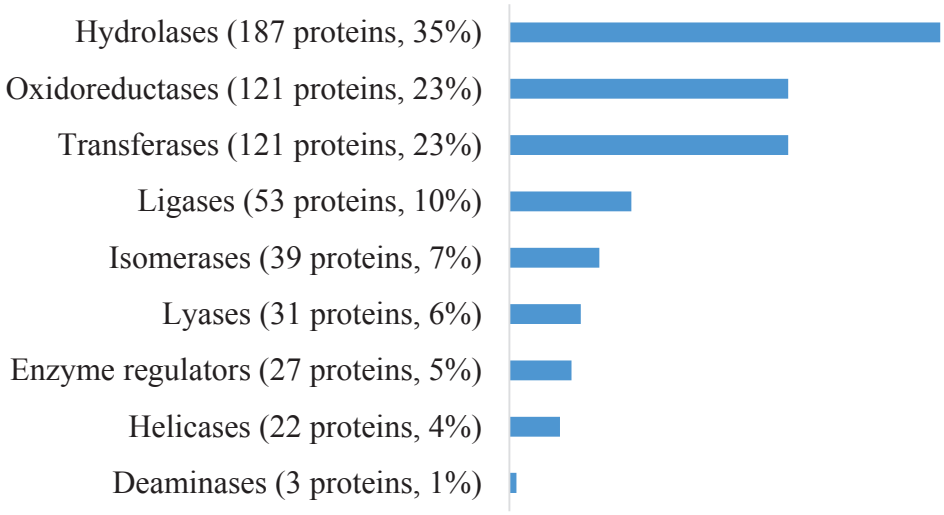


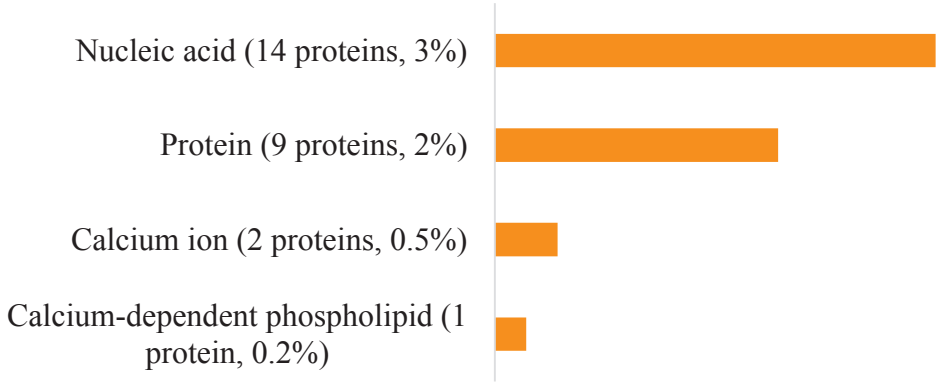
Figure 6. The functions of proteins from the *A. franciscana* diapause proteome. PANTHER was used to functionally characterize the proteins in the diapause proteome. The number of proteins in each category was divided by the complete number of proteins to give the percentage of proteins in each category which was then represented in a pie graph.

Figure 7. The different groups of proteins in the diapause proteome of *A. franciscana*. PANTHER was used to divide each protein category in the diapause proteome into subgroups. **A**, catalytic; **B**, binding; **C**, structural; **D**, transporter; **E**, translations; **F**, the last panel is composed of 4 categories including receptors, antioxidants, signal transducers, and channel regulatory. The percentage for each category indicates the number of proteins of each subgroup compared to the total number of proteins within the same category.

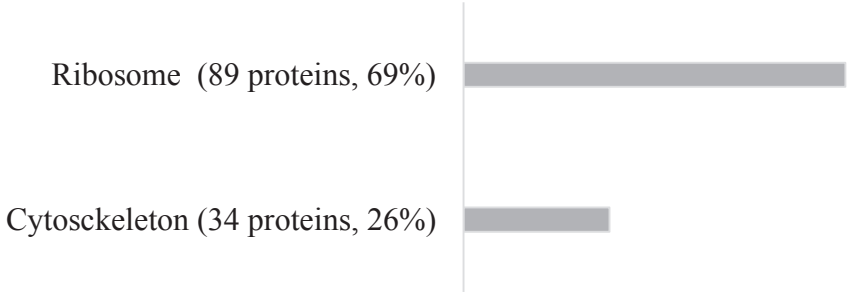
A


Hydrolases (187 proteins, 35%)
Oxidoreductases (121 proteins, 23%)
Transferases (121 proteins, 23%)
Ligases (53 proteins, 10%)
Isomerases (39 proteins, 7%)
Lyases (31 proteins, 6%)
Enzyme regulators (27 proteins, 5%)
Helicases (22 proteins, 4%)
Deaminases (3 proteins, 1%)

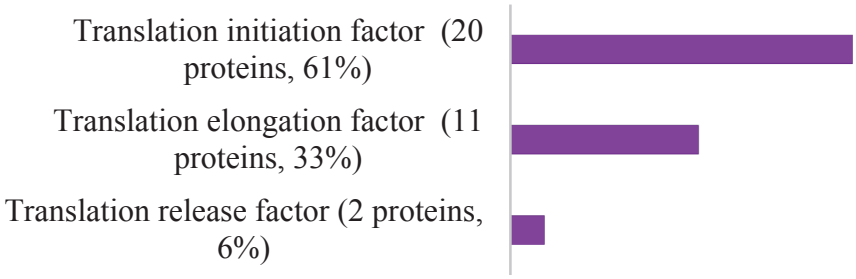
B


Nucleic acid (14 proteins, 3%)
Protein (9 proteins, 2%)
Calcium ion (2 proteins, 0.5%)
Calcium-dependent phospholipid (1 protein, 0.2%)

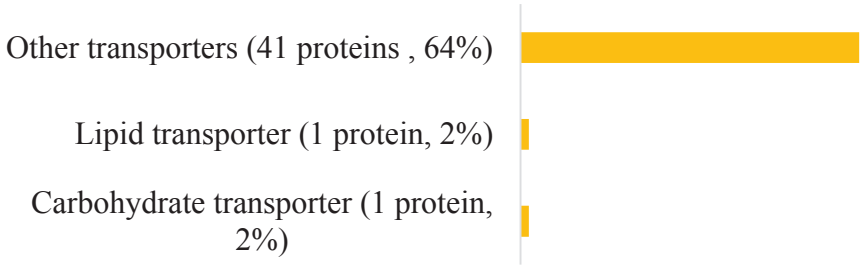
C


Ribosome (89 proteins, 69%)
Cytosckeleton (34 proteins, 26%)

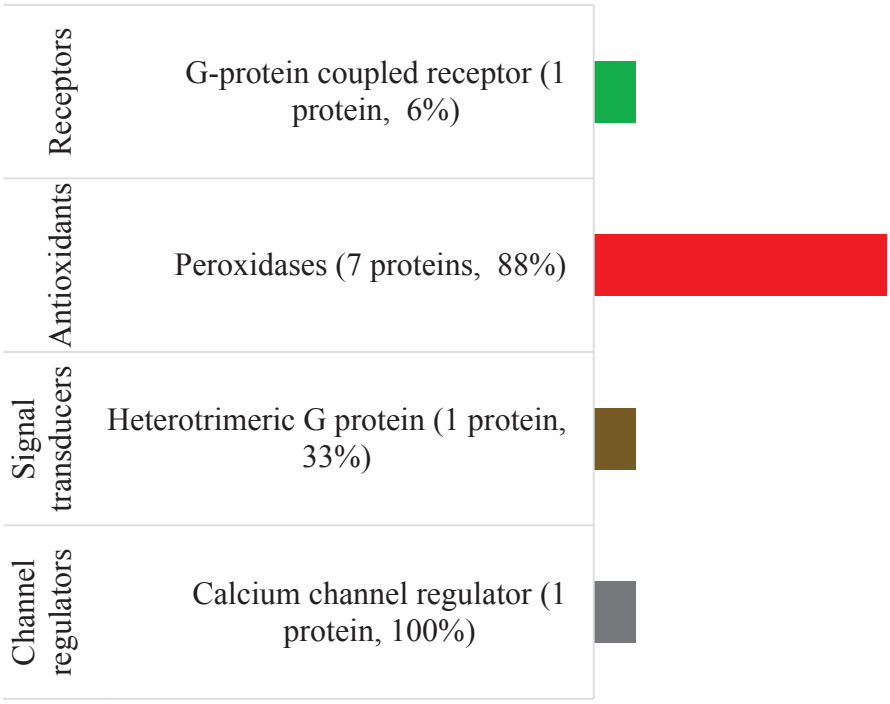
D


Translation initiation factor (20 proteins, 61%)
Translation elongation factor (11 proteins, 33%)
Translation release factor (2 proteins, 6%)

E


Other transporters (41 proteins , 64%)
Lipid transporter (1 protein, 2%)
Carbohydrate transporter (1 protein, 2%)

F


Receptors
G-protein coupled receptor (1 protein, 6%)
Antioxidants
Peroxidases (7 proteins, 88%)
Signal transducers
Heterotrimeric G protein (1 protein, 33%)
Channel regulators
Calcium channel regulator (1 protein, 100%)

involved in other transport activities (Fig. 7D). Of the total number of the diapause proteome proteins, 3% were involved in translation, 20 of which were initiation factors, 11 were elongation factors, and 2 were release factors (Fig. 7E). The last four functional categories within the diapause proteome of *A. franciscana* were composed of 17 receptors, 8 antioxidants, 3 signal transducers and one channel regulator (Fig. 7F). Of the 17 receptors, only one G-protein coupled receptor was identified, and of 8 antioxidant enzymes 7 were peroxidases. Of 3 signal transducers, one heteromeric G-protein was recognized. One channel regulator was identified.

3.4 *p26* knockdown modified the diapause proteome of *A. franciscana* cysts

Upon knockdown of *p26*, the amounts of most proteins were unchanged in the diapause proteome of *A. franciscana.* However, some proteins either disappeared to below LC-MS/MS detection limits or were reduced significantly when *p26* was knocked down; whereas other proteins appeared or increased in amount. Those proteins that varied quantitatively upon *p26* knockdown either have the potential to be p26 substrates in diapausing *A. franciscana* cysts or possibly have their synthesis affected by p26.

3.4.1 Proteins that disappeared from the diapause proteome upon *p26* knockdown

Ten proteins disappeared (became non-detectable) from the diapause proteome upon knockdown of *p26* (Table 5). All identifications of proteins that disappeared were based on a match to 1 unique peptide. Six of these proteins were involved in genetic information processing whereas 2 others were metabolic enzymes, one mediated cellular processing and one was unknown.

Table 5. Proteins that disappeared from the *A. franciscana* diapause proteome upon *p26* knockdown

[1]Protein designation	[2]Function
Genetic Information Processing	
RNA granule protein, invertebrate	Chaperoning activity & translation suppressor
*U2 snRNP-associated SURP motif containing protein	RNA processing
Elongation factor 1 alpha	Protein biosynthesis
Elongation factor-1 alpha	Protein biosynthesis
Elongation factor-1 alpha	Protein biosynthesis
*Ubiquitin conjugation factor E4 A	Protein ubiquitination
Metabolism	
Mitochondrial isocitrate dehydrogenase [NAD] subunit	Tricarboxylic acid cycle
*Probable glutamine dependent NAD (+) synthase	NAD biosynthesis
Cellular Process	
* Caspase	Apoptosis
Not Annotated	
artfr59618g00010	Unknown

[1]Proteins were identified by Proteome Discoverer software. * The identity of proteins assessed manually was accepted if their threshold match was ≤ 0.1.[2]Proteins were functionally categorized by using the UniProt database. Bold font indicates functional pathways based on the KEGG PATHWAY.

3.4.2 Proteins that decreased but did not disappear from the diapause proteome upon *p26* knockdown

Eleven proteins in the diapause proteome of *A. franciscana* decreased by 50% or more upon knock down of *p26* and these may be p26 substrates (Table 6). Six of these proteins were involved in genetic information processing, 3 mediated environmental information processes, and the functions of the other 2 proteins were unknown.

3.4.3 Proteins that appeared in the diapause proteome upon *p26* knockdown

Mass spectrometry revealed 35 proteins in the proteome of *p26* knockdown cysts that were lacking in the diapause proteome (Table 7). All identifications were based on a match to 1 unique peptide. Eighteen of these proteins were involved in genetic information processing, 10 were metabolic enzymes, 6 participated in environmental information processing and one protein was not annotated.

3.4.4 Proteins normally present in the diapause proteome that increased upon *p26* knockdown

Twenty-two proteins increased in the diapause proteome by at least 2-fold when p26 was eliminated (Table 8). Twelve of these proteins were involved in genetic information processing and 7 in metabolism. The functions of the remaining 3 proteins were not annotated.

Table 6. Proteins that decreased but did not disappear from the *A. franciscana* diapause proteome upon *p26* knockdown

[1]Protein designation	[2]Function	[3]Decrease -p26 : +p26	[4]Unique Peptides
Genetic Information Processing			
CG11700	Protein ubiquitination	0.5	2
Ubiquitin/ribosomal S27 fusion protein 2	Protein turnover	0.5	2
T-complex protein 1, theta subunit	Chaperoning activity	0.5	3
* T-complex protein 1, beta subunit	Chaperoning activity	0.5	2
*Heat shock 70 kDa protein 4	Chaperoning activity	0.5	2
* Shell gland specific protein	Cyst shell formation	0.2	5
Environmental Information Processing			
*Vacuolar ATPase subunit H	Proton transport regulator	0.4	2
*Uncharacterized protein B0WHJ5	Chitin metabolism	0.2	2
*GL19819	DNA binding	0.5	10
Not Annotated			
artfr5076g00040	Unknown	0.5	2
artfr1800g00080	Unknown	0.4	2

[1]Proteins were identified by Proteome Discoverer software. * The identity of proteins assessed manually was accepted if their threshold match was ≤ 0.1. [2]Proteins were functionally categorized by using the UniProt database. [3]Decrease –p26: +p26, the decrease amount of a protein in cysts lacking p26 as compared to cysts containing p26. [4]Unique peptides, the number of unique peptides sequenced by mass spectrometry that matched the protein of interest. Bold font indicates functional pathways based on the KEGG PATHWAY.

Table 7. Proteins that appeared in the diapause proteome of *A. franciscana* upon *p26* knockdown

[1]Protein designation	[2]Function
Genetic Information Processing	
* Coatomer subunit beta	Vesicle mediated protein transport
*Importin-4	Protein transport
*Hypoxia up-regulated protein 1	Cellular response to stress
U-box domain-containing protein 35-like	Stress response and ubiquitin system
E3 binding	Ubiquitination
*Heat shock 70 kDa protein 14	Stress response
E9GQY5	DNA and RNA secondary structure unwinding, promotion of transcription
E9GM63	Regulation of transcription initiation from RNA polymerase II promoter
*Mediator of RNA polymerase II transcription subunit 25	Regulation of transcription
RNA recognition motif, spliceosomal PrP8	Pre-mRNA splicing via spliceosome
* CWF19-like protein	mRNA splicing/cell cycle control
E9G6S3	Methionine synthase activity
*tRNA methyltransferase 11-2 homolog	RNA methylation
* Proteasome 26S subunit, non-ATPase 9	Assembly of the 26S proteasome/ transcription co-activator
26S proteasome regulatory complex, non-ATPase sub-complex, Rpn1 subunit	Proteasome regulator
Peptidase S1C	Peptide hydrolase
* 60S ribosomal protein L6	Ribosome large subunit assembly, translation
*Eukaryotic translation initiation factor 3, subunit B	Initiation of translation

Table 7(*Continued*)

[1]Protein designation	[2]Function
Metabolism	
Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	Fatty acid metabolism
* Hydroxysteroid dehydrogenase-like protein 2	Steroid biosynthesis
Pyrroline-5-carboxylate reductase	Proline biosynthesis
* Inosine 5' monophosphate dehydrogenase 2	Guanosine monophosphate/ purine biosynthesis
Adenosine/AMP deaminase domain	Glycosidase / purine metabolism
*Phospho-triesterase related protein	Glycosidase
*Phospho-glucomutase 2	Carbohydrate/ purine metabolism
Phosphoglucomutase	Glucose metabolism
*Pyruvate kinase	Glycolysis
* Glucose dehydrogenase	Fatty acid/ nucleotide and nucleic acids synthesis
Environmental Information Processing	
*Transmembrane 9 superfamily member 2	Small molecules transporter
*Chloride intracellular channel protein 2	Regulation of ion channel transmembrane activity
*Ras-related protein Rab-18	GTPase mediated signal transduction
* F1A3Y3	Signal transduction
Nicastrin	Proliferative signaling & cell-cell communication
GD21468	Phosphatidylinositol phosphorylation
Not Annotated	
artfr1743g00010	Unknown protein

Table 7(*Continued*)

[1]Proteins were identified by Proteome Discoverer software. *The identity of proteins assessed manually was accepted if their threshold match was ≤ 0.1.[2]Proteins were functionally categorized by using the UniProt database. Bold font indicates functional pathways based on the KEGG PATHWAY.

Table 8. Proteins normally present in the diapause proteome of *A. franciscana* that increased upon *p26* knockdown

[1]Protein designation	[2]Function	[3] Fold-increase -p26 : +p26	[4]Unique Peptides
Genetic Information Processing			
Small ribonucleoprotein particle U1 subunit 70K, isoform C	mRNA splicing via spliceosome	6.8	2
*40S ribosomal protein S8	Translation	2.5	3
*40S ribosomal protein S21	Developmental / ribosomal/ ribonucleo protein	4.0	3
*Lachesin	Developmental protein/ morphogenesis	2.7	2
*Vesicle-associated membrane protein associated protein A	Protein localization to ER	2.8	2
* Reticulon-1	Regulate ER structure and functions	4.9	2
*Similar to lola-like protein	Regulation of transcription	3.3	2
Importin subunit alpha	Protein import into nucleus	2.9	3
*Serine/threonine protein kinase	Protein phosphorylation	3.4	3
Nucleotide-binding alpha-beta plait	Ribosomal/ ribonucleoprotein	5.0	2
* Nucleoprotein TPR	Ribonucleoprotein / nucleocytoplasmic transporter	2.2	2
*Nuclear pore complex Nup50	Nuclear transport	3.8	3
Metabolism			
Male sterility, NAD-binding	Oxidation-reduction, ubiquinone-6 synthesis	2.1	2
Acyl-CoA oxidase / de-hydrogenase, central domain	Lipid metabolism	3.0	2
Aldehyde dehydrogenase C-terminal	One-carbon metabolism	2.2	2

Table 8 (*Continued*)

[1]Protein designation	[2]Function	[3]Fold-increase -p26 : +p26	[4]Unique Peptides
Orotidine 5' phosphate decarboxylase domain	De novo uridine biosynthesis	2.7	2
* 3-Hydroxyanthranilate 3, 4 dioxygenase	Pyridine nucleotide biosynthesis	2.1	2
Dolichyldiphospho oligosaccharide protein glycosyltransferase subunit 1	Protein glycosylation	3.4	2
Glucosamine-6-phosphate isomerase	Carbohydrate metabolism/energy generation	2.8	2
Not Annotated			
Mitochondrial glyco-protein	Unknown	2.3	2
Uncharacterized protein, R7W130	Unknown	4.1	5
* Uncharacterized protein, E9FU69	Unknown	2.2	2

[1]Proteins were identified by Proteome Discoverer software. *The identity of proteins assessed manually was accepted if their threshold match was ≤ 0.1. [2]Proteins were functionally categorized by using the UniProt database. [3]Fold-increase –p26/+p26, the amount increase of a protein in cysts lacking p26 as compared to cysts containing p26. [4]Unique peptides, the number of unique peptides sequenced by mass spectrometry that matched the protein of interest. Bold font indicates functional pathways based on the KEGG PATHWAY.

CHAPTER 4: DISCUSSION

4.1 Culture of *A. franciscana*

The *Artemia* cysts from the GSL used in this study are recognized as a heterosexual specie known as *Artemia franciscana* (Kellogg, 1906). However, I initially observed during my work an extremely male-biased sex ratio in the culture and females that continuously reproduced offspring in the absence of males, suggesting a mixed population including parthenogenetic organisms. The observation of a mixed population of *Artemia* from the GSL is in agreement with experimental data and field observations made previously (von Siebold, 1883; Packard, 1883; Jensen, 1918; Cuellar, 1990; Campos-Ramos *et al*., 2003). Therefore, careful attention was paid to the identity of the *Artemia* used in this study, and the results pose a caution that all commercially obtained cysts used in experiments should be examined closely to ensure they consist of only one population of organisms.

4.2 p26 was significantly reduced by RNAi in cysts of *A. franciscana*

The results in this thesis represent the second time that the injection of females with *p26 dsRNA* has been used to knockdown p26 in embryos of *A. franciscana*. The first RNAi knockdown was done by King and MacRae (2012) and the work showed that p26 affects the development of diapause-destined *A. franciscana* embryos and that it is required for maximal stress resistance of diapausing cysts. In the initial work the knock down of *p26* by RNAi in *A. franciscana* persisted for at least four broods (King & MacRae, 2012). Knockdown of *p26* through the fourth brood was confirmed by the immunoprobing of western blots in the work presented herein. However, protein extracts from the fifth to seventh broods showed very faint p26 bands on western blots upon probing with p26-specific antibody, indicating that the effects of

RNAi were wearing off. GFP is widely accepted as a control in RNAi studies of *Artemia* (Liu *et al*., 2009; Zhao *et al*., 2012; King & MacRae, 2012), because *Artemia* does not synthesize GFP and thus *GFP dsRNA* does not modify mRNA and protein in *A. franciscana.* Therefore, injection of females with *GFP dsRNA* would not likely have an impact on the proteome of diapausing *Artemia* cysts as characterized in this study.

My results also represent the first confirmation by MS, a technique that is much more sensitive for the detection of proteins than immunoprobing of western blots (Hu *et al.*, 2005), of the effectiveness of RNAi in knocking down *p26* in cysts of *A. franciscana*.

4.3 The *A. franciscana* diapause proteome

Artemia, used extensively as a model organism for cell/ molecular/ physiological studies, produce encysted embryos which survive extremely harsh environmental conditions for many years by entering diapause, a state of reduced metabolism and enhanced stress tolerance. The understanding of diapause in *Artemia* has suffered from the lack of analysis of the diapause proteome and most proteomic studies on *Artemia* have been done on embryos pre or post diapause (O'Connell *et al*., 2006; Qiu *et al*., 2007; Wang *et al.*, 2007). In this study, I took advantage of modern proteomic techniques, including 2D-LC and MS to investigate the proteome of diapausing *A. franciscana* cysts. Additionally, RNAi was used to evaluate the effects of knocking down p26, an abundant, diapause-specific protein in *A. franciscana* cysts, on the diapause proteome.

4.3.1 Functional classification of proteins in the diapause proteome of *A. franciscana*

MS analysis yielded 3212 proteins from diapausing *A. franciscana* embryos and demonstrated differences in proteins from cysts produced by females that received either *p26* or *GFP* dsRNA. The PANTHER functional classification system yielded, in comparison to other methods, the highest number of proteins in the diapause proteome. Thus, 1204 proteins within the *A. franciscana* diapause proteome were grouped into 9 categories including catalytic, binding, structural, transport, translation, channel regulatory, signal transduction, antioxidant and receptor. Accumulation of such a variety of proteins during diapause suggests that cysts devote cellular resources to these different biological activities which may ultimately contribute to diapause and their high stress tolerance. Even though not all of these proteins may be functioning during diapause, especially as diapause in *A. franciscana* is characterized by a profound reduction in biological activities, they may still contribute to cyst viability and stress tolerance.

Metabolic enzymes (catalysts) account for a large proportion of the proteins in the diapause proteome of *A. franciscana*, which agrees with the finding of Zhou and colleagues (2008) who examined the proteome of commercially obtained encysted diapause embryo of *A. sinica*, which were in post-diapause but had not undergone development. Also this finding is similar to that of Tu and colleagues (2015) who examined diapause and non-diapause eggs of the migratory locust, *Locusta migratoria* at transcriptional and translational levels. In *L migratoria* many of the genes and proteins up-regulated during diapause are involved in metabolism. Similarly, catalysts including hydrolases were found only at time 0 during the early

reactivation of *A. franciscana* cysts (Wang *et al*., 2007), and these hydrolases decreased over time during post diapause.

Because diapausing embryos of *A. franciscana* undergo a profound metabolic arrest (Clegg *et al*., 1996; Reynolds & Hand, 2004), the abundance of metabolic enzymes at first seems somewhat unusual. However, reduced ATP during diapause maintenance undoubtedly inhibits catalysts as metabolic activities are largely shut down. It is possible that during diapause maintenance catalysts are preserved in cysts for use post-diapause when embryos resume metabolic events (Browne *et al*., 1990).

Binding proteins account for the second largest group of proteins in the diapause proteome of *A. franciscana*, and of them PANTHER was able to allocate only a small number of proteins into biologically relevant groups, such is the limitation of PANTHER. The first group, representing 3% of the total number of binding proteins, consisted of nucleic acids binding proteins and the second group, accounting for 2% of the binding proteins was composed of protein binding proteins. This finding is similar to the previous finding of Zhou and colleagues (2008) who identified both kinds of binding proteins in the proteome of commercially obtained, early post-diapause encysted embryos of *A. sinica*. The nucleic acid binding proteins include RNA binding proteins, such as ribosomal/ribonucleoproteins, which play an integral part in regulating important biological functions such as DNA replication, gene expression, and metabolism of RNA, although these activities would be on hold during diapause in *Artemia*. The nucleic acid binding proteins would be largely required as cysts enter diapause and also upon diapause termination. Wang and colleagues (2007) observed that nucleotide binding proteins increase in amount during post diapause development of *A. franciscana* cysts, probably to meet the needs of transcription and translation in developing embryos.

The protein binding proteins in the *A. franciscana* diapause proteome included molecular chaperones. During stress such as that associated with diapause, when there is potential for protein denaturation, molecular chaperones bind many different substrates, thereby contributing to cell survival during diapause (Frydman *et al*., 1992; Parrotta *et al*., 2013; King & MacRae, 2015). In the current study the examples of molecular chaperones are subunit theta and beta of T-complex protein 1, HSP70-4 and p26. TCP-1-theta was tightly bound in stable complexes with 7 human sHsps, HspB1/B2/B3/B4/B5/B6 and HspB7, while HSP70-4 was tightly bound with 4 sHsps, HspB1, B2, B3 and B5 (Mymrikov *et al*., 2017), and this may reflect similar types of interactions in diapausing cysts.

Structural proteins form 11% of the *A. franciscana* diapause proteome, a finding similar to the 12% in the proteome of early post-diapause embryos of *A. sinica* (Zhou *et al*., 2008). Cytoskeletal proteins were abundant in both *A. franciscana* and *A. sinica* and they may play important roles in cell motility, macromolecule/organelle structure, cell division, intracellular transport, and cell differentiation, all potentially important as cysts develop and enter diapause. Structural proteins may contribute to the integrity of organelles during diapause preserving them from damage.

Transport proteins comprised the fourth largest group of proteins in the *A. franciscana* diapause proteome and the most abundant subgroup recognized by PANTHER transported compounds other than lipids and carbohydrates. Transporters may be important during diapause preparation because they transport molecules important for diapause initiation, maintenance and termination, but during *Artemia* diapause such activities would be largely shut down.

Translation factors including initiation, elongation, and release factors, were observed in the diapause proteome of *A. franciscana*. Translation factors bind to mRNA and ribosomes promoting protein synthesis, an ATP requiring process that is suppressed during diapause. Hence, despite their presence in cysts, translation factors are presumably inactive when the cysts are in diapause. However, during diapause preparation translation factors facilitate the synthesis of proteins associated with diapause, and they may also be stored for use during the synthesis of proteins following diapause termination.

It is likely that receptors are required for diapause entry, and possibly termination, by interacting with either environmental or physiological signals and transporting information across membranes. Ye and colleagues (2017) found receptors in diapausing embryos of parthenogenetic *Artemia* and knocking them down consistently inhibited diapause cyst formation. One G-protein coupled receptor was recognized in the *A. franciscana* diapause proteome by PANTHER. In the ovary of *B. mori*, G-protein coupled receptor cooperates in the synthesis of cryoprotectants, antifreeze proteins required to minimize freezing damage (Homma *et al*., 2006; Hagino *et al*., 2010). The findings presented above indicate that the receptors found in the *A. franciscana* diapause proteome modulate cyst formation, diapause initiation and termination and possibly contribute to changes that enhance stress tolerance.

Antioxidant enzymes were observed in the *Artemia* diapause proteome with peroxidases being the only type recognized by PANTHER. Wang and colleagues (2007) found peroxidases in newly emerged nauplii of *A. franciscana*, a post-diapause stage. Peroxidases are also up-regulated in the eggs of migratory locust during diapause (Tu *et al*., 2015). This group of enzymes is thought to repair diapause-induced oxidative damage in cysts of parthenogenetic *Artemia* exposed to stress.

Therefore, antioxidants are likely to be important components of the *A. franciscana* diapause proteome, probably enhancing diapause maintenance and the high stress tolerance by effecting repair of oxidative damage in cells.

Signal transducers, including a heterotrimeric-G protein, the only protein in this group that PANTHER recognized, were present in the *Artemia* diapause proteome. G proteins transduce signals from receptors to intracellular effectors, thereby regulating cell activities influencing systemic functions such as embryonic development and homeostasis (Neves *et al*., 2002). The identification of these signaling proteins in the *Artemia* diapause proteome suggests they are either involved in diapause entry by favouring oviparous development, or in diapause termination, by promoting exit from dormancy in response to exogenous diapause termination signals. This proposal is supported, albeit weakly, by Zhang and colleagues (2012) who found a heterotrimeric-G protein in diapause-destined larvae of the cotton bollworm, *Helicoverpa armigera*, during the diapause induction phase.

A calcium channel regulator was identified within the *A. franciscana* diapause proteome which agrees with the observation of Qiu and colleagues (2007) who found calcium channel protein in diapause-destined embryos of *A. franciscana.* Ion transport requires energy, so during diapause the exchange of ions with the external environment must be reduced because ATP is limiting. Accordingly, calcium channel regulator may be required to regulate ion exchange, functioning most likely to inhibit rather than stimulate ion transport either a cross intracellular membranes or between the inside and outside of the diapausing embryos, resulting in energy preservation and the maintenance of diapause.

4.3.2 *p26* knockdown modified the diapause proteome of *A. franciscana* cysts

The excel spread sheet of the cyst diapause proteome obtained by MS was used to compare proteins found in cysts containing and lacking p26. By this method, protein that either disappeared or were significantly reduced in amount (50% or more) but did not completely disappear were observed upon knockdown of *p26*. In contrast, other proteins not present in the diapause proteome appeared upon *p26* knockdown or they were significantly increased in amount (2-fold or more) from a basal level.

Many scenarios could be argued for the change seen in the protein abundance in response to the loss of a critical protein like p26. For example, proteins that disappeared upon the loss of p26 could possibly be below the level of detection by MS. The loss of p26 may cause the breakdown of a transcriptional down-regulator, resulting in overexpression of unregulated proteins which caused the appearance of and increase in amount of many proteins seen in this work. Another possible explanation for the increase in protein amount upon the loss of p26 is the inhibition of degradation because p26 is sequestering enzymes that degrade these proteins. Another scenario for all of the protein modifications upon the loss of p26 is that the cells are compensating for the loss of this important diapause protein, and thus changes in protein levels would not result from a direct interaction with p26. Further experiments such as affinity pull-down, cross linkage, and co-immunoprecipitation are needed to test these scenarios, especially the binding of these proteins to p26.

Nonetheless, the most likely hypothesis for the protein compositional changes observed in the work presented herein is direct interaction with p26. Thus, the potential relationship of these proteins to p26 and to the formation of diapause cysts, maintenance of diapause, diapause termination and post-diapause growth was a focus of this study. Interestingly, proteins were observed that changed upon the

loss of p26, but could not be identified by either homology or close similarity search. These proteins are apparently specific to *A. franciscana* and it will be very interesting to identify them in future work.

4.3.2.1 Proteins of the diapause proteome that disappeared or were reduced upon p26 knockdown

Gibert and colleagues (2012) showed that knocking down HSP27 in human cancerous cells eliminated three proteins, other than HSP27, and they argued that these proteins were substrates of HSP27. Accordingly, in the work presented herein, p26 substrates are most likely those proteins that either disappeared or were reduced in amount when *p26* was knocked down, which suggests that the preservation of these proteins depends on p26, at least during diapause. p26 plays a fundamental role in diapausing *A. franciscana* embryos and this may involve interaction with specific substrates, however there has been little investigation of p26 substrates since p26 was first reported by Clegg and colleagues (1994). One exception is the work on the binding of *A. franciscana* tubulin to p26 (Day *et al*., 2001). p26 substrates could be involved in embryo development, diapause initiation, maintenance and termination, and in stress tolerance (King & MacRae, 2012), suggesting that p26 interacts with many different proteins. Thus when p26 disappears, proteins that bind p26 during diapause may also vanish.

This, the first study to examine p26 substrates, yielded 21 proteins that either disappeared to below detection levels or were significantly reduced when p26 was knocked down (Tables 5 & 6) and these proteins are a main focus for further discussion. The potential relationship between each of these proposed p26 substrates

and the formation of diapause cysts, maintenance of diapause, diapause termination and post-diapause growth are considered in detail below.

A group of proteins completely disappeared upon the loss of p26, suggesting that their binding to p26 is required for their preservation, perhaps because they are needed at the same time as p26 for stress tolerance or they are required post-diapause. Examples are RNA granule protein which is induced by stress (Shiina *et al.*, 2005; Solomon *et al*., 2007). Stress granules recruit mRNAs encoding proteins involved in gene expression and suppress their translation, which contributes to stress-induced translational arrest, such as occurs during diapause (Solomon *et al*., 2007; Anderson & Kedersha, 2008, 2009). Also, mitochondrial isocitrate dehydrogenase [NAD] subunit and glutamine dependent NAD (+) synthase, enzymes involved in the TCA cycle and needed for diapause maintenance. The finding of Xu and colleagues (2012) of an increasing amount of mitochondrial isocitrate dehydrogenase [NAD] subunit during diapause in the *cotton bollworm, Helicoverpa armigera* may support such arguments.

Other proteins that disappeared upon the loss of p26 appear to be critical for embryo development, examples being those proteins that modulate protein homeostasis such as elongation factor 1 alpha, (EF-1α) and ubiquitin conjugation factor E4. Previous studies to support the argument that these proteins are required for homeostasis are those that show the presence of EF-1α during post diapause development in *A. salina* (Golub & Clegg, 1968; Clegg & Golub, 1969), and *A. franciscana* (O'Connell *et al*., 2006). Also, U2 snRNP, a component of the ATP-independent spliceosomal complex, catalyzes pre-mRNA splicing upon binding to pre-mRNA (Staley & Guthrie, 1998; Moore *et al*., 1993; Das *et al*. 2000; Wahl *et al*., 2009). Hence, as diapause develops in *A. franciscana* cysts, U2 snRNP is likely to

catalyze mRNA splicing but as ATP becomes limiting, U2 snRNP apparently binds to p26, potentially inhibiting its activity and preserving the protein for use post-diapause. At least one caspase completely disappeared from the A. *franciscana* diapause proteome upon the loss of p26. Caspases mediate programmed cell death (apoptosis) and can be activated by physiological stress (Creagh, 2014) as occurs during diapause. The presence of caspase suggests that apoptosis is required for the development of diapause cysts, possibly for the elimination of cells that if they remained would tend to promote ovoviviparous development rather than encystment and diapause. Disappearance upon the loss of p26 indicates that p26 binds caspase(s) during diapause, suppressing its activity and possibly protecting the protein for use post-diapause.

A second group of putative p26 substrates decreased in amount upon the loss of p26 but did not completely disappear, an indication of their partial dependency on p26 for preservation during diapause. These proteins may be functional before p26 is synthesized, such as during diapause initiation and they may bind p26 for preservation and subsequent use later in development. Within this group, a shell gland specific protein, with no apparent similarity to other known proteins, decreased 80% upon loss of p26. Shell gland specific proteins bind polysaccharides and other proteins participating in formation of the embryonic cuticle layer of the cyst shell which is essential for shell impermeability (Conte *et al*., 1977; Clegg & Conte, 1980; Busa *et al*., 1982). Shell gland specific proteins are synthesized specifically in the cells of the shell gland during the formation of *Artemi*a cysts (Liu *et al*., 2009; Dai *et al*., 2011). Perhaps this shell gland specific protein, which is unlikely to be required after cyst formation is complete, is inhibited upon binding to p26 leading to the cessation of shell formation. A second protein, B0WHJ5 that partially disappeared upon

knockdown of p26 may also be involved in cyst shell formation via a role in the synthesis of chitin, a major component of the cyst shell. BOWHJ5 may bind shell gland specific protein(s) and cooperate in formation of the cyst shell, but like the shell gland specific protein its relationship with p26 remains unsettled.

Vacuolar ATPase, a mediator of proton transport, may be an important regulator of diapause in *A. franciscana* cysts, because the formation of diapause cysts is accompanied by large shifts in intracellular pH (pHi) (Busa *et al*., 1982; Busa & Crowe, 1983; Cove & Hand, 2005). Such shifts in pHi may function as fundamental regulators in the transition between the active and inactive metabolic states in *Artemia* embryos. Up-regulation of V-ATPase H during diapause in locust eggs has been observed (Tu *et al*., 2015). Binding to p26 may represent a way for the regulation of V-ATPase during diapause and also serve to protect this protein for use post-diapause.

Subunit theta and subunit beta of T-complex protein 1, part of the chaperonin HSP60, and HSP70-4 are ATP-dependant molecular chaperones that assist in the folding of nascent and partially denatured proteins and they may sequester denaturing proteins during diapause as ATP declines (Yaffe *et al*., 1992; Frydman *et al*., 1992; Miklos *et al*., 1994; Sanchez *et al*., 1994; Chen *et al*., 2006; Mitra *et al*., 2007; Parrotta *et al*., 2013). These proteins may bind p26 during transition into diapause, a time when ATP is still high and cellular processes such as translation are being inhibited in preparation for diapause, which would result in their preservation in the cyst and their demise if p26 is missing. Moreover, binding to p26 would ensure these proteins are readily available for post-diapause development. Upon stress exposure TCP-1- theta subunit and HSP70-4 tightly binds human sHsps, supporting the idea that p26 binds other molecular chaperones (Mymrikov *et al*. 2017).

Other putative p26 substrates that appear to be important during diapause maintenance because they bind p26 in diapause-destined embryos are G11700 and ubiquitin/ribosomal S27 fusion protein 2, both of which are involved in the ubiquitin system and thus in protein homeostasis, vital during the stress of diapause. Both proteins were reduced 50% upon the loss of p26, suggesting that they may bind p26 in diapause-destined embryos, Ubiquitin/ribosomal S27 fusion protein is unnecessary for protein synthesis in *Arabidopsis thaliana* under normal growth conditions but its loss is lethal under stress (Revenkova *et al*., 1999). Thus it is possible that ubiquitin/ribosomal S27 fusion protein 2 behaves in the same way during the development of *A. franciscana* embryos.

4.3.2.2 Proteins that appeared or were present and increased upon p26 knockdown

The loss of p26 resulted in the appearance or an increase in the amount of 57 proteins, and such changes suggest that p26 suppresses the synthesis of these proteins. p26 loss could cause the breakdown of a transcriptional down-regulator that results in over-expression of unregulated proteins. A summary of the main observations related to these proteins is provided below.

The metabolic activity of newly released *A. franciscana* cysts containing or lacking p26 is similar (King & MacRae, 2012) but many metabolic enzymes changed in amount upon the loss of p26. This is especially true for proteins that appeared upon the loss of p26. Thus, it is possible that p26 modulates metabolism through inhibiting the synthesis of metabolic enzymes. This result agrees with a previous proposal that p26 is a metabolic regulator (Clegg *et al*., 1994; 1995). King (2013) observed that the development of diapause-destined embryos was slowed upon the loss of p26 which

agrees with my observations herein, indicating that p26 does not halt DNA duplication and cell division (Clegg *et al*., 1999). Thus, there must be other inhibitor(s) of development in the cyst diapause proteome that slow cell cycle progression and lead to cell growth inhibition. The findings in this study support such a proposal because several proteins that appeared upon the loss of p26 are involved in embryo development a process that is inhibited during diapause. Examples of these proteins are proteasome 26S subunit, non-ATPase 9, E3 binding protein, 26S proteasome regulatory complex, non-ATPase sub-complex, Rpn1 subunit, Ras-related protein Rab-18, eukaryotic translation initiation factor 3, subunit B and 60S ribosomal protein L6. All these proteins enhance embryo development in one or several ways including effects on oogenesis, embryogenesis, cell cycle regulation, cell growth, gene transcription, protein quality control and protein synthesis (Postlethwait & Giorgi, 1985; Bem *et al*., 2011; Kleiger &Mayor, 2014). When p26 is lost these proteins appeared, an indication that they are synthesized and utilized upon resumption of post-diapause development.

A second group of proteins in the diapause proteome increased when p26 was eliminated by RNAi, an indication that their synthesis could be partially controlled by p26. The up-regulation of these diapause proteome proteins upon the loss of p26 suggests a role in pre- and/or post-diapause development, but they may not enhance diapause maintenance and stress tolerance. Examples within this group are considered below.

Small ribo-nucleoprotein particle U1 subunit 70K, isoform C (snRNP-U1-70K), which increased 7- fold upon the loss of p26, regulates RNA splicing (Krämer, 1995) a process that requires ATP and thus is not likely to occur during diapause. Importin α, which transports proteins into the nucleus, increased three-fold upon the

loss of p26, suggesting that its synthesis is inhibited during diapause by p26, yet it is present in the cyst proteome. Possibly, p26 suppresses the synthesis of both snRNP-U1-70K and importin α during diapause, but once ATP is available and p26 is declining post-diapause RNA splicing and protein transport resume as they are required for subsequent development.

The 40S ribosomal protein S8 (RPS8), a protein also found in diapause and non-diapause eggs of the domesticated silkworm *B. mori* (Fan *et al*., 2013), and required for protein synthesis, increased three-fold upon the loss of p26. RPS8 inhibits the synthesis of other ribosomal proteins by affecting the translation of their mRNA (Dean *et al*., 1981), *In vitro* and *vivo* experiments confirmed that the suppression of protein synthesis and apoptosis requires RPS8 to interact with CDK11p46 (Hao *et al*., 2011) indicating that CDK11p46 controls RPS8, but CDK11p46 has yet to be characterized in *Artemia* so it is unknown if this process occurs in *Artemia*. CDK11p46 should it exist in *Artemia* cysts, may interact with RPS8 and cooperate with p26 to suppress protein translation and apoptosis which are shut down during diapause. Inhibition by p26 may be a way to control the synthesis and thus the activity of such proteins.

To sum up, p26 in cooperation with other regulator(s) may inhibit the synthesis of proteins and when p26 is eliminated these proteins either appear in the diapause proteome or they increase in amount.

4.4 Conclusions

This is the first research providing a global analysis of the *A. franciscana* diapause proteome and characterizing the major protein changes that occur upon knock down of p26, the most abundant, diapause specific protein in cysts, and which

mediates embryo development, diapause maintenance and stress tolerance. Modern proteomic techniques including RNAi, 2D-LC and MS/MS were used to identify proteins in the *A. franciscana* diapause proteome and to determine how p26 affects the proteome. MS analysis yielded 3212 proteins from diapausing *A. franciscana* embryos and demonstrated differences in proteins from cysts containing and lacking p26. Using the PANTHER functional classification system, 1204 proteins within the *A. franciscana* diapause proteome were grouped into 9 categories listed according to their abundance as follows: catalytic, binding, structural, transport, translation, channel regulatory, signal transduction, antioxidant and receptor.

Upon knockdown of p26 the amounts of most proteins were unchanged in the diapause proteome. However, 21 proteins either disappeared or were reduced in amount upon p26 knockdown, suggesting that they are p26 substrates. An additional 57 proteins either appeared in the diapause proteome or increased in amount upon the loss of p26 suggesting that p26, potentially cooperating with other unknown regulator(s), inhibits their synthesis during diapause. Due to the technical difficulty in obtaining diapausing cysts and the high cost of MS analysis, only a single proteomics analysis of one sample generated from several thousand diapausing cysts was performed. MS replicates would strengthen the data set in terms of protein identification and quantification. However, the findings provide further evidence that p26 is crucial during diapause in *A. franciscana*, regulating proteins that affect the formation of diapausing animals and their stress tolerance. Moreover, the research contributes to our understanding of the proteins that p26 influences during diapause induction, maintenance and termination, information that can be applied to other organisms exhibiting this physiological process, thereby benefiting industries such as

aquaculture and agriculture, where the ability of organisms to undertake diapause has important economic consequences.

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APPENDIX A. SOLUTIONS AND RECIPES

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

Solution for 1.2% agarose Gel electrophoresis

0.5 X TBE: 50 mM Tris, 50 mM boric acid, 1 mM EDTA, pH 8.0

Solutions for SDS-polyacrylamide gel electrophoresis

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

1B: 18.3 g Tris, 2.5 ml 20% (w/v) SDS, dH_2O to 100 ml, pH 8.8

1C: 0.2% (v/v) TEMED: 100 µl TEMED, dH_2O to 50 ml

1D: 5% (w/v) ammonium persulfate: 50 *m*g ammonium persulfate. dH_2O to 10 ml

2B: 6.0 g Tris, 2.5 ml 20% (w/v) SDS, dH_2O to 100 ml, pH 6.8

2C: 2% (v/v) TEMED: 100 µl TEMED, dH_2O to 5 ml

12.5% SDS Polyacrylamide Running Gel: 1A, 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 Loading Buffer (diluted four-fold for electrophoresis): 1.2 g Tris 250 mM, 3.2 g SDS 280 mM, 16 ml glycerol 40% (v/v), 8 ml β-mercaptoethanol 20% (v/v), 0.08 g bromophenol blue 0.2% (w/v) & dH_2O to 40 ml, pH 6.8

Running Buffer: 12.0 g Tris 25 mM, 57.6 g glycine, 8.0 ml with 0.04% (w/v) SDS 200 mM & dH_2O to 4 L

Solutions for western blotting

Transfer Buffer: 800 mL methanol 20% (v/v), 12.0 g Tris 25 mM, 57.6 g glycine 200 mM & dH_2O to 4 L)

2% Ponceau S Solution (100 mL):2.0 g Ponceau S, 30 g Trichloroacetic acid & Dilute 1:9 in distilled water

TBS: 1.21 g Tris 10 mM, 8.18 g NaCl 140 mM & dH_2O to 1 L, pH 7.4

8% milk solution (100 mL): 8.0 g no name skim milk powder & 100 mL TBS

TBS-T: 1.21 g Tris 10 mM, 8.18 g NaCl 140 mM, 1 mL 0.1% Tween-20 & dH_2O to 1 L, pH 7.4

HST: 1.21 g Tris 10 mM, 58.4 g NaCl 1M, 5 ml 0.5% Tween-20& dH_2O to 1 L, pH 7.4

Solutions for preparing cell free extract and samples for mass spectrometry

Denaturing buffer: for 1mL: 0.48 g urea 8 M (Sigma-Aldrich), 0.4 mL TEAB 1M (Sigma T7408) & H2O to 1mL

Trypsin solution: 0.02 µg trypsin in 1 µl 50 mM TEAB (Promega, V5113)

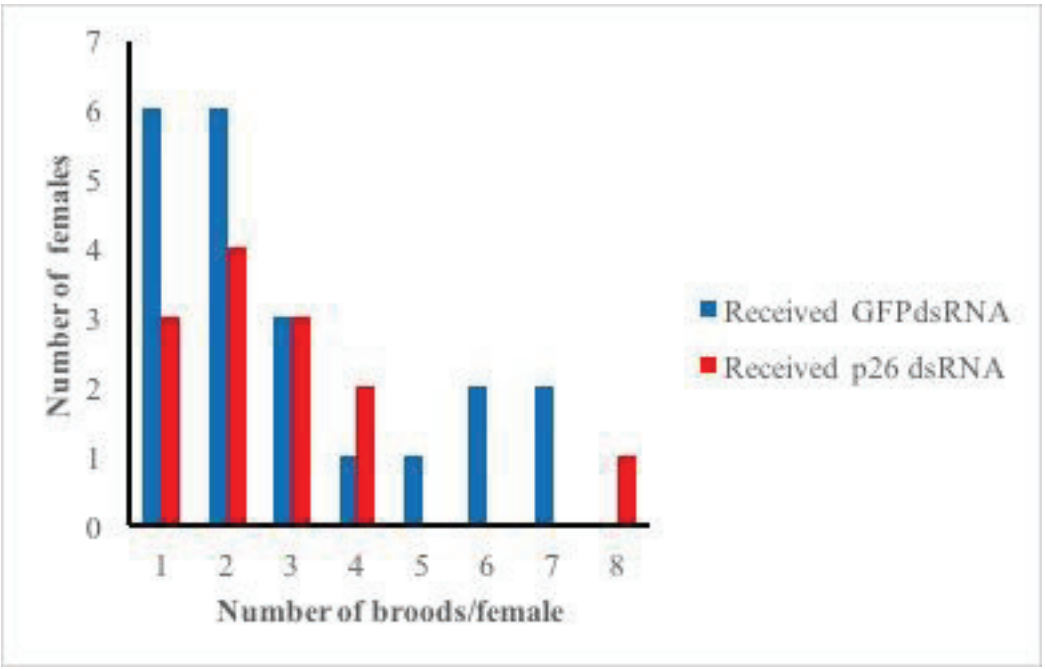
0.5 M DTT:77 *m*g in 1mL

0.7 $IAcNH_2$: 130 *m*g in 1mL

Suspension buffer used in the first LC separation: 5% CAN, 10 mM NH4HCO2, pH 8.0

Suspension buffer used in the second LC separation: 30 µl of 0.3% ACN and 5 µl 0.5% FA in 970 µl MS-grade H_2O, pH 2.0

APPENDIX B. Number of broods for females received *p26* dsRNA comparing with those received *GFP* dsRNA. Fertilized females receiving *GFP dsRNA* generally survived longer and produced more broods than females receiving *p26* dsRNA. On average females that received *p26 dsRNA* produced two broods whereas those received *GFP dsRNA* produced three broods.



APPENDIX C. Determination of protein concentration from cyst extracts. One thousand hydrated cysts were homogenized in varying volumes of buffer and the protein concentration of the extracts was measured. Optimal extraction of cyst protein occurred in 800 µl of buffer.