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RNA INTERFERENCE IN *ARTEMIA FRANCISCANA* APPLIED TO SEX DETERMINING GENES

Thesis submitted in fulfillment of the requirements for the degree of Doctor in Applied Biological Sciences (PhD).

Dutch translation of the title:
RNA-interferentie in *Artemia franciscana* toegepast op geslachtsbepalende genen.

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SUMMARY

Sex determination is an important research topic in many species. Understanding the sex determination mechanism allows to control sex-linked traits such as the size and the color. In insects, some models of sex determination mechanisms have been proposed based on the isolated sex-determining genes, and their functions were verified by silencing via the RNA interference mechanism. Many crustaceans are considered to be species of economic value, but the understanding of their sex determination mechanisms is still limited. In this study, the brine shrimp *Artemia franciscana*, as a crustacean model, was used to initially investigate the molecular mechanism of sex determination in crustaceans. With the goal of determining the function of sex-determining genes in the embryonic stage, RNA interference is considered as a suitable technique for this purpose.

To investigate functional RNAi in *A. franciscana*, we first characterized genes involved in RNA interference (RNAi) mechanism from the available genomic and transcriptome data of *A. franciscana*. It revealed that this species has at least nine genes involved in RNA interference including two genes encoding *Dicer* enzymes, three *Argonautes*, two dsRNA binding proteins (*TRBP*), *Drosha* and *Sid-1* genes together with evidence of a *Pasha* and a *Exportin-5* gene. The phylogenetic results confirmed the identity of these genes and revealed that homologous members of both the siRNA and miRNA pathway genes in arthropods are also present in this crustacean species.

For functional RNAi, for example to silence sex-determining genes at embryonic stage, dsRNA molecules need to be delivered to the *A. franciscana* embryo. Two strategies for dsRNA delivery, dsRNA microinjection and feeding of dsRNA-producing bacteria, have been tested on adult females to assess the possibility of silencing a particular gene at the embryonic stage of the progeny. The *caudal* gene, responsible for abdomen segmentation, was used as an

indicator gene due to the fact that effective RNAi can be evaluated by atypical RNAi phenotype of nauplii in the offspring, namely a shortened abdomen. The results showed that egg-sac dsRNA microinjection can induce transgenerational RNA interference in *A. franciscana*. However, the tested dsRNA feeding strategy failed to induce RNA interference in the progeny of treated females. Therefore, the egg sac dsRNA microinjection method was chosen to study the function of sex-determining genes. These results confirmed that indeed RNAi is functional in *A. franciscana,* providing an efficient delivery system facilitating functional genomic research in this crustacean species.

Regarding sex-determining genes in *A. franciscana*, two *fem-1* homologs and eight *doublesex* homologs were isolated and identified from the available transcriptome data. dsRNA microinjection of egg-sac of *A. franciscana* adult females was applied to silence those candidate genes for studying their relationship with the formation of hooked graspers in adult males and egg-sac in adult females of *A. franciscana.* Two *doublesex* transcripts were confirmed as sex-specific transcripts in *A. franciscana.* The results also suggested that the *doublesex* splicing mechanism, described in insects, is likely to be also present in *A. franciscana* and that therefore, these genes can be used in further studies to clarify the mechanism of the sex determining pathway in *Artemia* as well as in other crustaceans.

SAMENVATTING

Geslachtsbepaling is een interessant onderzoeksonderwerp in veel soorten. Inzicht in het geslachtsbepalingsmechanisme staat toe om de geslachts-gerelateerde eigenschappen zoals grootte en kleur te bepalen. In insecten zijn sommige modellen van geslachtbepalingsmechanismen voorgesteld op basis van de geïsoleerde geslachtsbepalende genen, en hun functionaliteiten werden geverifieerd door silencing via het RNA interference mechanisme. Veel crustaceeën worden aanschouwd als economisch waardevolle diersoorten maar het begrijpen van hun geslachtsbepalingsmechanisme is nog steeds beperkt. In dit onderzoek werd het pekelkreeftje, *Artemia franciscana*, gebruikt als model organisme, om initieel het moleculair mechanisme van de geslachtsbepaling in crustaceeën te onderzoeken. Om de functie van de geslachtsbepaling van genen in het embryonale stadium te bepalen, wordt RNA interference gezien als een geschikte techniek.

Om functionele RNAi te onderzoeken in *A. franciscana*, hebben we eerst de genen die betrokken waren bij RNA interferentie (RNAi) geannoteerd uit de beschikbare genoom- en transcriptoomdata van *A. franciscana*. Het is gebleken dat deze soort tenminste negen genen heeft die betrokken zijn bij RNA interferentie, waaronder twee genen die *Dicer*-enzymen coderen, drie *Argonautes*, twee dsRNA bindende eiwitten (TRBP), en ook *Drosha* en *Sid-1* homologen. Bovendien is er ook bewijs gevonden voor de aanwezigheid van een *Pasha* en een *Exportin-5* gen. De fylogenetische analyses bevestigden de identiteit van deze genen en onthulden dat homologe leden van zowel de siRNA als miRNA pathway genen bij geleedpotigen ook aanwezig zijn in deze schaaldieren.

Voor functionele RNAi, bijvoorbeeld om geslachtsbepalende genen in de embryonale fase uit te schakelen, moeten dsRNA moleculen aangeleverd

worden aan het *A. franciscana* embryo. Twee strategieën voor dsRNA aanlevering, dsRNA micro-injectie en vervoederen van dsRNA-producerende bacteria, werden getest op adulte vrouwtjes om te trachten een bepaald gen uit te schakelen in het embryonaal stadium van de nakomelingen. Het *caudal* gen, verantwoordelijk voor abdomen segmenatie, werd gebruikt als een indicator gen zodat effectieve RNAi makkelijk kan geëvalueerd worden aan de hand van een atypisch phenotype van de nauplii in de nakomelingen, namelijk een verkort abdomen. De resultaten toonden aan dat broedzak dsRNA micro-injectie een langdurig en transgenerationeel effect van RNA interferentie in *A. franciscana* kan induceren. De voederstrategie met dsRNA echter, resulteerde niet in succesvolle RNA interferentie in de experimentele dieren. Daarom werd de broedzak dsRNA micro-injectie methode gekozen om de functionaliteit van geslachtsbepalende genen in het embryonaal stadium te bestuderen. Deze resultaten bevestigden dat RNAi functioneel is in *A. franciscana* en voorzag ons op deze manier van een efficiënte toepassingswijze om functioneel genoomonderzoek in deze schaaldiersoort.

Wat betreft geslachtsbepalende genen in *A. franciscana* werden twee *fem-1* homologen en acht *doublesex* homologen geïsoleerd en gedetermineerd uit de beschikbare transcriptoom data. dsRNA micro-injectie in de broedzak van volwassen *A. franciscana* vrouwtjes werd toegepast om expressie van deze kandidaat-genen te onderdrukken om hun relatie met de vorming van de hooked graspers in volwassen mannetjes en de broedzak in volwassen vrouwtjes te bestuderen. De *doublesex* transcripts werden bevestigd als geslachts-specifieke transcripts in *A. franciscana*. De resulaten lijken ook aan te geven dat het *doublesex* splicing mechanisme van insecten waarschijnlijk aanwezig is in *A. franciscana* en dat deze genen daarom in verdere studies kunnen gebruikt worden om het mechanisme van de geslachtsbepalende pathway van *Artemia* alsook andere crustaceeën verder te verduidelijken.

%	Percentage
°C	Degree Celsius
×g	Relative centrifugal force (G force)
a.a	Amino acid
bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
CT	Cycle threshold
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
g	Gram
GenBank	Genetic sequence database of the National Institute of Health
h	Hour
L	Liter
LB	Luria-Bertani
mg	Milligram
miRNA	Micro ribonucleic acid
mL	Milliliter
mRNA	Messenger ribonucleic acid
OD	Optical density
PCR	Polymerase Chain Reaction
pH	Measure of the acidity of a solution
piRNA	Piwi-interacting ribonucleic acid
RACE	Rapid Amplification of cDNA ends
RNA	Ribonucleic acid
RT-PCR	Real time-polymerase chain reaction
SD	Standard deviation
siRNA	Small interfering ribonucleic acid
UTR	Untranslated region
µg	Microgram
µL	Microliter

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CHAPTER 1

Introduction & Research objectives

1.1 Biology of *Artemia franciscana*

The brine shrimp, *Artemia franciscana,* is a small crustacean zooplankton species and an important live food in larviculture due to their nutritional value and appropriate size. The classification of the *A. franciscana* is as follows (Regier *et al*., 2010):

Phylum Arthropoda

Subphylum Pancrustacea

Class Branchiopoda

Order Anostraca

Family Artemiidae

Genus *Artemia*

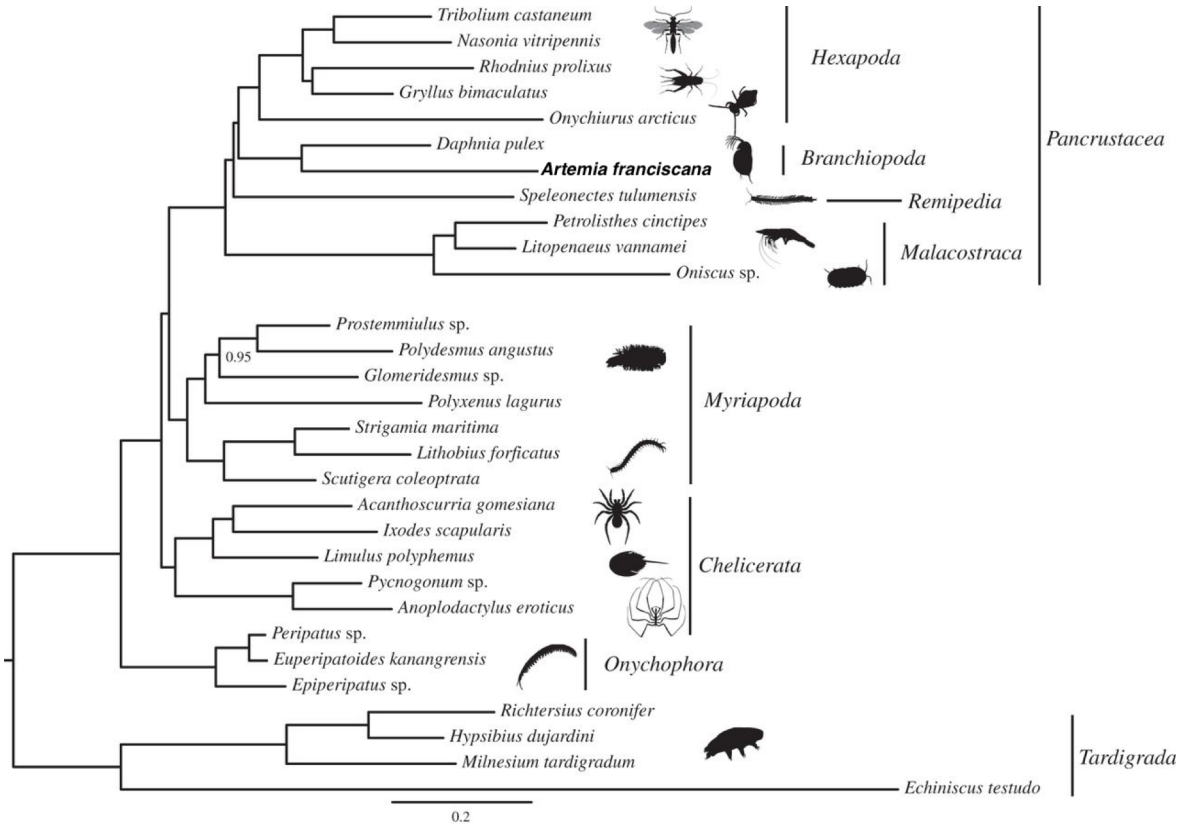

Figure 1.1. The phylogeny of Panarthropoda. Pancrustacea is a clade, comprising all crustaceans and hexapods (Lozano-Fernandez *et al.*, 2016).

The length of *Artemia* adults is usually around 8-10mm for males and 10-12mm for females and their thickness approximates 4mm for both sexes (Criel & Macrae, 2002a). This species is characterized by a remarkable resistance to high osmotic stress of hypersaline environments. The life span of *A. franciscana* varies from two to four months, depending on salinity and temperature of the environment. They are not only tolerant to high salinities but also to variable temperatures (6-35°C), ionic composition and they have a pH tolerance from neutral to highly alkaline.

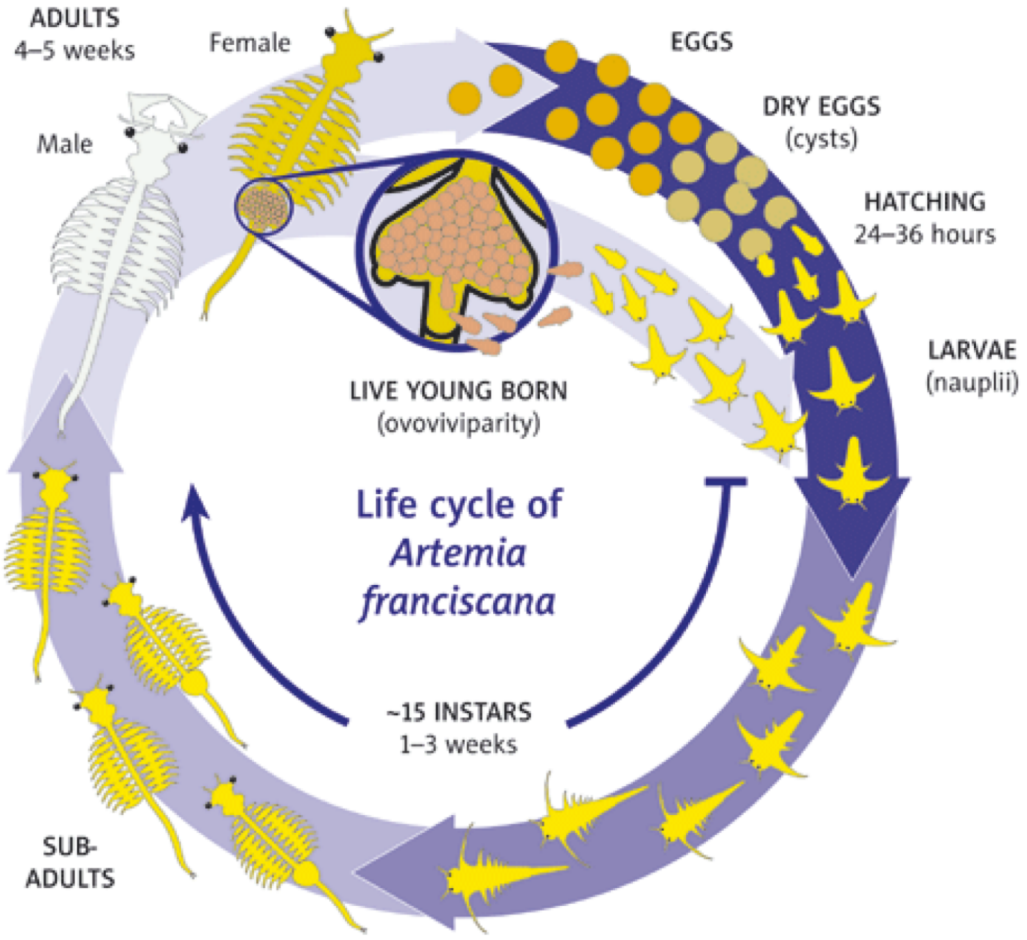

Figure 1.2. The life cycle and reproduction modes of *A. franciscana.* Adapted from *http://www.leloft.co.uk/artemia.html*

The male *Artemia* has a paired penis in the posterior part of the trunk region while female *Artemia* are characterized by the brood sac. Eggs develop in two tubular ovaries in the abdomen. Once ripe, they become spherical and migrate via two oviducts into the unpaired uterus (Lavens & Sorgeloos, 1996). In favorable living conditions, the fertilized eggs in the brood sac of the female develop into free-swimming *Artemia* nauplii (ovoviviparous reproduction). If living conditions deteriorate, they have an ability to produce dormant eggs (basically an embryo), known as cysts which may be stored for long periods and can be hatched into nauplii easily on demand (Lavens & Sorgeloos, 1987). At hatching, the nauplius larva (400 to 500 µm in length) has a brownish-orange color and they do not take up food as their digestive system is not functional yet and the mouth is not open. After about 8 h, the animal molts into the 2nd larval stage. Small food particles ranging in size from 1 to 50 µm are filtered out by the 2nd antennae and ingested into the functional digestive tract (Lavens & Sorgeloos, 1996). The clearance rate of 4 day old animals is 1.48-2.10 ml per individual per hour (Makridis & Vadstein, 1999). The larva grows and differentiates through 15 molts. From the 10th instar stage on, important morphological as well as functional changes are taking place; for instance the second antennae are enlarged and modified as claspers in males (Fig 1.2) (Lavens & Sorgeloos, 1996). *Artemia* nauplii have a high nutritious value and are small in size, which makes them suitable to feed fish and crustacean larvae. Therefore, this species is used as an important feed for industrial aquaculture. Besides, this species also has a short life cycle and is easy to culture in laboratory condition thus, making *A. franciscana* a model organism for many fundamental studies.

1.2 *Artemia franciscana* in the aquaculture research

Aquaculture is the fastest growing food-production sector in the world. However, disease outbreaks are the biggest impediment to aquaculture development, affecting the economic development in many countries (Bondad-

Reantaso *et al*., 2005). Therefore, understanding the host immune system against pathogens is considered as an important key to reduce the negative effects of disease outbreaks to aquaculture. *Artemia* has a small body size, short life cycle, and is well-adapted for a natural or artificial seawater environment. Moreover, their cysts can survive for many years in certain well-defined conditions, so it is very convenient for laboratory tests. Their short life cycle allows for short bioassays. Biologically, *Artemia* is closely related to many crustacean species, including several crustacean species with high economic value. Hence, this species is an excellent model organism to study the modes of action of probiotic and pathogenic bacteria, as it can be easily cultured under gnotobiotic conditions (Marques *et al*., 2004). The gnotobiotic *A. franciscana* rearing system has gradually become a powerful tool in the study of immune function and response to pathogenic agents (Norouzitallab et al., 2016 ; Marques et al., 2006). In addition, *A. franciscana* is also used in different fundamental researches such as epigenetic studies (Norouzitallab et al., 2014) and toxicity assessments (Rajabi et al., 2015 ; Neumeyer et al., 2015). Recently, the genome of *A. franciscana* was sequenced by Ghent University and the quality of this genome is gradually improving, which makes *A. franciscana* even more convenient to explore biological mechanisms at the gene and protein level.

1.3 Problem definition and research objectives

The RNA interference mechanism was first reported in *C. elegans* (Fire *et al*., 1998), and was later shown to be present in many different species. The RNAi core genes have been found in many crustaceans. These findings have revealed that RNA interference is not only involved in development but is also an important component of the antiviral and -bacterial immune system of crustaceans (Lai & Aboobaker, 2017). *Artemia* has been used as a model organism along with the use of RNAi to explore the mechanism of immunity (Iryani *et a*l., 2017), biological evolution (Copf *et al*., 2006) as well as their ability

to survive in extreme environments (MacRae, 2016). The findings from these studies can be useful in aquaculture to improve the productivity. The successful application of RNAi in the study of gene function in *A. franciscana* suggests that this species probably has a similar RNAi mechanism as other arthropod species. However up to now, the RNAi machinery core genes of this species have not been determined. The identification of RNAi machinery core genes could elucidate more about the mechanism of RNAi in crustaceans and could facilitate using RNAi more efficiently in *A. franciscana* research.

The RNAi technique has been used extensively in studies on the development of sex in many different crustaceans. One of the successes of this technique is the induction of sex-change in giant freshwater prawn *Macrobrachium rosenbergii*. This has brought about economic benefits due to the fact that males are bigger than females and are thus economically more interesting. The male sexual development of *M. rosenbegii* is divided into three main phases including sex determination, sexual differentiation and sexual maturation (Chandler *et al*., 2016). RNAi has been used to interfere with the expression of sex hormones at the sexual differentiation phase leading to sex-reversal events (Ventura & Sagi, 2012). Many sex-determining genes homologous to those found in insects, which play a role at the sex determination stage, have been found in *M. rosenbergii* as well as other crustaceans. However, the understanding of their function is still poor. Recently, evidence of potential sex-determination genes in *A. franciscana* was reported in a publication reporting the *A. franciscana* genome sequencing at Ghent University (De Vos *et al*., 2013). *A. franciscana* has a WZ-ZZ sex-determining system which has also been found in other crustacean species such as *Cherax quadricarinatus* (Parnes *et al*., 2003), *Penaeus monodon* (Staelens *et al*., 2008), *Macrobrachium rosenbergii* (Ventura, & Sagi., 2012) and *Eriocheir sinensis* (Cui *et al*., 2015). Although AFLP sex markers have been reported in *Artemia franciscana* (De Vos *et al.*, 2013), its sex-determining genes are still unclear. Due to the available transcriptome data, genome data and the similarity

of the WZ-ZZ sex-determining system to several crustacean species with high economic value, *A. franciscana* has become a suitable model organism in the study of the sex-determining mechanism. The findings can help to better understand the sex determination mechanism on other crustaceans and bring an economical benefit when traits that link to the sex can be controlled.

In this study, we use *A. franciscana* as a model organism to investigate the RNAi machinery as well as the sex-determining genes. To have an overview of RNAi pathways in *A. franciscana*, we first characterized the RNAi-related genes in *A. franciscana* using the available transcriptome database at Ghent University. Next, strategies for inducing RNA interference in embryo were evaluated. The most appropriate strategy was subsequently applied in elucidating the role of candidate sex-determining genes. The latter were identified in the available transcriptome and genome database of Artemia. The research is presented in four chapters:

Chapter 2: RNA interference in Crustacea and potential applications in aquaculture.

Chapter 3: Annotation of the core genes of the RNA interference machinery in *Artemia franciscana.*

Chapter 4: RNA interference in *Artemia franciscana* though microinjection and feeding.

Chapter 5: Cloning and RNAi of putative of sex-determining genes in *Artemia franciscana*

CHAPTER 2

RNA interference in Crustacea and potential applications in aquaculture

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Abstract

Since the mechanism of RNA interference was discovered, researchers have made remarkable strides in unraveling biological mechanisms at the gene level in a wide range of organisms. RNAi is a simple and rapid method that allows silencing gene expression at the post-transcriptional level and is triggered by the introduction of double-stranded RNA into the cell, leading to the degradation of the corresponding mRNA. In this chapter, we will provide an overview of the recent discoveries in RNAi pathways in crustaceans; the experimental procedures, success rates and challenges of RNAi techniques applied in Crustacea are also discussed. It showed that RNAi machinery in crustaceans shares similar core genes of RNAi as the insect models, the closest model organisms to Crustacea, but also reveals its own different features. As a model Crustacea, *Artemia franciscana* was widely used to study function gene using RNAi technique, however the core genes of RNAi of this species are still unidentified. Successful RNAi in Crustacea and *A. franciscana* shows various potential applications of RNA interference in the research and the aquaculture.

2.1 Introduction

RNA interference (RNAi) is a biological process where the expression of a specific gene is silenced post-transcriptionally by messenger RNA (mRNA) degradation or translational inhibition after the introduction of gene-specific double-stranded RNA (dsRNA) into a cell or organism (Fire *et al*., 1998 ; Meister & Tuschl, 2004 ; Marc *et al*., 2010). Since its discovery in 1998, RNAi has been used extensively in research, where it is applied as a functional genomic tool. Additionally, other possible applications, such as the use of RNAi in crop protection as well as its therapeutic use in animals and humans, are under development already (Huvenne & Smagghe, 2010). In Crustacea, RNAi has also already been used successfully as an anti-viral strategy in aquaculture (Lima *et al*., 2013). In this review, we start by giving a brief summary of the molecular mechanism and the RNAi machinery components that are involved. While the RNAi mechanism shows a high degree of evolutionary conservation throughout eukaryotes, differences in RNAi pathway components are found between different taxonomical groups. An overview of what is known about RNAi in Crustacea will be presented and compared to RNAi in other taxonomic groups such as insects, where it has been investigated extensively.

2.2 Molecular mechanism

For RNAi, double-stranded RNA (dsRNA) which is introduced into the cell is processed into small regulatory RNAs consisting of 20-30 nucleotides by a RNase-III-like enzyme called DICER (Zamore *et al*., 2000). These small regulatory RNAs are then taken up by the RNA-induced silencing complex (RISC), which is comprised of various proteins, and which unwinds the small regulatory RNAs into single stranded molecules. The antisense strand, called the guide strand, is loaded into the RISC complex. The other strand, called the passenger strand, is degraded as a RISC complex substrate. Finally, this complex is coupled to the target mRNA, based on specific base-pairing, to induce endonucleolytic cleavage causing the destruction of mRNA molecules.

A schematic overview of this cellular process is given in Figure 2.1. The RNAi mechanism in shrimp is known to play a role in antiviral immunity. When treated with different dsRNAs, which do not specifically target White spot syndrome virus (WSSV) and Taura syndrome virus (TSV), the injected *Litopenaeus vannamei* nevertheless showed increased resistance to these viruses. These results showed that dsRNA could induce antiviral protection in the marine crustacean (Robalino *et al*., 2004).

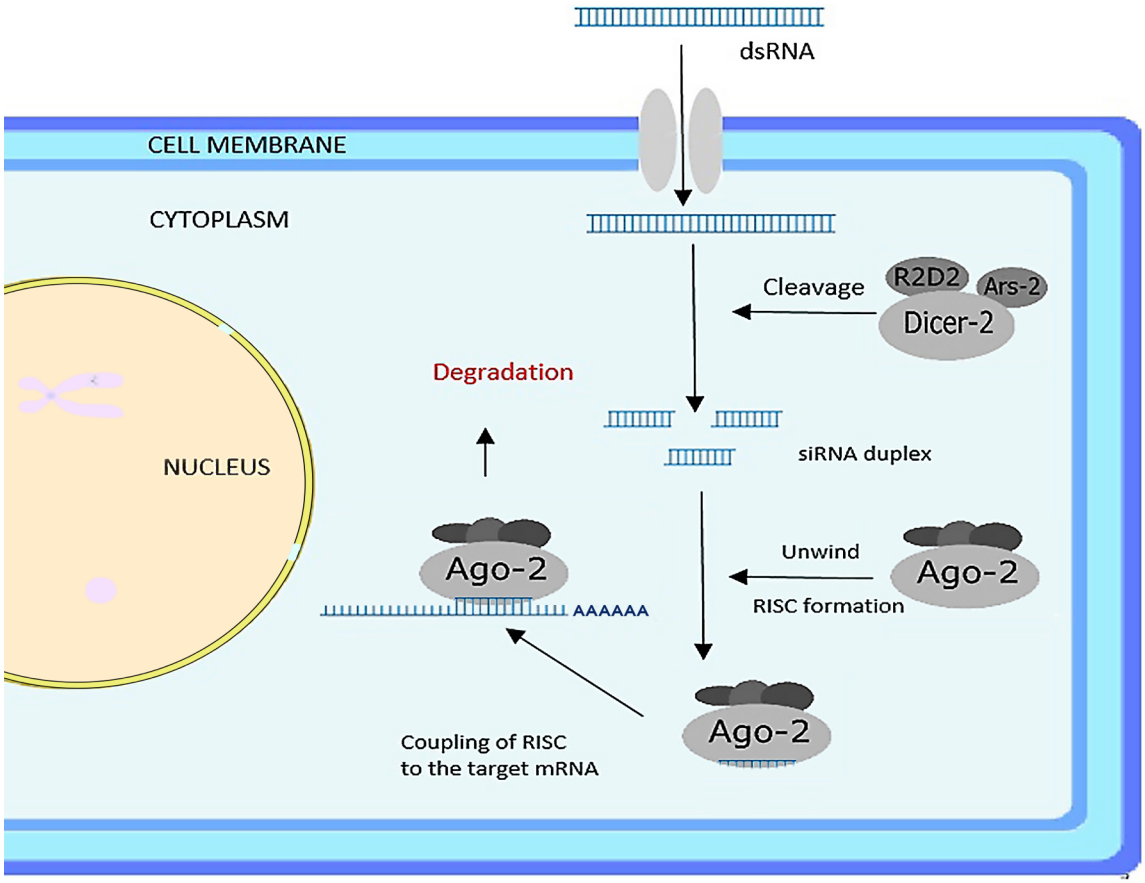

Figure 2.1 Schematic representation of the siRNA pathway in Crustacea. The long dsRNAs are processed in the cytoplasm by the DICER-2 RNase into siRNAs. The resulting siRNAs are taken up by the RNA-induced silencing complex (RISC). The duplexed siRNA, which is bound to the AGO-2 protein, the central component of RISC, is unwound and the passenger strand rapidly dissociates. Finally, this complex is coupled to the target mRNA, based on specific complementary base-pairing, to induce endonucleolytic cleavage causing the degradation of mRNA molecules.

The regulatory RNAs can be classified into three main classes: small interfering RNAs (siRNAs), micro RNAs (miRNAs) and P-element induced wimpy testis (PIWI)-interacting RNAs (piRNAs). siRNAs originate from exogenous dsRNAs, from endogenous transcripts from repetitive sequences or from transcripts that can form long hairpins. The siRNA pathway is known to be mainly involved in the immune response against viral infections. miRNAs are derived from endogenous, single stranded noncoding RNA transcripts or introns that fold into imperfect stem-loop structures and are known to be important for internal gene regulation. The piRNAs guide PIWI proteins to complementary RNAs derived from transposable elements for cleaving the transposon RNA, playing a role in the protection of the genome.

Most vertebrates and roundworms have only one DICER protein, which generates both miRNAs and siRNAs. In contrast, insects, fungi and plants have more than one DICER or DICER-like proteins (Sasaki & Shimizu, 2007). DICER enzymes in *Drosophila melanogaster* (Meigen, 1830) are classified as DICER-1 and DICER-2 in terms of their specialized functional activities. DICER-1 can process pre-miRNA to mature miRNA, while DICER-2 is responsible for the cleavage of dsRNA precursors into siRNA molecules (Kalidas *et al*., 2008). In the black tiger shrimp *(Penaeus monodon*) (Su *et al.*, 2008 ; Li *et al.*, 2013b), pacific white shrimp (*Litopenaeus vannamei*) (Yao *et al*., 2010 ; Chen *et al.*, 2011) and kuruma shrimp *Marsupenaeus japonicus* (Bate, 1888) (GenBank accession numbers GU265733, JQ349041), two DICER enzymes, *Dicer-1* and *Dicer-2*, have been reported (Table 2.1).

Table 2.1. Overview of the RNAi core genes identified in shrimp species

Species	Genes	Tissues	References
L. vannamei	*Dicer-1*	Fertilized eggs	Yao *et al*., 2010
	Dicer-2	Gill, heart, hepatopancreas, stomach, intestine, nerve, pyloric caecum, and epidermis	Chen *et al.*, 2011
	Ago-1	Gill	Labreuche *et al*., 2010
	Ago-2	Gill	Labreuche *et al*., 2010
P. monodon	*Dicer-1*	Hemolymph and lymphoid organ tissues	Su *et al*., 2008
	Dicer-2	Gill, hemocyte and hemolymph, but not in muscle	Li *et al*., 2013b
	Ago-1	Ovary and lymphoid organs, gill, muscle, hepatopancreas and nerve cord	Dechklar *et al.*, 2008
	Ago-2	Lymph, hemocytes, eyestalk, gill, hepatopancreas, ovary, intestines, stomach, muscle, heart, epidermis, nerve and brain	Yang et al., 2014a
	Ago-3	Hemocytes, ovary, hepatopancreas, lymphoid organ, gill, heart, nerve cord, thoracic ganglia, brain and eyestalks	Phetrungnapha *et al*., 2013
	TRBP	Lymphatic organs and ovaries	Yang *et al*., 2013b
F. chinensis	*TRBP*	Hemocytes	Wang *et al*., 2012
M. japonicus	*TRBP*	Hemocytes	Wang *et al.*, 2012

The Argonaute (AGO) protein family members are the catalytic components of the RNA-induced silencing complex (RISC). Based on amino acid sequence similarities and phylogenetic analyses, they are classified into three categories: AGO, PIWI and worm-specific Argonautes (WAGO) (Carthew & Sontheimer, 2009). Several different AGO proteins have been identified in shrimp species (Table 2.1). The *L. vannamei* AGO-1 (LvAGO-1) shares 99% amino acid identity with *P. monodon* AGO-1 *(*PmAGO-1), while the LvAGO-2 protein displayed 39% similarity with PmAGO-1. Monitoring *LvAgo-1* and *LvAgo-2* transcript accumulation by qPCR following dsRNA injection into shrimp showed a strong induction of *LvAgo-2* mRNA expression only. This suggested that *LvAgo-2* may be specifically implicated in the RNAi pathway (Labreuche *et al*., 2010).

The transactivation response RNA-binding protein (TRBP), which belongs to the dsRNA-binding protein family, interacts with Dicer and binds to double-stranded RNA as a critical component of the RNA-induced silencing complex. By co-immunoprecipitation assays and pull-down assays, it has been proven that DICER-2, AGO-2 and TRBP in *L. vannamei* interact with each other (Chen *et al*., 2011). Sequence homology and phylogenetic analysis indicated that *P. monodon* TRBP (PmTRBP) is evolutionarily closest *to* LvTRBP1. TRBP is also found in other shrimp species (Table 2.1).

The Janus kinase / signal transducers and activators of transcription (JAK/STAT) signaling pathway appears to be very important in the antiviral immunity response of vertebrates and invertebrates. In a STAT silencing experiment, *Ago-2* and *Dicer-2* of *L. vannamei* were significantly upregulated upon white spot syndrome virus (WSSV) injection, indicating the activation of the siRNA pathway. However, the miRNAi pathway-related genes *Ago-1* and *Dicer-1* showed no change in mRNA level. These results proved that *Stat* dsRNA injection initiated the siRNA pathway but not the miRNA pathway (Wen *et al*., 2014). Another RNAi-related component that has been studied recently

is the Tudor staphylococcal nuclease from *P. monodon* (*PmTSN*). Since PmTSN interacts with PmAGO-1, but not with PmAGO-2 or PmAGO-3, this nuclease could be one of the components of PmAGO-1-RISC, indicating the involvement of PmAGO-1 and PmTSN in the shrimp RNAi pathway (Phetrungnapha *et al.*, 2013). Furthermore, Chen *et al*. (2012) managed to isolate and characterize two other components of the RNAi pathway in *L. vannamei*, namely *Arsenite* resistance gene 2 (*LvArs2*) and partner of drosha (*LvPasha*). The authors also demonstrated, by co-immunoprecipitation assays, that LvARS2 interacted with LvDICERr2 and LvPASHA, suggesting that *LvArs2* may be involved in regulating the miRNA/siRNA pathways and in the defense against RNA viruses in Crustacea. Similar interactions have been reported in *Drosophila*, where *Ars-2* could be involved in both siRNA and miRNA pathway regulation through association with *Dicer-2* and *Pasha*. Knockdown of *Ars2* in cells and adult flies renders them highly susceptible to infection with several RNA viruses.

In *C. elegans*, transmembrane transporter proteins, named SID proteins, that play an important role in the cellular uptake and transportation of dsRNA have been identified. *Sid-1* is responsible for transportation of dsRNA in between cells , while *sid-2* is necessary for the uptake of dsRNA from the food (Whangbo & Hunter, 2008). S*id-1* or *sid-1-like* genes are present in most insect genomes (except in *Diptera*) and a homolog of *sid-1* has also been identified in white shrimp *L. vannamei* (*Lv-sid1)* (Labreuche *et al*., 2010).

The above studies have proven the presence of genes related to the RNAi machinery in shrimp. However, the information on these RNAi-related genes was still fragmentary, until Christiaens *et al*. (2015) made a major step in providing an overview of a large number of RNAi-related genes in *Crangon crangon* through a transcriptome analysis and annotation of all RNAi-related transcripts. Core machinery genes from the three main RNAi pathways were identified, including *Dicer-1*, *Ago-1*, *drosha*, *Pasha* and *Loquacious* genes for

the miRNA pathway, *Dicer-2*, *Ago-2A* and *Ago-2B* for the siRNA pathway, and *Piwi1*, *Piwi2* and *Ago-3* genes for the piRNA pathway. Fifteen genes related to the RISC complex and four genes involved in dsRNA uptake were also identified in *C. crangon*, including *FBX011*, *SR-C*, *Sid-1A* and *Sid-1B* (Christiaens *et al*., 2015). This transcriptome analysis revealed a set of RNAi core genes that is similar to that which is present in most insect species. However, some differences were observed as well. For example, *R2D2* was absent in the transcriptome of *C. crangon*, while it has been found in several insect species (Christiaens *et al*., 2015). In insects, the DICER-2/R2D2 complex binds duplex siRNA, forms the RISC loading complex, and facilitates the transfer of siRNA onto AGO-2. Further information on other Crustacea will have to show whether this gene is insect-specific, since to the best of our knowledge, it has not been found outside of the class of Insecta.

2.3 Experimental procedures for functional RNA interference in Crustacea

2.3.1 The dsRNA synthesis

One of the first steps before applying RNAi is the production of siRNA or long dsRNA. In general, dsRNA molecules can be produced either *in vitro*, using a reverse transcriptase, or *in vivo* using a bacterial or plant system. *In-vitro* production, using one of the commercially available kits, allows for a fast production of dsRNA of a high purity. The main disadvantage of this production method is the cost, since RNAi applications on aquatic animals require large amount of dsRNA. Bacteria-based production systems allow for a dsRNA production at a much lower cost, especially for large amounts of dsRNA. The *Escherichia coli* HT115 (DE3) strain, which lacks the double-strand-specific RNase III, allows transcription and the accumulation of large amounts of dsRNA upon introduction of a dsRNA expression vector (Fire *et al.*, 1998). The fragment that is cloned in the vector contains the forward and reverse fragments

of the target sequence. Upon expression of this RNA, both the forward and reverse part will self-anneal, forming the dsRNA molecule (Yodmuang *et al*., 2006). Using another strategy, target DNA is cloned inbetween two T7 promoter sequence on a vector such as Litmus28i, Litimus38i or L4440 (Chen et al., 2013 ; Treerattrakool et al., 2013 ; Sarathi et al., 2008). The transformed HT115 (DE3) strain can eventually produce up to 45 µg dsRNA/1-OD600/ml *E. coli* culture (Posiri *et al*., 2013), and large-scale *in-vivo* dsRNA production is approximately one fourth the cost of production using a commercial *in vitro* transcription kit (Saksmerprome *et al*., 2009). However, an important issue with this production method is the purity of the dsRNA. Whether synthesized by an *in vitro* or an *in vivo* strategy, dsRNA should always be purified and analyzed on agarose gel before calculating the dose for every RNAi experiment. In the case of dsRNA synthesized by *E. coli*, the dsRNA purification method plays an important role in removing components originating from the *E. coli* bacteria that might affect the efficiency of RNAi, such as endogenous bacterial RNAs and bacterial toxins. Most likely however, the resulting dsRNA will not be as pure as when synthesized *in vitro* since small amounts of bacterial remnants might always remain. Finally, siRNA oligo can also be chemically synthesized (Chu et al., 2014 ; Hayakijkosol & Owens, 2012 ; Xu et al., 2007). The chemical synthesis method allows siRNA production with a high quality and purity, and allows incorporating modifications to the backbone and/or RNA bases to improve siRNA target specificity, stability in the presence of nucleases.

2.3.2 The dsRNA delivery strategy

The first step to trigger RNAi is the introduction of the dsRNA into the research subject. Depending on the species and organ, different dsRNA delivery strategies can be used, such as injection, oral delivery, and electroporation.

The first experiment attempting to introduce foreign DNA into shrimp by electroporation was conducted in 1999. Electroporation is a technique in which an electrical field is applied to cells in order to increase the permeability of the cell membrane, allowing DNA to be introduced into the cell. The DNA vector of 6.2kb, bearing a DNA fragment that includes a bacterial alkaline phosphatase gene driven by a human cytomegalovirus (CMV) promoter, was introduced into *P. monodon* zygotes by electroporation, a technique which…. The hatching rate of electroporated zygotes (46%) was significantly lower than that of zygotes in the untreated group (89%). Based on dot blot analysis, the rate of gene transfer was 37% in the mysis-stage, 23% in postlarva 15 (PL15), 19% in postlarva 45 (PL45), and 21% in 4-months-old shrimp (Tseng *et al*., 2000). Introduction of nucleic acid fragments by electroporation of single-cell white shrimp (*Litopenaeus schmitti*) embryos has also been performed successfully, using a pulse amplitude of 12 kV for 6 seconds to introduce a DNA plasmid bearing the β-galactosidase gene. As a result, β-galactosidase activity was detected in 19.4% of shrimp embryos after electroporation (Amílcar *et al*., 2000). The electroporation method was optimized for delivery of DNA vectors into decapsulated *Artemia sinica* cysts and to generate stable lines of transgenic *A. sinica* (Chang *et al*., 2011). Recently, this technique has been applied to deliver siRNA molecule into embryos of this model shrimp in the *As-sumo-1* knockdown experiments. Embryos of *A. sinica* were electroporated in buffer containing siRNA, which is designed for targeting *As-sumo-1* from *A. sinica*. Resulting deformations appeared at the nauplii stage and the deformed individuals all continued to survive (Chu *et al*., 2014). Electroporation allows the delivery of nucleic acid into embryos at an early stage and can be done with large numbers of zygotes or embryos at the same time.

The microinjection method is widely used to introduce nucleic acids, including dsRNA, into arthropods at different stages of development, including zygotes, embryos, larval stages, pupae and adults. Delivery of a DNA vector containing *lacZ* by microinjection was successful in fertilized eggs of *L. schmitti.*

All injected embryos survived and 30% of these embryos developed to the nauplii stage and were positive for β-galactosidase activity (Cabrera *et al*., 1995). Microinjection was also used to deliver dsRNA specific to the *Distal-less* (*Dll*) gene into ovulated eggs of the small cladoceran crustacean (*Daphnia magna*) (Kato *et al*., 2011b) and common water flea (*Daphnia pulex*) (Chizue *et al*., 2013). This method is especially suitable for exploring gene function at *in vivo* conditions on the small model shrimp, *Artemia*. The dsRNA solution can be injected in different stages and tissues of this model shrimp, including post-diapause embryos (Dai *et al*., 2008), the hemocoel of young larvae (Copf *et al*., 2004 ; Copf *et al*., 2006), the body cavity (Dai *et al*., 2011 ; Yang *et al*., 2013a), reproductive segments (Liu *et al*., 2009 ; Zhao *et al*., 2012) and the egg sac of adult shrimp (King & MacRae, 2012). Copf *et al*. (2004; 2006) have developed a protocol for microinjecting dsRNA of *caudal* gene into the haemocoel of young *A. franciscana* larvae. The surviving larvae injected with *caudal* dsRNA showed a strong phenotype with a short body that is missing some segments, whereas injections with control dsRNA gave no adverse effects (Copf *et al*., 2004). The injection dose depends on the size and the RNAi sensitivity of the target species and tissues and usually ranges from about 5 ng to 1000 ng per injection. Hemolymph injection (Yodmuang *et al*., 2006 ; Ongvarrasopone *et al*., 2011) and intramuscular injection (Kim et al., 2007a ; Hou *et al*., 2014 ; Loy et al., 2013) are used to deliver dsRNA into bigger shrimp species (Table 2.2).

Finally, oral administration delivery of dsRNA, either in the form of naked dsRNA or in the form of bacteria that contain the specific dsRNA, has been successful in many arthropod species as well (Baum *et al*., 2007 ; Mao *et al*., 2007 ; Whyard *et al*., 2009 ; Wuriyanghan *et al*., 2011). When *P. monodon* and *L. vannamei* were fed with inactivated bacteria expressing shrimp-specific dsRNA targeting *Rab7* and *Stat*, a significant reduction of *Rab7* as well as *STAT* transcripts was confirmed by real-time PCR at 48h after 6 days of continuous feeding, not only in the hepatopancreas but also in the gills (Attasart *et al*., 2013). *Artemia* enriched with dsRNA-expressed bacteria could be used as a

dsRNA-delivery system for these shrimp species. As a proof of concept, *Artemia* nauplii, enriched with *E. coli* expressing dsRNA targeting the Leam Singh virus (LSNV) were used in a feeding experiment with *P. monodon* postlarvae. Quantitative RT-PCR indicated that the numbers of LSNV copies in most of the treated shrimp were at least 1000-fold lower than in the untreated controls (Thammasorn *et al*., 2013), effectively proving that this approach could be used in an antiviral treatment of shrimp. A dsRNA-feeding strategy was also used to knock-down the gonad-inhibiting hormone (*GIH*) gene of *P. monodon* by oral feeding with adult *Artemia salina* that was enriched with dsGIH-expressing *E. coli*. However, the suppression of GIH transcripts by such oral feeding was less effective than the injection method (Treerattrakool *et al*., 2013). The dsRNA can be mixed with chitosan to form dsRNA chitosan complex nanoparticles in order to enhance protection of the dsRNA in the intestinal tract of the shrimp, as well as improve uptake by the gut epithelial cells. The WSSV challenge experiment wherein *P. monodon* were fed with feed coated with inactivated bacteria containing dsRNA of the WSSV VP28 gene, showed higher survival than those fed with food coated with VP28dsRNA-chitosan complex nanoparticles (Sarathi *et al*., 2008).

Recently, symbiont-mediated RNAi has been demonstrated in the kissing bug (*Rhodnius prolixus)* and the western flower thrips (*Frankliniella occidentalis*) (Whitten *et al*., 2016). Through oral administration, the engineered bacteria, producing dsRNA specific for the host, colonized the digestive tract of the insects and caused a sustained systemic knockdown. This delivery could be a good way to overcome certain challenges involved with oral dsRNA delivery in insects, such as degradation of naked dsRNA in the digestive system. Although symbiont-mediated RNAi has not yet been applied in shrimp, this feeding approach might also be suitable for certain practical applications, such as antiviral treatment of shrimp, or large-scale experiments, rather than the more time-consuming injection method.

Table 2.2. Overview of the recent RNAi studies on several crustacean species for functional genomics and to control viral diseases

Species	Target genes	dsRNA Producing	dsRNA length	Delivery method	Doses	Phenotypes	Confirm method	References
sDaphnia melanica	Phenoloxidase	*in-vivo* by *E. coli*	245bp	Feeding	Filtrated water lake (*100ml*) contained 10 *D. melanica* and 1.2 × 10^8 *E. coli* cells.	Loss of melanin pigmentation and significant reduction in the ability to survive after UV exposure.	RT-PCR	Schumpert et al., 2015
Artemia franciscana	Group 1 late embryogenesis abundant (LEA)	*in-vitro* kit	463bp	Injection	80 ng dsRNA was injected into the egg sac	Embryos lacking group 1 LEA proteins showed significantly lower survival than control embryos after desiccation and freezing	qRT-PCR	Toxopeus et al., 2014
Macrobrachium rosenbergii	Insulin-like receptor (Mr-IR)	*in-vitro* kit	506bp	Injection	5 µg/g body weight	The individuals in the RNAi-treatment group develop male- specific secondary trait earlier than members of the control.	qRT-PCR	Sharabi et al., 2015
Litopenaeus vannamei	Lv-galectin	*in-vitro* kit	No data	Injection	2.5 µg/g shrimp	Increased mortality and hemocyte apoptosis	qRT-PCR	Cha et al., 2015
	ORF 1a and ORF 1b from IMNV	*in-vitro* kit	593bp, 600bp, 598bp	Injection	2 µg dsRNA/g body weight	Inhibition of infectious myonecrosis virus replication, resulted in 90 and 83% shrimp survival.	qRT-PCR	Feijo et al., 2015
Penaeus monodon	VP28 or WSSV051 from White spot virus	*in-vivo* by E.coli	181bp, 393bp	Feeding	Treated shrimp received 1.2 and 2.4 µg dsRNA per meal	Reduced % cumulative mortality and delayed average time to death	No data	Thammasorn et al., 2015
	PmDNV ns1 and vp	*in-vivo* by *E.coli*	600bp, 400bp	Feeding	Shrimp (200-300 mg) were fed with 30 mg of the diet-ns1+vp or commercial food twice a day for 14 days	Significant reduction of PmDNV was observed in the shrimp fed with dsRNA-ns1+vp in comparison to the control shrimp	qRT-PCR	Chimwai et al., 2016
Procambarus clarkii	Toll-like receptors PcToll	*in-vitro* kit	No data	Injection	50 µg of dsPcToll RNA/crayfish (9-10g)	The expression of Cru1, Cru2, ALF2 and Lys1 were inhibited.	qRT-PCR	Wang et al., 2015
Metapenaeus ensis	Neuroparsin (MeNPLP)	*in-vitro* kit	320bp	Injection	3 µg dsRNA/shrimp of 25-35 g	Significant decrease of vitellogenin transcript level in the hepatopancreas and ovary.	Northern blot	Yang et al., 2014c

So far, there is no chemical transfection method for dsRNA delivery in shrimp. However, a DNA transformation experiment in *L. vannamei* was successfully performed with fertilized eggs at the one-cell stage by using microinjection, electroporation and the jetPEI transfection reagent method. Expression of the inserted gene of adult transgenic shrimp was determined by RT-PCR and showed that the transfecting reagent jetPEI seems to be the most efficient method for introducing foreign genes into shrimp eggs due to its simple procedures and high hatching rate, gene transfer efficiency, and survival rates (Sun *et al*., 2005). The jetPEI was also used to transfect a DNA vector construct allowing the stable expression of an antisense Taura syndrome virus-coat protein (TSV-CP) gene construct in shrimp zygotes. The transgenic shrimp exhibited increased resistance to the TSV infection as compared to control animals (Lu & Sun, 2005). Therefore, these studies showed that jetPEI could potentially be used to transfer dsRNA into embryos of the shrimp species.

2.4 Applications

2.4.1 Disease control

Aquaculture, and more specifically the shrimp industry, is often faced with outbreaks of disease caused by bacteria and viruses. The discovery of RNAi has enabled studies on immune mechanisms and the function of genes involved in the process of fighting bacteria in shrimp species. Mechanisms that prevent bacteria from proliferation are believed to be related with prophenoloxidase. Knockdown of the inactive precursor of prophenoloxidase by RNAi resulted in a significantly increased bacterial load in *M. japonicus* (Fagutao *et al*., 2009). In another study, the *QM* gene from *L. vannamei*, encoding a tumor suppressor protein, was knocked down, resulting in a dramatic decrease of prophenoloxidase transcripts and activity in shrimp hemolymph, while the mortality was significantly increased in a bacterial challenge test with *Vibrio anguillarum* (Liu *et al*., 2014). Knockdown of p38 mitogen-activated protein kinases in *L. vannamei* by RNAi resulted in a higher

mortality of *L. vannamei* under *V. alginolyticus* and *S. aureus* infection, as well as a reduction in the expression of three antimicrobial peptides shrimp genes, namely, *pen4*, *crustin* and *alf2* (Yan *et al*., 2013). The expression of *MjGal*, encoding for a galectin of *M. japonicus*, was upregulated mostly in hemocytes and hepatopancreas tissue, and the protein was bound to both Gram-positive and Gram-negative bacteria. *MjGal*-silenced shrimp had significantly higher levels of bacteria in the hemolymph than the control shrimp, which confirmed that *MjGal* plays a key role in the shrimp defense against bacterial infections (Shi *et al*., 2014). Furthermore, the role of *M. japonicus* crustin like peptide (MjCRS) was examined *in vivo* by RNAi and this research suggested that MjCRS is involved in antibacterial defense and might not have a critical function against viral infections (Hipolito *et al*., 2014). Viral disease outbreaks are a major concern in the development of the shrimp aquaculture industry. Investigations into host-pathogen interactions might give new insights to viral infectivity and defense mechanism. RNAi also has been widely used as a powerful tool in identifying the genes that participate viral infection (Li *et al*., 2007) as well as defense from the host (Wang et al., 2013 ; Píndaro et al., 2013). Recently, antimicrobial peptides belonging to the anti-lipopolysaccharide factor (ALF) family from *P. monodon* have been investigated using RNAi. The results suggested that the ALFPm3 gene was regulated by Toll and IMD pathways, while the ALFPm6 gene was regulated by the Toll pathway (Kamsaeng *et al*., 2017).

One of the potential antiviral therapeutics to be considered is using RNAi to inhibit the replication of the RNA viruses as well as DNA viruses by knocking down virus-specific genes or downregulating host genes that are related to viral replication mechanisms. *Rab7*, a small GTPase protein of *P. monodon* and *L. vannamei*, plays a role in vesicular transport during viral infection. Silencing of the gene encoding Rab7 dramatically inhibited the replication of WSSV, Yellow head virus (YHV), LSNV and TSV in infected shrimp (Ongvarrasopone et al., 2008 ; Ongvarrasopone *et al.*, 2011 ; Ongvarrasopone et al., 2010). The direct

targeting of viral genes also allows us to inhibit viral replication in the host. This strategy has been demonstrated for targeting different kinds of viral genes, such as the genes encoding for viral coat protein in WSSV and TSV (Xu *et al.*, 2007 ; Zhu & Zhang, 2011 ; Wu et al., 2007 ; Lu & Sun, 2005), or viral enzymes such as RNA-dependent RNA polymerase in LSNV (Saksmerprome *et al*., 2013). RNAi has also been used successfully to inhibit the replication of YHV (Tirasophon et al., 2005 ; Saksmerprome *et al.*, 2009 ; Assavalapsakul et al., 2009). These results showed that dsRNA targeting nonstructural genes (proteases, polymerases, helicases) can effectively inhibit the viral replication, while the targeted structural genes were the least effective. While the studies on the applicability of RNAi to control the virus were mostly conducted on a small scale, the success of these experiments clearly showed that RNAi has enormous potential in inhibiting the replication of the virus and improve the survival of shrimp. In fact, researchers in Thailand recently succeeded in using RNAi by oral delivery against WSSV infection in *P. monodon*, leading to reduced percentages in cumulative mortality and delayed average time of death (Thammasorn *et al*., 2015).

2.4.2 Sex control

Using RNAi for sex control of shrimp is a remarkably practical application in aquaculture. The giant freshwater prawn (*Macrobrachium rosenbergii*) is considered an important freshwater shrimp in many countries and due to their bigger size and higher economic value, male prawns are preferred over females. In this species, the androgenic gland plays a role in male sex differentiation (Nagamine *et al*., 1980a ; Nagamine *et al*., 1980b), where a specific gene, namely insulin-like AG (*Mr-IAG*) is highly expressed (Ventura *et al*., 2009). *In vivo* silencing of *Mr-IAG*, by injecting the shrimp with Mr-IAG specific dsRNA, at an early developmental stage of juvenile males induced a full and functional sex reversal of males into neo-females. Additionally, crossing neo-females with untreated males produced all-male progeny (Ventura & Sagi,

2012). These experiments show that we can use RNAi in controlling the sex of giant freshwater prawn without changing the genetic structure of animals and therefore without creating transgenic animal species.

2.4.3 Functional genomics

RNAi can also be applied for research on the physiology of many different species of shrimp. Such studies have resulted in the discovery of genes involved in the molting of crayfish *Cherax quadricarinatus* (Von Martens, 1868) (Pamuru *et al*., 2012) and *P. vannamei* (Sangsuriya et al., 2014 ; Qian et al., 2014), and have allowed studying the reproductive functions of a gonad-stimulating hormone from sand prawn *Metapenaeus ensis* (Tiu & Chan, 2007), evaluating the function of the gonad-inhibiting hormone in *P. monodon* (Treerattrakool *et al.*, 2013 ; Treerattrakool et al., 2008 ; Treerattrakool et al., 2011) and determining genes related to growth regulation of *P. monodon* (De Santis *et al*., 2011). Recently, RNAi was used to investigate the function of Mandible alanine rich structural protein in *C. quadricarinatus.* The results of this study suggested that it play an important role in the organization of the chitin fibers oriented vertically to the surface of the mandibular incisor tooth (Abehsera et al., 2017).

The number of RNAi studies on *Artemia* (model shrimp) has risen rapidly recently (Table 2.2). *Artemia* species have a short life cycle and survive in extreme conditions. RNAi studies on *Artemia* gradually unravel mechanisms of stress resistance and diapause cysts. RNAi knockdown experiments on shell gland-specific genes and the Ste20 gene indicated that these genes are required for cyst shell formation and are involved in protecting the encysted embryos from environmental stress (Dai *et al.*, 2011 ; Zhou et al., 2014 ; MacRae, 2016). RNAi was also used to prove the involvement of p90 ribosomal S6 kinase in termination of cell cycle arrest during development of *Artemia* encysted embryos (Dai *et al*., 2008) and to prove that acetylation of chromatin-

associated histone H3 lysine 56 inhibits the development of encysted *Artemia* embryos (Zhou *et al.*, 2013). When the mechanism of cell quiescence regulation in *Artemia* was investigated using RNAi, it showed that a histone lysine methyltransferase is expressed abundantly in *Artemia* diapause embryos, in which cells are in a quiescent state (Dai *et al.*, 2017). The occurrence of autophagy was found to be increased before the diapause stage but dropped to an extremely low level in diapause cysts in *Artemia*. Silencing of transcription factor p8 gene revealed that this gene regulated autophagy during diapause formation in *Artemia* (Lin *et al*., 2016).

2.5 Conclusions

Since the first publication on the presence of the RNAi machinery in shrimp, the number of studies on shrimp RNAi has rapidly increased. These have identified the core components of the RNAi machinery in shrimp and this understanding has brought a positive impact and promises great potential for use in research and on many different applications in shrimp. The dsRNA molecules production process is evolving from *in vitro* to *in vivo* synthesis with lower cost, allowing more practical applications. In addition, newly developed dsRNA delivery techniques allow manipulations at the cellular and tissue level, especially; and studies have shown initial success in dsRNA delivery simultaneously on many individuals. RNAi can now be applied to control the sex commercialization shrimp, and promises widespread application enhancing animal production and prevention of shrimp viral diseases.

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CHAPTER 3

Annotation of the core genes of the RNA interference machinery in *Artemia franciscana*

Abstract

Artemia and the RNA interference technique were used in research to explore the function of genes in crustaceans. In this study, we study genes involved in RNA interference in *Artemia franciscana*. We annotated and characterized nine putative genes involved in RNA interference including two *Dicer*, three *Argonauts*, *two dsRNA binding proteins*, *Drosha* and *Sid-1* genes together with evidence of a *Pasha* and *Exportin-5* gene. The phylogenetic results suggested the identity of these genes and revealed that homologous members of both the siRNA and miRNA pathway genes in arthropods are also present in *Artemia*. This study provides the first look at the RNAi machinery of *A. franciscana* with its distinctive features compared to insects.

3.1 Introduction

In the last decade, *Artemia sp.* has been used as a model organism to increase our understanding of the biological processes in crustacean species. Researchers have explored many biological mechanisms at the molecular level, such as stress resistance and diapause (Liu *et al.*, 2009), cell division (Chen *et al*., 2016), development and differentiation (Copf *et al.*, 2004), and reproduction (Dai et al., 2010) using RNA interference (RNAi) as powerful tool. Moreover, the RNAi mechanism is known to be involved in the protection of cells against the entry of genetic material coming from e.g. viruses in shrimps (Labreuche *et al.*, 2010). Two parallel and closely related RNA pathways exist in eukaryotes: the siRNA (small interfering RNA) and miRNA (micro-RNA) pathways. In *Drosophila melanogaster*, the Dicer1, Loquacious and Ago-1 proteins are involved in the miRNA pathway, whereas Dicer2, R2D2 and Ago-2 protein are involved in the siRNAi pathway. A number of other proteins are also involved, such as Drosha, Pasha and Exportin-5, which are involved in the processing and transport of miRNAs from the nucleus to the cytoplasm (Denli *et al*., 2004 ; Bohnsack, 2004). The miRNAs are a class of small noncoding RNAs that function as regulators of gene expression. This process helps to regulate the level of mRNA for protein translation. Furthermore, a number of other proteins involved in the RNAi machinery are those responsible for the cellular uptake of dsRNA from the environment, such as SID-2, or the dsRNA transport from cell to cell by SID-1 in *C. elegans*, which can help spread the signals needed to activate the process of RNAi in target tissues.

Although studies have used RNAi as a tool to explore the biological processes in *Artemia*, the RNAi core genes of *Artemia* are still unidentified. Therefore, identification of the RNAi core genes of brine shrimp is necessary. To have an overview of the genes that may be involved in the RNAi of *A franciscana*, candidate genes from the transcriptome database of *A. franciscana* were identified, using homologs from others arthropod species as

reference sequences. Subsequently evidence for their transcription was generated. . The complete coding sequence of the candidate genes were then isolated by RACE PCR. Finally, their homology to homologs available in the GenBank, displayed by phylogenetic analysis, was used to confirm their identity. The information on these genes allows studying the molecular mechanism of RNAi in *Artemia* as well as the dsRNA delivery strategy. The latter will be discussed in Chapter 4 of this thesis.

3.2 Materials and methods

3.2.1 Culture of *Artemia franciscana*

A. franciscana cysts *(Vinh Chau strain, Vietnam)* used in this study, were obtained from the Artemia Reference Center of Ghent University. Cysts were incubated at 28^{o}C with strong aeration in autoclaved natural sea water (salinity is 28 – 30 ppt) until hatched. The harvested larvae were cultured and maintained at 28^{o}C with aeration in autoclaved sea water under fluorescent light (300 lux, 24/24 hours) and fed with green alga *Tetraselmis suecica*.

3.2.2 Searching potential transcript of RNAi core genes

Amino acid sequences of homologs for the RNAi core genes were selected from several species including *Penaeus monodon, Litopenaeus vannamei, Marsupenaeus japonicus, Caenorhabditis elegans, Drosophila melanogaster* and *Fenneropenaeus chinensis*, available in GenBank (*https://www.ncbi.nlm.nih.gov*) (Table 3.1). The selected sequences were used as reference sequences to search for hits (contigs) in the *A. fransciscana* transcriptome database using the TBLASTN algorithm (*http://bioinformatics.psb.ugent.be/orcae/overview/Artfr*). The selected contigs were then translated to peptide sequence by the Translate tool (*http://web.expasy.org/translate/*). The obtained putative peptide sequences

were subsequently used in Protein BLAST searches to verify their identity (*https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins*). The contigs showing a match to RNAi core genes were finally used to design PCR primers for isolating cDNA of RNAi core genes.

3.2.3 RNA extraction and cDNA synthesis

A pool of *A. franciscana* (5 day-old larvae to adult animal) was used to extract total RNA using the RNeasy mini kit (Qiagen, Germany). The total RNA was used to synthesize cDNA in a 20 µL reaction using an oligo-dT primer and the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Science,

Table 3.1 Amino acid sequences of homologs for the RNAi core genes

Group	Gene	Species	GenBank accession number	Reference
miRNA pathway	*Ago1*	*Penaeus monodon*	ABC68592.1	Unajak *et al*., 2006
		Litopenaeus vannamei	ADK25180.1	Labreuche *et al*., 2010
		Marsupepanaeus japonicus	AFQ31557.1	Huang & Zhang, 2012
	Dicer 1	*Penaeus monodon*	ABR14013.1	Su *et al*., 2008
		Litopenaeus vannamei	ACF96960.1	Yao *et al*., 2010
	Drohsa	*Marsupepanaeus japonicus*	ADB65770.2	Huang *et al*., 2012
	Pasha	*Litopenaeus vannamei*	AEI83216	Chen *et al*., 2012
	Exportin	*Crassostrea gigas*	EKC39725	
siRNA pathway	*Ago2*	*Litopenaeus vannamei*	ADK25181.1	Labreuche *et al*., 2010
	Dicer 2	*Penaeus monodon*	AGL08684.1	Li *et al*., 2013b
		Litopenaeus vannamei	AEB54796.1	Chen *et al*., 2011
dsRNA uptake	*SID1*	*Litopenaeus vannamei*	ADK25179.1	Labreuche *et al*., 2010
	SID2	*Caenorhabditis elegans*	NP_499823.2	McEwan et al., 2012
dsRNA binding protein	*TRBP1*	*Litopenaeus vannamei*	AEB54790.1	Chen *et al*., 2011
	TAR	*Marsupenaeus japonicus*	AEE36481.1	Wang *et al*., 2012
		Penaeus monodon	AGD81191	Yang *et al*., 2013b
		Fenneropenaeus chinensis	ACM17808.1	Wang *et al*., 2012
	Loqs	*Penaeus monodon*	AGC95230.1	Unpublished
	R2D2	*Drosophila melanogaster*	NP_609152.1	Hoskins et al., 2007

USA). Briefly, a 20 μL of reaction mixture containing 4 μL of 5X reaction buffer, 2 μL of 10 μM dNTPs mix, 20 units of Ribonuclease inhibitor, 200 units of RevertAid H Minus M-MuLV Reverse Transcriptase, 1 μL of oligo-dT primer and 500 ng of total RNA was mixed. Subsequently, the reaction mixture was incubated for 70 minutes at 42 °C. The reaction was terminated by heating at 70 °C for 5 minutes and then cooled to 4 °C. Complementary deoxyribonucleic acid (cDNA) was then used as template in PCR for isolating RNAi core genes in the further steps.

3.2.4 Isolating and sequencing CDS of RNAi core genes

3.2.4.1 First PCR of RNAi core genes

The gene specific PCR primers of each gene were designed to amplify the longest possible fragment of the selected contig (the potential transcript of RNAi core gene). The same conditions of the first PCR were applied for all genes in this study. Briefly, the PCR reaction contained 2.0 μL cDNA, 5 μL reaction buffer, 1.0 μL dNTPs (10 mM), 0.5 mM gene specific forward primer, 0.5 mM gene specific reverse primer (first primer, table 3.2) and 0.25 μL DreamTaq DNA polymerase (Thermal Scientific, USA). The PCR reaction was carried out in a thermal cycler (2720 Thermal Cycler, Applied Biosystems, USA) programmed for 94 °C for 4 minutes; 40 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 5 minutes; followed by 72 °C for 10 minutes. The amplicons were then separated on agarose gel and cleaned using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). These cleaned amplicons were directly used for sequencing by Sanger method using same PCR primers at LGC Genomics (http://www.lgcgroup.com). For long amplicons, the complete sequence was obtained by repeated sequencing with walking primers. The complete cDNA sequence of each gene was compiled by Vector NTI software

(Invitrogen, USA), while the open reading frame was predicted by ORF Finder from NCBI.

The missing 5'UTR or 3'UTR of the obtained sequence which encode the in-completed protein were then isolated by Rapid amplification of cDNA ends (RACE) in the further steps.

3.2.4.2 Rapid amplification of cDNA ends

Based on the sequences that were obtained from the first PCR in the step mentioned above step, the gene specific primers (Table 3.2) were designed to amplify the UTR sequence of each gene using SMARTer RACE Kit (Clontech, USA) with a modified protocol. Briefly, the cDNA reaction for isolation of 5'UTR: 20 μL cDNA reaction contains 4 μL 5X First-Strand Buffer, 0.5 μL DTT (100 mM) 1 μL dNTPs (20 mM) 5'-CDS Primer A, 10 μL total RNA, 1 μL of the SMARTer-II-A Oligonucleotide, 0.5 μL RNase Inhibitor (40 U/μL), 2 μL SMARTScribe Reverse Transcriptase (100 U). This mixture was incubated at 42 °C for 90 minutes. The cDNA reaction for the isolation of 3'UTR is the same as for the isolation of 5'UTR but SMARTer-II-A Oligonucleotide was not added and 5'-CDS Primer was replaced by 3'-CDS Primer from the kit. Subsequently, a PCR reaction was carried out to amplify UTR sequence: 1.0 μL cDNA (for 5' end UTR cDNA or 3'end UTR cDNA), 5 μL PCR buffer, 1.0 μL dNTPs (10 mM), 5.0 μl 10X UMP primer (Clontech Kit), 0.5mM gene specific primer (for 5'UTR or 3'UTR) and 0.25 μL DreamTaq DNA polymerase. The PCR thermal cycle was followed by denaturation for 3 minutes at 95 °C and subsequently, a 35 cycles amplification was carried out for 30 seconds at 95 °C; 30 seconds at 55 °C; 2 minutes at 72 °C. The reaction was extended for 10 minutes at 72 °C and then cooled to 4 °C. The UTR PCR products were purified from agarose gel using Wizard® SV gel and PCR Clean-up System (Promega, USA) and then ligated into the pGEM®-T easy vector (Promega, USA) using T4 ligase (LigaFast™ Rapid DNA Ligation System, Promega, USA). The ligation reaction

contains 5 µL 2X Ligase buffer, 1 µL pGEM®-T Easy vector, 3 µL 5'UTR or 3'UTR sequence and 1 µL T4 ligase. The ligation mix was incubated at room temperature for 3 hours to form the recombinant vector. The recombinant vector was subsequently transformed into the TOP10 chemically competent *E. coli* (ThermoFisher Scientific, USA). The positive clones were selected through ampicillin resistance selection and confirmed by PCR cloning analysis using a gene specific primer (RACE primer) combined with a vector primer (T7 primer, Promega, USA). The recombinant vector bearing the UTR sequence from positive transformants was purified by Wizard® Plus SV Minipreps DNA Purification System kit (Promega, USA) for sequencing both strands, using T7 and SP6 primer. The full length of UTR sequence was assembled by Vector NTI software (Invitrogen, USA).

3.2.4.3 Transcription evidence of full CDS

After amplifying and sequencing the UTR sequence using the RACE kit, the complete mRNA sequence from the 5UTR to 3UTR of each gene was confirmed by a PCR using gene specific primers which were designed after obtaining the UTR sequence. The PCR reaction (50 µL) contained 100 ng of total cDNA, 0.5 mM of each gene specific primer (Confirming primer, Table 3.2), 0.2 mM of dNTPs and 1.0 U of Phusion DNA polymerase in 1X of Phusion Green HF buffer (F-534S, Phusion Green High-Fidelity DNA polymerase, Thermal Scientific, USA). The PCR reaction was programmed for 98 °C for 30 seconds; 40 cycles at 98 °C for 12 seconds, 60 °C for 30 seconds, and 72 °C for 6 minutes; followed by 72 °C for 10 minutes then cooled to 4 °C. The amplicons were then separated on 0.8% agarose gel using 0.5X TAE electrophoresis buffer.

Table 3.2. The primers used in this study

Gene name	Primer names	Sequences (5'-3')	Information
Ago 1	F5	CTGGTCCCTTGGGCCCTAGTAC	First PCR
	R3	CGACCATTATCAAGAGAATCAGTTCTAAGC	First PCR, confirming primer
	F2	AAATGAGGCGGAAATACCGCG	Sequencing
	F4	TGCTGGTCTACAGCTTGTTGTTGTTG	Sequencing
	R4	TTGGTGCCTCTTGAGGAGAAGATGC	Sequencing
	R5	CGTGGGCATTTATCAGGTTGAATGGACACATC	5'RACE primer
	F8	CGGTTAGCACTACTTATAAGGGTGAGGATG	Confirm PCR
Ago 2	F2	CACAAAAAAAGCATATCACTATGACGTAGAA	First PCR
	R3	GGCAAGAAGGTGAGCATAATAGCTCGGAGC	First PCR
	F3	CCACCGGGTACAGTCGTTGACTCCTTG	Sequencing
	R5	ACGACCTCAAATTCCCTAGCTCTCTCTTCG	Sequencing
	R6	CTCCCTGAGGTTGCGTAGGTCTAGGTCCCC	Sequencing
	Ago2.5nRACE	AGTAATAGTGTTGAGATCGATCTCAGCAGC	5'RACE primer
	Ago2.3nRACE	CCACCGGGTACAGTCGTTGACTCCTTG	3'RACE primer
	ArAgo2.3URTF	CAGCCAGGTGCATGGGGACAAGGACCTCAG	confirm primer
	ArAgo2.3URTR	GACTGGACCATGAGTTTTGACACACACGAC	confirm primer
Ago 3	ArAgo3aF	ATGGGAGCTCATACGCAACAATCC	
	ArAgo3aR	TGAGCTGAAATTGTTCTCATCAAAAAGGACAC	
Dicer 1	Dicer1-F4	CGATGCGAAACAGCTAGTGATATTG	First PCR
	Dicer1-3UTR1	TCAACTCATTTTCACAAATTGTATGGGATG	First PCR
	Dicer1F1	CAAGCTCTGACAATGTCAAATGC	Sequencing
	Dicer1F5	AAGGTTCTGAGACTTTTAGAGATC	Sequencing
	Dicer1F8	GATGATTACTGTCATGAAGATGATG	Sequencing
	Dicer1R3	CCGAGTCCATCAATCATAGTTAAATTG	Sequencing
	Dicer1 R5	TCTTTTCGTACTGTGGAAGACAATCTG	Sequencing
	Dicer1.5nRACE	TCAGTTGGATCTGGCAAGCTATTC	5'RACE primer
	F	GAAAAGTCTCGACGCTGCATCTTGAG	confirming primer
	R	ACAAAGAATAGCCAAAGTAAGTAATTTATTGCTCC	confirming primer
Dicer 2	ArDicer2F1	CTTATATCCACGAGTGTATTGGAAGAAGGG	First PCR
	ArDicer2UTRR	GAGGGCACTAACGTCATTGAAAGATTGTGG	First PCR
	Dicer2F2	CTACAATAACCCGCGAAAATTTGGCCCTGG	Sequencing
	Dicer2R1	GGAATCTAGGTATACAGCACCGCATATAG	Sequencing
	Dicer2R2	GACTGCGTCACCAAGGAACTCTAGCCTCTG	Sequencing
	Dicer2R3	GGACAGTTAAATCAGGCCAAGCACTGAC	Sequencing
	Dicer2R4	CCAGCAACGCCACGTAAGTAGTTTCCATC	Sequencing
	Dicer2.5nRace	CCAGCAACGCCACGTAAGTAGTTTCCATC	5'RACE primer
Drosha	F1	GAACAAATGACGAAAACAGCAGCTTTCGGAC	First PCR
	R1	GTCCTTCATCACTAAGAATAGAAGC	First PCR
Sid-1	F	GTCCATCATTTGGGAGGGATGAGG	First PCR
	R	CATGTACACTTTCCACTTTCGCTTCC	First PCR
dsRNA binding protein	TRBPCDS-F	GGGATCCATGTTTGAGGTGAAAAG	First PCR
	TRBPCDS-R	AGCCATACAGGCTGAGTATTTCTC	First PCR
	StaufenCDS-F	CATCTTTAAGACAATCTTCTTTAAGCTAACG	First PCR
	StaufenCDS R	GCCAATAAAGAAATGCAAGGAAGGC	First PCR

3.2.5 Signal peptide, domain prediction and phylogenetic analysis

The Scan-Prosite database currently contains patterns and profiles specific for more than a thousand protein families or protein domains. The domain architecture of the isolated putative proteins in this study was analyzed by Scan Prosite (*http://prosite.expasy.org/prosite.html*) (De Castro *et al*., 2006). The signal peptide sequence is predicted by the SignalP 4.1 Server (*http://www.cbs.dtu.dk/services/SignalP*). The phylogenetic tree was generated from *http://www.phylogeny.fr/* by using PhyML, a phylogeny software based on the maximum-likelihood principle (Guindon *et al*., 2010). The usual bootstrapping procedure is replaced by a fast approximate likelihood ratio test (aLRT), which has proven to be a good and faster alternative to the (time-consuming) bootstrap analysis (Anisimova *et al*., 2006). The putative polypeptide sequence from the isolated transcripts and known polypeptide of homologs from NCBI were introduced under FASTA format.

3.3 Results

3.3.1 DICER protein

Two cDNA molecules have been isolated and sequenced from *A. franciscana* by RACE and successfully amplified in a PCR (Fig. 3.2A). The predicted ORF of these cDNA fragments encode polypeptides with several domains corresponding to the structure of Dicer proteins. These two cDNA were named *ArDicer-1* and *ArDicer-2* (Fig. 3.1). The cDNA of *ArDicer-1* (GenBank accession number: KY609157) is 5973 bp in length with an ORF that encoded a polypeptide of 1.932 aa residues, while the cDNA of *ArDicer-2* (GenBank accession number: KY609158) is only 4.875 bp in length, bearing an ORF encoding a polypeptide of 1.518 aa residues.

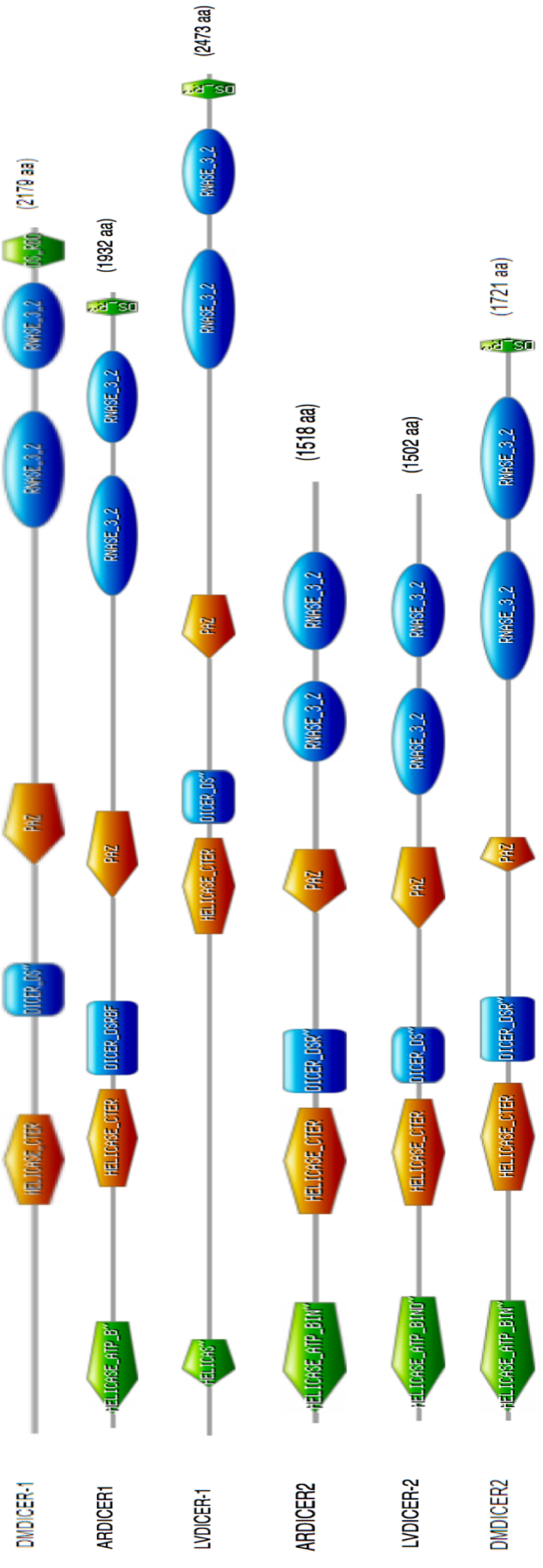


Figure 3.1. The structure of Dicer proteins from *Artemia franciscana*. ArDicer1 and ArDicer2 are Dicer protein from *A.franciscana*. LvDicer-1 and Lvdicer-2 are Dicer from *Litopenaeus vannamei*. DMDicer-1 and DMdicer-2 are Dicer from *Drosophila melanogaster*. The domains are in order from the left to the right: ATP-binding helicase type-1 (HELICASE_ATP_BIND_1), C-terminal helicase (HELICASE_CTER), Double-stranded Dicer-binding fold (DICER_DSRBF), PAZ, two Ribonuclease III (RNASE_3_2) and Double stranded RNA-binding domain (DS_RBD).

A phylogenetic analysis of the retrieved polypeptide sequences was performed to confirm the correct relationship and homology of ArDICER-1 and ArDICER-2 to DICER protein from others crustacean and insect species (Fig. 3.2B). It showed that ArDICER-2 belonged to the DICER-2 protein cluster, while ArDICER-1 is grouped in the DICER-1 cluster but it not close to any shrimp or insect species.

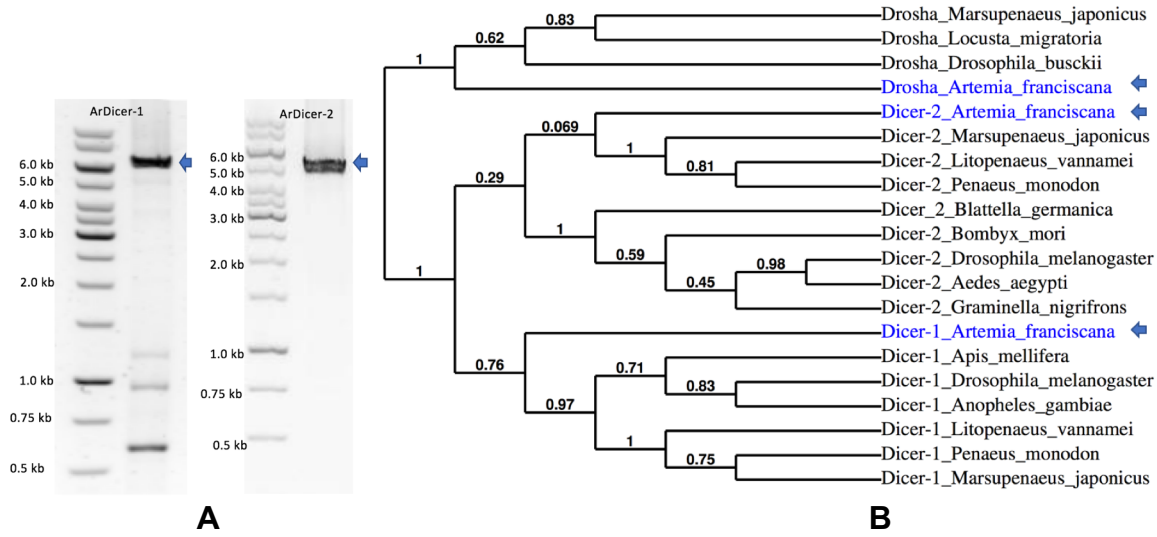


Figure 3.2. The evidence of transcription and the phylogeny tree of the DICER and DROSHA proteins from *Artemia franciscana* **A**: Agarose gels showing the evidence of amplification of the complete transcripts of *ArDicer-1* and *ArDicer-2* by PCR. The arrows indicate the specific PCR products. **B**: A phylogenetic tree representing DICER and DROSHA proteins from *Artemia franciscana* with other shrimp species (*P. monodon; L. vannamei; M. japonicus*) and insect species (*A. mellifera; G. nigrifrons; A. aegypti; B. mori; B. germanica; D. melanogaster; D. buskii; L. migratoria*). The numbers on the branches represent the branch support values.

3.3.2 Argonaute proteins

Based on transcriptome data of *A. franciscana*, specific primers were used in the PCR and RACE method (Table 3.2), resulting in three cDNA sequences which have been amplified and sequenced. They were named *ArAgo-1*, *ArAgo-2* and *ArAgo-3*. The RACE method was used to isolate *ArAgo-1* (3875 bp, KY609160) and *ArAgo-2* (3515 bp, KY609161), while *ArAgo-3* (2772 bp, KY661908) was successfully isolated in the first-round PCR. The full cDNA sequences of *ArAgo-1* and *ArAgo-2* were also confirmed by a PCR (Fig 3.3A). The sequence analysis showed that for *ArAgo1* and *ArAgo-2*, a full CDS was obtained but for *ArAgo-3* only a partial CDS could be retrieved. However, they all encode different putative proteins bearing typical Argonaute domains including one PAZ and one Piwi domain (Fig 3.3B). Among these three Argonautes, the polypeptide of *ArAgo-2* has a glycine-rich region at its N-

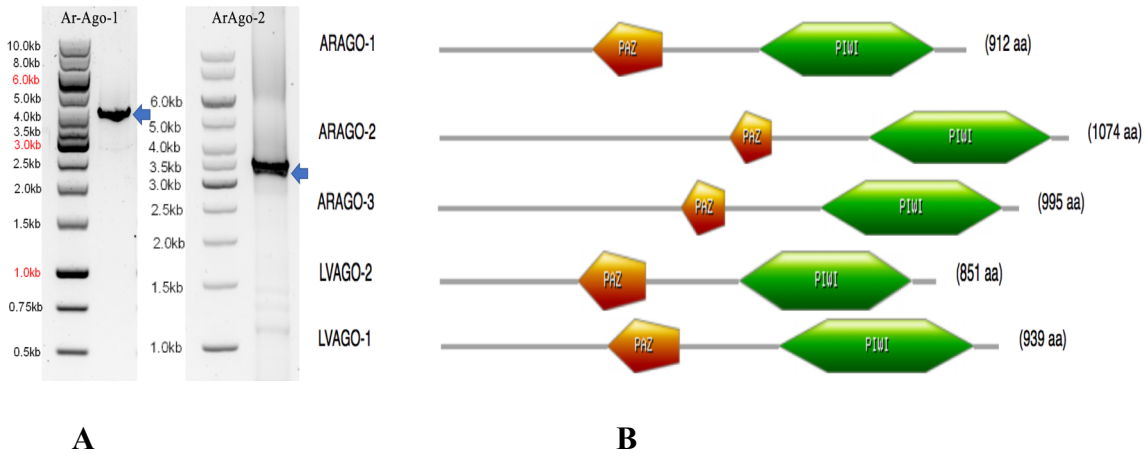


Figure 3.3. The evidence of transcription and structure of three Argonaute proteins in *A. franciscana.* **A**: The PCR product from the complete transcript of *ArAgo-1* and *ArAgo-2* in *A. franciscana*, the arrows indicate the specific PCR products. **B**: The structure of Argonaute from *A. franciscana*. Argonaute proteins of *A. franciscana* are complete ArAGO-1, complete ArAGO-2 and partial ArAGO-3; LVAGO-1 and LVAGO-2 represent the structure of Argonaute proteins in *L. vannamei*. The PAZ domain is represented in orange, the PIWI domain in green.

terminal. According to the conserved domains database of NCBI, this Glycine-rich domain is frequently found at the N-terminal of Argonaut-like proteins.

The phylogenetic tree confirmed that the three isolated putative *Argonaute* candidates in this study belong to the cluster of AGO proteins and did not cluster together with the related and similar Aubergine/Piwi protein clade (Fig. 3.4). The ArAGO-1 was placed in the cluster of AGO-1 proteins. Moreover, the phylogenetic tree suggested that ArAGO-1 protein is more closely related to the AGO-1 protein of insects than that of other crustacean species, particularly several shrimp species. In contrast, ArAGO-2 and ArAGO-3 separately branched into one cluster, not close to AGO proteins which have been isolated and identified before in crustaceans and insects. Moreover, amino acid sequences of ArAGO-2 and ArAGO-3 share only 60% identity, indicating a considerable degree of divergence.

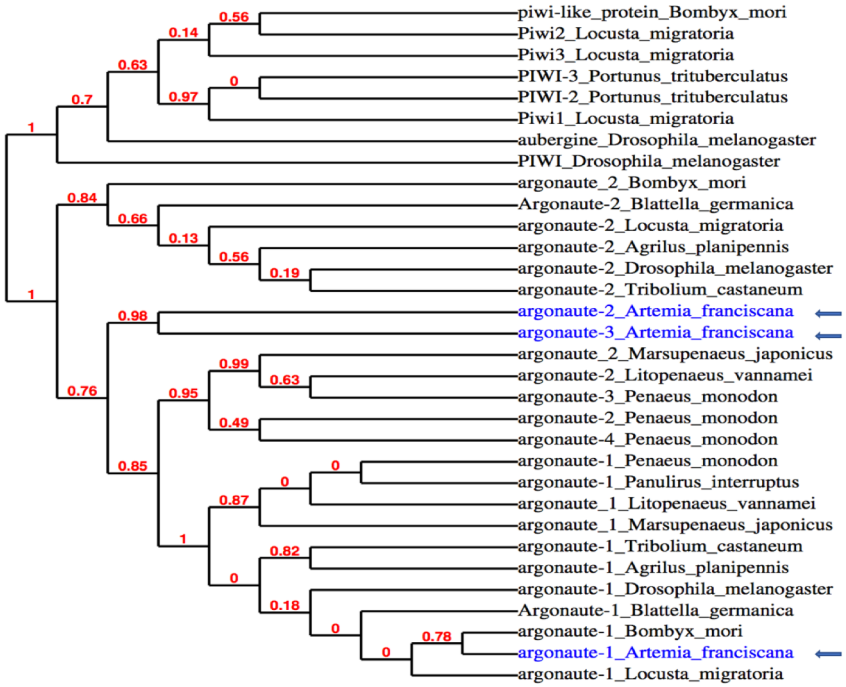


Figure 3.4. The phylogenetic tree of three isolated putative Argonaute proteins from *A. franciscana* with other shrimp and insect species. The numbers are the branch support values. The arrows indicate the putative Argonaute proteins that were found in this study.

3.3.4 The double stranded RNA binding proteins

The *A. franciscana* transcriptome database was searched for the dsRNA binding protein using homologs of RNA-binding protein (TRBP), Loquacious (Loqs) and R2D2 and several good hits were found that match to TRBP homologs. From those good hits, two cDNA sequences were isolated and identified as encoding for proteins containing dsRNA binding domains. The first putative protein ArTRBP-1 (KY661910) has three dsRBDs and the second putative protein ArStaufen (KY680793) comprises four dsRBDs and one Staufen C-terminal domain (fam16482). In addition, another contig designed *as ArTRBP-like* (KY996554) that encodes for a dsRNA binding protein also was cloned for sequencing. The *ArTRBP-like* encodes a

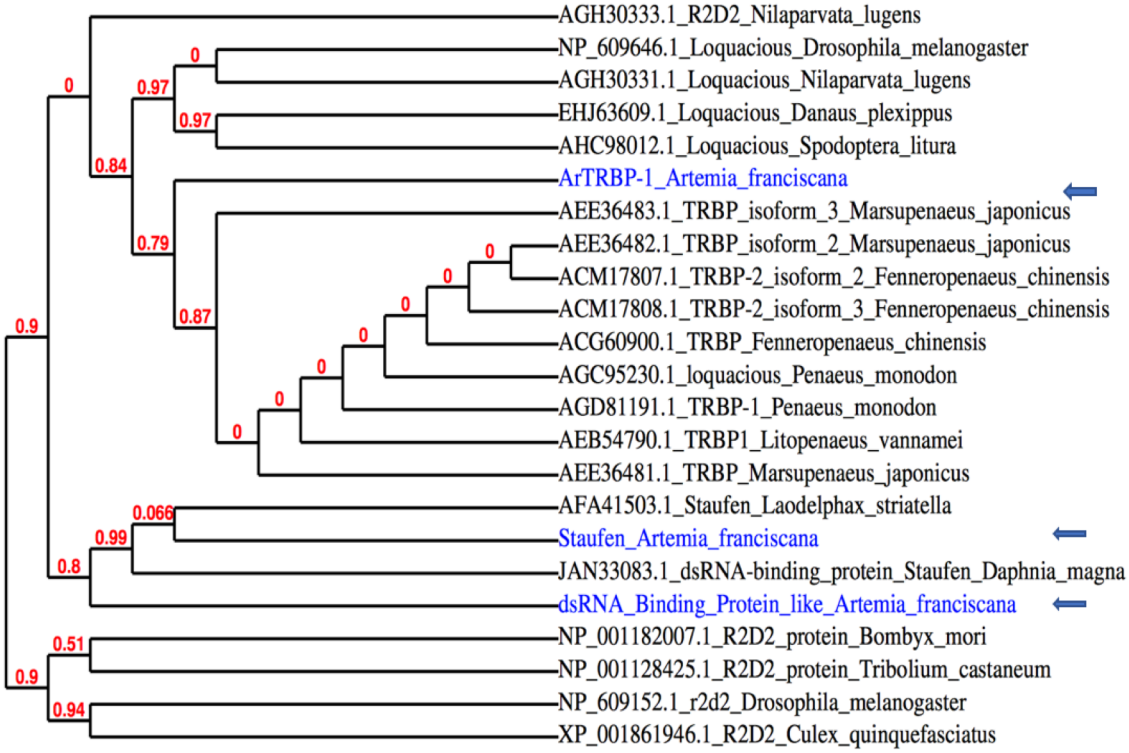


Figure 3.5. The phylogenetic tree of three isolated dsRNA binding proteins from *A. franciscana* with other shrimp and insect species. The numbers are the branch support values. The arrows indicate the putative RNA binding proteins that were found in this study

polypeptide bearing three dsRBDs. The phylogenetic tree was used to identify the ArTRBP-1 and ArTRBP-like proteins by comparing them to RNAi-related proteins previously identified in other species. It shows that the ArTRBP-1 is located in the same cluster with TRBP of other shrimps, while the ArTRBP-like protein is close to the cluster of Staufen proteins (Fig. 3.5).

3.3.5 Drosha, Pasha and Exportin-5

One cDNA sequence of 4342 nucleotides, coding for a putative protein of 1358aa, was also isolated from cDNA of *A. franciscana* (KY609159). This cDNA sequence encodes a putative protein containing two RNase III domains and a dsRNA-binding domain at the C-terminal. Based on domain structural and phylogeny tree analysis, it suggested that this is a *Drosha* homolog (namely *ArDrosha*) (Fig. 3.2). However, proline-rich and arginine/serine-rich domains were not present at the N-terminal of this putative ArDROSHA protein.

Using *Pasha* from *L. vannamei* as a homologous sequence in the Blast search, a transcript was found in the *A. franciscana* transcriptome database (contig_3385). This contig was not confirmed by PCR, however, the computational translation showed that it encodes a polypeptide of 589 a.a; and consists of an amino acid sequence “SYICILHEYTQHIIRKLPKYE FSELENSSSPYGCTISIDNVKYGAAQGASKAKLEAAKKALEILIP”, containing a dsRNA-binding motif of *Pasha* homolog. This result suggests that contig_3385 could be a candidate for *A. franciscana Pasha*. Additionally, we also found evidence of *Exportin-5* on another contig (contig_26227). It bears a partial CDS that encodes a polypeptide which shares 27% identity with *Crassostrea gigas Exportin-5* gene.

3.3.6 The dsRNA uptake and transportation

The polypeptide *sid-1* of *C. elegans* was used in a BLAST search, resulting in a good hit to contig_39778 in the *A. franciscana* transcriptome database. This transcript was confirmed by PCR, the sequencing result of PCR product showed that it is a partial cDNA *ArSid-1* (KY661909) encoding for a C-terminal polypeptide of 560 a.a bearing a complete RNA-gated channel SID-1 domain.

3.4 Discussion

Genes involved in RNAi have previously been isolated in different shrimp species, including *P. monodon, L. vannamei M. japonicus, and F. chinensis*. Based on these studies, models of the RNAi pathways in Crustacea have been proposed. However, many questions still need to be answered in order to get the full view of the mechanism of RNAi activity on crustaceans (He *et al*., 2015). RNAi has recently been used in several studies on the immune system, biological development and stress resistance of *A. franciscana* in different environmental conditions. This study was conducted in order to have a view of the RNAi core genes in *A. franciscana*.

The DICER protein is seen as the first component in the mechanism of RNAi; DICER will bind to the dsRNA molecule and cut it into smaller dsRNA molecules (siRNAs) before transferring them to the Argonaute protein. DICER proteins have similar structures and are highly conserved between species. They normally consist of seven main domains; the N-terminal ATP-binding Helicase type-1 domain, a second helicase domain, double-stranded Dicer binding fold, PAZ, two Ribonuclease III and a C-terminal double stranded RNA-binding domain (Gao *et al*., 2014b). The arthropod Dicers can be divided into the *Dicer-1* or *Dicer-2* subfamilies (Gao *et al.*, 2014b). In *Drosophila*, *dmDicer-1* or *dmDicer-2* generate different classes of small RNAs where *dmDicer-1*

processes pre-miRNAs originating from the nucleolus while *dmDicer-2* processes dsRNAs into siRNAs in the cytoplasm (Lee *et al*., 2004 ; Liu *et al*., 2007). The number of DICER proteins vary among different organisms, for example four dicer proteins are present in *Arabidopsis thaliana*, two in *D. melanogaster*, and one in *Caenorhabditis elegans* and fission yeast *Schizosaccharomyces pombe*. DICER proteins are usually conserved in terms of domain structure in most species. However, some domains may be lost during the evolution such as the loss of the DEAD domain in DICER-1 of mollusks, annelids, platyhelminths and most arthropods (Gao *et al.*, 2014b). In *Gardia intestinalis,* the DICER protein lacks the N-terminal helicase domain and the dsRBD at its C-terminal (Svobodova *et al*., 2016). Here, we found one *dicer-1* and one *dicer-2* gene in *A. franciscana*. Domain prediction showed that the C-terminal dsRBD is only present in ArDICER-1 and that this domain is absent in ArDICER-2. In the human DICER protein, the dsRBD is involved in the binding of siRNAs and longer dsRNAs with overhangs (Zhang *et al*., 2004). The C-terminal dsRBD of human Dicer may also have properties of a nuclear localization signal (NLS) (Doyle *et al*., 2013). Interestingly, analyzing all DICER-2 proteins from crustaceans available in the NCBI GenBank, including *L. vannamei* (AEB54796.1) *M. japonicus* (BAM37458.1) and *P. monodon* (AGL08684.1) revealed that the dsRBD is absent in DICER-2 proteins from those crustacean species (data not shown).

Argonaute family proteins are characterized by the PAZ and Piwi domains and their role in RNA silencing is well studied and described. They can be classified into three subfamilies: AGO, PIWI and worm-specific Argonautes (WAGO) (Carthew & Sontheimer, 2009). Argonaute family members *dmAgo-1* and *dmAgo-2,* belonging to the *Ago* subfamily, were identified in *D. melanogaster*. *DmAgo-1* is required for mature miRNA production in the siRNA pathway, while *dmAgo-2* is part of the RISC complex of the siRNA pathway (Okamura *et al*., 2004). In this study, we identified three genes that encode for different Argonaute protein in *A. franciscana*. The phylogenetic tree showed that

these three proteins belong to the Ago subfamily, together with those of some insects and crustaceans. Unlike insects, some crustacean species are characterized by a variable number of AGO subfamily proteins, based on available genome sequencing data. For instance, three were found in *Daphnia pulex* (McTaggart *et al*., 2009) and four found in *Parhyale hawaiensi* (Kao *et al*., 2016) and *P. monodon* (Unajak *et al.*, 2006 ; Yang et al., 2014a ; Phetrungnapha *et al.*, 2013 ; Leebonoi et al., 2015). Three *Ago* genes were isolated in *L. vannamei* using the RACE method for isolation. These species share only a single-copy homolog of *Ago-1*, and a variable set of multiple copies of *Ago-2* homologs. Among the three isolated AGO proteins of *A. franciscana*, only ArAGO-1 was found in the same cluster with other AGO-1 homologs. Hence, we presume that ArAGO-1 of *A. franciscana* is homologous to AGO-1 homologs in other arthropods, suggesting a role in the miRNA pathway. In *P. monodon*, *PmAgo1*, *PmAgo2* and *PmAgo3* were reported to be activated by bacterial and viral infection, and involved in dsRNA-mediated gene silencing. *PmAgo4* may not play a role in dsRNA-mediated gene silencing or antiviral defense, but it appeared to participate in the control of transposons (Leebonoi *et al*., 2015). This showed that the *Ago-2* subfamily in crustacean is not only operating in silencing of exogenous RNA but may have other activities in the development. *ArAgo-2* and *ArAgo-3* were found in a separate cluster in the phylogenetic tree, which is located inside a bigger crustacean and insect *Ago-2* homolog clade. Thus, we suppose that these *Artemia* Ago's belong to the *Ago-2* subfamily. Important to note is that the genes involved in the piRNA pathway were not investigated in detail in this study. Therefore, there are still many proteins containing PAZ and Piwi domains in the transcriptome data of *A. franciscana* that need to be further identified such as PIWI and worm-specific Argonautes (WAGO).

To recruit Argonaute proteins and form the core of the RNA-induced silencing complex (RISC), DICER proteins have been shown to act in cooperation with several proteins which have dsRNA binding domains

(dsRBDs), such as *Loquacious* (*LOQS*) and *R2D2* in *D. melanogaster* (Okamura et al., 2011 ; Forstemann et al., 2005); or HIV-1 transactivating response (TAR) RNA-binding protein (TRBP) in humans (Chendrimada *et al*., 2005 ; Haase *et al*., 2005). In *D. melanogaster*, the LOQS protein contains three dsRBDs; dsRBD1 and dsRBD2 are classical RNA-binding domains, which interact with the dsRNA during DICER processing (Jakob *et al*., 2016). In contrast, R2D2 protein has only two dsRBDs and is the sensor for choosing the guide strand of the siRNA duplex to be loaded into the RISC. The DICER-2/R2D2 complex binds to siRNA and enhances sequence-specific messenger RNA degradation mediated by the RNA-initiated silencing complex (RISC) (Liu et al., 2006). In humans, TRBP plays a role in the recruitment of the DICER complex to AGO-2 (Chendrimada *et al.*, 2005). TRBP consists of three dsRBDs (Daviet *et al*., 2000 ; Haase *et al.*, 2005). In our research, we isolated *ArTRBP-1* and *ArTRBP-like* from *A. franciscana* that encode for a polypeptide containing three dsRBDs. The phylogenetic analysis showed that putative ArTRBP-1 is more closely related to the TRBP of crustaceans rather than LOQS and R2D2 of insects. This result suggests that ArTRBP-1 might interact with ArDICER-2 and could play a similar role as the TRBP of other shrimp species such as *L. vannamei and F. chinensis*. TRBP-1, DICER-2 and AGO-2 of *L. vannamei* have been shown to interact by co-immunoprecipitation assays and pull-down assays (Chen *et al.*, 2011). Evidence of interaction between TRBP and RISC complex has also been found indirectly in *F. chinensis* (Wang *et al*., 2009). Interestingly, so far there is no evidence for a *R2D2* homolog in *A. franciscana.* The absence of this *R2D2* was also reported in the analysis of the Brown shrimp transcriptome (Christiaens *et al.*, 2015). Besides, the putative ArTRBP-like of *A. franciscana* contains three dsRBDs but is not closely related to any TRBP known in other crustaceans. We also could not find any protein from other crustaceans that has a similar structure of ArTRBP-like by Protein BLAST in NCBI. In addition, signal peptide prediction confirmed the absence of a signal peptide in the primary polypeptides of ArTRBP-like which suggests that ArTRBP-like may play its role only in the cytoplasm. Given these facts, it is not

unlikely that ArTRBP-like could have a similar function as R2D2 in *D. melanogaster*, interacting with ArDICER-1 to assist in loading of siRNA to the RISC complex. Further research, investigating the interaction between *ArTRBP-like* and *ArDicer-1*, will have to confirm this. If correct, these results suggest that RISC complex with its dsRNA binding protein has diverged considerably, or even evolved differently in crustaceans and insects.

The initiation of the siRNAi pathway is triggered by the presence of extracellular dsRNAs in the cell. These dsRNAs can be transported or taken up into the cell through systemic RNAi and environmental RNAi mechanisms. Environmental RNAi comprises the uptake of dsRNAs from the environment such as the digestive system of the shrimp, while cell to cell or tissue to tissue transportation of dsRNA is called systemic RNAi. The identification of these proteins allows researchers to explore suitable dsRNA delivery strategies to activate the RNAi mechanism. In *C. elegans*, RNAi signal spreading is coordinated by *sid-1* (Feinberg & Hunter, 2003) the intercellular transporter of dsRNAs, while *sid-2* play a role in uptake of dsRNA from the environment (McEwan et al., 2012). The *sid-1* homolog is also considered as a key component in the uptake of dsRNA in certain arthropods (Gu & Knipple, 2013), however some insects have no *sid-1* homologs such as *D. melanogaster*. Here, receptor-mediated endocytosis most probably plays a role in the dsRNA uptake mechanism (Saleh *et al*., 2006). *Sid-1* homologs have been identified in some crustacean species, such as *L. vannamei* (Labreuche et al., 2010) and *C. crangon* (Christiaens et al., 2015), but were not found to be present in *Parhyale hawaiensis* (Kao *et al.*, 2016). In this study, one copy of an *ArSid-1* homolog was found in the available *A. franciscana* transcriptome, which might explain the success of RNAi by using dsRNA injection in this species (Copf *et al.*, 2004).

In several crustacean species, the complete set of RNAi core genes have been identified based on transcriptome data such as those from the European shore crab (*C. maenas*) (Verbruggen *et al*., 2015), Brown shrimp (*C. crangon*)

(Christiaens *et al.*, 2015) and the amphipod crustacean *Parhyale hawaiensis* (Kao *et al.*, 2016). Together with two *ArDicer,* three *ArAgo* and two *ArTRBP c*opies, there are several of RNAi core genes including Ar*Drosha, ArPasha, ArSid-1* and *ArExportin-5*, which have been isolated fully or partially in *A. franciscana* in this study. They revealed, for the first time, a clear view of the genes that may be involved in RNAi interference of *A. franciscana*. However, the identity of the isolated genes was only based on sequence and phylogenetic analysis. Further studies on the biological functions of these genes should be carried out in the future to confirm their role in the RNAi pathway and the innate immune response.

CHAPTER 4

RNA interference in *Artemia franciscana* through microinjection and feeding

Abstract

Artemia, known as brine shrimp, is a potential crustacean species model. In *A. franciscana*, the *caudal* gene is responsible for abdomen segmentation and nauplii with a shortened abdomen can be produced by RNAi silencing of this gene in nauplii. In this chapter, we aim to standardize an RNAi protocol that can be applied to silence a gene at the early stage of embryonic development, allowing us to silence sex determining genes (chapter 5 of this thesis). Two strategies for dsRNA delivery were evaluated on adult females to assess the possibility of silencing the *caudal* gene in their offspring. Firstly, dsRNA specific to *caudal* was injected into the egg-sac of the mother, leading to a successful silencing for up to 17 days and in 2 successive broods with expected phenotypes in RNAi-nauplii. These results showed that, using this method, a long-lasting and possibly even a transgenerational RNAi silencing effect can be achieved in *A. franciscana.* This allows us to use the RNAi technique to investigate the function of genes in early development, such as genes involved in sexual differentiation. Secondly, inducing RNAi using a feeding strategy with dsRNA-caudal producing bacteria was attempted, but this strategy failed to cause the expected RNAi phenotype in the offspring.

4.1 Introduction

RNAi technology has recently been used in studying aquatic animals such as fishes and crustaceans (Boonanuntanasarn *et al*., 2003 ; Kelly & Hurlstone, 2011 ; Ma *et al*., 2016a ; Yang *et al.*, 2014b). To activate the RNAi mechanism, dsRNA or siRNA need to be introduced into the experiment animal by strategies such as injection, immersion in dsRNA solution, or uptake through the digest tract (Whyard *et al*., 2015 ; Yang *et al.*, 2014b ; Sarathi *et al.*, 2008). In shrimp, delivery of dsRNA is mainly done by feeding and injection. In the feeding method, a large amount of dsRNA is produced in bacteria and subsequently inactivated bacteria are used to coat feed pellets, given to shrimp (Sarathi *et al.*, 2008 ; Saksmerprome *et al.*, 2009 ; Zhu & Zhang, 2011). In another approach, dsRNA produced by bacteria was mixed with chitosan nanoparticles as a delivery vehicle (Sarathi *et al.*, 2008). Feeding animals with RNAi-coated feed seems a promising method for high throughput delivery to a large number of animals.

Recently, *Artemia* species are being used as an animal model for the study of gene function in crustaceans (Qiu et al., 2006 ; Hsia et al., 2010 ; Chen et al., 2009). Many studies have focused on the knockdown of a specific gene to explore its function during all developmental stages. Up to now, only microinjection has been used to deliver dsRNA into *Artemia* species. The microinjection technique was first applied in *Artemia* to study the role of the *caudal* gene (*Afcad*, a homeodomain transcription factor) during the larval development of *Artemia* (Copf *et al.*, 2004). RNAi was done by injecting dsRNA of *Afcad* directly into larvae at an early stage. However, the effect of RNAi could only be observed a few days later, prohibiting the study of the effect of eliminating *A. franciscana caudal* mRNA during the early stages of the development. In the study of p90 ribosomal S6 kinase (RSK1 is a family of serine/threonine kinase that mediates signal transduction downstream of mitogen-activated protein kinase cascades), the researchers successfully

injected *RSK1* dsRNA directly into the de-capsulated embryos to investigate the requirement of *RSK* activity during early embryonic development of *Artemia* (Dai *et al.*, 2008), demonstrating that RNAi can be successfully used to investigate gene function at an early stage of embryonic development. Since 2011, the body cavity microinjection technique was introduced in *Artemia* before the egg sac is formed, allowing the researchers to obtain eggs with a RNAi phenotype. For example, cysts of SGEG1 RNAi-treated females became transparent and their surface was rough while SGEG2 knockdown led to cysts which were more fuscous and opaque, and their surface was more uneven. Moreover, the double RNAi-treated cysts were shrunken and transparent, and the surface adhesive and coarse (Dai *et al.*, 2011). The egg sac with its large size allows for easy and quick injection. The egg sac microinjection technique has allowed to investigate the function of *Group 1 LEA* and *heat shock p26* genes through embryonic development. The results suggest that *Group 1 LEA* is required for maximum resistance of cysts to freezing and desiccation (Toxopeus *et al.*, 2014), while heat shock p26 prevents spontaneous diapause termination and protects against stress (King & MacRae, 2012). However, it should be noted that the RNAi silencing efficiency can be variable between individuals in the offspring.

In this chapter, we aim to standardize an RNAi protocol that can be applied to silence a gene at the early stage of embryonic development, allowing us to silence sex determining genes (chapter 5 of this thesis). Herein, two strategies for dsRNA delivery, dsRNA microinjection and feeding of dsRNA-producing bacteria were applied on adult females to assess the possibility of silencing a particular gene from the embryonic stage onwards. In both strategies, we used *A. franciscana caudal* as an indicator gene. It was anticipated that an effective *caudal* RNAi silencing can be evaluated by the typical phenotype of nauplii in the offspring, which is characterized by a short body, distinct from wild phenotypes (Copf *et al.*, 2004). The ability to achieve RNAi in *Artemia* adult

females by feeding them with dsRNA producing *E. coli* HT115 was also investigated through the analysis of RNAi *caudal* phenotype of released nauplii.

4.2 Material and methods

4.2.1 *Artemia* culture

Artemia franciscana cysts were incubated at room temperature with strong aeration for 24h in autoclaved seawater. The hatched nauplii were transferred to a 4 L plastic tank for rearing at room temperature with aeration. They were fed with *Tetraselmis suecica* and maintained for 30 days until maturation.

4.2.2 RNA extraction and cDNA preparation

Total RNA was extracted from cysts or nauplli of *A. franciscana* using an RNeasy RNA extraction kit (Qiagen, Germany). Briefly, the sample was homogenized in 600 µL RLT reagent from the RNAeasy kit (Qiagen, Germany) with a glass bead beater and centrifuged at 12.000 ×g for 3 minutes. The supernatant was then transferred to fresh tubes prior to RNA isolation with the RNAeasy kit (Qiagen, Germany). cDNA was produced with First Strand cDNA Synthesis kit (Invitrogen, USA) and oligo-dT primer according to manufacturer's instructions.

4.2.3 The transcription of the *caudal* gene

The specific primers afcadF (CAACAGACCTATGACAAGAG) and afcadR (CCGACAATCCTAACACAGT) were designed to amplify a 481bp fragment of *Artemia caudal* gene. The one-step reverse transcription polymerase chain reaction (RT-PCR) was used to detect the transcript of *caudal* mRNA. Briefly, 2 ng of total RNA from each sample was mixed with 25 µL one-

step RT-PCR mix (1X Colorless GoTaq® Flexi buffer, 0,5 mM afcad primer, 0,5 µM afcadR primer, 2 mM MgCl2, 0,2 mM dNTPs, 2% trehalose, 5 U AMV reverse transcriptase (Promega, USA) and 1,25 U GoTaq® Hot Start Polymerase (Promega, USA)). The PCR reaction was carried out in a thermal cycler programmed for 1 cycle at 45 °C for 60 minutes, 95 °C for 2 minutes; 35 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 35 seconds; followed by 72 °C for 10 minutes. The *caudal* PCR product (481bp) was separated by electrophoresis in a 2%(w/v) agarose gel.

4.2.4 Microinjection of dsRNA

dsRNA preparation: The dsRNA-*caudal* template was produced by PCR using a cDNA template and *caudal*-specific primers (Afcaf-for ATCTGTATATACAACCCGATACTTG and Afcad-rev CAACAGACCTATAA CAAGAG) with a T7 promoter sequence (TAATACGACTCACTATAGGGAGA) added to the 5' end of each primer. After cleaning up with a PCR cleanup kit (Promega, USA), the PCR product was used for *in vitro* dsRNA synthesis using the MEGAscript RNAi kit (Ambion, USA). The quantity and quality of the dsRNA was confirmed by NanoDrop and agarose gel electrophoresis. The dsRNA was stored at -20 °C prior for further use. For the negative control, dsRNA which is specific to green fluorescent protein gene (*gfp*) was used. The protocol for dsRNA-*gfp* production was the same as the *caudal* dsRNA. Specific primers for *gfp* were GFP355F CTGATCGCGCTTCTCG and GFP355R AGGAGCGCA CCA TCTTCTTC.

Injection assays: The injected dsRNA solutions (*caudal* or *gfp*, at a concentration of 320 ng/µL), were prepared by diluting dsRNA in 0.5% phenol red in Dulbecco's phosphate buffered saline (Sigma-Aldrich, USA). Just before the injection, one month old and unfertilized adult females were laid on 2% agarose gel. The females' big brood sac at this age is suitable for microinjection and allows analysis of the phenotype in a higher number of nauplii. Seawater

surrounding the *Artemia* was removed using tissue paper. Approximately, 250 nL of the dsRNA solution containing 80ng dsRNA was injected into the egg-sac (Fig. 4.3A). Injection was executed with a Nanoject II microinjector (Drummond Scientific Co., Broomall, PA, USA) using a micropipette prepared with preset program 45 on a P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Co., Novato, CA, USA). Forty females were used per treatment and after injection, the shrimp females were kept in 6-well plates. Only *Artemia* which still had a red color after 2 hours were used for mating two days post-injection (the injected females need the time to heal the wound at the injected site). Each couple was reared separately in 15 ml sea water using the 6-well plate, and fresh sea water with algae was added every day. Nauplii morphology was investigated under stereoscope.

Real time PCR: qPCR was used as tool to confirm *caudal* downregulation. The transcription levels of *caudal* were quantified by RT-qPCR on both treated and control groups using gene specific primers for *caudal* and using *tubulin* as reference gene (King *et al*., 2013). To confirm the qPCR efficiency, standard curves for *caudal* and *tubulin* gene were prepared by serial dilution of known copy number of PCR product (Fig 4.4C). The pooled nauplii which originated from the second brood of three different females (considered as three biological samples) were separately used for RNA extraction and cDNA preparation. Two technical replications were used in the qPCR experiment on each cDNA sample. Briefly, 30-50 nauplii from the same brood were pooled for total RNA extraction using the Purelink RNA minikit (Thermo Fisher Scientific, USA). At the last step of RNA purification, 40 µL nuclease free water was added for elution. The cDNA was then prepared by SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific, USA) in a reaction of 20 µL using 300 ng total RNA as template. The relative qPCR was carried out using QuantiFast SYBR Green PCR kit (Qiagen, Germany) on Rotor-Gene Q Platform (Qiagen, Germany).

4.2.5 dsRNA feeding experiment

Construction of dsRNA-producing vector: To construct the dsRNA producing vector, two rounds of cloning were done using a pGEM-T Easy vector. In the first round of cloning, a 524 bp *caudal* fragment was amplified by PCR using gene specific primer afcadF CAACAGACCTATGAACAAGAG and afcadR CCGACAATCCTAACACAGTA GCCAG. After cleaning up from agarose gel, the first PCR product was ligated to pGEM-T Easy vector (Promega, USA), following the manufacturer's instructions. The recombinant vector was then transformed into competent Top 10 *E. coli* using a heat shock protocol. The positive colonies were selected by colony PCR using a T7 primer and afcadR. The first purified recombinant vectors were purified using the Miniprep kit (Promega, USA) for the second cloning. In the second cloning, the reversed complement DNA fragment of 387 bp from *caudal* was generated by primers afcaf-F-PstI AGACctgcagATCTGTATATACAACCCGATACTTG and afcad-R-SalI (AGACgtcgacCAACAGACCTATAACAAGAG). This second PCR product was then ligated into first recombinant vector at *Pst*I and *Sal*I site. Briefly, the 387 bp PCR product and the first recombinant vector were digested with *Pst*I and *Sal*I (NEB) in a double digestion reaction. Subsequently, they were joined to form pGEM-T dsRNA-AfCad (Fig. 4.1) vector using T4 ligase (Promega, USA) following the manufacturer's instructions. The pGEM T-dsRNA-afCad was then transformed into competent Top 10 *E. coli*. The positive colonies were selected by PCR using afcaf-F-PstI and SP6 primer (promega, USA). The purification of the pGEM T-dsRNA-Afcad vector was conducted using Minipreps DNA Purification System (Promega, USA) from positive clones and the inserted sequence was confirmed by sequencing at LGC company (www.lgcgroup.com) using the Sanger sequencing method. To produce the dsRNA-CaudaI of 387bp, pGEM-T-dsRNA-AfCad was transformed into the RNase III-deficient *E. coli* strain (HT115) (Timmons & Fire, 1998).

The *E. coli* HT115 producing dsRNA-*GFP* of 355 bp were prepared using the same strategy as dsRNA-*caudal*. The *E. coli* HT115 strain is an RNase III deficient *E. coli* strain, allowing the *afcad* and *gfp* dsRNA to accumulate in the *E. coli* cell upon induction by IPTG. The 455 bp of the sense GFP fragment was produced by PCR with primer (GFP527F TGATCGCGCTTCTCGT TGGGG) GFP527R (CTGACCCTGAAGTTCATCTG C). This PCR product was then cloned into the pGEM-T vector (Promega, UAS), and transformed to competent Top10 *E. coli* cells. The positive clones carrying the recombinant vector (pGEM-T/GFPfor) were confirmed by PCR using the T7 and GFP455R primer, which amplified a product of 532 bp. The reverse sequence of GFP (355 bp) was amplified by PCR using the GFP355F-SalI (CACCGTCGACTGATCGCGCTTC TCG) and GFP355R-PstI (GACCTGCAGAGGAGCGCACCATCTTCTTC) primers. Next, they were digested with *Pst*I and *Sal*I restriction enzyme. The digested reverse GFP fragment was ligated to pGEM-T/GFPfor, which was also opened by the same enzymes. The recombinant vector (pGEM-T-GFP) was

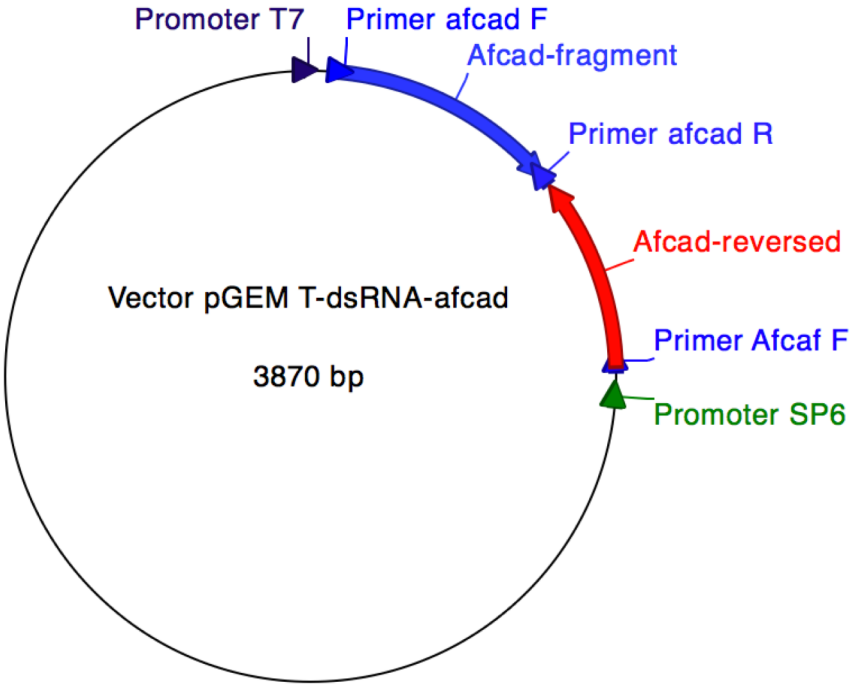


Figure 4.1. The structural Vector pGEM T easy transcripts containing a *Afcad-dsRNA* construct.

transformed into *E. coli* Top10 and confirmed by cloning analysis PCR using SP6 and GFP355R-PstI primer. For the production dsRNA-GFP of 355 bp, pGEM-T-dsRNA-caudal was transformed to the HT115 *E. coli* strain.

Bacteria producing dsRNA: An overnight culture was prepared by inoculating a transformant of the HT115 DE3 *E. coli* strain containing the pGEM-T easy dsRNA expression vector into 4 mL selective 2X YT medium broth (16 g tryptone, 10 g yeast extract and 5 g NaCl were dissolved to 1 litter water, pH 7.2) containing ampicillin (100 µg/mL) and tetracycline (12.5 µg/mL), incubated at 37 °C in a shaking incubator at 250 rpm for overnight. After overnight incubation, 250 µL was transferred into a falcon tube containing 25 mL selective YT2X broth containing Ampicillin (100 µg/mL) and Tetracycline (12.5 µg/mL), and was incubated at 37 °C with shaking. When the OD600 value of the culture reached 0.4, 12 µL of 1M IPTG was added to the culture for inducing dsRNA synthesis. Induction of dsRNA transcription was performed at 37 °C and shaking. After 4 hours of incubation, the induced cells pelleted were harvested by centrifuging at 6.000 ×g for 10 minutes. Physiological solution (8 ml) was used to re-suspend cell pellets and aliquoted 1 mL/1.5 mL tube. The cell pellets were then harvested by centrifugation at 6000 ×g for 5 minutes.

Subsequently, dsRNA was extracted from these dsRNA-production induced bacteria pellets by TRI-reagent (Sigma, USA) and checked for quantitation by spectrophotometric method (using Nanodrop) and quality by gel electrophoresis. Briefly, the cell pellet from the previous step (cell pellet of 3 mL bacterial culture) was suspended in 220 µL TES buffer (10 mM Tris pH 7.5, 10 mM EDTA and 0.5% SDS) by pipetting and incubated for 3 minutes at 100 °C. The lysate was incubated at room temperature for 5 minutes. Subsequently, endogenous RNA of *E. coli* as well as single-stranded RNA in the loop region of the hairpin dsRNA were eliminated by adding 25 µL of 10X RNase A buffer (4 M NaCl, 0,1 M Tris-HCl) and 5 µL RNase A (1000 U/µL) and then incubated at 37 °C for 30 minutes. To extract dsRNA, 750 µL of TRI-Reagent LS (Sigma,

USA) was added to the lysate, vortexed for 30 seconds, and then kept for 5 minutes at room temperature. Subsequently, 200 µL chloroform was added and vortexed before incubated at room temperature for 15 minutes. Then, the solution was centrifuged at 12.000 ×g for 15 minutes at 4 ^{o}C. Next, 500 µL of the aqueous solution was transferred to a new tube containing 500 µL isopropanol, mixed well and then kept at room temperature for 10 minutes before centrifuging again (12.000 ×g for 8 minutes at 4 ^{o}C). The pellet was washed with 1 mL of 75% ethanol by vortexing, followed by centrifuging at 7.500 ×g for 5 minutes at 4 °C. Finally, the supernatant was collected by pipetting with a small tip and the dsRNA pellet was dried at room temperate for 5 minutes before being dissolved in 50 µL nuclease free water. To confirm the quality, 3 µL of 1/100 diluted dsRNA was separated on a 1.5% agarose gel.

Feeding assay: Ten days before the experiment, 1 month old and unfertilized females were reared in separated tanks. Twenty adult females of *A. franciscana* were kept in a glass bottle containing 200 mL sterilized seawater. The pelleted bacteria producing dsRNA (*caudal* or *GFP*) from fresh bacterial culture were added to a final OD600 of 0.2 for feeding in the morning, the algae were then added in the afternoon of the same day. During the first ten days of the feeding experiment, seawater was changed in the morning just before new bacteria were added. After ten days of feeding dsRNA-bacteria, the females were transferred to 6-well plate for mating. The nauplii of these treated females were collected and abnormal phenotype was observed under stereoscope.

4.3 Results

4.3.1 Expression of *caudal* in *Artemia*

The expression of *afcad* was detected by reverse transcription PCR method. It showed that transcription of this gene starts at 0h (after de-capsulating within 1 hour of rehydration) and has a strong expression peak at

the umbrella-stage (12 hours after hatching), which is before the instar I stage (26h after hatching) and instar 2 (36 hour after hatching) (Fig. 4.2B). To double check the presence of *afcad* mRNA in cysts, the number of amplification cycles was increased to forty cycles and 20 ng of total RNA from cyst were used as PCR template. The result showed that *afcad* mRNA was stored in cysts (Fig. 4.2A).

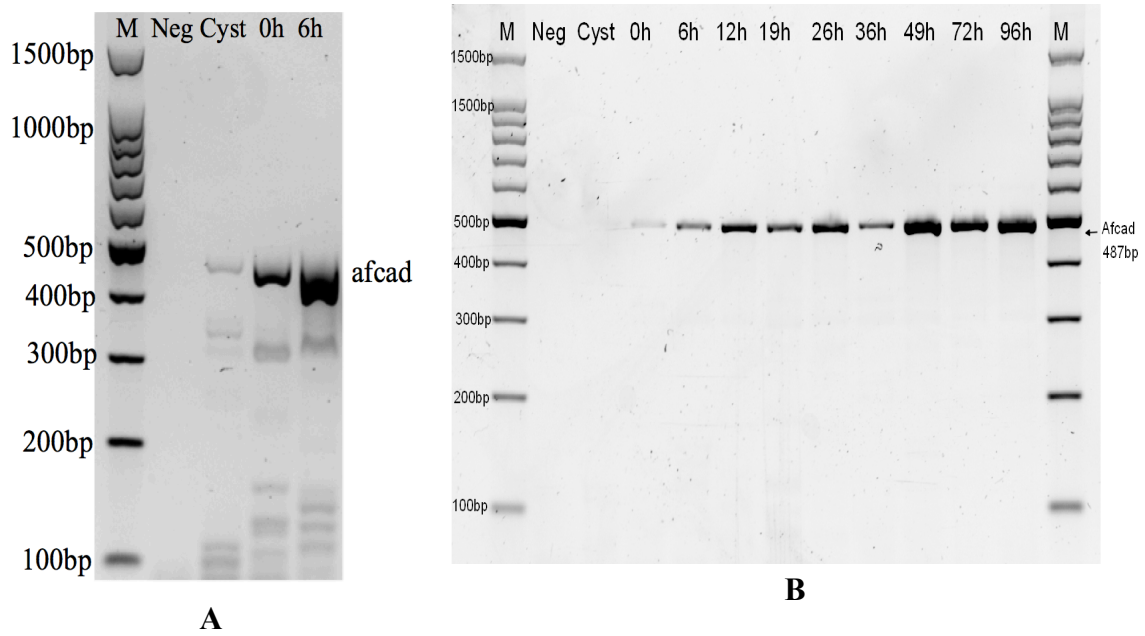


Figure 4.2. Detection of expression of afcad by RT-PCR. A: 40 cycle RT-PCR to detect afcad mRNA. B: 30 cycles RT-PCR in detection of afcad mRNA; M: 100bpDNA marker, Neg: negative control, Cyst: *Artemia* cyst, 0h-72h: time point 0-96h post hatching.

4.3.2 Injection assay

The egg-sac microinjection was done on forty females. However, only 24 animals survived the injection procedure and released nauplii in the *Afcad*-RNAi treatment. They were divided into three group based on the number of brood of nauplii that they released. There were four females in group A, which released only one brood of abnormal nauplii. Subsequently, these females produced

cysts or died. Group B contains the thirteen females which died or started producing cysts after the second brood. Of those thirteen, eleven females produced nauplii with abnormal phenotypes in two broods and two females produced normal nauplii only, without an RNAi phenotype, in both broods. The last group (group C) containing seven females produced three broods of nauplii, after which they died or started producing cysts. The average brood size of 1st, 2nd, 3rd were approximately 36, 28 and 25 nauplii, respectively. The abnormal nauplii of the treatment group exhibited a short body and spontaneously sank to the bottom (Fig. 4.3 B, C and D).

Figure 4.3. The egg sac microinjection in maternal *Artemia* and the RNAi phenotype induced in nauplii, the arrows indicate individuals with *Afcad* RNAi-phenotype. **A**: The needle penetrating in the egg sac, the pink color indicates that the dsRNA solution was injected successfully. **B** and **C:** The *l Afcad* RNAi phenotype of released nauplii from RNAi maternal *Artemia.* **D**. Larval phenotype at 9 day old, there are two small deformed nauplii from *AfCad* RNAi maternal *Artemia* and one big nauplii with normal wild phenotype from the *gfp* RNAi maternal *Artemia*.

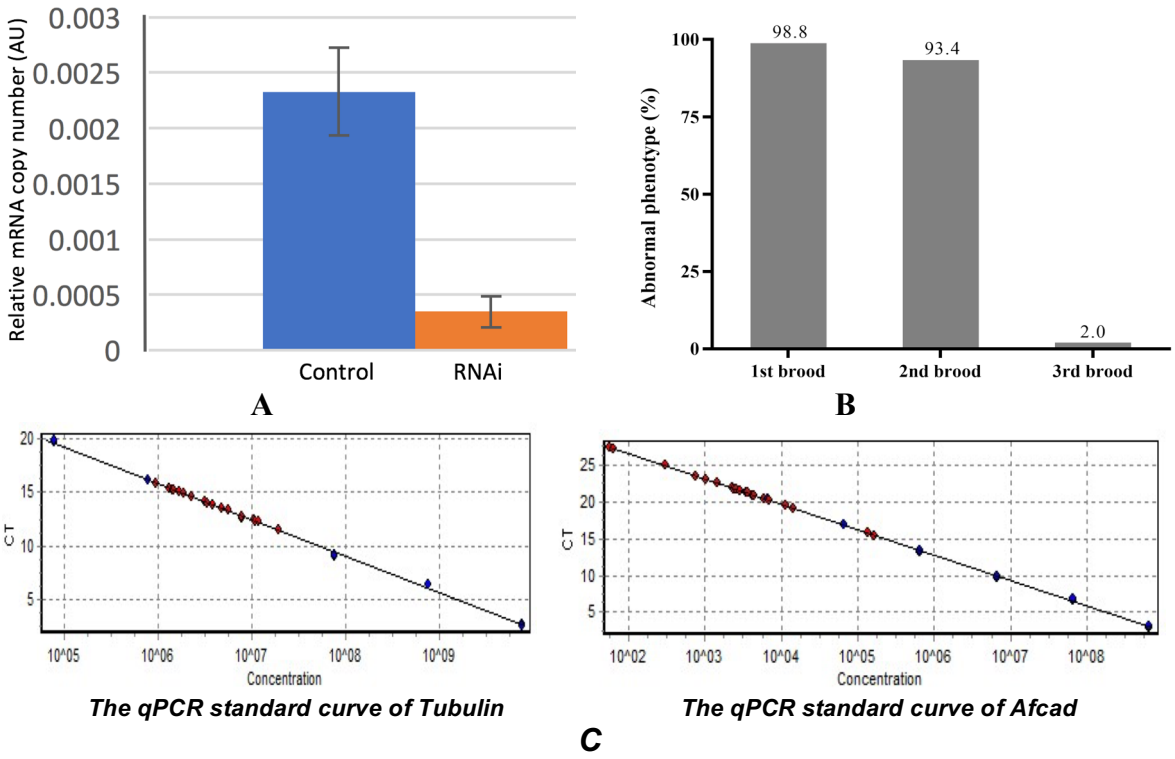


Figure 4.4. Silencing efficiency and the ratio of abnormal phenotypes of nauplii from the first to the third brood. **A**: The qPCR shows different silencing efficiencies for *afcad* RNAi treatment (orange) relative to control group (blue) (mRNA copy numbers of *afcad* were compared with copy numbers of *tubulin* mRNA, AU: arbitrary units). Error bars represent the standard deviation. **B:** The percentage of abnormal phenotype of nauplii in first brood (released at day 1 to 8) and second brood (released from day 14 to 17) is different compared to the third brood (released at day 20 to 21). **C**: The qPCR standard curves were built from known copy number of PCR product. qPCR efficiency of *tubulin* and *afcad* were 98% and 95% respectively.

The observation showed that *afcad* dsRNA treated maternal *Artemia* from group A, B and C released 95.8±1.5% nauplii with an abnormal phenotype (762/795 nauplii) in the first brood. Similarly, the *afcad* dsRNA injected maternal *Artemia* from groups B and C released 487/511 abnormal nauplii (93.4±1.6%) in the second brood in the first 17 days. However, the ratio of nauplii with abnormal phenotype of the third brood from group C was only 2% (5/254 nauplii) (Fig. 4.4B). Additionally, the abnormal nauplii were kept in new sea water but

most of them died after seven days. Only two surviving nauplii showed absence of body segments and missing appendages (Fig. 4.3D). The quantification of *afcad* mRNA from the RNAi-treated nauplii group (second brood), where we observed approximately 90% silencing, revealed that RNAi silencing of *afcad* in *A. franciscana* was still very efficient, even in the second brood (Fig. 4.4A).

4.3.3 Feeding assays

E. coli cells *(*strain HT115) were transformed with the vector constructs pGEM-T-dsRNA-afcad and pGEM-T-dsRNA-gfp that were designed for the expression of dsRNA of *afcad* and *gfp*. The dsRNA of *afcad* and *GFP* that was extracted from these transformants showed that they could produce dsRNA of *afcad* and *gfp* correctly with a yield of 15µg dsRNA/4mL bacteria culture (Fig 4.5).

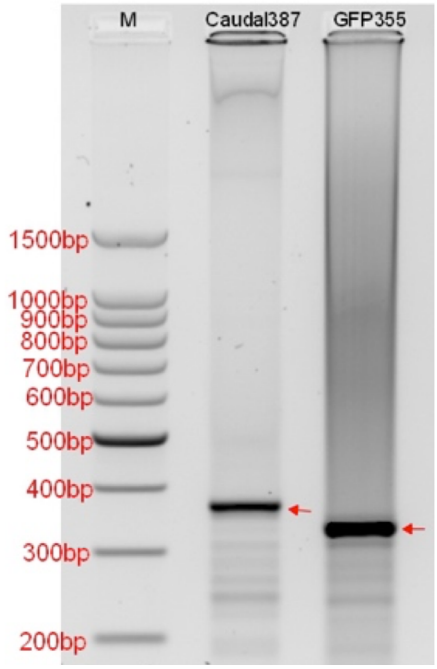


Figure 4.5. The purified *caudal* dsRNA (387bp) and GFP dsRNA (355bp) from dsRNA-producing transformants *E. coli* (HT115 strain).

After a period of ten days, feeding with bacteria producing dsRNA of *afcad* and *gfp*, ten surviving females of the treated group and eleven females of the control (*gfp* dsRNA) group were transferred to 6 well plate for mating. After 7-10 days, all females from both treatments produced nauplii, the average brood size was 56 nauplii. However, no abnormal phenotype was observed in nauplii from the a*fcad* treatment population.

4.4 Discussion

In *Artemia* development, *afcad* expression persists in the growth zone until the early-mid stage of larval development. During later larval stages, *afcad* is also expressed in a small number of mesodermal and ectodermal cells and in developing anal structures of the adult (Copf *et al*., 2003). In this study, the transcription profile of *afcad* was analyzed in cysts by semi-quantitative RT-PCR. We found that its transcription quickly increased at least during the first four days of the early larval stage. This result suggests that *afcad* may play its role from the early embryonic development of *A. franciscana* onwards. The knockdown of *afcad* during early larvae stages using microinjection resulting in larvae with a normal head and anterior thoracic segments but which failed to develop posterior thoracic, genital, and post-genital segments after 1-2 weeks development (Copf *et al.*, 2004). In our results, the *afcad* dsRNA treated maternal *Artemia* released nauplii that exhibited an abnormal shape with a curved upward tail to the back, shorter than normal. These nauplii could not survive longer than seven days, most likely because of failure to develop body segments. This result showed that *afcad* also plays an important role in development of the anterior thoracic segments during the embryonic stage.

dsRNA injection is routinely used to study gene function in *Artemia*. These results indicate that dsRNA which is introduced into the body cavity or egg-sac of females can also induce the RNAi knockdown mechanism in the oocytes and lead to silencing in the progeny. Although it is not clear whether

the dsRNAs directly enter the embryo or the RNAi signal is spread out from the female body to the embryo for inducing RNAi of the target gene. *Sid-1* gene was found in *A. franciscana* in the first study (chapter 3), which might be involved in the transportation of dsRNA and might maintain the signal for RNAi in the host. In *C. elegans*, the SID-1 protein transports extracellular dsRNA into the germline where it can silence maternally deposited mRNAs and segregate to embryos to silence embryonically expressed mRNAs. Extracellular dsRNA is also endocytosed into oocytes. The endocytosed dsRNA then requires SID-1 and SID-5 protein in embryos to silence embryonically expressed genes (Hinas *et al*., 2012 ; Wang & Hunter, 2017). In *Artemia*, the effectiveness of RNAi is usually evaluated through quantitative PCR or western blotting. In some cases, it can be evaluated visually through scoring phenotypes of cysts (Dai *et al.*, 2011 ; Yang *et al.*, 2013a) or abnormal embryonic development (Zhao *et al.*, 2012). However, the longevity of the RNAi effect in dsRNA-treated *Artemia* females as well as the efficiency of RNAi on living nauplii released from females had not been reported before. Due to the typical RNAi phenotype at the nauplii stage, the *afcad* gene can be considered as a good candidate gene to investigate transgenerational RNAi from *A. franciscana* adult females to their offspring. It has been reported that the interbrood intervals of *A. franciscana* reared in the laboratory at 25 °C is 4.4 day (Wurtsbaugh and Gliwicz 2001). The interbrood intervals were observed around 5-6 days in our experiment at 28 °C, this allows expecting three nauplii broods could be produced by each female within 21 days of experiment. RNAi as evaluated through the abnormal *afcad* phenotype of released nauplii, demonstrated that *afcad* RNAi-treated maternal *Artemia* could produce a high ratio of nauplii with a strong RNAi phenotype for at least two broods within about 17 days post injection. In contrast, the abnormal phenotype ratio in the third brood, which was released 4-5 days later was significantly reduced, indicating that recovery of expression of the target gene might have occurred at that point. These results could indicate that, at least for this gene, an efficient RNAi effect can be achieved in *A. franciscana* larvae for at least 17 days, but not longer than 21 days. In the round worm *C. elegans*,

ingestion of dsRNA by larval later stage resulted in silencing of 100% of the progeny (Marré *et al*., 2016). It showed that dsRNA can be directly transferred between generations in *C. elegans*. In insects, for instance the red flour beetle *Tribolium castaneum*, injection of dsRNA into the mother's haemocoel also results in knock down of zygotic genes and RNAi phenotypes in nearly 100% of offspring embryos(Bucher *et al*., 2002). Intergenerational transfer of dsRNA occurs even in animals that lack any DNA of matching sequence, and dsRNA that reaches progeny can spread between cells to cause gene silencing (Marré *et al.*, 2016). In conclusion, although biochemical evidence for intergenerational transfer of dsRNA was not documented here, the data provide circumstantial evidence for intergenerational RNAi transfer, facilitating RNAi research in *Artemia.*

The presence of the RNAi-*afcad* phenotype in *A. franciscana* nauplii (in the progeny) suggests that RNAi treatment in *Artemia* sustained for at least up two weeks. The prolonged duration of RNAi has been reported in *L. vannamei* in WSSV challenges. It showed that animals treated with dsRNA targeting viral genes (*vp26* and *vp28*) gradually lost the antiviral effect verified by virus challenge 10 days after RNAI treatment (Mejía-Ruíz *et al*., 2011 ; Nilsen *et al*., 2017). In *P. monodon*, protection against WSSV lasts thirteen days upon RNAi treatment via viral specific dsRNA injection (Westenberg *et al*., 2005). However, if the shrimp received several doses of dsRNA, protection lasted 28 days (Anil *et al*., 2015). In this study, the abnormal phenotype in released nauplii suggests that RNAi effectively occurs during embryonic development stages in the egg-sac. Additionally, the qPCR of *afcad* in released nauplii (Fig 4.4A) and the presence of abnormal nauplii (Fig 4.3) which could not survive beyond day 7 suggest that RNAi is still happening in nauplii after being released from the females.

The protocol for microinjecting dsRNA into the haemocoel of young larvae showed that 51% of the treated individuals have RNAi phenotype defects

in at least some of their appendages (Copf *et al.*, 2006). Recently, nauplii released by dsRNA-treated maternal *Artemia* have been used to study the involvement of HSP70 protein against pathogenic bacteria (Iryani *et al.*, 2017) or to study stress-resistance related proteins such as Ste20-Like kinase (Zhou *et al.*, 2014). The high rate of nauplii RNAi phenotypes obtained from *afcad* dsRNA-treated maternal *Artemia* demonstrates the reliability of the egg-sac injection method. Hence it can be used to produce nauplii with RNAi-phenotype for developmental gene function studies. The nauplii, which are released within the first 17 days post injection by this method, are suitable for studying different genes, including for example immune system genes, stress resistance genes, etc.

In several species, including some crustacean species such as *P. monodon* (Attasart *et al.*, 2013), *L. vannamei* (Thammasorn *et al.*, 2015 ; Sanitt et al., 2014), *Daphnia pulex* (Schumpert *et al.*, 2015), RNAi can be induced effectively to silence specific gene expression through ingestion of bacteria producing the corresponding dsRNA. This strategy has been shown to be successful in silencing viral genes (Sarathi *et al*., 2008) as well as host genes (Ongvarrasopone *et al.*, 2011). However, the efficacy of dsRNA uptake into shrimp cells through oral administration was shown to be lower than injection (Attasart et al., 2013). The *E. coli* strains were confirmed to produce *afcad* dsRNA correctly. However, the females in these feeding assay did not release any abnormal nauplii contrary to the microinjection delivery. Therefore, oral feeding of bacteria producing *afcad* dsRNA could perhaps not induce a strong enough RNAi-response in *A. franciscana* females. Another possibility is that *Artemia* lacks a strong systemic RNAi system, allowing the silencing signal to pass from the digestive system to the egg sac of female *Artemia*. In *P. monodon*, oral feeding of bacteria producing dsRNA targeting Gill-Associated Virus has also been reported as being ineffective, despite good results using microinjection (Sellars *et al*., 2011). Probably, there are some causes for this failure such as degradation of dsRNA in the gastrointestinal tract by nucleases,

pH, or the lack of a functional dsRNA uptake mechanism in the gut, two important issues which were also observed as being involved in RNAi efficiency in some insect species. An additional explanation could be that transgenerational RNAi after feeding, in the sense that it requires transport of the silencing signal from somatic cells to the germline within the body, is not functional in *Artemia* and that the effects we see after microinjection are due to direct uptake of injected dsRNA by the oocytes in the brood sac. These results could indicate that either environmental RNAi in the gut or systemic spread of the silencing signal to the germline is not working or not working very efficiently in *Artemia*. This might lead to the presence of much lower concentrations of dsRNA in the environment to which the oocytes or embryos are exposed to. Further research will have to elucidate these issues.

Besides these physiological factors which could explain the lack of successful feeding RNAi gene silencing, there are also some technical reasons which might play a role, and which might be improved or investigated further in the future. For example, the dose of dsRNA to which the shrimp were exposed to could play a role. Unfortunately, using this feeding assay and delivery method, it is impossible to find out the exact dose the treated animals were exposed to. However, an estimation could be made, based on *in vitro* purification and quantification of the dsRNA produced in the *E. coli*. In *Artemia*, approximately 12 hours after hatching, the nauplii molt into the second larval stage and they start filter feeding on different kinds of particles, such as various microalgae, bacteria, and detritus. The clearance rate of 4 day old *A. franciscana* is around 1.48 - 2.10 ml per individual per hour. In our feeding assay, we reared 20 adult females in 200 ml of seawater and *E. coli* were added to reach a final OD of 0.2 in the seawater. From 1ml *of E. coli* at OD of 0.2, we were able to purify 1.5µg of dsRNA. Therefore, the total amount of dsRNA (in *E. coli*) contained in 200ml of cultural seawater was 300µg dsRNA every day. Based on visual observation of the seawater, we can assume that twenty females could filter most *E. coli* in 24 hours. This means that each individual

could eat around 15 ug per day maximally. Given the fact that the experiment ran for 10 days, the amount of dsRNA taken up orally is high, especially compared to the doses which were needed with microinjection (a single 80ng dose). A second technical reason we had considered was the use of live bacteria, rather than dead or inactivated bacteria. Using the latter to feed the shrimp might lead to a higher or more rapid release rate of dsRNA by the bacteria inside the shrimp body. However, purification of dsRNA from dead bacteria indicated that the amount of dsRNA is very low (data not shown). For this reason, we decided to use live bacteria.

So far, no information is available in Crustacea as to whether oral RNAi could lead to parental RNAi. It would be useful to investigate whether this is specific for RNAi targeting the offspring of the treated females, or whether oral RNAi does not work at all in *A. franciscana.* In insects, most proof of parental RNAi was the result of injection experiments (Bucher *et al.*, 2002), but recently, parental RNAi after oral uptake of dsRNA has been reported in the western corn rootworm (Vélez *et al*., 2017).

CHAPTER 5

Cloning and RNAi of putative sex-determining genes in *Artemia franciscana*

Abstract

Our understanding of the sex of crustaceans has recently made remarkable strides through the use of advanced research tools. However, the genetic mechanism of sex determination in Crustacea is still unclear. In this study, we isolated and identified two *fem-1* homologs and eight *doublesex* homologs from *Artemia franciscana.* The phylogenetic tree showed that four out of eight *doublesex* transcript are closely related to the sex specific *doublesex* gene in insects. In addition, two transcripts were confirmed as sex-specific transcript in *A. franciscana.* The results suggest that the *doublesex* splicing mechanism described in insect is likely also to be present in *A. franciscana*. Therefore, these genes can be used in further studies to clarify the mechanism of the sex determination pathway of *Artemia* as well as in other crustaceans. In this study, we decided to use RNA interference to induce knockdown of these genes, in order to study their relationship with the formation of hooked graspers in adult males and the egg-sac in adult females. However, injection of dsRNA specific to our target genes did not result in any effect on sex ratio, using these morphologies as markers. Several possible reasons for the lack of phenotypical changes have been identified. It is possible that the RNAi silencing was not efficient enough at the transcript level, which could lead to an insufficient depletion at the protein level. Another important factor is that for some of the silenced genes, we later identified the presence of different isoforms. dsRNAs able to discriminate between these isoforms could be necessary to influence sex ratio in *A. franciscana*.

5.1 Introduction

The *Artemia* genus contains species with sexual as well as parthenogenetic reproduction modes. *Artemia* has a short life cycle, and is cultured easily under laboratory conditions. This species is being used as an animal model to discover genes involved in biological processes. It has also been used in ecotoxicity assays (Nunes *et al*., 2006 ; Rajabi *et al*., 2015 ; Nguyen *et al*., 2016). *Artemia* has a WZ-ZZ sex-determining system which means that males are homogametic (ZZ), while females are heterogametic (ZW) (De Vos *et al*., 2013 ; Bowen, 1965). The WZ-ZZ sex-determining system has also been found in other crustacean species such as *Cherax quadricarinatus* (Parnes *et al*., 2003), *Penaeus monodon* (Staelens *et al*., 2008), *Macrobrachium rosenbergii* (Ventura & Sagi, 2012) and *Eriocheir sinensis* (Cui *et al*., 2015). The *Artemia* males have a translucent body (Stephen & Leighton, 2009), while the females are brown-red in color and have a egg-sac which receives ripe oocytes from the ovaries via two oviducts. Embryos in the egg-sac of the female develop into free-swimming nauplii (ovoviviparity) in favorable reproductive conditions. However, under stressful environmental conditions, the females release cysts in diapause (oviparity). Cysts (when out of diapause), after being activated, develop into nauplii when they are placed in favorable conditions (Criel & Macrae, 2002b). In the males, the antennae develop into hooked graspers, while in females are characterized by an egg-sac, and their antennae degenerate into sensorial appendages.

The primary sex determining genes and their molecular mode of action have been explored in the nematode *Caenorhabditis elegans* and fruit fly *Drosophila melanogaster*. It was shown that a mutation in one gene could directly influence the sex ratio in a population. In *C. elegans*, three sex-

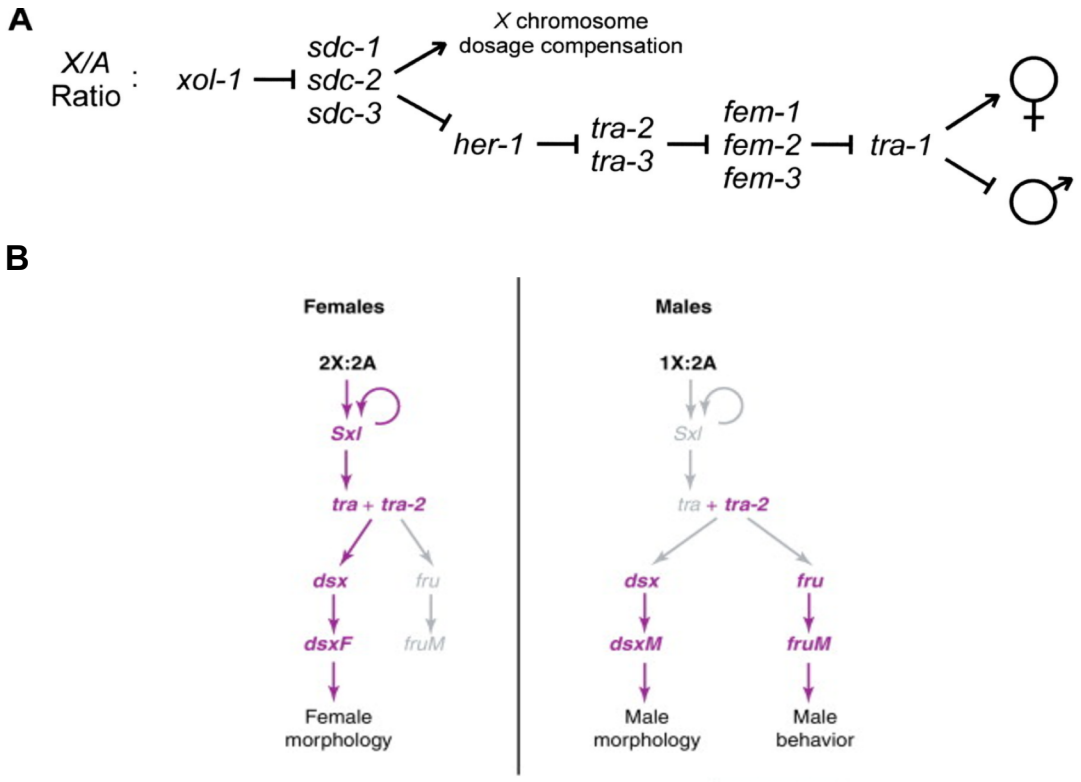


Figure 5.1. The simplified genetic pathway for sex determination in the *Caenorhabditis elegans* (David *et al*., 2000) and *Drosophila melanogaster* (Kopp, 2012). The ratio between X chromosomes and autosomes determines to the on/off state of *XO lethal (xol*) or Sex lethal (*sxl) in C. elegans* and *D. melanogaster* respectively. **A**: In female *C. elegans* (XX), *Transformer-2* (*tra-2)* negatively regulates the *fem* genes, allowing *tra-1* to promote female development. In the males (XO). Elevated *HERmaphrodization* (*her-1*) activity inhibits *tra-2* and permits the *fem* genes to bring about male development by negatively regulating *tra-1*. The *fem* genes have additional targets in the germ line, because they are required for spermatogenesis irrespective of the state of *tra-1*. **B**: In *D. melanogaster* females, the presence of functional SXL and TRA proteins leads to female-specific splicing of *dsx* and suppresses male-specific splicing of *fru*. In males, the absence of SXL and TRA allows the default splicing of *dsx* and *fru* to produce male-specific isoforms.

determining genes (*feminizer*, *fem*) have been isolated. *Fem-1* is required for development of the male body as well as for spermatogenesis (Fig 5.1A). Additionally, the sex in *C. elegans* is normally determined by the X chromosome to autosome (X:A) ratio, with XX being hermaphrodites while XO are males. Mutants in *fem-1* cause both XO and XX animals to develop as females (Zanetti & Puoti, 2013 ; Doniach & Hodgkin, 1984). In *D. melanogaster*, the *doublesex* (*dsx*) gene is transcribed to produce a common primary transcript that is alternatively spliced and poly-adenylated to yield male (dsxM) and female (dsxF) specific mRNAs (Burtis & Baker, 1989) (Fig 5.1B). The *D. melanogaster dsx* gene has been proven to be related to the *mab-3* gene in *C. elegans* which also plays a role in male sexual development (Christopher *et al*., 1997). In crustaceans, *dsx* genes have also been isolated and characterized in *Macrobrachium rosenbergii* (Jung et al., 2016 ; Yu et al., 2014), *Macrobrachium nipponense* (Ma *et al*., 2012), *Eriocheir sinensis* (Zhang & Qiu, 2010) and four *Daphnia* species (Toyota *et al*., 2013). Two *dsx* genes (*DapmaDsx1* and *DapmaDsx2*) from *D. magna* were identified, which are expressed in the males but not in the females. *DapmaDsx1* has been reported as a sex determination gene which is responsible for the male trait development (Kato *et al*., 2011a). In *E. sinensis,* the expression of *dmrt-like* was successfully inhibited by RNAi, resulting in a reduced testis size, showing that this gene plays a role in testicular development (Ma *et al.*, 2016a). There is no evidence that *dsx gene* plays a role in phenotypic gender differences in *M. rosenbergii*. Instead, the insulin-like AG (*Mr-IAG*) gene has been isolated from the androgenic glands. *In vivo* silencing of *Mr-IAG* temporarily prevented the regeneration of male secondary sexual characteristics (Ventura *et al.*, 2009 ; Ventura *et al*., 2012).

The first sex-linked gene of *Artemia* has been reported in *Artemia salina,* showing that the eye color trait depends on a sex-linked gene (Bowen, 1963). Sex-specific proteins were also identified in the adults and cysts of *A. salina*. The female sex-specific proteins may be associated with the cyst-forming

process in the females (Schwab, 1974). In an effort to explore the molecular mechanism of sex differentiation in *Artemia*, a few molecular biology techniques have been employed to understand this mechanism. Eight sex-linked AFLP marker alleles have been reported in *Artemia franciscana* that were inherited from the female parent (De Vos *et al.*, 2013). Recently, a *Masculinizer* gene (*Ar-Masc*) was identified in *A. franciscana.* RNAi experiments in females showed that gene silencing of *Ar-Masc* could slightly change the female-male ratio of progeny (Li *et al*., 2017). In the silkworm *Bombyx mori*, the MASC protein encoded in the Z chromosome activates a sex determining gene that regulates the splicing *of dsx* gene into the male isoform (Kiuchi *et al*., 2014).

The RNAi targeting of *fem-1* and *dsx* has shown that those genes strongly affect the transforming of sexual morphology in *C. elegans* and several insects. Thus, these two genes are good candidates to start studying sex determination mechanism in *A. franciscana*. We found transcription evidence for *fem-1* and *dsx* in the transcriptome database of *A. franciscana*. However, these sequences were too short to translate into putative protein. Therefore, RACE PCR was used to determine the complete sequence of *fem-1* and *dsx*. Two gene copies of *fem-1* and six gene copies of *dsx* were isolated from *A. franciscana*. The expression profile of those genes was evaluated in both genders using PCR. RNAi was also applied to determine the effect of those gene on gender ratio.

5.2 Material and methods

5.2.1 Animals culture

Artemia franciscana cysts (Great Salt Lake, Utah, USA) were hydrated in autoclaved sea water with strong aeration at 28^{o}C until hatching (approximately 20-24 hours). The nauplii (larvae) were then transferred to a 5 L plastic tank with autoclaved sea water for rearing. The larvae were cultured at 28^{o}C in autoclaved sea water with aeration under fluorescent light and were fed

with alive marine microalgae *Tetraselmis suecica*. At DAH (days after hatching) 15-20, the female juveniles (before egg-sac formation) were identified by their brown-red colour. The adult males and females are completely different in morphology at DAH 20-25 with the hooked grasper phenotype in males and the egg-sac appearance in females.

5.2.2 Isolation of *feminizer* and *doublesex* genes

Searching of potential transcripts: Primary amino acid sequences of *feminizer (fem-1)* and *doublesex* (*dsx*) from *Daphnia magna* were used for BLAST against *A. fransciscana* transcriptome database (Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium). The selected contigs (good hits) were subsequently used to verify their identity using the BLASTX algorithm (*https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins*). The contigs that showed a match to *fem-1* and *Doublesex* and *mab-3* genes were selected for coding DNA sequence (CDS) analysis. The amino acid sequence of the selected contigs were determined by the translate tool from the website *http://web.expasy.org/translate/.* For contigs that contain a full CDS, PCR primers were designed for isolating and sequencing the CDS. In contrast, for contigs containing only a partial CDS, their respective full-length cDNAs were identified by 3′ and 5′ rapid amplifications of cDNA ends (RACE).

Rapid amplifications of cDNA end: Based on contigs that contained only a partial CDS, gene specific primers (Table 5.1) were designed to isolate the UTR using SMARTer RACE Kit (Clontech, USA) with a modified protocol. Briefly, the 20 µL cDNA reaction for the isolation of the 5'UTR contained 4 µL 5X First- Strand Buffer, 0.5 µL DTT (100 mM), 1 µL dNTPs (20 mM) 5'-CDS

Table 5.1 Primers used to confirm the full cDNA of *A. franciscana* putative *feminizer*, *doublesex* genes and for producing DNA template used for making dsRNA.

Genes	Gene specific primers (5'-3')	*Size*	*Used for*
Fem-1A	For: AAGAACAAGATGATAATGGAGTGATAC Rev: AGCTTCAGTTAATGATTAAACCGCATGG	*2028*	*Isolating*
	dsRNA-For: G CAT AGG TCA CGG GAT G *dsRNA*-Rev: T CGG CTC CAG CCT CCA G		*RACE and dsRNA*
Fem-1B	For: CAGAAAATTTCTTGTTTATTATTTGAGTATATGC Rev GGCACCGCCCTTCTACCCAGAAATACG	*1429*	*Isolating*
	dsRNA-For C TTA GCA GAC CAG GGG C dsRNA-Rev C AAG CGC TTC TTA CTG G		*RACE and dsRNA*
Doublesex-1	For: ATCCTTTGGGACAATGGATGTTACACTC Rev CTTTCATAGTGTTGTCAAAACGCGC	*903*	*Isolating*
	dsRNA-For CCATGGTGTCATTGCTTGGC *dsRNA*-Rev CTTTGTCAATACCTGGAGC		*RACE and dsRNA*
Doublesex-2	For: TGGGAGAGAGACAGGAATGTAAATACATAC Rev GCAGCTCTGAGTGATACCAAAAAATTAAG	*1459*	*Isolating*
	dsRNA-For A CGA TAC CAG CGT ACG C dsRNA-Rev T TGA CGG CCA GCC TGG T		*RACE and dsRNA*
Doublesex-3	For: ATATGAAGTGAGCAATGGTGATACGC Rev CGAATTCGTTTAATCACACATTTAAGGCCAC	*1169*	*Isolating*
	dsRNA-For A GGT GTG ACT TCT CG *dsRNA*-Rev T GTC CTC GTA TGA GCA		*dsRNA*
Doublesex-4	Alpha-For: TCTTCAACCATGAAAACAAACAGGCATCTACC Alpha-Rev-A: CAGGCCAATTTTTTGAATATTATCTAGAAGC	*920*	*Isolating*
	Alpha-For: TCTTCAACCATGAAAACAAACAGGCATCTACC Alpha-Rev-B: CAGGCCAATTTTTTGAATATTATCTAGAAGC	*1010*	*Isolating*
	Beta-For: AGTTCGGTTTGTGGTTCCTCACGG Beta-Rev-C: CAGGCCAATTTTTTGAATATTATCTAGAAGC	*1124*	*Isolating*
	dsRNA-For T ACT GCA GTT TCC GCC *dsRNA*-Rev T GCC TAT GAT CCC AGC		*RACE and dsRNA*
Doublesex-5	For: CTACTATCTTACACCTAAGTGCGCCTGAG Rev: GCAACGATTGAAGAGAAATGGGAAAGGATC	*918*	*Isolating*
	dsRNA-For T TAT GGC GCA AGC CA *dsRNA*-Rev G GCA ATG CCG CAT CTG		*dsRNA*
Doublesex-6	For: GATTAAACGAACTAAGGCCAGAGAGA Rev: CACAAAGAACGAAAATAAGAAACTAACACAC	*966*	*Isolating*
	dsRNA-For A AAT AAT GGC GGC AC *dsRNA*-Rev A GTA GCT TTC GCT GAA		*dsRNA*
Tubulin	*For: GCAGTGGTCTACAAGGTTTC* *Rev: TGCATTGACGTCTTTTGGTACGACATCTC*	*605*	*Internal control*

Primer A, 10 μL total RNA, 1 μL of the SMARTer II A Oligonucleotide, 0.5 μL RNase Inhibitor (40 U/μL) and 2 μL SMARTScribe Reverse Transcriptase (100 U). The mixture was incubated at 42 °C for 70 minutes. The cDNA reaction for the isolation of the 3'UTR followed the same protocol as used for the isolation of the 5'UTR, except that SMARTer II A Oligonucleotide was not added and the 5'-CDS Primer was replaced by 3'-CDS Primer from the kit. Subsequently, a PCR reaction (50 μL) was carried out to amplify the UTR sequence: 1 μL cDNA (for 5' end UTR cDNA or 3'end UTR cDNA), 5 μL PCR buffer, 1 μL dNTPs (10 mM), 5 μL 10X UMP primer, 0.5 mM gene specific primer (for 5'UTR or 3'UTR) and 0.25 μL Dream*Taq* DNA polymerase. The PCR thermal cycle was conducted by denaturation for 3 minutes at 95°C, following by 35 cycles of amplification were carried out; 30 seconds at 95 °C; 30 seconds at 56 °C; 2 minutes at 72 °C. The reaction was extended for 10 minutes at 72 °C and then cooled to 4 °C. The UTR PCR products were purified from agarose gel using Wizard® SV gel and PCR Clean-up System (Promega, USA) for subsequent direct sequencing. The full length of UTR sequence was assembled by Vector NTI software (Invitrogen, USA).

Phylogenetic analysis: The phylogeny tree was generated from *http://www.phylogeny.fr/* using PhyML, a phylogeny software based on the maximum-likelihood principle (Guindon *et al.*, 2010), wherein the usual bootstrapping procedure is replaced by a fast approximate likelihood ratio test (aLRT), which is proven to be a good alternative to the (time-consuming) bootstrap analysis (Anisimova *et al.*, 2006). The putative polypeptide sequence from the isolated transcripts and known polypeptide sequences of homologs from NCBI were introduced under FASTA format.

RNA extraction and cDNA preparation: The total RNA of the samples (the hydrated cysts, nauplii and adults) were prepared using the RNeasy mini kit (Qiagen**,** Germany). The total RNA was used to synthesize cDNA in 20 μL reaction by using poly-T primer and RevertAid H Minus First Strand cDNA

Synthesis Kit (Thermo Science, USA). Briefly, a 20 µL of reaction mixture containing 4 µL of 5X reaction buffer, 2 µL of 10 µM dNTPs mix, 20 units of ribonuclease inhibitor, 200 units of RevertAid H Minus M-MuLV Reverse Transcriptase, 1 µL of oligo-dT primer and 500 ng of total RNA. Subsequently, the reaction mixture was incubated for 70 minutes at 42 °C. The reaction was terminated by heating at 70 °C for 5 minutes and then cooled to 4 °C. Complementary deoxyribonucleic acid (cDNA) was then used as template in PCR for further steps.

Confirming transcription and sequencing: The gene specific PCR primers of each gene were designed to amplify the complete cDNA of each gene (Table 5.1). The cDNA from different stages in the lifecycle, were used as template in a PCR reaction, to increase the chance of gene amplification. The applied PCR conditions were the same for all genes in this study. Briefly, the PCR reaction contained 150 ng cDNA, 5 µL reaction buffer, 1 µL dNTPs (10 mM), 0.5 mM gene specific primers, (Table 5.1) and 0.25 µL DreamTaq DNA polymerase (Thermal Scientific, USA). The PCR reaction was carried out in a thermal cycler (2720 Thermal Cycler, AB, USA) programmed for 94 °C for 4 minutes; 35 cycles at 94 °C for 30 seconds, 55 °C for 60 seconds, and 72 °C for 5 minutes; followed by 72 °C for 10 minutes. The amplicons were then separated on agarose gel and cleaned using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). These cleaned amplicons were used directly for sequencing by the Sanger method using the same PCR primers.

5.2.3 RNAi

Double stranded RNA preparation: The dsRNA of each gene was produced by PCR using a cDNA template and gene-specific primers with a T7 promoter sequence (TAATACGACTCACTATAGGGAGA) added to the 5' end of each primer (Table 5.1). After cleaning up using the PCR cleanup kit

(Promega), the cleaned-PCR product was used for *in vitro* dsRNA synthesis using MEGAscript RNAi kit (Ambion, USA). The quantity of dsRNAs was measured by Nanodrop and evaluated using agarose gel electrophoresis for specificity. The dsRNA was stored at -20 °C prior to further use. For the controls, the *afcad* dsRNA was used as positive control, while dsRNA-gfp was used as negative control (chapter 4).

***Injection assays*:** The dsRNA injection solutions with a concentration of 320 ng/µL were prepared by diluting of dsRNA in 0.5% phenol red in Dulbecco's phosphate buffered saline (Sigma-Aldrich, USA). Injection was performed with a FemtoJet® Microinjector (Eppendorf, USA) using Femtotips II microinjection capillary tips (Eppendorf, USA). Just before the injection, the females were laid on 2% agarose gel and the surrounding seawater was removed using tissue paper. Approximately 250 nL dsRNA (80ng) was injected into the egg-sac. For each gene, eighteen dsRNA-treated females were kept in a 6-well plate. Only females still retaining red color after 2 hours were used for mating two days later. Each couple was reared separately in 10 mL sea water using the 6-well plate. New seawater with algae was added every day. Released nauplii from each RNAi-female were separately transferred to bottles of 500 mL of sterilized seawater for rearing until the sex ratios could be observed. The released nauplii from RNAi treated females have not been checked for efficiency of silencing of the respective target genes as living nauplii were needed to establish the sex ratios statistically.

5.3 Results

5.3.1 Isolating *fem-1* genes

The BLAST results against the *A. franciscana* transcriptome database showed two contigs containing a sequence with a high homology to the *fem-1* gene. The full cDNA sequence of these two contigs were then determined by

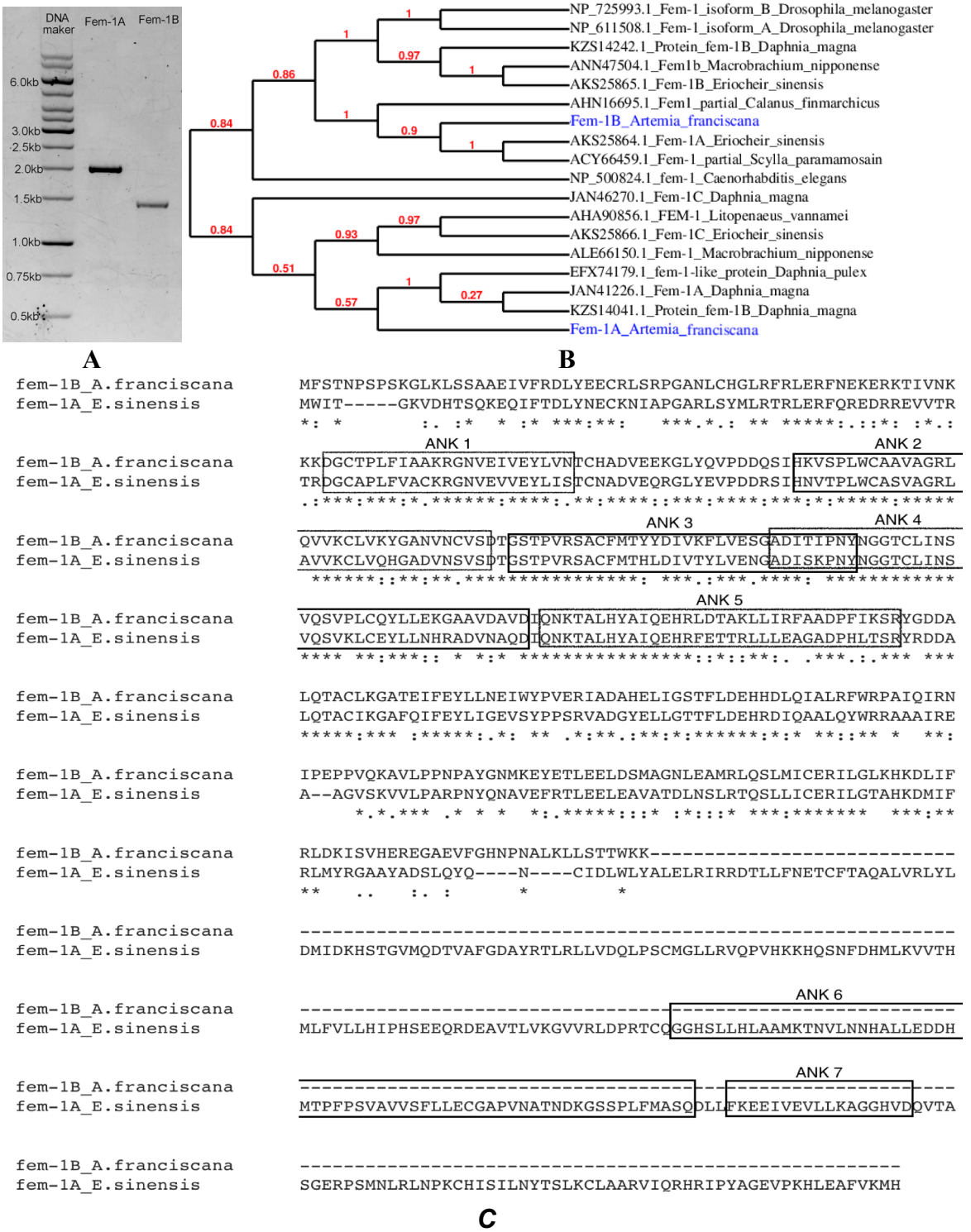
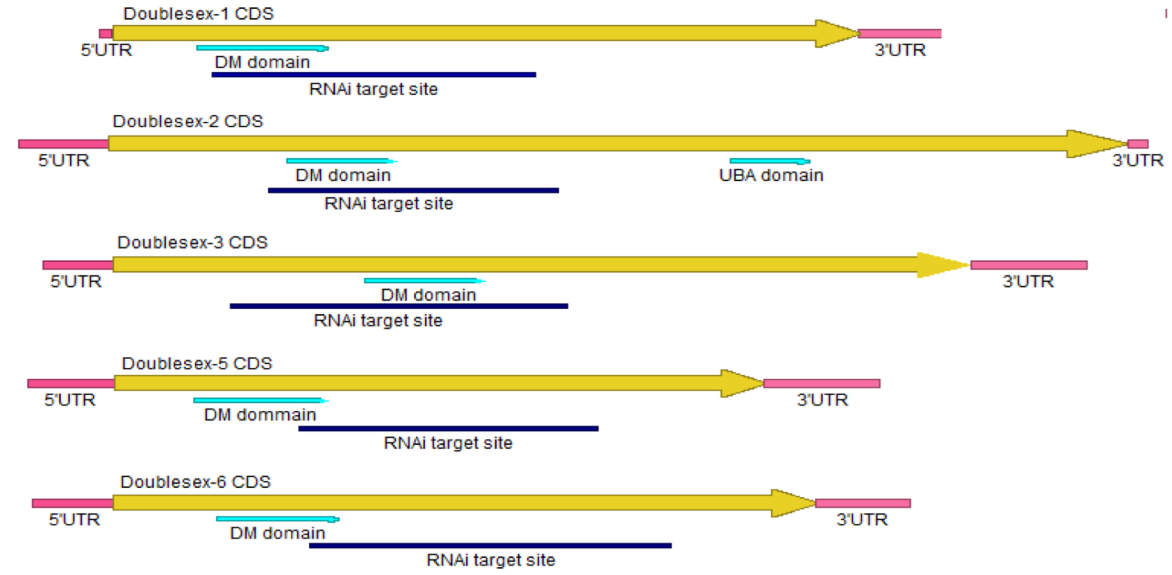


Figure 5.2. Isolation of *fem-1* homologs in *A. franciscana*. **A**: The PCR result of *fem-1* genes from cDNA of six hours hydrated *A. franciscana* cyst, ***B****:* the phylogenetic tree of *FEM-1* homologs, the red numbers are the branch support values. **C**: the polypeptide alignment of *A. franciscana* FEM-1B against *E. sinensis* FEM-1A. The ankyrin repeat motif was predicted by using NCBI's conserved domain database.

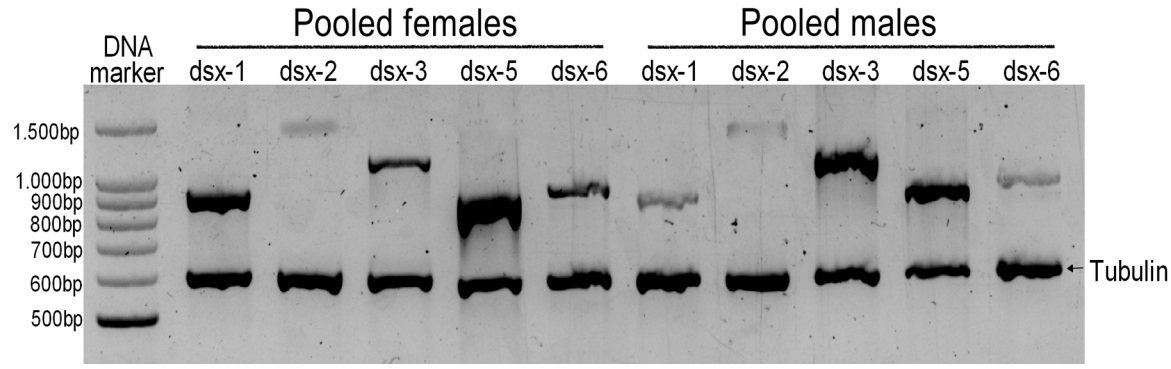
using the RACE method and evidence of their transcription were obtained by PCR from the cDNA of hydrated cysts (Fig 5.2A). These *fem-1* genes were submitted to NCBI GenBank, designated *fem-1A* (MF287964) and *fem-1B* (MF287965). *Fem-1A* encodes a polypeptide of 627 aa and contains six continuous ankyrin (ANK) motif repeats in the N-terminal and one in the C-terminal. Compared with *fem-1A*, *fem-1B* encodes for a polypeptide of 392 aa, consisting of only five continuous ANK motif repeats all located in the N-terminal. The phylogenetic tree showed that *A. franciscana* ArFEM-1A and ArFEM-1B belong to the family of feminizer proteins which have been reported in some crustaceans (Fig. 5.2B).

5.3.2 Isolating of *doublesex* genes

Six contigs that encode for a polypeptide with a Doublesex/Mab-3 domains (DM domain) (Yi & Zarkower, 1999) were found in the *A. franciscana* transcriptome data. These contigs were named doublesex-1, doublesex-2, doublesex-3, doublesex-4, doublesex-5 and doublesex-6. Based on the nucleotide sequence, only doublesex-3, doublesex-5 and doublesex-6 contained full coding DNA sequence (CDS) which can encode for a full putative polypeptide of the protein, while the others contained only a partial CDS. Therefore, the full doublesex-1, doublesex-2, and doublesex-4 cDNA sequences were then identified by RACE method. Interestingly, RACE results revealed three different isoform cDNAs from the doublesex-4 sequence, named doublesex-4 alpha isoform A of 920bp, doublesex-4 alpha isoform B of 1010bp; and doublesex-4 beta isoform C of 1156bp. Their nucleotide sequences were submitted to NCBI GenBank as *doublesex-1* (*dsx-1*) (MF287957), *doublesex-2* (*dsx-2*) (MF287958), *doublesex-3* (*dsx-3*) (MF287959), *doublesex-4*-alpha isoform A (*dsx-4αA*) (MF287960), *doublesex-4*-alpha isoform B (*dsx-4αB*) (MF993793), *doublesex-4*-beta isoform C (*dsx-4βC*) (MF287961), *doublesex-5* (*dsx-5*) (MF287962) and *doublesex-6* (*dsx-6*) (MF28796). All isolated *dsx* genes encode different putative DSX proteins



A



B

Figure 5.3. The mRNA scheme and the evidence of transcription of *A. franciscana doublesex-1, 2, 3, 5 and 6* mRNA. **A**: The mRNA scheme, the pink bars represent the untranslated region (UTR); the yellow arrows represent the coding DNA sequence; the green bars indicate the region encoding the DM domain; the dark blue bars indicate the target site for RNAi. **B**: The evidence of transcription of *A. franciscana* dsx 1, 2, 3, 5, and 6 mRNA *by* semi quantitative PCR (*tubulin* was used as the internal control) from cDNA sample of pooled females and pooled males. The size of amplicons is indicated in table 5.1.

bearing one DM domain (Bayrer et al., 2005 ; An et al., 1996) (Fig. 5.3A and 5.4A). Among those, the DSX protein that is translated by d*oublesex-2* bears an additional ubiquitin - associated domain (UBA domain) at its C-terminal, which is a common domain found in DSX proteins in insect species (Bayrer *et al.*, 2005).

The open reading frame prediction from dsx-4αA and dsx-4αB cDNA showed that they contain different 3'UTR sequences (named 3'UTR-A and 3'UTR-B, respectively). Therefore, they are predicted to encode for a same putative polypeptide of 186 aa long, namely DSX-4 Alpha. In contrast, dsx-4βC cDNA is predicted to encode a polypeptide of 289 aa long, namely DSX-4 Beta. The sequence at the 5'end of the cDNAs (consisting of a 5' untranslated region (5'UTR) and a partial CDS encoding for the N-terminal) of dsx-4α (A, B) and dsx-4βC were completely different (namely alpha and beta sex specific sequence, respectively). Additionally, the dsx-4βC cDNA has a different 3'UTR sequence, namely 3'UTR-C, in comparison to the 3'UTR-A and 3'UTR-B sequence. The dsx-4αA, dsx-4αB and dsx-4βC cDNAs share a common sequence of 477 nucleotides encoding a common polypeptide including the DM domain (Fig. 5.4A). The polypeptide alignment suggested that DSX-4 beta is probably an isoform of DSX-4 Alpha but bearing an extended polypeptide at its N-terminal (Fig 5.5B). Taken together, these results suggested that dsx-4αA, dsx-4αB and dsx-4βC cDNA probably were the result of alternative splicing events from *dsx-4*.

The transcription of eight isolated *dsx* genes was confirmed in *A. franciscana* adults using animals which were reared separately since the juvenile stage until the fully matured stage. The PCR was done on cDNA from pooled male or female adults. The PCR products showed the presence of Dsx-1, Dsx-2, Dsx-3, Dsx-5 and Dsx-6 cDNA in both sexes (Fig. 5.3B). However, dsx-4βC cDNA could only be seen in pooled males, while dsx-4αA cDNA could

only be found in pooled females. Interestingly, dsx-4αB cDNA was present in both sexes (Fig. 5.4B).

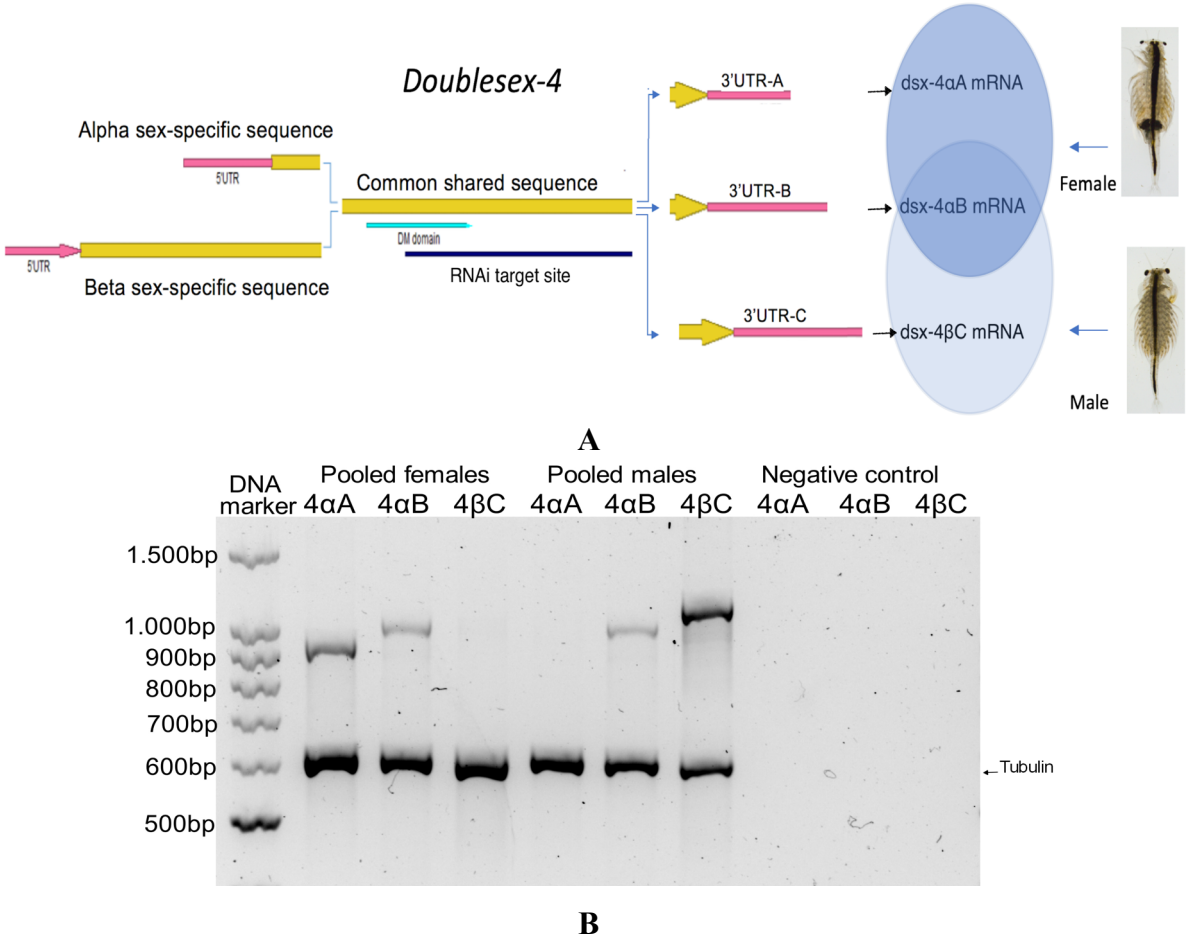
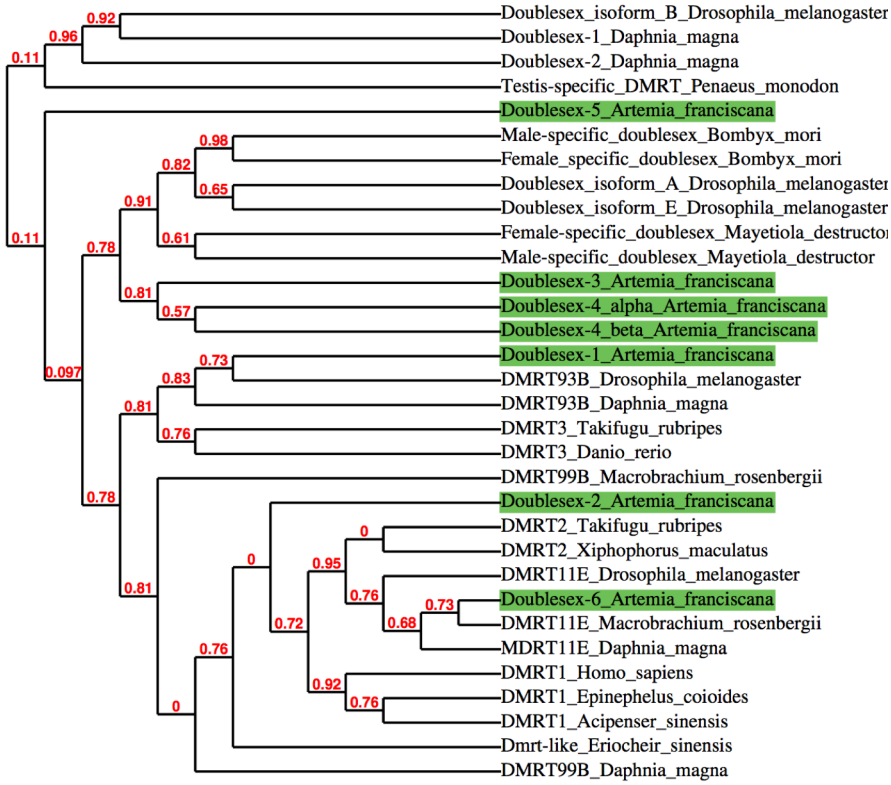


Figure 5.4. The mRNA scheme and the evidence of transcription of *A. franciscana* doublesex-4 mRNA. **A**: The mRNA scheme of *doublesex-4*; the pink bars represent the untranslated region (UTR); the yellow arrow is the location of coding DNA sequence; the green bar indicates the region encoding DM domain; The dark blue bar indicates the target site for RNAi. *Doublesex-4* produces dsx-4αA, dsx-4αB and dsx-4αB mRNAs. dsx-4αA mRNA is the combination of alpha sex-specific, common and 3'UTR-A sequence, while dsx-4αB mRNA is joined from alpha sex-specific, common and 3'UTR-B sequence. Dsx-4βC mRNA is the combination of beta sex-specific, common and 3'UTR-C sequence. **B**: The evidence of transcription of *A. franciscana* dsx-4αA, dsx-4αB and dsx-4βC mRNA isoform by PCR from cDNA sample of pooled females and pooled males. The amplicon size is indicated in table 5.1.



A

```
Dsx-4.alpha       ------------------------------------------------------------
Dsx-4.beta        MVIQTLKHRADRERMEEIQQTQAIVTSSTEKTTNESNSNSAAPDSVTEYAGSGHVVQQMP

Dsx-4.alpha       ---------------------------------------MNRRIFEKYPDFPIIPGFI
Dsx-4.beta        SASHTYTDASYSPDEPRDLSRKRRHESDENEDDIQIKIGNMSNEKDDESLSDM--GDMSD
                                                          *.:  *.  *:

Dsx-4.alpha       SGSQGDKMGKMRMPTCARCRNHGQVVKLRGHKRYCSFRHCLCDRCALTSEKQRVMAAQVA
Dsx-4.beta        AGSQGDKMGKMRMPTCARCRNHGQVVKLRGHKRYCSFRHCLCDRCALTSEKQRVMAAQVA
                  :************************************************************

Dsx-4.alpha       LRRAQKQDEENGIVRPVPITPKRPEIIYPPPRIPSPPSAPLPLQLPIPPQPLNYIDSYLK
Dsx-4.beta        LRRAQKQDEENGIVRPVPITPKRPEIIYPPPRIPSPPSAPLPLQLPIPPQPLNYIDSYLK
                  ************************************************************

Dsx-4.alpha       FSLLSMKDELKLGEHNLPLLYLAYSFVGWDHRHLIKELHVGKLGSSS----
Dsx-4.beta        FSLLSMKDELKLGEHNLPLLYLAYSFVGWDHRHLIKELHVARNSLVKLGLL
                  ************************************.: .  .
```

B

Figure 5.5. A: The phylogenetic tree of DSX proteins. **B:** The primary polypeptide alignment of *Artemia franciscana* DSX-4 Alpha and DSX-Beta. The box indicates the DM domain sequence; the asterisks indicate the conserved amino acids. The numbers are the branch support values.

5.3.3 Silencing of sex determining genes

Based on the number of injected females that could survive and produce progeny during the RNAi experiment, five individual females that could release nauplii were chosen for each RNAi treatment group: RNAi *gfp* as the control, RNAi *dsx-1*, RNAi *dsx-2*, RNAi *dsx-3*, RNAi *dsx-4*, RNAi *dsx-5*, RNAi *dsx-6*, RNAi *fem-1a*, and RNAi *fem-1b*. The number of nauplii/brood varied from 12 to 118 for the different females. Under the described culture conditions, the ratio of male to female was 1:1 in the negative control group. The hooked graspers and egg-sac morphology were used as sex-specific male and female markers respectively. There was no significant difference in the male/female ratio in any

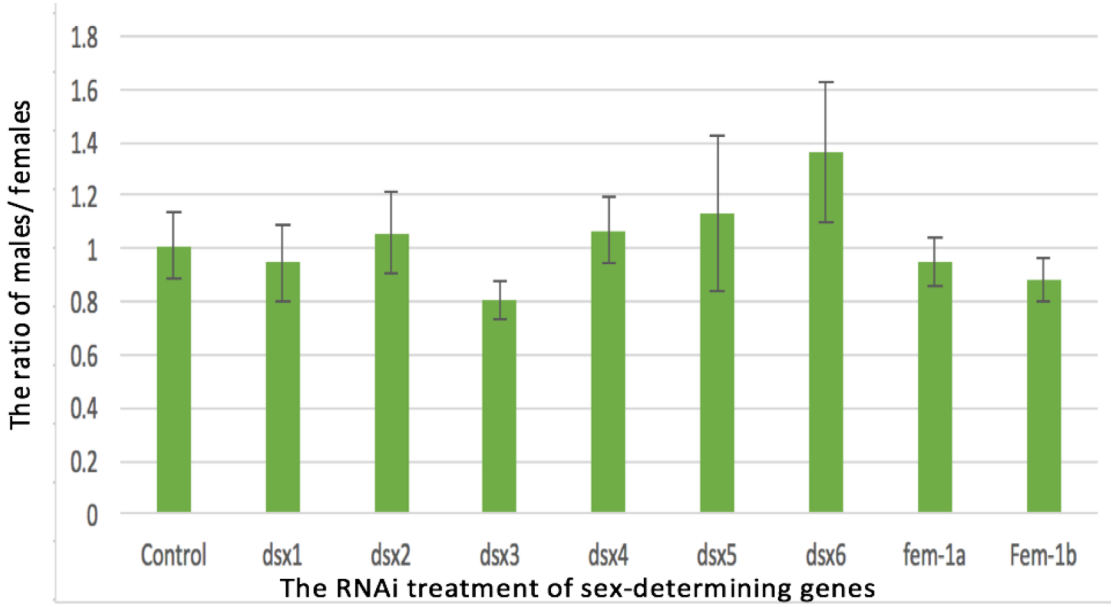


Figure 5.6. Effect of silencing putative sex-determining genes on gender ratio in *A. franciscana*. The number of reproducing female are five animals in each RNAi treatment: RNAi *gfp* as the control, RNAi *dsx-1*, RNAi *dsx-2*, RNAi *dsx-3*, RNAi *dsx-4* (all of transcripts), RNAi *dsx-5*, RNAi *dsx-6*, RNAi *fem-1a*, and RNAi *fem-1b*. The error bar is the standard deviation. The columns represent the ratio of males/females in the progeny released from RNAi-treated females. The number of nauplii used for analysis in each treatment were: Control = 201; *dsx-1* = 314; *dsx-2* = 272; *dsx-3* = 292; *dsx-4* = 493; *dsx-5* = 299; *dsx-6* = 378; *fem-1a* =229, *fem-1b* = 289.

of the RNAi treatments between the control and each treatment in this experiment (student's t-test, $p>0.05$) (Fig. 5.6).

5.4 Discussion

The sex determination mechanism has been studied extensively in invertebrate animal models such as *C. elegans* and *D. melanogaster*. It showed that sex is determined in the embryonic stage by the involvement of so-called sex-determining genes. In *C. elegans*, *fem-1* is considered an important gene required for promoting the male body phenotype. *Dsx* genes have been identified in many insects to be linked to the sex determination pathway (Verhulst & van de Zande, 2015). Recently, sex-determining genes homologous to those of insects have been found in crustaceans. However, the involvement of these genes in the sex determining mechanism of crustaceans has not been elucidated. In *A. franciscana*, the available RNAseq databases suggested that this species has many genes that are similar to sex-determining genes in fruit flies including *fem-1* and *doublesex* (De Vos, 2014). Considered as a model animal, understanding the sex determining mechanism of this species may help to understand sex determination in *e.g*. commercial crustaceans.

Fem-1, which is considered a signal-transducing regulator, was reported to be involved in the sex determination pathway in *C. elegans*. FEM-1 contains repeated motifs called Ankyrin (ANK) repeats which are required for promoting male development in *C. elegans* (Spence *et al*., 1990). The ANK repeat is an approximately 33-residue motif in proteins consisting of two alpha helices separated by loops. It was first identified in yeast cell cycle regulators and *Drosophila* signaling proteins (Breeden & Nasmyth, 1987 ; Mosavi *et al*., 2004). In the FEM-1 protein family, the number of ANK repeats is varied. *C. elegans* FEM-1 has seven ANK repeats (Spence *et al*., 1990) whereas eight, nine, and eight repeats have been determined in FEM-1a*,* FEM-1b*,* and FEM-1c of Mitten crab *E. sinensis*, respectively *(*Song *et al.*, 201*5)*. Here, two *fem-1* homologs

from *A. franciscana* have been isolated and sequenced. We found that their primary polypeptides contain seven and five ankyrin repeat motifs in ArFEM-1A and ArFEM-1B*,* respectively. The phylogeny analysis (Fig 5.2B) and protein BLAST result from NCBI confirmed that the *A. franciscana* ArFEM-1B polypeptide has 63% identity to the N-terminal of *E. sinensis* ArFEM-1A protein (AKS25864.1*)* (Fig 5.2C), which has a primary polypeptide structural with five ANK repeats at the N-terminal and two ANK repeats at the C-terminal. However, *A. franciscana* ArFEM-1B only contains five ANK repeats at the N-terminal sequence, but no ANK repeat sequences at its C-terminal. Thus, *A. franciscana* FEM-1B contains the lowest number of ANK repeats in any FEM-1 that has so far been isolated from crustaceans (Robinson *et al*., 2014). In this study, only two *A. franciscana fem-1* homologs were found in the *A. franciscana* transcriptome database. The number of *fem-1* genes was reported to be different between species using transcriptome analysis. It revealed that there were at least four *fem-1* genes in *M. nipponense* (Jin *et al*., 2013 ; Rahman *et al*., 2016), one in Green mud crab (*Scylla paramamosain*) (Gao *et al*., 2014a), one in *Penaeus monodon* (Robinson *et al.*, 2014), three in *E. sinensis* (Liu *et al*., 2015), seven in *L . vannamei* (Peng *et al*., 2015) and three in *D. magna (GenBank).*

Doublesex genes are related to the family of doublesex and mab-3 related transcription factor (DMRT) proteins bearing Doublesex/Mab-3 domains (Yi & Zarkower, 1999). The *DMRT* were reported to be very highly conserved, as well as essential for sexual development in mammal, fish, and insects (Zhang et al., 2014 ; Kim et al., 2007b ; Verhulst & van de Zande, 2015). The number of proteins bearing DM domains is different between species and divided into several different groups (Picard *et al*., 2015). In this study, we identified eight cDNA sequences encoding seven putative DSX proteins in *A. franciscana*. The phylogenetic tree showed that *A. franciscana* DSX-1, DSX-2 and DSX-6 were related to DMRT93B, DMRT99B and DMRT11E protein from *D. magna*, *E. sinensis* and *M. rosenbergii,* respectively (Fig 5.5A). In contrast,

putative *A. franciscana* DSX-3, DSX-4 alpha, DSX-4 beta and DSX-5 polypeptides are closely related to sex-specific DSX protein from *D. melanogaster*, *B. mori*, and. *M. destructor* (Fig 5.5A), thus they might involve the sex-determining as other *dsx* genes in insects.

In crustaceans, the transcripts of *fem-1* were found at different developmental stages in different tissues *e.g.* heart, hepatopancreas, brain, muscle, eyestalk, gill, and androgenic gland of *Macrobrachium nipponense* (Rahman *et al.*, 2016), testis and ovary of *E. sinensis* (Qiao *et al*., 2015 ; Song *et al.*, 2015 ; Ma *et al*., 2016b). The transcript of *fem-1* and its protein level showed no significant difference in both sexes of adult *M. nipponense* (Qiao *et al.*, 2015). Transcripts of two *A. franciscana fem-1* homologs were found at the early embryonic stage (six hours of hydrated cyst) and in both adult females and males, suggesting that they might play a biological role from the very early stage onwards in the development of *A. franciscana*. Thus, their particular biological function in relation to sex determination needs to be further investigated.

Doublesex is a critical transcription factor considered to be at the end of the sex determination cascade in *D. melanogaster* and many other insect species as a master gene in genotypic sex determination pathway. *Dmrt* and *dsx* are involved in the formation of sex-linked traits including sexual behavior, gonadal development and sex-specific morphology in many insects (Verhulst & van de Zande, 2015). Until now, *dsx* and *dmrt* have been characterized in only a few crustacean species, however, their involvement in the sex determination is not well understood. In *D. magna*, the transcription of *dmrt11E* and *dmrt99B* is higher in ovaries than in the testes (Kato *et al*., 2008). In *M. rosenbergii*, the transcription of both *dmrt11E* and *dmrt99B* is found in spermatogonia and spermatozoa during spermatogenesis *(Yu et al., 2014).* In *D. magna*, the dmrt93B mRNAs were detected only in the testes, and not in the ovaries (Kato *et al*., 2008*)*. Based on phylogenetic analysis, *A. franciscana* DSX-1, DSX-2

and DSX-*6* are very close to DMRT93B, DMRT99B and DMRT11E, respectively, thus they are hypothesized to play a similar role in development of gonadal tissue in *Artemia*.

In insects, sex-specific splicing generates sex-specific transcripts from the sex-specific *dsx* gene controlling sexually-dimorphic traits. CRISPR/CAS9 was exploited to create the mutants in male-specific *dsx* in *Bombyx mori*. It showed that disruptions of gene function produced either male-specific sexually-dimorphic defects or intersexual phenotypes (Xu *et al*., 2017). The sex-specific *dsx* splicing in *B. mori* depends on the presence of MASC protein in the embryo to promote the production of the male-specific splicing variants (Kiuchi *et al.*, 2014). Recently, a *masc* gene was characterized in *A. franciscana*. Masc RNAi during embryonic development of sexual *A. franciscana* could slightly change the ratio of females/males (Li *et al.*, 2017). It suggested that *A. franciscana* probably also has a similar sex determining mechanism in which the splicing of *dsx* plays an important role in sex determination. In this study, the RACE method has not been applied on *A. franciscana dsx-3* and *dsx-5*, thus potential variant transcripts of these two genes remain unknown. However, three isoforms of *dsx-4* were isolated in *A. franciscana* suggesting that a specific splicing mechanism of *dsx* also exists in *A. franciscana*. In crustaceans, up to now, evidence for alternative splicing of *dsx* gene could only be found in *D. magna*, involved in environmental sex determination. *Doublesex-1* of *D. magna* was identified to directly determine the gender via the splicing at 5'end of mRNA to produce doublesex-1 alpha or doublesex-1 beta mRNA. Therefore, they have different 5'UTR sequences in their structural mRNA, which leads to the control of the translation of key regulator protein of the male phenotype (Kato *et al.*, 2011a). In this study, the dsx-4βC isoform mRNA has a different 5'UTR sequence in comparison to the dsx-4αA, and dsx-4αB isoforms mRNA, thus this may influence the efficient translation of corresponding protein. In comparison to the DSX-4 alpha primary polypeptide sequence, DSX-4 beta has a longer N-terminal sequence, this difference could affect the formation of the dimer

structure that is needed for its function (Bayrer *et al.*, 2005 ; Zhang *et al*., 2006). Moreover, the transcription of dsx-4βC mRNA could only be found in the adult males, and *dsx-4αA* mRNA is only present in the females (Fig. 5.4). This reinforces the hypothesis that sex specific transcripts of doublesex-4 are related to sex determination in *A. franciscana*. The presence of dsx-4αA and dsx-4αB mRNA in the adult female, and dsx-4βC and dsx-4αB mRNA in the adult males has led to the hypothesis that the males have DSX-4 alpha and DSX-4 beta protein, while the adult females have only DSX-4 alpha protein. Therefore, the presence of the putative DSX-4 beta protein could be a key factor in male specific differentiation of *A. franciscana*.

Some sex-linked traits can be easily observed when sex-specific *dsx* transcript is silenced by RNAi, such as the sexual behavior in *D. melanogaster* (Billeter *et al*., 2006*)*, or for instance the sex-specific horn length in Japanese rhinoceros beetle *Trypoxylus dichotomus* (Ito *et al*., 2013), mandible size in stag beetles *Cyclommatus metallifer* (Gotoh *et al*., 2016b) or anterior morphological phenotypes in *Gnatocerus cornutus* (Gotoh *et al*., 2016a). In the sawfly *Athalia rosae*, effects of *dsx* silencing on the development of internal reproductive organs caused incomplete spermatogenesis, resulting in absence of mature sperm in the *dsx* silencing sawfly males. *Dsx* silencing sawfly females have phenotypically normal and had normal fertility (Mine *et al*., 2017). In this study, the RNAi method was used to investigate the biological function of isolated *fem-1* and *dsx* in sex determination via sex-linked morphology in *A. franciscana* (the hooked graspers characterized for the male and the egg-sac characterized female). No significant difference in sex ratio in the RNAi groups compared to the control group was observed. In insects, complete sex reversal phenotypes have not been documented when *dsx* is silenced, however, small changes in external and internal genital organs can be observed, e.g. testes and the horn (Mine *et al.*, 2017 ; Kijimoto et al., 2012). Thus, the effect of *dsx-4* silencing in *A. franciscana* can affect other organs rather than the formation of hooked graspers and egg-sac. *Dsx-4* is considered as the most probably sex-

determining gene of *A. franciscana* due to the fact that the alternative splicing found on this gene. However, the RNAi experiment of *dsx-4* has been done before the alternative splicing of *doublesex-4* was characterized, thus, the RNAi target site was designed on the common shared sequence of *dsx-4.* Therefore, RNAi probably has modified all dsx-4 cDNAs variants, resulting potentially in the absence of change in the ratio of DSX-4 alpha/DSX-4beta protein which is suggested to be relevant for gender of *A. franciscana*. Another potential reason to consider is that the RNAi silencing was not efficient enough to lead to a sufficient depletion of protein. This might also be related to the half-life of the targeted proteins, which is unknown.

Additionally, in the gene silencing experiments, the released nauplii from treated females have not been checked for efficiency of silencing of the respective target genes. This has not been done as alive nauplii were needed to establish the sex ratios statistically. Thus, it remains to be confirmed that all genes were effectively silenced by the applied RNAi strategy and how long the silencing has been effective. Hence it cannot be excluded that the absence of gender effect by RNAi on the respective genes is caused by the absence of effective silencing.

In this study, two *fem-1* and six *dsx* homologs have been isolated and sequenced from *Artemia franciscana*. The expression profiles showed that they are all expressed in both sexes, except *dsx-4*. We determined that *dsx-4* is potentially transcribed into three different mRNA isoforms, probably due to splicing event. Dsx-4αA and dsx-4αB mRNAs were found only in the adult female animals, whereas Dsx-4βC and dsx-4αB were detected in the adult male animals. These three mRNAs encode two types of DSX-4 proteins which are characterized by large differences in amino acid sequence at the N-terminal and minor changes at the C-terminal. Herein, we hypothesize that *dsx-4* is good candidate for further investigations into sex differentiation in *Artemia franciscana.*

CHAPTER 6

General discussion and conclusions

6.1 General discussion

6.1.1 *Artemia franciscana* RNA interference

RNA interference has been found in many species, including some model organisms and is in fact expected to be present in all eukaryotes. The genes involved in the RNAi mechanism have been well studied. For instance, the RNAi machinery in the insect models, such as *D. melanogaster* consists of two distinct RNAi pathways: the small interfering RNA (siRNA) and the microRNAs (miRNAs) pathways. *Dicer-1*, *Loquacious* (*Loq*) and *ago-1* are involved in the miRNA pathway, while *Dicer-2*, *R2d2* and *Ago-2* are involved in the siRNAi pathway. Interestingly, accumulating evidence suggests that both the miRNA and siRNA pathways play an important role in immune response against viruses as well as bacteria, in both insects and crustacean species. Understanding the RNAi mechanisms may lead to useful strategies and applications in animal health management, especially for species that lack adaptive immune mechanisms such as shrimp.

By using the available *A. franciscana* transcriptomic database, we have isolated and fully sequenced crucial genes involved in the miRNA and siRNA pathways of *A. franciscana*. Two *Dicer* homologs and three *Ago* homologs have been identified from *A. franciscana*. The phylogenetic analysis revealed that these *ArDicer-1* and *ArAgo-1* are closely related and homologous to *Dicer-1* and *Ago-1* of other crustacean species, which are probably involved in the miRNA pathway. Furthermore, *ArDicer-2, ArAgo-2* and *ArAgo-3*, homologous to the crucial elements in the siRNA pathway in insects and crustaceans were identified in *A. franciscana* as well. In some crustaceans, the presence of more than one copy of the *Ago* homolog belonging to *Ago-2* group has been reported (Kao *et al.*, 2016 ; Leebonoi *et al.*, 2015). Thus, the number of *Ago-2* homologs in *A. franciscana* may also be higher than one. Interestingly, the *Ago* homologs belonging to the *Ago-2* group in crustaceans are not only play the role in the siRNA pathway but also participate in the control of transposons such as *Ago-*

4 of *P. monodon* (Leebonoi *et al*., 2015). Therefore, *A franciscana ArAgo-2* and *ArAgo-3* can play different functions as well.

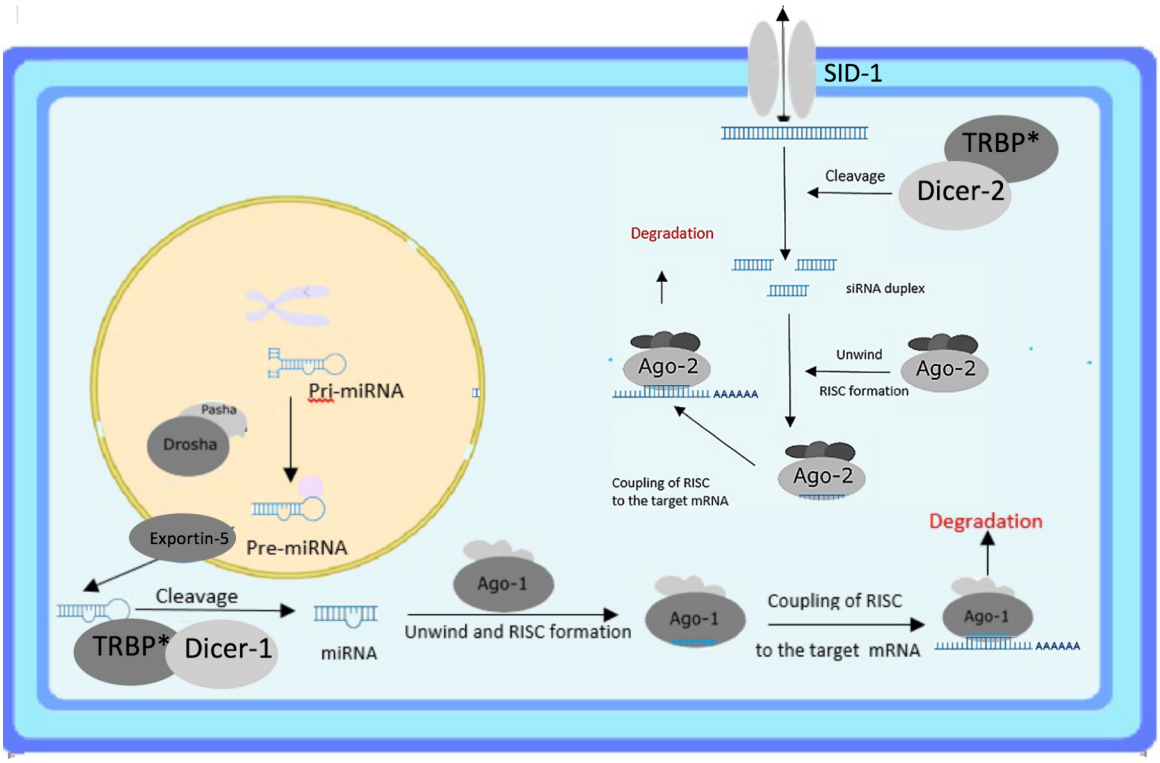


Figure 6.1. The isolated core components in the miRNA and siRNA pathway of *A. franciscana*. The TRBP* represents the putative ArTRBP-1 and ArTRBP-like proteins. Ago-2 homologs probably include putative ArAGO-2 and ArAGO-3 protein.

Regarding the dsRNA-binding proteins (*Trbp*), only one type of *Trbp* homolog had previously been reported in crustacean species. However, we identified two distinct *Trbp* homologs from *A. franciscana*, namely *ArTrbp-1* and *ArTrbp-like*. Both putative *A. franciscana* ArTRBP homologs contain three dsRBDs. Whereas *ArTrbp-1* is closely related to the *Trbp* homologs found in other crustacean species, and *ArTrbp-like* is more closely related to the ArStaufen homolog, which was also identified in the *A. franciscana* transcriptome. DICER-associated dsRNA-binding proteins are important regulatory factors that contribute both substrate and cleavage specificity during miRNAs and siRNAs production. Moreover, putative *A. franciscana* ArDICER-

2 has no dsRBD at its C-terminal; therefore, the function of the *A. franciscana ArTrbp-1* as well as of *ArTrbp-like* in the miRNA and the siRNA pathway needs to be investigated. Additionally, other RNAi components were also isolated including *ArDrosha, ArExportin-5* and *ArSid-1*. These isolated genes reveal a first view on the RNAi machinery of *A. franciscana* (Fig 6.1).

From the immune system point of view, RNAi pathways have been viewed as an important component of the innate immune system in crustaceans, which plays an important role in the immune response against viruses (Wang & Zhu, 2017 ; He *et al.*, 2015 ; Labreuche & Warr, 2013 ; Xu et al., 2014). Recently, miRNAs were identified from *E. sinensis, Scylla paramamosain*, and *M. japonicus* when these species were exposed *Spiroplasma eriocheiris* and *Vibrio alginolyticus* (Zhu et al., 2016 ; Wang & Zhu, 2017 ; Li et al., 2013a). In *M. japonicus,* for instance, miRNAs might mediate the regulations of phagocytosis, apoptosis or pro-phenoloxidase system by targeting different genes resulting in strong effects on the innate immunity (Yang et al., 2012). Knockdown of miRNAs expression can decrease mortality in WSSV-infected shrimp WSSV and enhance mortality of *V. alginolyticus*-infected shrimp due to an affected phenoloxidase activity and superoxide dismutase activity (Zhu *et al.*, 2016 ; Wang & Zhu, 2017 ; Li *et al.*, 2013a). These evidences suggest that the miRNA pathway is indirectly involved in eliminating the invasion of pathogens in shrimp. In the siRNA pathway, RNAi can directly inhibit the replication of different viruses such as WSSV, IMNV, TSV, YHV via the dsRNA or siRNA of viral genes. The siRNA pathway also inhibits viral infection via the influence of host gene expression, for example silencing of *PmRab7*, a *P. monodon* small GTPase protein, could inhibit replication of Leam Singh virus (Ongvarrasopone et al., 2010). In *Drosophila,* the Janus kinase/signal transducers and activators of transcription (JAK–STAT) pathway is required for antiviral responses (Dostert *et al*., 2005). In *L. vannamei,* RNAi silencing of STAT in shrimp showed that *LvAgo-2* and *LvDicer-2* (siRNA pathway related genes) were significantly up-regulated upon WSSV injection. It

proved that STAT dsRNA injection initiated the siRNA pathway (Wen *et al.*, 2014). In this study, the core components of the siRNA and miRNA pathway have been characterized, so they can be used as a supplementary tool to carry out research related to the immune system in *A. franciscana* as well as to better understand the native immune response of crustacean.

One of the important applications of the siRNA pathway is its use in determining the function of genes. In crustaceans, RNAi can be achieved by delivering dsRNA into animal via injection or feeding. For instance, to investigate the function of *PmRab7*, the double-stranded RNA of *Rab7* was injected into a juvenile, which dramatically inhibited WSSV-VP28 mRNA and protein expression (Ongvarrasopone *et al.*, 2008). Feeding of *E. coli* expressing dsRNA targeting Rab7 (dsRab7) or dsRNA targeting the protease gene of YHV (dsYHV) resulted in inhibition of YHV replication and lowered shrimp mortality. dsRab7 could reduce shrimp mortality by 70% whereas dsYHV showed a 40% reduction in mortality compared to the control (Sanitt *et al.*, 2014). In *A. franciscana*, RNAi has been used as a useful tool to study various aspects of biological processes in the development of this species. So far, microinjection of dsRNA is the only delivery method leading to successful RNAi in *A. franciscana*. However, the time span of the RNAi activity and the efficiency by which silencing is achieved is poorly documented. That makes the interpretation, in terms of phenotypic effects of the RNAi treatment, difficult. Therefore, we set up an RNAi experiment to investigate RNAi efficiency by microinjection in *A. franciscana* and to validate our experimental setup. When the *afcad* gene was used as a target gene, the effect of RNAi on the offspring could be clearly observed visually without using biochemical or histological analyzes. Herein, a 387 bp dsRNA fragment targeting *caudal* was injected into the egg-sac of big adult *A. franciscana* females. During the injection, each female received about 250-300 µL dsRNA solution (containing 80-96 ng of dsRNA). The egg-sac microinjection of dsRNA allowed generating more than 95% of alive nauplii with the RNAi phenotype from 22/24 experimented females.

Only 2/24 females failed to produce offspring with the expected phenotype. Additionally, the RNAi time span was determined to last approximately 17 days, resulting in females releasing two broods of nauplii exhibiting the RNAi phenotype. This experiment served as a validation of our experimental RNAi setup and proved that RNAi silencing could be induced efficiently in the offspring of injected females. The dsRNA microinjection technique in *A. franciscana* can be applied to silence different genes such as structural genes, enzymes, as well as immune genes (Iryani *et al.*, 2017 ; Duan et al., 2014 ; Liu *et al.*, 2009). The results suggested that the dsRNA microinjection could be a highly reliable technique of obtaining alive nauplii with a functional RNAi silencing and the desired RNAi phenotype, facilitating the study of the function of gene in many early development pathways.

In insects, an efficient intestinal dsRNA uptake can be observed in some species, but not all species can apply this strategy to induce RNAi. The delivery of dsRNA by oral administration does not only depend on the target genes but also depends on the environment of the digestive tract which may be responsible for the (nucleolytic) degradation of dsRNA. In the migratory locust *Locusta migratoria* for instance, RNAi is highly efficient through dsRNA injection, but oral delivery of dsRNA is much less effective due to the presence of a double-stranded RNA degrading enzyme in the midgut (Song et al., 2017 ; Luo et al., 2013). In this study, the dsRNA feeding experiment showed that under the described conditions the desired phenotype could not be obtained, indicating that dsRNA could not be delivered efficiently to the embryos in the egg-sac by oral uptake of the mother. The exact mechanisms of cellular uptake and systemic spread of dsRNA in arthropods is not clear yet. In *C. elegans*, a gene called *sid-1* has been implicated in cellular uptake and systemic spread of dsRNA in somatic cells, but also into the germline (Winston *et al*., 2002; Devanapally *et al*, 2015). Research in insects has indicated that a homolog of the *C. elegans sid-1* gene is also present in many insect genomes and is likely also involved in cellular uptake of dsRNA in several insect species, together

with endocytotic uptake (Cappelle *et al*., 2016). Additionally, *sid-2* in *C. elegans* was found to be involved in specific cellular uptake of dsRNA from the intestinal environment (McEwan *et al*, 2012; Winston *et al*., 2007). However, *sid-2* homologs have never been identified in arthropods. In *A. franciscana*, *ArSid-1* was predicted as a *sid-1* homolog that could play a role in the intercellular transport of dsRNA, but a *sid-2* homolog was not found in our transcriptome database, therefore *Artemia* could lack a strong dsRNA uptake RNAi system. Additionally, genes involved in endocytosis-mediated dsRNA uptake were not investigated in this study. Thus, the existence of a dsRNA uptake mechanism from the environment remains unknown in *A. franciscana.*

6.1.2 *Artemia franciscana* sex-determining genes

Gender is a research topic that receives a lot of attention as the understanding of sex determination mechanism could lead to the control of the sex ratio of populations. Such applications could bring some benefits to agriculture and aquaculture. The sex determination mechanism has been discovered in model nematodes and insects. It has been shown that sex determination mechanisms in these different species share common genes. One of the most important genes in this mechanism encodes for transcription factor proteins which are encoded by *Mab-3* of *C. elegans* and by *doublesex* of *D. melanogaster* and *B. mori* (Fig 6.2). In *C. elegans,* the ratio between X chromosomes and autosomes leads to the on/off of *xol* state, that will control the expression of several genes resulting in the on/off of *xol* state of *mab-3*. In *D. melanogaster,* a ratio between X chromosomes and autosomes determines the on/off state of *sxl* resulting into sex-specific splicing of *dsx* to produce *dsxF* or *dsxM* under control by *tra* and *tra-2* (Herpin & Schartl, 2015). In ZW sex chromosome system of *B. mori*, sex-specific transcripts of *doublesex*

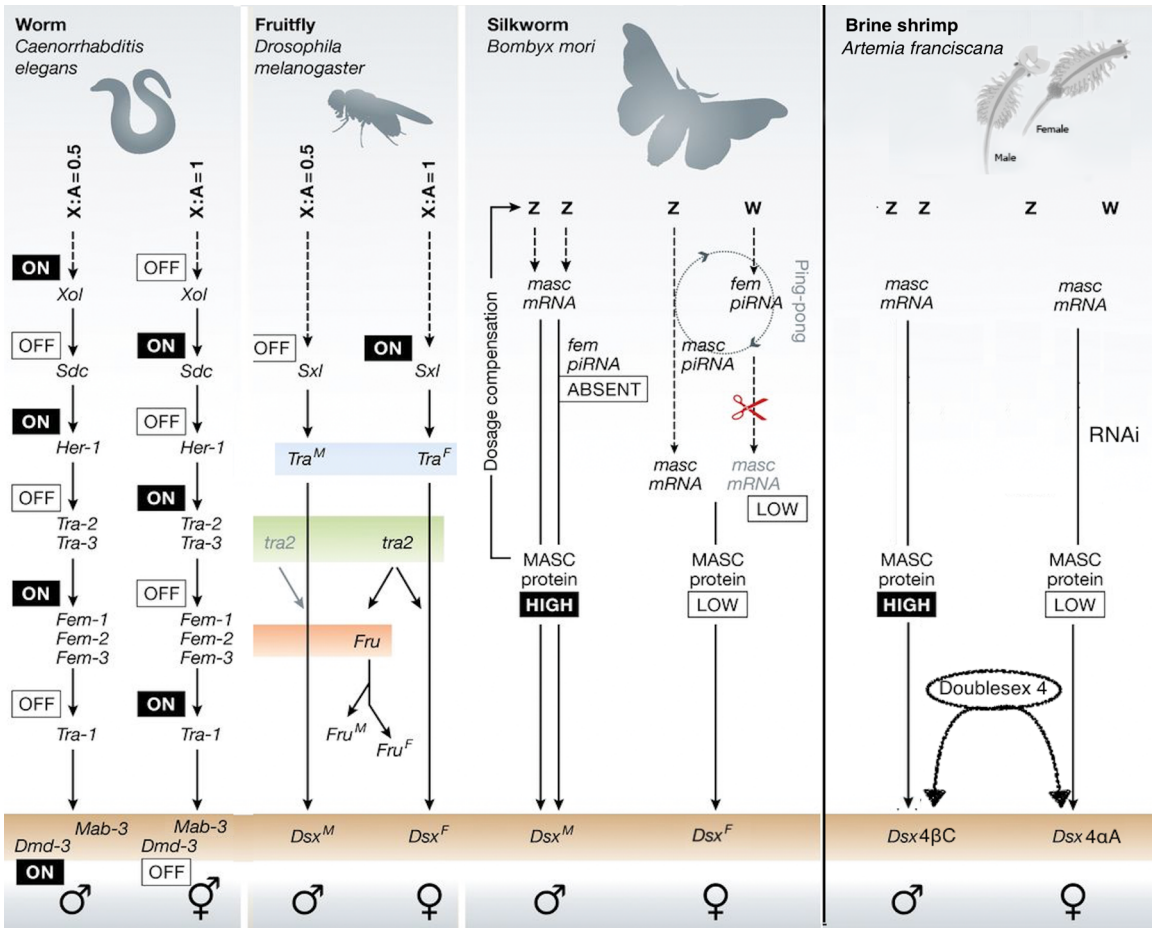


Figure 6.2 Genetic pathways and genes involved in sex determination of some invertebrates and *Artemia franciscana*. **A**: Sex mechanism in *C. elegans*, *D. melanogaster* and *B. mori* adapted from Herpin & Schartl (2015). In *C. elegans* and *D. melanogaster*, a ratio between X chromosomes and autosomes determines the sex. This leads to on/off state of *xol* or *sxl*, respectively. Sex in the silkworm *B. mori* is controlled by a ZW sex chromosome system. Produced only from the sex-determining locus on the W, the piRNAs suppress the male sex-determining factor Masc. **B**: The evidence of genes related the sex determination in *A. franciscana*. Silencing of *masc* in females cause more females to be produced in the progeny, rather than males. Two sex-specific transcript isoforms of *dsx4* were found; dsx-4αA mRNA is a female-specific transcript and dsx-4βC is a male-specific transcript.

producing *dsxF* and *dsxM* are controlled by the level of MASC protein. There is a female-specific piRNA precursor on the W-chromosome of *B. mori,* which is named *fem*. This provides a source of piRNAs for piRNA pathway targeting on *masc* mRNA. In female *B. mori* (WZ), the piRNA pathway uses fem-piRNAs from the W-chromosome to silence masc mRNA which originated from the Z-chromosome, resulting in the MASC protein being maintained at a low level due to a low level of masc mRNA. At a low level of MASC protein, *dsx* will be spliced to produce dsxF transcripts which will then promote a female phenotype. In male *B. mo*ri (ZZ), there is no presence of this piRNA precursor, and the MASC protein is translated at a high level. Here, this protein has an influence on the splicing of *dsx* to produce *dsxM* transcripts for the promotion of a male phenotype (Kiuchi *et al.*, 2014 ; Sakai *et al.*, 2015). At protein level, both DSX-M and DSX-F proteins form asymmetric homodimers at low concentration and tetramers and higher oligomers at higher concentrations (Cho & Wensink, 1996). However, only DSX dimers can bind on specific DNA sequence to control the transcription of multiple genes linked to the sex-traits (Verhulst & van de Zande, 2015 ; Erdman et al., 1996).

In this study, two *fem-1* and six *dsx* homologs were identified from *A. franciscana*. Among those genes, *dsx-4* is transcribed into three different isoforms, namely dsx-4αA, dsx-4αB and dsx-4βC mRNAs. Dsx-4αA mRNA is a female-specific transcript, while dsx-4βC mRNA is a male-specific transcript. In *A. franciscana*, *masc* homologs has also been found expressed in embryonic stage (Li *et al.*, 2017). Silencing of *masc* in *A. franciscana* females caused the female:male ratio of progeny to be 2.19:1. It indicated that more females are produced than males when the MASC protein level is low (Li *et al.*, 2017). A high level of MASC protein in *A. franciscana* related to the male phenotype shows a similarity with the sex determination in comparison with this mechanism in *B. mori* (Fig. 6.2).

During this study, *A. franciscana* genomic data were not available yet;

Thus, the structure of this *dsx-4* locus is still unknown. However, at the transcription level, dsx-4αB mRNA was found present in both sexes, since dsx-4αA and dsx-4αB mRNAs probably encode for the same DSX-4 Alpha protein, females are predicted to have only DSX-4 Alpha homodimer. In contrast, the male has both dsx-4βC and dsx-4αB mRNA; therefore DSX-4 Alpha and DSX-4 Beta protein probably are present together, resulting in three different types of protein dimer (DSX-4 Alpha homodimer, DSX-4 Beta homodimer and DSX-4 Alpha/DSX-4 Beta heterodimer). In *B. mori*, the complication of cis- and trans-spliced transcripts of *dsx* has also been reported to generate seventeen alternatively spliced forms and eleven putative trans-spliced variants. However, all transcripts encode for only four female-specific, two male-specific DSX proteins and one DSX protein common to males and females (Duan *et al*., 2013a ; Duan *et al*., 2013b). Therefore, the alternative splicing of *A. franciscana doublsex-4* may need to be investigated further to unravel its precise role in the sex determination.

6.2 Conclusions

In this study, we have identified RNA interference machinery core genes in *A. franciscana*. These genes belong to two RNAi pathways of this species, including the miRNA and the siRNA pathway. In crustaceans, only one copy of a dsRNA binding homolog has previously been reported. In this study, two dsRNA binding protein genes were found in *A. franciscana.* The ArTRBP-like is considered to be a new candidate in this protein family. Additionally, the RNA interference is defined as a component of the immune system in crustacean, so the RNA interference machinery core genes can be used in immunological studies *on A. franciscana* such as *Argonaute* which is known to play a role during the infection of virus and bacteria.

The egg-sac microinjection method showed that it could be used to prepare a nauplii population with the desired RNAi phenotype. A high ratio of

these nauplii with the desired RNAi phenotype are released from RNAi-treated female within the first 17 days after dsRNA injection. This corresponds with the first 2 broods. In the third brood, the RNAi phenotype is mostly absent. The resulting nauplii phenotype suggests that RNA interference molecules are transgenerationally transmitted. The dsRNA delivery via feeding dsRNA-producing bacteria failed to induce the RNAi in *A. franciscana* adults.

We isolated and sequenced some genes related to sex determination pathway in *A. franciscana*, including two *fem-1* and six *dsx* homologs. The transcription of these genes has been investigated in adult *A. franciscana*. The transcription of those genes was confirmed in both male and female adults. However, *dsx-4* produces different transcripts in males and females. Among those, dsx-4αA mRNA is a female-specific transcript, dsx-4βC mRNA is a male-specific transcript, while dsx-4αB mRNA is found in both male and female adults. It suggests that this phenomenon may be closely linked to the sex determination in *Artemia*.

6.3 Future perspectives

The RNA interference pathways including miRNA, siRNA and piRNA are found in many eukaryote species such as fungi, plants, insects, crustaceans, birds, fishes and mammals. Among these, the miRNA and the piRNA pathways have been known to play crucial roles in key biological processes such as controlling epigenetic modifications, cell proliferation, tissue differentiation, apoptosis and sexual differentiation. Additionally, the siRNA pathway has emerged as an important regulator of the host innate immune response and host pathogen, as it acts as a defence system for the destruction of foreign nucleic acid. These all indicate that the RNAi mechanism plays an important role during the development of organisms in general, and crustaceans in particular. With many of the biological advantages such as its small size, its short life cycles and different reproduction modes, *Artemia* is becoming an

interesting model organism for the order of Crustacea. This species could be used to study various aspects, including toxicity assessment of nanoparticles, larviculture, management of policies in aquatic resources and trained immunity. The available genomic and transcriptome data of *A. franciscana* (at Ghent University) and the characterized core genes of RNAi in this study may together contribute as the research material for accelerating the study of biological processes in this species as well as other crustaceans, particularly at gene level.

The mechanism of sex determination and sexual differentiation have been studied in many different species, especially insects. Up to date, sex reversal in crustaceans has only been successful in *M. rosenbergii*. Sex reversal in this species was performed using RNAi or by the removal of the androgenic gland to eliminate the effect of insulin-like hormone prior to gender differentiation. In insects, *dsx* plays a central role in sex determination through splicing events, resulting in direct control of some of the internal and external phenotypes of the organism. In some crustaceans, in which sex is controlled by genetic sex-determination, *dsx* homologs have also been isolated. However, there was no evidence of splicing events producing sex-specific transcripts. The existence of *dsx-4* and its sex-specific alternative splicing in *A. franciscana* indicate that crustacean species may, at least partially, share the sex determining mechanism with insects. Thus, studying the sex-specific alternative splicing of *dsx-4* in *A. franciscana* may provide insights to control sex-linked traits in crustaceans. In this context, the egg-sac microinjection method can be applied to silence the *doublesex-4* isoforms specifically in embryos and observe the gender ratio in the progeny. The goal is to eliminate dsx-4βC mRNA from the zygote stage. Therefore, it is possible to use a beta-sex-specific sequence and 3'UTR-C sequence of dsx-4βC mRNA to prepare dsRNA for inducing RNAi. In addition, it is possible to use RNAi to simultaneously remove dsx-4βC mRNA and masc mRNA to investigate the effect on gender ratio. The correlation between *masc* and sex-specific *dsx*-

4 transcripts should also be determined to clarify their function in the sex determination mechanism of *A. franciscana*.

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Curriculum vitae

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Education

2013 to 2017	PhD candidate in Applied Biological Sciences Laboratory of Animal Production and Artemia Reference Center, Faculty of Bioscience Engineering, Ghent University.
2003-2005	Master of Science in Molecular Biology Laboratory of Gene Technology Department of Biosystems (Division of Animal and Human Health Engineering), University of Leuven - KU Leuven, Belgium.

1995-1999	Bachelor of Microbiology and Molecular biology University of Natural Sciences, Ho Chi Minh city, Vietnam

Skills and awards

Research experience:

- qPCR, PCR, SDS-PAGE, immunoblotting.
- Genome annotation
- Cloning, recombinant protein and protein purification.
- RNA interference

Scholarship and travel grants:

2013 – 2017	Vietnam International Education Development VIED scholarship, Vietnam for full time PhD study at Ghent University, Belgium
2003 – 2005	VLIR- scholarship for full time Master study at University of Leuven - KU Leuven, Belgium

Previous working experiences

2006 – 2013	Head of Laboratory of Molecule biology of Southern Monitoring Center for Aquaculture Environment and Epidemics, Research Institute for Aquaculture No. 2, Ho chi Minh city, Vietnam.
1999 – 2006	Researcher at Aquatic pathology research unit of Research Institute for Aquaculture No. 2, Ho chi Minh city, Vietnam.

Training and conferences

Specialist courses, and Skills Training:

2013: Basics of Biology for Engineers at University of Leuven, Belgium.

2016: Precision Genome Engineering at VIB, Ghent, Belgium.

2016: Genome Engineering and Synthetic Biology at VIB, Ghent, Belgium

2017: qPCR-course – Biogazelle at Ghent University, Belgium.

2017: Efficient academic communication in cross-cultural environments at Ghent University, Belgium.

Conferences:

2016: Poster at VLIZ Marine Scientist Day in Brugge, Belgium.

2017: Oral presentation at LARVI 2017 at Ghent University, Belgium.

Publications

Das M, Li X, **Dung NV**, Bossier P, Tinh NTN (2017) No effect of N-acyl homoserine lactones disruption by lactonase enzyme on the virulence of Vibrio anguillarum towards sea bass (*Dicentrarchus labrax*) and brine shrimp (*Artemia franciscana). Aquaculture International*. https://doi.org/10.1007/s10499-017-0214-2

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