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RESEARCH ARTICLE

Artemin, a diapause-specific chaperone, contributes to the stress tolerance of *Artemia franciscana* cysts and influences their release from females

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ABSTRACT

Females of the crustacean *Artemia franciscana* produce either motile nauplii or gastrula stage embryos enclosed in a shell impermeable to nonvolatile compounds and known as cysts. The encysted embryos enter diapause, a state of greatly reduced metabolism and profound stress tolerance. Artemin, a diapause-specific ferritin homolog in cysts has molecular chaperone activity *in vitro*. Artemin represents 7.2% of soluble protein in cysts, approximately equal to the amount of p26, a small heat shock protein. However, there is almost twice as much artemin mRNA in cysts as compared with p26 mRNA, suggesting that artemin mRNA is translated less efficiently. RNA interference employing the injection of artemin double-stranded RNA into the egg sacs of *A. franciscana* females substantially reduced artemin mRNA and protein in cysts. Decreasing artemin diminished desiccation and freezing tolerance of cysts, demonstrating a role for this protein in stress resistance. Knockdown of artemin increased the time required for complete discharge of a brood of cysts carried within a female from a few hours up to 4 days, an effect weakened in successive broods. Artemin, an abundant molecular chaperone, contributes to stress tolerance of *A. franciscana* cysts while influencing their development and/or exit from females.

KEY WORDS: Artemin, Molecular chaperone, Diapause, Stress tolerance, *Artemia franciscana*

INTRODUCTION

Diapause, which is most common in insects (Poelchau et al., 2013; Mizoguchi et al., 2013; Fan et al., 2013) but occurs in organisms as diverse as rotifers (Clark et al., 2012), tardigrades (Guidetti et al., 2011), crustaceans (Tarrant et al., 2008; King and MacRae, 2012; Clark et al., 2013), killifish (Meller et al., 2012) and mammals (Murphy, 2012; Cha et al., 2013), entails decreased metabolism and augmentation of stress tolerance (Denlinger, 2002; Koštál, 2006; MacRae, 2010; Hahn and Denlinger, 2011). Photoperiod, temperature and crowding induce diapause, an integral part of the life cycle of many animals (Xiao et al., 2010; Meuti and Denlinger, 2013). Diapause involves several phases (Koštál, 2006), starting with initiation when gene expression is altered and accumulation of sugars and lipids may occur. Initiation is followed by maintenance, with metabolism generally lower and stress resistance higher than in the rest of diapause. Termination, the final phase of diapause, is triggered by species-specific signals including temperature change and desiccation (Clegg and Trotman, 2002; Robbins et al., 2010). If termination takes place in unfavorable conditions, a post-diapause dormancy termed quiescence follows, but with appropriate temperature, sufficient water and aeration, growth resumes. Diapause is usually limited to one life stage per organism which displays varying degrees of metabolic suppression and stress tolerance, occurring in embryos of the silkworm, *Bombyx mori* (Moribe et al., 2010), larvae of the moth *Sesamia nonagrioides* (Gkouvitsas et al., 2009a; Gkouvitsas et al., 2009b), pupae of the flesh fly *Sarcophaga crassipalpis* (Li et al., 2007) and adults of the mosquito *Culex pipiens* (Kim et al., 2010).

Ovoviviparously developing embryos of *Artemia franciscana* Kellogg 1906 emerge from females as swimming nauplii, whereas oviparous development yields gastrulae encased in chitinous shells, termed cysts (Jackson and Clegg, 1996; Liang and MacRae, 1999; MacRae, 2003; Ma et al., 2013) (Fig. 1). Cysts exiting females enter diapause, where metabolism is greatly diminished and stress tolerance is extreme, including resistance to repeated freezing and thawing, desiccation, heat and years of anoxia (Clegg et al., 1996; Clegg, 1997; Clegg et al., 2000; Clegg, 2005; King and MacRae, 2012; King et al., 2013). During development of diapause-destined *A. franciscana* embryos, at least three small heat shock proteins (sHsps) are produced (Jackson and Clegg, 1996; Liang and MacRae, 1999; Qiu et al., 2007; Qiu and MacRae, 2008a; Qiu and MacRae, 2008b). The sHsp p26, upregulated during embryo development, contributes to the profound stress tolerance of *A. franciscana* cysts and modulates embryo behavior (Liang and MacRae, 1999; Villeneuve et al., 2006; King and MacRae, 2012), whereas ArHsp21 contributes little, if any, to stress tolerance and development (King et al., 2013). A fourth diapause-specific molecular chaperone called artemin is found in diapause-destined embryos and cysts (Warner et al., 2004; Tanguay et al., 2004; Chen et al., 2007; Hu et al., 2011; Clegg, 2011).

Artemin, an abundant, ATP-independent, diapause-specific molecular chaperone shown to protect transfected mammalian cells from stress and prevent protein denaturation *in vitro*, is a ferritin homolog (Chen et al., 2003; Warner et al., 2004; Chen et al., 2007; Hu et al., 2011; Shirzad et al., 2011; Shahangian et al., 2011). Artemin assembles oligomers of ~600–700 kDa formed from 24 monomers, each ~26 kDa (De Graaf et al., 1990; Chen et al., 2007; Clegg, 2011; Hu et al., 2011). Artemin fails to bind iron because the carboxyl terminal extension of protein monomers thought to have a role in chaperoning (Shirzad et al., 2011) fills the oligomer cavity which is equivalent to the space where iron resides in ferritin. Additionally, artemin lacks all but a single residue of the di-iron ferroxidase center employed by ferritin in iron binding (Harrison and Arosio, 1996; Chen et al., 2003; Chen et al., 2007; Crichton and Declercq, 2010). Artemin is very thermotolerant, surviving at 90°C for 30 min, and heated artemin binds non-polyadenylated RNA (Warner et al., 2004). High stability, chaperone activity *in vitro*, and abundance suggest that artemin contributes to cyst stress tolerance,

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List of abbreviations

dsRNA	double-stranded RNA
GFP	green fluorescent protein
RNAi	RNA interference
sHsp	small heat shock protein

a proposal supported herein. Moreover, the knockdown of artemin extended the time for a brood of cysts to exit a female from hours to days, implying a role for this novel, species-specific molecular chaperone in embryo development and/or cyst release.

RESULTS

Quantitation of artemin mRNA and protein in *A. franciscana* cysts

Artemin mRNA was almost twofold higher in amount than was p26 mRNA and much higher than ArHsp21 mRNA (Fig. 2). Copy numbers for p26 and ArHsp21 mRNAs determined herein were consistent with previous results (King et al., 2013). In spite of the 2-fold difference in mRNAs for artemin and p26, both proteins represented approximately 7% of soluble cyst protein (Fig. 3). Values for p26 and ArHsp21 protein abundance were generated previously (King et al., 2013) and are included to facilitate comparison of these diapause-specific sHsps with artemin.

Knockdown of artemin mRNA and protein in *A. franciscana* cysts

Artemin cDNA and double-stranded RNA (dsRNA) were of the expected size, with the dsRNA slightly larger than the cDNA (Fig. 4A). As determined by RT-PCR the injection of *A. franciscana* females with dsRNA completely knocked down artemin mRNA in cysts (Fig. 4B). Performing PCR in the absence of reverse transcriptase demonstrated that cyst mRNA was not contaminated with DNA. The injection of females with either dsRNA for green fluorescent protein (GFP) or control solution had no effect on artemin in cysts (Fig. 4C, lane 1), whereas injection of artemin dsRNA resulted in almost complete knockdown with very small amounts of artemin detectable on western blots (Fig. 4C, lane 2).

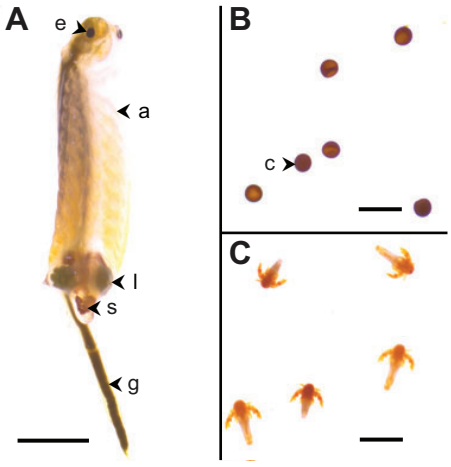

Fig. 1. Life history stages of *Artemia franciscana*. Light micrographs of an *A. franciscana* adult female immobilized on cold 5% agar (A), cysts in seawater (B) and nauplii immobilized in seawater with Lugol's solution (Sigma-Aldrich) (C). a, appendage; c, cyst shell; e, eye; g, gut; l, lateral egg sac containing oocytes; s, shell gland. Scale bars, 1 mm (A,C) and 0.5 mm (B).

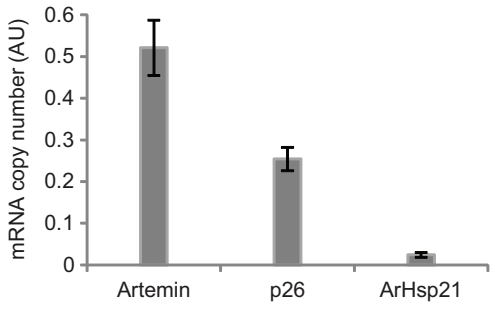

Fig. 2. Quantitation of artemin mRNA in *A. franciscana* cysts. RNA extracts from commercially obtained *A. franciscana* cysts were reverse transcribed and the copy numbers of artemin, p26 and ArHsp21 were determined by qPCR as described in Materials and methods. The ratios of chaperone mRNA copy number to α-tubulin were averaged for three independently prepared cyst RNA extracts. AU, arbitrary units. Error bars indicate ±s.d.

Artemin contributes to cyst stress tolerance

The hatching of cysts after diapause termination by desiccation and freezing, used as a measure of cyst viability and stress tolerance although it was not possible to know with certainty whether non-hatched cysts were in fact dead, was reduced when artemin was

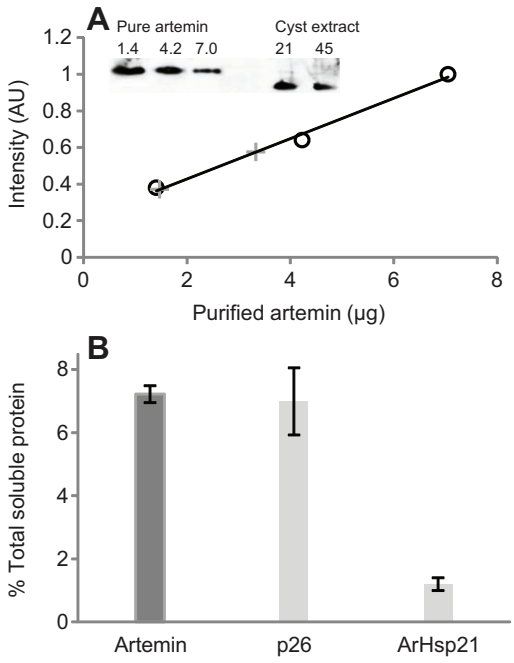

Fig. 3. Quantitation of artemin protein in *A. franciscana* cysts. (A) Purified artemin produced in *Escherichia coli* and protein extracts from *A. franciscana* cysts were resolved in 12.5% SDS polyacrylamide gels and blotted to nitrocellulose. Membranes were probed with antibody to artemin followed by HRP-conjugated goat anti-rabbit IgG antibody. Visualization of immune-reactive proteins was by chemiluminescence (insets, representative results) and the fluorescence intensity of each band was determined with ImageJ. The values obtained for purified artemin (open circles) were curve-fitted with a linear regression and the proportion of soluble cyst protein represented by artemin was determined from the fluorescence intensity of cyst extract (+). (B) The proportion of total soluble protein composed of artemin was calculated from fluorescence data and averaged for each cyst sample. Data for p26 and ArHsp21 were previously determined (King et al., 2013) and are included to facilitate comparison. The experiment was performed in triplicate with independently prepared cyst extracts. Error bars represent ±s.d.

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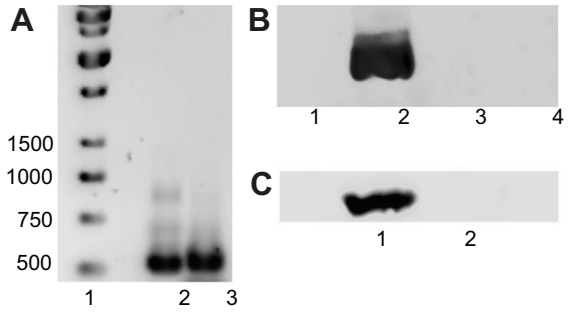

Fig. 4. Knockdown of artemin RNA and protein by RNAi. (A) cDNA and dsRNA specific to artemin were resolved by electrophoresis in 1.2% agarose gels and visualized with Gelstar®. Lane 1, Generuler 1 kb DNA size markers; 2, cDNA used to generate dsRNA; 3, artemin dsRNA. Marker size on the left is in bp. (B) RT-PCR was performed using RNA from 34 cysts released by females injected with artemin dsRNA (1, 3) and control solution (2, 4). RNA samples were incubated with (1, 2) and without (3, 4) reverse transcriptase. Electrophoresis was in 1.2% agarose and gels were stained with Gelstar®. (C) Protein extracts from 42 cysts produced by females injected with either dsRNA for GFP (1) or dsRNA for artemin (2) were resolved in 12.5% SDS polyacrylamide gels, blotted to nitrocellulose and probed with antibody to artemin. Proteins were visualized by chemiluminescence.

lowered by RNA interference (RNAi). Of cysts containing normal and reduced amounts of artemin, 73% and 43%, respectively, survived diapause termination by desiccation and freezing (Fig. 5). Nauplii arising from cysts with normal and reduced artemin attained sexual maturity and the adults reproduced normally.

Artemin knockdown extends the time for complete release of cyst broods

Artemia franciscana females, subsequent to injection with GFP dsRNA or control solution and thus containing normal amounts of artemin, exhibited typical behavior and at 5 days post-fertilization complete broods of cysts were released from females in a few hours. However, when artemin was knocked down, the exit of a complete brood of cysts took on average ~2.6 days from discharge of the first cyst (Fig. 6). The extension of release time was most pronounced for the first brood of cysts and diminished with successive broods (Fig. 6). Unlike the situation with loss of p26, the rate of embryo development, determined as the time after fertilization to liberation of the first cyst in a brood, was similar for embryos with normal and reduced artemin (P=0.94), with

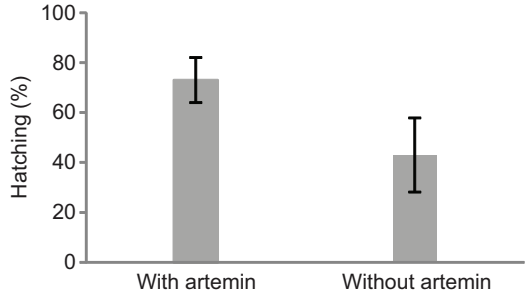

Fig. 5. Artemin maximizes stress tolerance in *A. franciscana* cysts. Diapause in *A. franciscana* cysts either containing or lacking artemin was terminated by desiccation and freezing. The cysts were incubated in seawater, hatched nauplii were counted and the percentage of viable cysts corresponding to hatched nauplii was plotted. The experiment was performed in duplicate and the error bars indicate ±s.e.m.

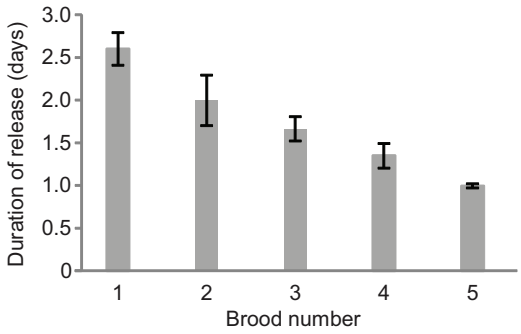

Fig. 6. Artemin is required for the synchronous liberation of cysts from females. Successive releases of cyst broods from females injected with dsRNA specific to artemin were tracked and the time from release of the first to the last cyst in each brood was recorded. The data from five females were pooled and the average release time for each successive brood is graphed. The experiment was performed in triplicate and the error bars indicate ±s.e.m.

discharge from females initiated at ~5 days post-fertilization (not shown). Cysts with reduced artemin incubated 90 days in seawater at room temperature and then flushed into hexagonal weigh boats failed to hatch, a result contrasting that obtained with cysts lacking p26 (King and MacRae, 2012). Control cysts containing artemin also failed to hatch.

DISCUSSION

Artemin is a heat-tolerant, cysteine-enriched, diapause-specific protein found in *A. franciscana* cysts that protects other proteins from stress-induced denaturation *in vitro* (Chen et al., 2007; Hu et al., 2011; Shirzad et al., 2011; Shahangian et al., 2011) and binds non-polyadenylated RNA when heated (Warner et al., 2004). Assuming that artemin and p26 transcripts are equally available within the cytoplasm for translation, then artemin mRNA is translated less efficiently than p26 mRNA, a conclusion occasioned by the twofold difference in the amount of mRNAs but similar abundance of their protein products. As determined by immunoprobing of western blots, artemin constitutes 7.2% of the total soluble protein in cyst extracts, a value lower than an earlier estimate of 12% (Warner et al., 2004). Variation in the reported amounts of artemin is likely due to the use of different methods for quantitation.

Artemin exhibits chaperone activity in turbidimetric assays and in transfected mammalian cells (Chen et al., 2007), but the function of this protein had not been examined *in vivo*, hence the use of RNAi in the present study. Artemin mRNA was not detectable in cysts after injection of *A. franciscana* females with dsRNA, but probing of western blots unexpectedly revealed a small amount of residual artemin. This finding differs from those for p26 and ArHsp21, which are not detectable on blots following knockdown by RNAi (King and MacRae, 2012; King et al., 2013). After breakage of diapause by desiccation and freezing, more cysts with normal, as opposed to reduced, amounts of artemin hatched upon incubation in seawater. These data demonstrated that artemin contributes to cyst stress tolerance, in agreement with data obtained *in vitro* (Chen et al., 2007; Hu et al., 2011; Shirzad et al., 2011; Shahangian et al., 2011). Approximately 40% of cysts with reduced artemin hatched after desiccation, freezing and incubation, whereas knockdown of p26 almost completely eliminates post-diapause cyst hatching under the same conditions (King and MacRae, 2012). p26 appears, therefore, to have a greater role in stress resistance during diapause

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than does artemin, even though their chaperone activities are similar *in vitro* (Sun et al., 2004; Sun and MacRae, 2005; Sun et al., 2006; Chen et al., 2007). In contrast, artemin exerts a greater effect on stress tolerance than does ArHsp21 (King et al., 2013). The respective contributions of artemin and p26 to stress tolerance do not reflect the amounts of these proteins in cysts as they are equally abundant. The dissimilarity in stress resistance conferred by each protein may mean that artemin and p26 protect different proteins in cells, some of which are more important during diapause than others. Whatever the case, p26, artemin and ArHsp21 clearly have different physiological and developmental functions encompassing processes in addition to stress tolerance. Nevertheless, these molecular chaperones may work in concert to protect organisms during diapause, possibly by binding different populations of substrate macromolecules or by functioning as an integrated complex of effector molecules.

The discharge of cysts with diminished artemin started on the fifth day post-fertilization, the normal time for initiation of cyst exit from females under the culture conditions employed. A brood of cysts is usually completely liberated within hours of the time the first cyst departs the female; however, when artemin is reduced the average time for total brood release is prolonged to ~2.5 days. There is a downward trend in the extension of release times between successive broods of cysts from the same female, implying the renewed production of artemin, but this was not examined. Artemin synthesis, should it occur in successive broods, would contradict the situation for p26, which is not produced by cysts in the first four broods released by females after injection of dsRNA for p26. The prolonged expulsion of cysts in a single brood indicates that artemin is required for the normal exit of cysts from females and suggests that artemin influences the synchronization of embryo development. That is, cysts with reduced and perhaps varying amounts of artemin as a consequence of knockdown develop at different rates, and those taking the most time to reach maturity are the last to leave the female. Cysts produced in females injected with dsRNA for artemin are morphologically similar to one another and to cysts produced in animals that receive either dsRNA for GFP or control solution. The cyst shell (Liang and MacRae, 1999; Dai et al., 2011; Liu et al., 2009) appears to be assembled in developing embryos with reduced artemin at the same time as embryos with abundant artemin. Thus, shell formation does not seem to be a factor in the protracted release of cysts with reduced artemin. Because artemin is an RNA binding protein, RNA normally sequestered may be free in the cytosol when artemin is limited, and these RNAs interfere with development and slow the liberation of cysts. p26 is the only other molecular chaperone known to affect the development of diapause-destined embryos. The loss of p26 delays release of the first cyst from a brood, but all cysts within a brood exit within hours of the time the first cyst is discharged (King and MacRae, 2012).

To summarize, the use of RNAi methodology demonstrated that artemin contributes to stress tolerance in *A. franciscana* cysts, an observation supported by its abundance and stability and the results from several published experiments performed *in vitro*. When artemin is knocked down, the time for complete release of a cyst brood is extended, showing that artemin modulates the discharge of cysts from females, and perhaps influences embryo development by as yet undetermined mechanisms.

MATERIALS AND METHODS

General procedures

The culture of *A. franciscana* in seawater, injection of *A. franciscana* females with 60–80 ng of dsRNA, diapause termination by desiccation and freezing, evaluation of cyst stress tolerance and monitoring of cyst hatching were described previously (King and MacRae, 2012; King et al., 2013).

Quantitation of artemin and sHsp mRNAs in *A. franciscana* cysts

mRNAs for artemin and the sHsps p26 and ArHsp21 in commercially obtained *A. franciscana* cysts (INVE Aquaculture, Inc., Ogden, UT, USA) were quantitated using the QuantiTect® SYBR® Green PCR Kit (Qiagen, Mississauga, ON, Canada) in a Rotor-Gene RG-3 (Corbett Research, Sydney, Australia) with 0.5 µl cDNA and primers for α-tubulin, artemin, ArHsp21 and p26 (Table 1) at 10 µmol l^{-1}. Each cDNA copy number was determined from a standard curve produced in Rotor-Gene 6 software (Corbett Research). Each standard curve of cycle threshold (C_t) values was prepared using a range of known concentrations of DNA fragments representing each protein of interest (King et al., 2013). The copy numbers of artemin, ArHsp21 and p26 were normalized against α-tubulin and these values were plotted. Melting curve analysis was performed for each experiment to assess primer fidelity. mRNA for each molecular chaperone was quantitated in three independently prepared samples from cysts.

Quantitation of artemin in *A. franciscana* cysts

Artemin was quantitated in *A. franciscana* cysts as described for sHsps (King et al., 2013) except that purified, bacterially produced artemin was used to generate standard curves. To prepare artemin, the 6xHN-tagged prokaryotic expression vector pPROTet.E133 (BD Biosciences Clontech, Mississauga, ON, Canada) containing a full-length artemin cDNA insert

Table 1. Primers used for RT-PCR, qPCR and dsRNA synthesis

	Primer (5′ to 3′)	
cDNA amplified	Forward	Reverse
qPCR		
Artemin	AGATGCCTTTTCCCATTGTG	CTTGTGAACCGATGCAGTGT
p26[a]	GCACTTAACCCATGGTACGG	TCATCAGCTGTGTCCCTCAA
ArHsp21[a]	TGTTCAAAGCGTTACCATCG	CATCAGATGTTTTCTGCTCGC
α-tubulin[a]	CGACCATAAAAGCGCAGTCA	CTACCCAGCACCACAGGTCTC
dsRNA production		
Artemin	TAATACGACTCACTATAGGGAG ATGCATCGGTTCACAAGGA	TAATACGACTCACTATAGGGAG ATCAAGGCACGATTGATGG
GFP[b]	TAATACGACTCACTATAGGGAG ACACATGAAGCAGCACGACTT	TAATACGACTCACTATAGGGAG AAGTTCACCTTGATGCCGTTC
RT-PCR		
Artemin	TCTATGCCAGACGCCAAGGCTG CCGTGG	CCACGGCAGCCTTGGCGTCTCT GGCATAGAA

All primers were from Integrated DNA Technologies (Coralville, IA, USA). The T7 promoter is shaded.
[a]King et al., 2013.
[b]Zhao et al., 2012.

(Chen et al., 2007) was transformed into *Escherichia coli* BL21PRO (BD Biosciences Clontech) and induced with anhydrotetracycline (BD Biosciences Clontech) at 100 ng ml^{-1} for 8–16 h (Hu et al., 2011). Recombinant artemin was purified from bacterial homogenates on BD TALON metal affinity columns (BD Biosciences) (Hu et al., 2011) and protein extracts were prepared from commercially obtained *A. franciscana* cysts (Liang et al., 1997). Varying, but known, amounts of purified artemin and cyst extract protein were resolved in 12.5% SDS polyacrylamide gels, transferred to nitrocellulose membranes and reacted simultaneously with antibody to artemin (Chen et al., 2007) diluted 1:20,000 in 10 mmol l^{-1} Tris containing 140 mmol l^{-1} NaCl, pH 7.4 (TBS), followed by HRP-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich, Oakville, ON, Canada) (King et al., 2013). Visualization of immune reactive proteins and analysis of fluorescence intensity were as described previously (King et al., 2013).

Preparation of artemin dsRNA

The prokaryotic expression vector pPROTet.E133 (BD Biosciences Clontech) containing a full-length artemin cDNA was harvested with a miniprep kit from overnight cultures of *E. coli* BL21PRO (BD Biosciences Clontech) (Chen et al., 2007). cDNAs were amplified by PCR using Platinum® Taq DNA polymerase (Invitrogen, Burlington, ON, Canada) and forward and reverse primers containing the T7 promoter (Table 1). The PCR reaction conditions were 5 min at 94°C, 30 cycles of 94°C for 30 s, 61°C for 30 s and 72°C for 1 min, followed by 10 min at 72°C. The PCR products were employed as templates in 3 h incubations with primers containing the T7 promoter (Table 1) to generate dsRNA using the MEGAscript® RNAi kit (Ambion Applied Biosystems, Austin, TX, USA). PCR products were visualized in a DNR Bio-imagining Systems MF-ChemiBIS 3.2 gel documentation system after electrophoresis in 1.2% agarose gels in 0.5× TBE buffer (50 mmol l^{-1} TRIS, 50 mmol l^{-1} boric acid and 1 mmol l^{-1} EDTA, pH 8.0) and staining with Gelstar® (Lonza, Basel, Switzerland).

dsRNA specific to GFP was generated using GFP cDNA cloned in the vector pEGFP-N1 as template (Clontech, Mountain View, CA, USA) and forward and reverse primers containing the T7 promoter (Table 1) (Zhao et al., 2012). The conditions described above were used to produce GFP dsRNA, except the annealing temperature was 60°C and $MgCl_2$ was 25 mmol l^{-1}.

Detection of artemin mRNA and protein in cysts after RNAi

RNA was prepared from 34 hydrated *A. franciscana* cysts produced in females injected separately with dsRNA for artemin and GFP and with control solution [elution solution from the dsRNA kit mixed 1:1 (v/v) with 0.5% Phenol Red in Dulbecco's NaCl/Pi (Sigma-Aldrich, Oakville, ON, Canada)] using the RNeasy kit (Qiagen). cDNA was synthesized with Superscript III® reverse transcriptase and oligo-dT primers according to the manufacturer's instructions (Invitrogen), except the transcriptase was refreshed and incubation was extended 1 h. The resulting cDNA was amplified using forward and reverse primers for artemin (Table 1) with reaction conditions of 94°C for 5 min, 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, followed by 10 min at 72°C. PCR products were visualized as described above.

Cell-free protein extracts from 42 cysts produced in females injected separately with dsRNA for artemin and GFP and with control solution were resolved in SDS polyacrylamide gels and transferred to nitrocellulose. Membranes were incubated for 20 min with antibody to artemin (Chen et al., 2007) diluted 1:20,000 in TBS and then washed, exposed to HRP-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich), washed again, and visualized with a DNR Bio Imaging Systems MF – Chemi BIS 3.2 gel documentation system (Montreal Biotech, Montreal, QC, Canada).

Competing interests

The authors declare no competing financial interests.

Author contributions

All of the authors contributed to the conception, design and execution of the experiments, interpretation of the findings published, and drafting and revising the article.

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