Genetic Transformation of *Artemia franciscana* by Electroporation

A thesis presented by

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Alberto Constantino Rodriguez-Sanchez
Monterrey Nuevo León, May 25, 2018

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Dedication

Dedicated to God,
for all I have and can be

Dedicate to my parents, brothers and sister
you give those wings and I promise I will come back
Acknowledgements

To my parents for the opportunity to study in Monterrey their leading and their constant support over my live.

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To CONACyT for financial support, without which my stay in Monterrey would be impossible or at less very difficult
Genetic Transformation of *Artemia franciscana* by Electroporation

by

Alberto Constantino Rodriguez-Sanchez

Abstract

*Artemia franciscana* also known as brine shrimp, and sea monkey is a halophilic crustacean used in aquaculture as living food and had a little role in research as toxicological model. Despite its easy handling and availability, Artemia barely has genetic engineering. The objective of this work is to develop a protocol for the genetic transformation of *Artemia franciscana* using electroporation and diapause cysts. Decapsulated cysts were electroporated with exponential decay voltage and square wave pulse, the efficiency was calculated. The effect of the plasmid DNA used, and the voltage were evaluated. Square wave pulse shows a better efficiency compared to the exponential. The concentration of DNA has no effect on the efficiency of transfection. Voltage at one level (1000 V·cm⁻¹) had the best efficiency of transfection, but also the worst hatching. With protocol and the information generated, it will be possible to evaluate genetic regulatory elements to the future develop of a tool box for genetic manipulation.

Keywords: *Artemia* – *Artemia franciscana* – Genetic Manipulation
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Chapter 1 Introduction

*Artemia* genus is worldwide distributed in salty lakes with low predators and competitors (Robbins et al., 2010). Salt concentration in lakes, in contrast with sea, changes over season, for this reason *Artemia* genus develop adaptative strategies to survive.

Brine shrimp can survive in high salt concentration only tolerable for some bacteria and microalgae, it is possible due to its osmoregulatory system (Bartolomé, 2004; Jimenez, Gelabert, & Brito, 2006; Pacheco, 2011). This characteristic, combine with exceptional oxygen capacity of hemoglobin (Dhont & Sorgeloos, 2002) make possible to adult brine shrimp survive from 10 to 300 g/L of NaCl and 6 to 35 °C (Sarabia, 2002).

1.1 Biological and ecological development adaptation

The most relevant characteristic of *Artemia* is its ability to produce cystic eggs in extreme conditions (Sarabia, 2002). Like other invertebrate, *Artemia* are ovoviviparous, females can release swing larvae or cysts (Robbins et al., 2010). Cysts can resist a variety of extreme temperatures, -271°C for several hours and 60-70°C for 5-9 hours, and desiccation for several years (Bartolomé, 2004; Cisneros Burga, 2002; Cohen, 2006; Sarabia, 2002). The cyst has a semi-permeable shell, capable of absorb 200% of water and is responsible of its stress resistance (Anderson, Lochhead, Lochhead, & Huebner, 1970). Cysts accumulate several chaperons in order to prevent apoptosis and protein denaturalization (Qiu, Bossier, Wang, Bojikova-Fournier, & MacRae, 2006; Qiu, Tsoi, & MacRae, 2007; Robbins et al., 2010) This mechanism makes possible the specie preservation. In summer, salty lakes bodies increase its salinity killing the adult Artemia but cyst enter in a diapause stage and can survive several years (Bartolomé, 2004; Cisneros Burga, 2002; Sarabia, 2002).

1.2 *A. franciscana* life cycle

1.2.1 Cyst

The most notable characteristic of *Artemia* genus is its ability to produce both, swing larvae or diapause cyst in response to environment conditions (e.g. salt concentration, food availability) (Cohen, 2006). Diapause cysts has three layers: the first one (known as shell or chorion) is an alveolar layer consisting of lipoproteins and chitin, this layer can be from pale to brown color due to haematin concentration. This layer is the principal responsible of cyst
resistance and can be removal with hypochlorite (Van Stappen, 1996). The second layer is an outer cuticular membrane impermeable to lead, this layer is the limit between chorion and the last layer, the inner cuticular membrane, followed by a fibrous layer of the embryonic cuticle (Clegg & Trotman, 2002) (Fig 1.1).

**Figure 1.** Ultrastructure of a post-diapause (activated) encysted embryo of *A. franciscana*. The panels to the right illustrate major organelles. L (lipid droplets), N (nuclei), yp (yolk platelets), gly (glycogen), M (mitochondria), * (inner embryonic cuticle) (Modified from Clegg et al. (1999))

Dry cysts are the most common commercialization presentation. In this form, cyst only has 10% of water, but due to its high hydroscope, can absorbed 200% of water, (Van Stappen, 1996).
1.2.2 Nauplii (Oviparity and ovoviviparity)

*Artemia franciscana* nauplii can be born from a female brine shrimp or a diapause cyst. Independently of its origin (oviparity or ovoviviparity), *A. franciscana* nauplii has the same morphological structure, but significant biochemical differences (Clegg & Trotman, 2002).

Nauplii development is divided into stages known as instar, each one is divided in 15 molts. Nauplii instar II (8 hrs approx.) can filter small food particles of 1 to 50 µm (Van Stappen, 1996).

Nauplii instar I is the most used for feeding of larvae shrimp and fish due to high content of lipids (Patrick Sorgeloos, Lavens, Lè, Tackaert, & Versichele, 1996). Instar II is commonly used for bioencapsulation. Instar III and posterior stages are less used due to its high mobility that makes them harder to hunt.

![Figure 2. Nauplii Instar I (left) and Instar V (right)](image)

1.2.3 Adult

Full development of brine shrimp takes a month or eight days in optimal conditions. In this state the males and females are clearly distinguishable. The males have antennas that allow them to hold to females during mating. The females lack antennas and have a sac where they keep the oocytes.
1.2.4 Reproduction

Upon reaching maturity, the male performs copulation with the females clinging to them using their antennas in a position called “riding position”. Male and female swing synchronized. Females can release swimming nauplii under favorable environmental conditions. In extreme salinity conditions the embryonic development stops at gastrula stage and the formation of the chorion begins. In hypersaline conditions the cysts float to the shore and dry (Patrick Sorgeloos et al., 1996).
1.3 Use of *Artemia ssp.* in aquaculture

*Artemia* ssp., specially *Artemia franciscana* has an important role in aquaculture, and a less known role in salt production. In aquaculture, *Artemia* ssp. has an important role as living and fresh food. In its cysts state, it can be transported without special requirements. Cysts can be rehydrated and develop to nauplii stage in around 20 hrs.

*Artemia* nauplii are appreciate as living food, due to its high content of acid fatty eicosapentaenoic acid (EPA: 20:5n-3) and docosahexaenoic acid (DHA: 22:6n-3) (Van Stappen, 1996). A common practice is feeding *Artemia* with highly unsaturated fatty acids to increase its essential fatty concentration. This practice is called bioencapsulation, *Artemia* enrichment or boosting. It is possible due to no-selective filter feeding. Enrichment *Artemia* increase the survive of different species in larvae stage like shrimps (Seenivasan, Bhavan, Radhakrishnan, & Shanthi, 2012) (Immanuel & Palavesam, 2001), fish (Villalta, Estévez, Bransden, & Bell, 2005) and octopus (Okumura, Kurihara, Iwamoto, & Takeuchi, 2005), due to its fatty acid content.

*Artemia* has been enriched with different compound. Oil from different source (P. Sorgeloos, Dhert, & Candreva, 2001) and characteristics (Villalta et al., 2005) is the most common and show an improve in the survive of different marine species in early development stages (Immanuel & Palavesam, 2001; P. Sorgeloos et al., 2001; Villalta et al., 2005) Probiotic microorganisms, like *Lactobacillus sporogenes* has been used too showing similar results (Seenivasan et al., 2012).

1.4 Disease impact in aquaculture

In Mexico, retail Sales of Fish and Seafood was $56736.3 million MXN in 2017, $ 9118.3 million were crustaceans (Euromonitor International, 2018). Crustaceans have been the most cultivated in aquaculture, followed by fish (Food and Agriculture Organization of the United Nations, 2017). In 2013, a demand for more than 80% of shrimp production was reported due to Acute Hepatopancreatic Necrosis Disease (AHPND) caused by vibrio infections (Ibarra Gámez, 2015). Currently, infections in fish and shrimp by vibrio and other bacteria are prevented with strict controls and treated with antibiotics.
Due to growing concern about antibiotics used in meat, several companies have chosen to use antibiotic-free products. This trend has reached aquaculture. One case is the supposed
change of Chilean salmon by Norwegian salmon. Aquaculture in Chile uses many antibiotics contrary to Norway (Baroke, 2015).

The antibiotics used in aquaculture are administered directly in the pond, this produces an enormous chemical contamination. An alternative is the use of vehicles for drugs in food or others. A transgenic line of *Artemia* capable of produce antimicrobial molecules could be a solution.

### 1.5 Transgenesis of *Artemia* ssp.

Transgenic transformation of *Artemia* ssp. is uncommon. The first report of Artemia transformation occurred in 1995. Gendreau, Lardans, Cadoret, and Mialhe (1995), report the transient expression of luciferase in *A. franciscana*, using gen gun. The authors proposed Artemia as a good model due to “the increasing availability of relevant genetic data and its overall importance in aquaculture as a food”. Gendreau et al. use luciferase as report gen under the control of heat shot protein of 70kDa (*hsp70*) promoter from *Drosophila melanogaster*, *hsp70* promoter was used because it effective in a large range of organism. Luciferase expression can be detected 12 and 24 hrs before the bombardment but can be detected 48 hrs.

Beside gene gun, electroporation was used for the transgenesis of *Artemia* ssp. Chang, Lee, Chen, Lee, and Tsai (2011) used electroporation for the production of an *Artemia sinica* transgenic line. This line produces yellowfin porgy growth hormone (*ypGH*) from *Oncorhynchus tshawytscha*, under the control of a hybrid promoter between cytomegalovirus (CMV) and β-actin promoter from *Oryzias latipes*. After four generations, *A. sinica* still produce functional ypGH in zebra fish.

Jung and Kim (2013) test the use of β-galactosidase gen (*lacZ*) as a reporter gene using gen gun. *lacZ* under the control of Rous Sarcoma Virus Long Terminal Repeat Promoter (promoter RSV). The β-galactosidase was detected in all live stages and can be detected with PCR. Despite the potential of *lacZ* as a reporter gen, it is unsuitable to distingue between endogenous and exogenous activity due to β-galactosidase activity in *Artemia*.

Jheng et al. (2015), is the most recent article about *Artemia* transformation, Artemia sp. from Great Salt Lake was used as delivering of epinecidin-1, an antimicrobial peptide from *Epinephelus coioides*. Jheng et al. (2015) produce a transgenic *Artemia* with enhanced green fluorescent protein (EGFP)-epinecidin-1 fusion gene under the control of CMV.
promoter. Transgenic *Artemia* was offered to zebra fish as food and challenge to *Vibrio vulnificus*. This treatment increases in 70% the survive rate and reduce the bacterial number in liver and intestine.

1.6 Electroporation

Electroporation is a technic that use electric fields in a sample, electric field intensity depends of propose. The electric field can be use in exponential decaying, oscillatory or square pulses. Electroporation is used in food processing for inactivation of microorganism with 20-80 kV·cm⁻¹ intensity (Alzamora, Welti-Chanes, Guerrero, & Gómez, 2012). In molecular biology can be used as a transfection method of DNA material with electric field of 3000 to 100 V cm⁻¹, depending of the cell type and media (Potter & Heller, 2011).

![Different electroporation modes used for transfection](image)

**Figure 7** Different electroporation modes used for transfection: square pulse a), exponential decaying b) and oscillatory c).

1.7 Hypothesis

Bioencapsulation show that *Artemia ssp.* have a high potential as a vehicle of bimolecular compound (e.g. hormone, antibodies), but there are few reports of transgenic manipulation. The transfection of plasmid DNA in to *A. franciscana* cysts by electroporation is possible.
1.8 Objective

The general objective is to develop a protocol for the genetic transformation of brine shrimp. To achieve the general objective the following objectives were established:

1. Define a buffer for the electroporation of cysts
2. Determine the best wave type for electroporation
3. Determine the best amount of DNA
4. Determine the effect of voltage on efficiency and hatching
Chapter 2 Materials and Methods

2.1 Artificial Seawater

Artificial Seawater (ASW) was prepared according with the recipe of Kester, Duedall and Connors (1967).

Table 1 Recipe of Artificial Seawater

<table>
<thead>
<tr>
<th>Gravimetric salts</th>
<th>g per liter</th>
<th>Volumetric salts</th>
<th>g per liter</th>
<th>Salts solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>23.926</td>
<td>MgCl₂·6H₂O</td>
<td>10.831</td>
<td>1 M solution</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>4.008</td>
<td>CaCl₂·2H₂O</td>
<td>1.519</td>
<td>µL per liter</td>
</tr>
<tr>
<td>KCl</td>
<td>0.677</td>
<td></td>
<td></td>
<td>H₂BO₃</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.196</td>
<td></td>
<td></td>
<td>420.50</td>
</tr>
<tr>
<td>KBr</td>
<td>0.098</td>
<td></td>
<td></td>
<td>NaF</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>71.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SrCl₂·6H₂O</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>90.00</td>
</tr>
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</table>

Gravimetric salts were dissolved in 400 mL of MilliQ water. Volumetric salts were dissolved in 400 mL of MilliQ water. Gravimetric and volumetric salts were mixed, salts solutions were added, and pH was adjusted at 8.5 with NaOH 1.0 N and HCl 1.0 N. ASW were filtered through 0.22 µL PES membrane filter and stored until use. Before use for *A. franciscana* culture, pH was adjusted to 9.5.

2.2 Vector

Plasmid DNA pEGFP-N1 from Clontec was used as expression vector. Plasmid content the gene *egfp* that encode enhanced green fluorescent protein (EGFP) under the control of promoter of cytomegalovirus for mammalian expression.
Materials and Methods

2.2.1 Plasmid production

For plasmid production was produce in *E. coli* Top 10, by midiprep of Sambrook and Russell (2001) with some modifications. 15 mL of LB medium (Sigma Aldrich, St. Louis, Missouri USA) was inoculated with *E. coli* and incubated at 37 °C and 250 rpm 16 hrs. Culture was centrifuged at 3200 Relative Centrifugal Field (rcf) or G-force 10 min at 4 °C, and supernatant was discard. 200 µL of cool solution I (Tris-HCl 25mM, EDTA 10mM) was add, resuspend by vortex, transfer to a 1.5mL centrifugal tube and incubated at room temperature by 5 min. 400 µL of solution II (NaOH 0.2 N, SDS 41.9 mM) and 1.0 µL of PureLink RNAse (Thermo Fisher Scientific, Waltham, MA, USA) was add, mix by inverting rapidly five times and incubated 5 min at room temperature. 300 µL of cool solution III (acetic acid 2.0 M, sodium acetate 3.0 M) mix by inverting rapidly and incubate by 10 min at -20 °C. Result was centrifugated at 14000 rcf by 10 min at 4 °C. supernatant was recover and transfer to a new tube. 1 volume of cool phenol:chboroform:isoamyl Alcohol 25:24:1 (Sigma Aldrich, St. Louis, Missouri USA), mix by vortex and centrifugated at 14,000 rcf by 10 min. 600 µL of upper phase was recovered. 600 µL of cool isopropanol was add vortex and incubated at -20°C by 15 min. Mix was centrifugated at 14000 rcf by 4 °C by 10 min and supernatant was discarded. To wash pellet 1 mL of ethanol 70% was add vortex, centrifugated at 14000 rcf by 5 min and supernatant discard. The wash step was repeat one more time. Supernatant was discarded and incubated at 37 °C until ethanol was evaporated. Pellet was resuspended with 100 µL of nuclease free water. Plasmid DNA concentration was quantified with Take3 Micro-Volume plate (BioTek Instruments, Inc., Winooski, VT, USA) and Epoch Microplate
Materials and Methods

Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) and storage at -20°C until use.

2.3 Cyst electroporation

To achieve the transfection of plasmid DNA, cyst was decapsulated and electroporated

2.3.1 Hydration

For hydration of cyst a 50 mL centrifuge tube and cut 2 mm on the bottom, a micropipe tip of 200 µL with filter was cut in half, put in the bottom of 50 mL of centrifugal tube and stick with cyanoacrylate (Figure 9). Filtered air was supplied from centrifugal tube bottom with an aquarium air pump Elite 800 (Grupo Acuario Lomas, CDMX, Mexico). To reach a 28°C temperature, fluorescent lamp HEL-26W/65-T2 (Tecno Lite, Zapopan, JAL, Mexico) was used and temperature was monitored.

![Figure 9 Centrifugal tube modified for cysts hydration (left) and incubator (right)](image)

300.0 mg of commercial cysts of A. franciscana (Artemia 85% 185,000 NPG Biogrow, ProAqua, Mazatlán, SIN, Mexico) was weight. 30 mL of artificial seawater and cysts was mix in modified centrifugal tube with air for 2 hrs in incubator.

2.3.2 Decapsulation

Conical bottom and part of cap of a 50 mL centrifugal tube was cut and discard (Figure 10). Fabric was put in the cap and closed.
Hydrated cyst was recovered with modified centrifugal tube. For decapsulation modified tube with hydrated cyst was immerse and mix in 70 mL of decapsulation solution (NaOCl 2.5%, NaOH 0.268N) for 4 min. Decapsulated cyst was washed in 100 mL of water and neutralized by immerse in HCl 0.1N for 30 seg. Finally, wash with water was repeated twice.

2.3.3 Recover and hatching

Immediately after electroporation cyst hatching was done by transferred to 30 mL of ASW in petri dishes. Cysts were incubated at 28°C and light form fluorescent lamp at 2000 lm·m$^{-2}$ with a photoperiod of 16 hours light and 8 hours dark.

2.4 Effect of pH in hatching and survive

To determine pH of ASW for hatching and recover, 50 decapsulated cysts were place in ASW at pH 8.5 (recommended for A. franciscana) and pH 9.5. Number of living nauplii (with motion) and death nauplii (without motion) were counted at 16, 19, 22, 24, 40, 48 and 72 hrs. The experiment was done three times.

Hatching rate was calculated with the equation 1:

$$ Hatching\ rate = \frac{number\ of\ nauplii\ (living\ or\ death)}{cyts\ at\ the\ begining} \times 100 $$

(1)

Survive rate was calculated with the equation 2

$$ Survive\ rate = \frac{number\ of\ living\ nauplii}{number\ of\ nauplii\ (living\ or\ death)} \times 100 $$

(2)
2.5 EGFP detection

Sample of 100 µL of nauplii were take 24, 48, and 72 hrs after electroporation and place in cavity slides, excessive water was removed. To reduce evaporation during transport, samples were place in ice.

Expression of EGFP was detected through AXIO Imager A2 microscopy (Carl Zeiss, Oberkochen, Germany), using filter set Fs 09 (excitation: BP 450-490 nm, emission: LT 515nm). *A. franciscana* nauplii, and nauplii expressing fluoresces were counted in each sample. Transfection efficiency was calculated with the equation 3.

\[
\%\text{ efficiency} = \frac{\text{fluoresces nauplii}}{\text{total nauplii}} \times 100
\]

2.6 Effect of electroporation buffer

Different buffer for electroporation was test. Low resistance buffer, PBS and high resistance medium, sorbitol, was compared. PBS buffer 10x (NaCl 1.37 M, KCl 27.0 mM, Na₂HPO₄ 100.0 mM, KH₂PO₄ 20.0 mM, pH 7.4) was prepared and filtered through 0.2 µm membrane. A solution of sorbitol 2M was prepared.

For transformation by electroporation, 200 mg of decapsulated cyst, was mix in an electroporation cuvette of 0.2 cm with 100 µL of plasmid DNA at 5.0 µg/µL in sorbitol 1M and PBS 4x. Cyst and plasmid DNA was electroporated in Gene Pulser Xcell (Bio-Rad, Hercules CAL, USA) in two conditions.

**Table 2 Treatments for PBS buffer and sorbitol transfection**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voltage</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Voltage</td>
<td>500</td>
<td>250</td>
</tr>
<tr>
<td>Pulse number</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Pulse length</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Interval between pulses</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Materials and Methods

Treatments were choose based in results of Jheng et al (2015). To compensated transfer pulse, three extra pulses were added. Due to equipment limitations, interval between pulses were increased to 0.1 seconds.

2.7 Effect of exponential and square wave pulse

To compare exponential and square wave pulse, 200 mg of decapsulated cyst was mix with 100 µL of plasmid DNA in HEBS buffer. HEBS buffer 10x (glucose 60mM, HEPES 250 mM, KCl 50 mM, NaCl 1.4 M, Na₂HPO₄ 6 mM, pH 7.05) was prepared and filtered through 0.2 µm membrane. Plasmid DNA was mixed with HEBS buffer to a final concentration of 1.0 µg/µL and HEBS 1x in 100 µL. For square wave test parameters were same that previous experiment (Table 2). For exponential wave pulse, parameters proposed by Potter et al. (2011) for electroporation (α) and electroporation for sensible cells (β) was used.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>α</th>
<th>β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voltage</td>
<td>600</td>
<td>125 V</td>
</tr>
<tr>
<td>Voltage</td>
<td>3000</td>
<td>625 V·cm⁻¹</td>
</tr>
<tr>
<td>Capacitance</td>
<td>25</td>
<td>960 µF</td>
</tr>
</tbody>
</table>

2.8 Effect of plasmid DNA concentration

Electroporation to different concentration was performed to measure the effect of plasmid DNA in transfection efficiency. 100 µL of buffer HEPES with DNA concentration of 1.0, 1.5, 2.0, and 2.5 µg/µL was mixed with 200 mg previously washed with HEPES decapsulated cyst and electroporated at parameters in Table 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Voltage</th>
<th>Pulse number</th>
<th>Pulse length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>150 V</td>
<td>5</td>
<td>100 ms</td>
</tr>
<tr>
<td></td>
<td>750 V·cm⁻¹</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Materials and Methods

Interval between pulses 0.1 s

Samples of 100 µL were taken at 24, 48, and 72 hrs after electroporation, and analyzed under fluorescence microscopy to calculated efficiency.

2.9 Effect of electroporation voltage

To evaluated effect of voltage in transfection efficiency and hatching, electroporation at different voltage was performed. 100 µL of buffer HEPES with DNA at 1.0 µg/µL was mixed with 200 mg of decapsulated cysts in an electroporation cuvette, and electroporated at parameters in Table 5.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voltage</td>
<td>50</td>
<td>100</td>
<td>150</td>
<td>200</td>
</tr>
<tr>
<td>Voltage</td>
<td>250</td>
<td>500</td>
<td>750</td>
<td>1000 V·cm⁻¹</td>
</tr>
<tr>
<td>Pulse number</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Pulse length</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Interval between pulses</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

A homogeneous sample of 1.0 mL was take 48hrs after electroporation and a subsample of 200µL from sample to measure hatching rate. Hatching rate was calculated with equation 4

\[
\text{% hatching} = \frac{\text{number of nauplii}}{\text{number of nauplii + cyst}} \times 100
\]
Chapter 3 Results and Discussion

3.1 Cysts electroporation

3.1.1 Hydration

Cysts show an increase in size after 2 hrs of hydration in ASW, also show an increase in volume due to absorption of water Figure 11. This phenomenon is widely known and is essential in cyst resistance. Hydration step is necessary to prevent damage by absorption of hypochlorite and a uniform decapsulation.

Figure 11. Cysts before (left) and after (right) 2 hrs of hydration

3.1.2 Decapsulation

After 4 min in decapsulation solution, cyst change color form brown to orange (Figure 12), this change in coloration is due to chorion degradation, principally composted of haematin. Decapsulated cysts show a bright layer due to cuticular membrane compost of chitin.
3.2 Effect of pH in hatching and survival

Nauplii hatching and survive was affected by pH media. ASW with pH 9.5 improve hatching and survive in the experiment time. Hatching rate in pH 9.5 was 81.3 ± 4.6 %, compared with 60.0 ± 4.0 % in pH 8.5. *A. franciscana* cysts had a pH switch, internally, cysts have an acid pH in anoxia conditions and increase in presence of oxygen. In this acid environment, protein and RNA synthesis is inhibited and several key enzymes in trehalose consumption (major carbon source for cysts) (Clegg & Trotman, 2002). High pH in AWS can contribute in the activation of pH switch and increase hatching.
Survival in pH 9.5 reach the highest level 95.9 ± 2.7% compare with 87.7 ± 2.6 % in pH 8.5 over the experiment time. In hatching *A. franciscana* cysts release glycerol in the medium and increase the presence of microorganisms, basic media can act as a bacteriostatic and improve nauplii survival.

![Figure 14](image)

Figure 14 Survival rate in different pH. Survival in same time with same letter are equal $p \leq 0.05$.
Table 7 Survival rate in different pH

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>pH 8.5</th>
<th>pH 9.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>100.0 ± 0.0%</td>
<td>100.0 ± 0.0%</td>
</tr>
<tr>
<td>19</td>
<td>100.0 ± 0.0%</td>
<td>100.0 ± 0.0%</td>
</tr>
<tr>
<td>22</td>
<td>100.0 ± 0.0%</td>
<td>100.0 ± 0.0%</td>
</tr>
<tr>
<td>24</td>
<td>100.0 ± 0.0%</td>
<td>100.0 ± 0.0%</td>
</tr>
<tr>
<td>40</td>
<td>91.9 ± 7.3%</td>
<td>97.6 ± 2.4%</td>
</tr>
<tr>
<td>48</td>
<td>87.7 ± 2.6%</td>
<td>95.9 ± 2.7%</td>
</tr>
<tr>
<td>72</td>
<td>87.7 ± 2.6%</td>
<td>95.9 ± 2.7%</td>
</tr>
</tbody>
</table>

3.3 EGFP detection

EGFP under microscope was detected 48 hrs after electroporation and decay 78 hrs after electroporation. Transformation was transient, due to plasmid was transfected in supercoil and do not was integrated in genomic DNA and finally it was degraded.

![Figure 15 A. franciscana under fluorescent microscope. Wild type 24hrs a), 48hrs b) 72hrs c). Transformed A. franciscana 24 hrs d) 48 hrs e) 72 hrs f).](image-url)
3.4 Effect of electroporation buffer

Electroporation in PBS 4x and sorbitol 1 M do not show transfection effect at any voltage. Sorbitol is used in electroporation of *Pichia pastoris* (Wu & Letchworth, 2004) other sugar as mannitol have used for electroporation of plant cells, like left, root and stem cells, but with the addition of KCl to decrease resistance.

Transfection efficiency by electroporation in buffer with salt is higher than nonionic solutions like mannitol or sorbitol (Sambrook & Russell, 2001). Sorbitol solution was test due to its use in *P. pastoris* transformation. Theoretical, PBS is a good option for electroporation due to ionic characteristics and its ability to sequester divalent ions, but in this case, was incapable of transfection. According to Potter (2011), PBS or HEBS buffer used is arbitrary, but some cells can be more easily transfected in HEBS. Based in this information PBS 4x was replaced with HEPES buffer.

3.5 Effect of exponential and square wave pulse

Transfection in exponential wave, in both cases (α and β) was less effective than square wave (A and B). Electroporation time can explain this, in α and β treatment total electroporation time was 0.9 and 34.3 ms respectably, compared with 500 ms of total electroporation in A and B treatment. Due to square wave electroporation is easiest to control than exponential, first one was used for next experiments.

![Figure 16 Transfection efficiency of exponential and square wave](image-url)
Table 8 Efficiency of exponential and square wave

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>15.06 ± 0.35</td>
</tr>
<tr>
<td>B</td>
<td>20.71 ± 3.80</td>
</tr>
<tr>
<td>α</td>
<td>4.92 ± 2.04</td>
</tr>
<tr>
<td>β</td>
<td>8.96 ± 0.30</td>
</tr>
</tbody>
</table>

3.6 Effect of DNA concentration

Plasmid DNA concentration do not influence in efficiency. This is result is similar to Jheng et al (2015). Apparently, a high plasmid concentration only affects fluorescent intensity, but not efficiency. Fluorescent were not measure in this experiment.

![Figure 17 Transfection efficiency. Column with same letter are equal p ≤ 0.05](image)
Table 9 Efficiency at different DNA concentration

<table>
<thead>
<tr>
<th>[DNA] µg·µL⁻¹</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>42.79 ± 9.49%</td>
</tr>
<tr>
<td>1.5</td>
<td>46.56 ± 14.24%</td>
</tr>
<tr>
<td>2.0</td>
<td>49.22 ± 6.39%</td>
</tr>
<tr>
<td>2.5</td>
<td>46.55 ± 8.63%</td>
</tr>
</tbody>
</table>

3.7 Effect of voltage in electroporation

Electroporation voltage was statistical significant in transfection efficiency and hatching only at 1000 V·cm⁻¹ compared with other treatments. Voltage at 1000 V·cm⁻¹ result in the biggest transfection efficiently 53.2 ± 3.34%. This efficiency is bigger that efficient reported by Jheng et al. (2015) of 38.20 ± 1.76 %, with double of plasmid DNA and voltage of 100 V (cuvette cell size not reported).

Figure 18 Efficiency percentage. Column with same letter are equal p ≤ 0.05
Table 10 Efficiency at different voltage

<table>
<thead>
<tr>
<th>Voltage (V·cm⁻¹)</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>28.7 ± 7.03%</td>
</tr>
<tr>
<td>500</td>
<td>25.2 ± 7.29%</td>
</tr>
<tr>
<td>750</td>
<td>34.8 ± 9.15%</td>
</tr>
<tr>
<td>1000</td>
<td>53.2 ± 3.34%</td>
</tr>
</tbody>
</table>

In all treatments, hatching rate was inferior compared to control without treatment. Voltage at 1000 V·cm⁻¹ resulted in the smallest hatching rate (21.2 ± 5.39%), reduction of 32.17% compared with control. Jheng, obtained a hatching rate of 41.92 ± 2.16 in their best transfection efficiency.

Voltage used in electroporation reduce hatching rate drastically but increase efficiency. In future works, it will be necessary to carry out an analysis on which of the two parameters will be of greater utility for the experimental objective to be achieved.

Figure 19. Hatching percentage. Column with same letter are equal p ≤ 0.05
Table 11 Hatching at different voltage

<table>
<thead>
<tr>
<th>Voltage (V·cm⁻¹)</th>
<th>Hatching</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>65.9 ± 7.81%</td>
</tr>
<tr>
<td>250</td>
<td>44.7 ± 2.79%</td>
</tr>
<tr>
<td>500</td>
<td>51.0 ± 7.84%</td>
</tr>
<tr>
<td>750</td>
<td>46.2 ± 3.23%</td>
</tr>
<tr>
<td>1000</td>
<td>21.2 ± 5.39%</td>
</tr>
</tbody>
</table>
Chapter 4 Conclusions

It was possible to develop a protocol for the transient transformation of brine shrimp by electroporation of commercial cysts. The protocol included the decapsulation by a chlorinated solution and supercoil plasmid DNA. PBS buffer used by Jheng et al. (2015) was ineffective. HEBS buffer was effective for transformation. According to Potter (2011), PBS or HEBS buffer used is arbitrary, but some cells can be more easily transfected in HEBS.

The use of square electric pulses was more effective compared to exponential decay with the parameters that were evaluated. The exponential decay cannot be ruled out in future experiments, since other voltage and capacitances were not evaluated, but square pulses are easier to control. Takahashi et al. (1991) report similar differences into leukemia cell lines K562.

The concentration of DNA that were evaluated were not significant for efficiency, this may be because this is the maximum efficiency that can be achieved even though for this it would be necessary to evaluate at lower concentrations.

The higher voltage evaluated resulted in a statistically significant higher efficiency. The high transformation efficiency (53.2 ± 3.34%) is bigger than previous reported efficiency by Jheng et al. (2015).

Voltage have a positive effect in efficiency and negative in hatching. Chang et al. (2011) and Jheng et al. (2015) reported same effect, but there was also a significant effect on hatching. The efficiency achieved by the lower voltages were lower but cannot be discarded, so the use of voltage will have to respond to the target for transgenesis.
Chapter 5 Future Work

With the protocols and the information generated, it will be possible to evaluate genetic regulatory elements. To increase the genetic tools for genetic modification of Artemia and develop a tool box, the following genetic regulatory elements will be evaluate.

For future work, the expression of promoters of the white spot syndrome virus of shrimp, previously reported in insect cells, will be evaluated. These promoters promise a greater expression compared to others already established due to their origin and function in the cycle of virus infection.

Also, as future work is planned the use of the IRES sequence, to obtain bicistronic expression, faster, and better stable clone selection.

Another system to be evaluated in A. franciscana is the transposal system of the sleeping beauty, which is used for the insertion into the genome of expression cassettes in mammalian cells. Also, to obtain an insertion in the genome, the GYPSY isolates used in Drosophila would be evaluated.

With a genetically modified line, the use of the CRISPR-Cas9 system in A. franciscana could be evaluated, which has been used in several organisms.
## Appendix A

### Abbreviations and acronyms

#### Table 12. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>LB medium</td>
<td>Luria-Bertani medium</td>
</tr>
</tbody>
</table>

#### Table 13. Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp70</td>
<td>Heat shot protein of 70 kDa</td>
</tr>
<tr>
<td>AHPND</td>
<td>Acute Hepatopancreatic Necrosis Disease</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>ypGH</td>
<td>Yellowfin porgy growth hormone</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>lacZ</td>
<td>β-galactosidase gen</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>RSV</td>
<td>Rous Sarcoma Virus Long Terminal Repeat</td>
</tr>
</tbody>
</table>
## Appendix B

Variables and Symbols

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voltage per cm</td>
<td>Temperature</td>
<td>V·cm⁻¹</td>
</tr>
<tr>
<td>T</td>
<td>Temperature</td>
<td>°C</td>
</tr>
<tr>
<td>m</td>
<td>Mass</td>
<td>g, mg</td>
</tr>
<tr>
<td>pH</td>
<td>Potential of hydrogen</td>
<td></td>
</tr>
<tr>
<td>t</td>
<td>Time</td>
<td>s, ms</td>
</tr>
</tbody>
</table>


Curriculum Vitae

**Alberto Constantino Rodriguez-Sanchez** was born in Dolores Hidalgo, Guanajuato, Mexico. He is the second son of the marriage of J. Constantino Rodriguez Galarza and Marcelina Sanchez Rodriguez, Andrea Ivett is the first one and Cesar Enrique and Jassiel Melicio are the third and fourth respectively. He lives in Monterrey, Nuevo Leon where he studies his master’s Degree. He receives his college degree in Biotechnology Engineering at Tecnologico de Monterrey in 2016. In college he was research fellow at Centro del Agua para America Latina in the Molecular Biology area. Additionally, he was research fellow in Centro de Biotecnologia. During his Mater Degree, he works in *Artemia franciscana* genetic transformation.