Chapter 6

Microinjection-Based RNA Interference Method in the Water Flea, *Daphnia pulex* and *Daphnia magna*

Kenji Toyota, Shinichi Miyagawa, Yukiko Ogino and Taisen Iguchi

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/61485

Abstract

It is well known that most daphnid species have several attractive life history characteristics such as cyclical parthenogenesis, environmental sex determination, and predator-induced defense formation. Recent advances in high-throughput omics technologies make it easy to obtain a huge number of potential candidate factors involved in environmental stimuli-triggered phenotypic alterations. Furthermore, our group has developed a microinjection system to introduce foreign materials such as nucleotides and chemicals into the early-stage (one-cell stage) egg of *Daphnia pulex* and *Daphnia magna*. Consequently, we established a microinjection-based RNAi system that allows arbitrary gene functions to be investigated. However, this microinjection system does not seem to have pervaded in the daphnid research community due to its low throughput and high level of skills required. In this chapter, we review the microinjection method and its RNAi system in water fleas, *D. pulex* and *D. magna*, providing some technical tips and making challenging proposals for the development of novel high-throughput RNAi methods. Finally, we provide an overview of recently developed gene functional analysis methods such as overexpression and genome-editing systems.

Keywords: *Daphnia pulex*, *Daphnia magna*, genome editing, microinjection, RNAi-related gene

1. Introduction

The cladoceran crustacean water fleas are representative zooplankton ubiquitously found in freshwater habitats around the world [1]. Among them, species of the family Daphniidae, particularly genus *Daphnia*, have been well studied. All age classes of daphnids are principle consumers of algae and thus play an important role in the food web in freshwater ecosystems. In addition to this ecological significance, over the last decade, daphnids have drawn consid-

erable attention as a good indicator organism for aquatic toxicology and have thus been used in ecotoxicological studies [2]. Moreover, for over 100 years, they have shown various adaptive phenotypic alterations in response to external environmental stimuli, including environmental sex determination (ESD) [3, 4], cyclical parthenogenesis, in which the mode of reproduction changes between parthenogenesis and sexual reproduction [3, 4], and inducible defense, which is a predator-triggered alteration of body shape [5, 6]. The acquisition of such sophisticated life history strategies has enabled daphnids to prosper around the world. These environmental stimuli-triggered phenomena in daphnid species have attracted many scientists involved in ecological, developmental, and evolutionary biology [4, 7–12]. Although recent progress in sequencing technology facilitates the deciphering of genome and transcriptome information using 'nonmodel organisms', analytical methods for arbitrary gene functions are still insufficiently developed. In studies involving daphnids, the whole-genome sequencing project of *D. pulex* has been completed [12]. Furthermore, a microinjection system using early-stage embryos has been established, allowing gene functional analyses, including RNA interference (RNAi), to be possible in daphnids [13, 14]. Even though this microinjection-based experimental method can be used in two representative daphnid species, *D. pulex* and *D. magna*, some experimental aspects are different between them due to the difference in size of their early-stage embryos.

This chapter introduces *Daphnia* species as attractive models for eco-evo-devo studies and summarizes technical methods and tips for microinjection-based RNAi in *D. pulex* and *D. magna*. Finally, we review recent advances in the application of microinjection methods in daphnids such as genome editing and transgenesis.

1.1. Life cycle

Daphnids produce offspring either by parthenogenesis or by sexual reproduction in response to external environmental conditions. This mode of reproduction is referred to as cyclical parthenogenesis. They have a short generation period that lasts approximately 1 week under constant laboratory conditions, and their lifetime spans over 2 months or as much as 1 year when reared under colder temperatures [15]. Under optimal growing conditions, daphnids parthenogenetically produce offspring that expand their population consisting almost exclusively of females. This results in the exponential growth of genetically identical clone clusters. Mother daphnids produce several dozen eggs in their own brood chamber as a clutch just a few minutes after molting. Embryonic development occurs in the brood chamber. Subsequently, neonates are released to the outside just before the mother molts. Then individual mothers in the parthenogenetic phase repeat molting, spawning, and the release of neonates throughout their lifetime (Figure 1, parthenogenetic phase).

On the other hand, when environmental conditions deteriorate, for instance, a short day-length, lower temperature, food shortage, overcrowding, and the presence of predators, males are induced by parthenogenesis and are, therefore, genetically identical to their sisters and mother [4, 7–9, 16, 17] (Figure 1, sexual reproductive phase). In other words, an individual parthenogenetic mother has the potential to produce female and male offspring in response to changes in external environmental conditions. After copulation, sexual eggs, referred to as

http://dx.doi.org/10.5772/61485

resting eggs that are enclosed in an ephippium (modified carapace that is darkly pigmented by melanin), are produced. Resting eggs can tolerate extreme conditions such as drying, freezing, and digestion by fish and can remain viable for over 100 years [18]. When favorable conditions are restored, female neonates hatch out from resting eggs and reinitiate the parthenogenetic phase, thus building up a new population (Figure 1, sexual reproductive phase).

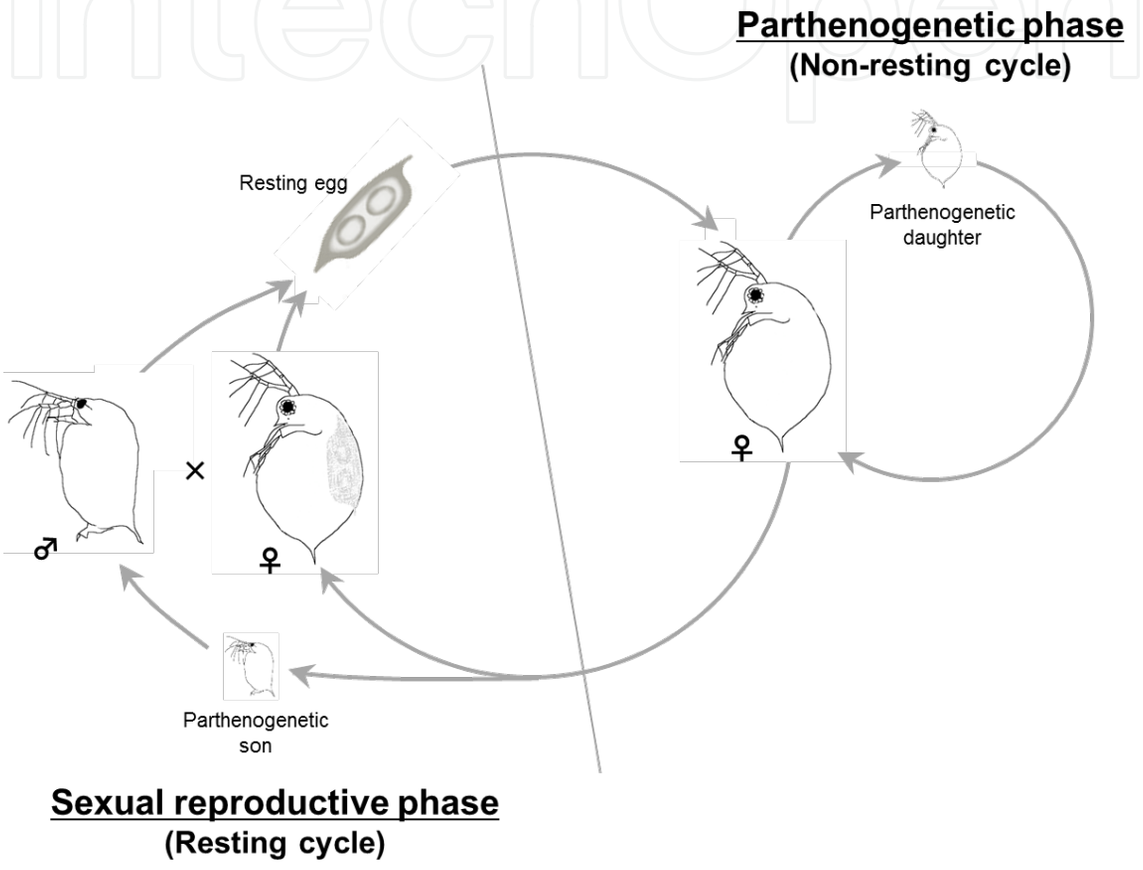

Figure 1. Schematic drawing of cyclical parthenogenesis in daphnids.

1.2. *Daphnia* as model species for ecological, evolutionary and developmental biology: eco-evo-devo

Although several details are still controversial, recent molecular phylogenetic analyses of the Arthropoda have revealed that the Crustacea clade is not monophyletic and is divided into at least three clades (Ostracoda, Malacostraca, and Branchiopoda) that include daphnids (Figure 2). Also, the current phylogenetic hypothesis supports the notion that Branchiopoda and Hexapoda form a sister group (Figure 2). This suggests that a growing body of findings involving daphnids has accumulated and can contribute to our understanding of the evolutionary and developmental aspects of Arthropoda, connecting knowledge between well-studied Hexapoda and more primitive Arthropoda clades.

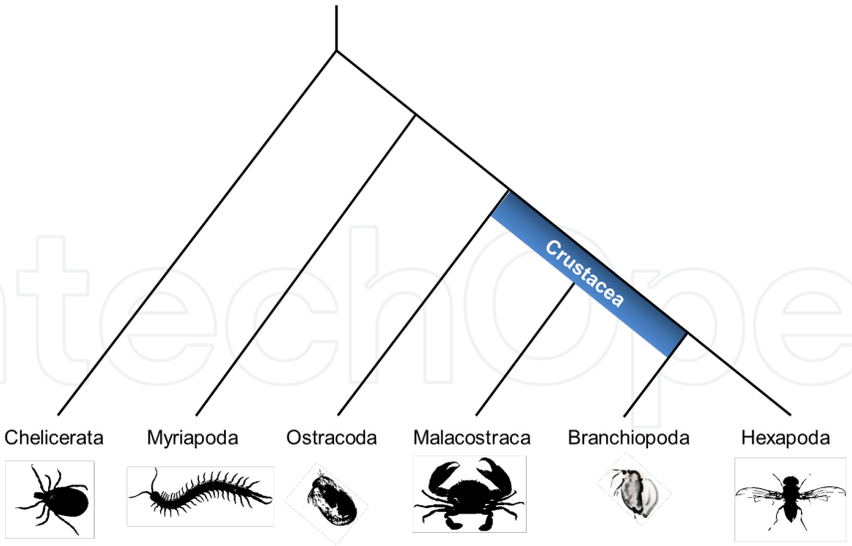

Figure 2. Phylogenetic tree of the Arthropoda. The branching pattern is based on Oakley *et al.* [19] with some modifications [20].

D. pulex and *D. magna have* long been used in ecological, evolutionary, developmental, and ecotoxicological studies as representative model daphnid species for the following reasons: *D. pulex* is ubiquitously widespread around the world, including Japan (Figure 3A), and shows striking phenotypic alterations in response to predator-released chemicals, forming 'neckteeth' [5]; *D. magna* has a huge body size among cladoceran species (maximum length of approximately 10 mm, Figure 3B); they are easy to maintain and rear under laboratory conditions; they propagate rapidly because of their short generation time and reproductive cycle; embryonic development can be observed *in vitro*; male offspring can be induced by administration of juvenile hormone agonists [21, 22]. In addition, individuals within a single strain are most likely genetically identical due to the diploidy of the parthenogenetic eggs that are maintained by mitosis-like meiosis, which skips a part of the first meiosis [23], allowing the environmental effects on their physiological and developmental processes to be investigated under a constant genetic background. Furthermore, we established a reliable induction system for environmental sex determination (ESD) studies using the *D. pulex* WTN6 strain, in which the sex of the offspring can be controlled by changing the day-length conditions in which long-day (14 h light:10 h dark) and short-day (10 h light:14 h dark) conditions can induce female and male offspring, respectively [17], and for inducible defense in several *D. pulex* strains where the incidence and number of neckteeth vary in response to different concentrations of *Chaoborus* kairomone [24].

In addition to the aforementioned advantages, useful experimental tools are available, for example, embryonic developmental staging [25, 26], whole-mount *in situ* hybridization and immunostaining using developing embryos [27], immunofluorescence and fluorescence *in situ* hybridization (FISH) [28], an expressed sequence tags (ESTs) database [29], and genetic linkage maps [30–32]. Furthermore, the whole-genome sequencing of *D. pulex* is complete [12, 32], although that of *D. magna* is still ongoing (https://wiki.cgb.indiana.edu/display/magna/Home). In addition, recent growing omics and bioinformatics technology enables daphnid

http://dx.doi.org/10.5772/61485

researchers to investigate cells, tissues, and organisms from a multilevel perspective such as the transcriptome [12, 33–35], proteome [36], or metabolome [37]. Thus, various excellent experimental tools and an increasingly huge accumulation of omics data make *D. pulex* and *D. magna* attractive model organisms for studying the molecular mechanisms underlying phenotypic alterations that depend on external environmental conditions. These reliable induction systems of focal phenotypes are indispensable for analyzing their physiological and developmental mechanisms.

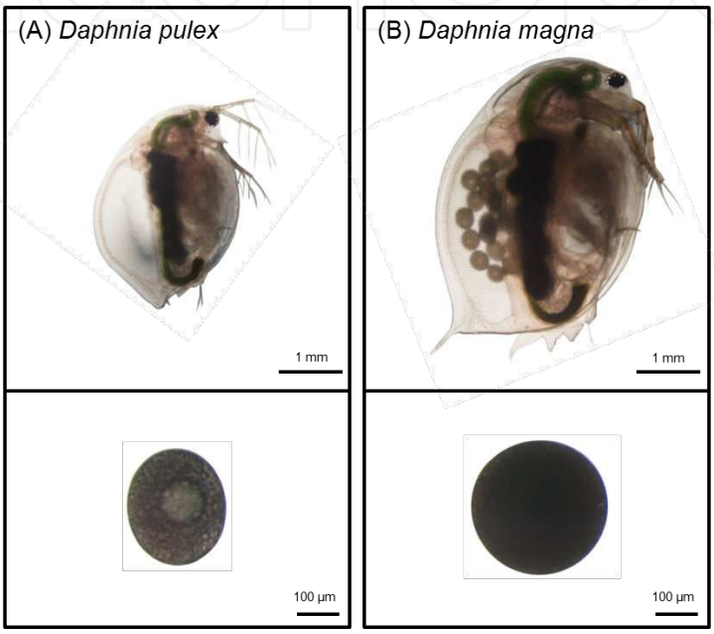

Figure 3. *Daphnia pulex* (A) and *D. magna* (B). Upper and lower parts indicate the adult and embryo just after ovulation, respectively.

2. Microinjection-based RNA interference (RNAi) in daphnid species

As mentioned above, recent high-throughput sequencing technologies have enabled biologists to use nonmodel organisms to easily access genomic information. However, the development of experimental methods for gene functional analysis still hampers their reverse genetics approach. To date, the RNAi method in *D. pulex* and *D. magna* has been established by a microinjection method using early-stage embryos. Although our previous reports described detailed methodology for microinjection and the tips, tricks, and traps associated with this methodology [13, 14], there are slight differences for each daphnid species. Furthermore, descriptions of genes involved in the RNAi machinery is insufficient. Therefore, we comparatively summarize the tips to manipulate the microinjection methods in detail prior to focusing on the current status of daphnid RNAi. We then introduce the gene repertoires involved in the RNAi mechanism in *D. pulex* and *D. magna* genomes.

2.1. Microinjection system

The daphnid microinjection system uses parthenogenetic eggs just after ovulation into the mother's brood chamber. The individual mother begins to lay eggs into the brood chamber a few minutes after releasing neonates and molting. To obtain many healthy eggs, eggs from 2 to 6-week-old daphnids should be collected.

There are two major technical issues in microinjection of daphnids. One is the rapid hardening of the egg membrane just after ovulation [38], which is caused by enzymatic activity of peroxidase. The second problem is a substantial difference between the internal and external osmotic pressures of the egg. The former issue hampers the penetration of the egg membrane by a needle while the latter causes the leakage of egg components after injection. To overcome these problems, Kato et al. [13] and Hiruta et al. [14] established improved protocols for microinjection in *D. magna* and *D. pulex*, respectively. They succeeded in the transient inhibition of the egg membrane hardening by ice-cold treatment just after ovulation. They also found the best culture conditions after injection by increasing external osmotic pressures: M4 medium [39] with 80-mM sucrose in *D. magna* [13] and a 2% agar plate covered with 60-mM sucrose dissolved in M4 media in *D. pulex* [14].

In addition, since the fineness of the needle is critical for the success of microinjection, we next describe a detailed preparation method. A glass needle is made from a glass capillary tube (GD-1; Narishige, Tokyo, Japan) by a micropipette puller (P-97/IVF; Sutter Instrument, Novato, CA, USA). The programmed P-97 parameters are as follows: heat: 845; pull: 50; velocity: 120; time: 200; pressure: 500. The value of the 'heat' parameter required for the ramp test is based on the manufacturer's protocol because this value depends on a combination of the filament and the glass capillary. In our case, using a combination of a regular P-97 filament and a GD-1 glass capillary, the 'heat' parameter value ranges between 845 and 870.

Based on the aforementioned information, we describe next the manipulation procedure of microinjection using daphnids eggs.

1. The setting of tools for microinjection and surgery are shown in Figure 4A-C. A glass Petri dish is prepared by placing two cover glasses side by side with M4-sucrose at ambient temperature.

2. The synthesized double-strand RNA (dsRNA) is mixed with an equal amount of 2-mM Chromeo 494 fluorescent dye (Active Motif Chromeon GmbH, Tegernheim, Germany), which is used as a visible marker for injection. When using *D. pulex* eggs, it is possible to confirm whether injection has succeeded by visual observation since the eggs are more transparent than *D. magna* eggs (Figure 3). A visible marker is thus not essential.

3. The dsRNA solution (1.0–1.5 μL) is loaded into the needle. The tip of the needle is then manually cut off using forceps under a stereomicroscope.

4. Mother daphnids just before molting (brood chamber is empty) are collected and observed until spawning begins. They are transferred to ice-cold M4-sucrose medium just before spawning is complete (4–5 eggs remain in each ovary).

http://dx.doi.org/10.5772/61485

5. Eggs are surgically obtained from the mother daphnid and placed in ice-cold M4-sucrose medium.

6. One to three eggs are immobilize by placing them at the edge of the left cover glass and are injected by using a microinjector (Femtojet, Eppendorf, Hauppauge, NY, USA) and a micromanipulator (MN-153, Narishige, Tokyo, Japan ; Figure 4D–F). The right cover glass is used as a holder when the needle is not withdrawn from the egg. Microinjections can be carried out within 1 h after ovulation.

7. The injected eggs are transferred into a plastic 6-well plate with 80-mM M4-sucrose medium for *D. magna* [13] or a 2% agar plate on a 6-well plate with 60-mM M4-sucrose medium for *D. pulex* [14] (Figure 4G) and subsequently incubated under constant temperature (18–20°C).

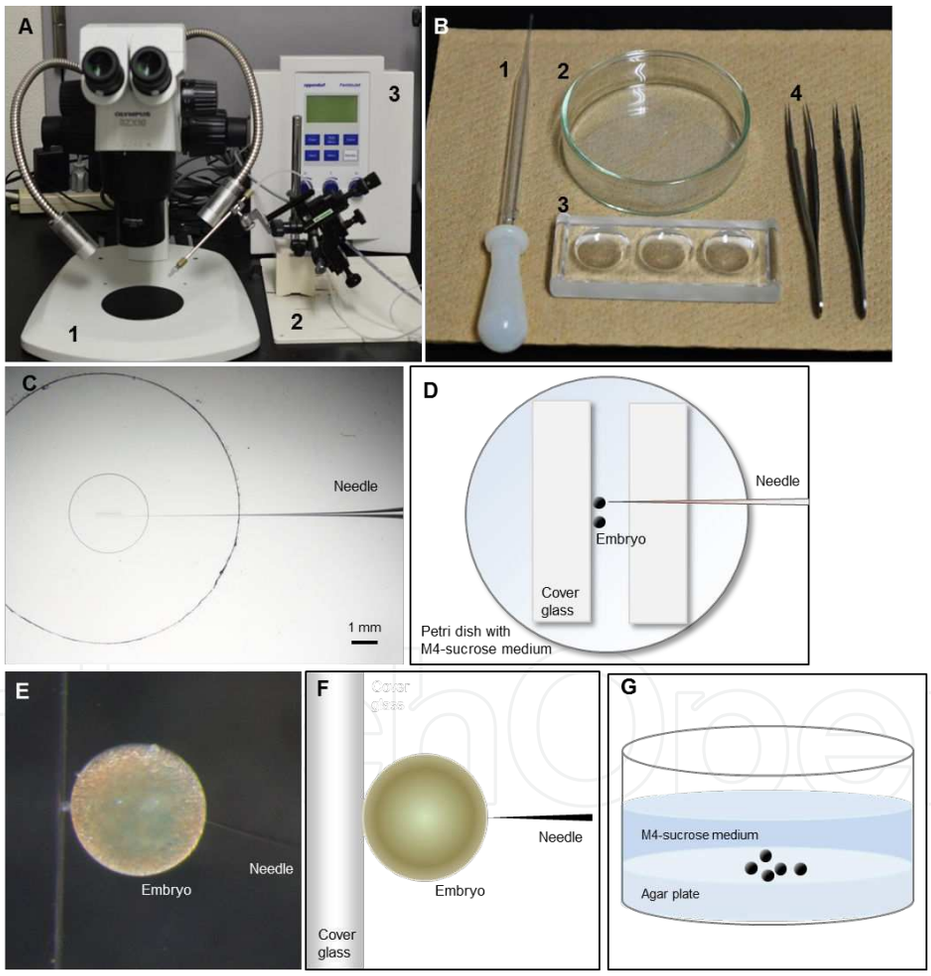

Figure 4. Microinjection of daphnid egg. (A) Experimental equipment. 1: stereomicroscope; 2: micromanipulator (MN-153, Narishige, Tokyo, Japan); 3: electronic microinjector (Femtojet, Eppendorf, Hauppauge, NY, USA). (B) Tools for surgical manipulation. 1: Pasteur pipet (Thomas Scientific, Swedesboro, NJ, USA); 2: glass dish with two cover glasses; 3: plate for blood test; 4: forceps (Dumoxel #5 Biologie). (C) A glass needle is made from a glass capillary tube (GD-1; Narishige, Tokyo, Japan). (D) Overview illustration of microinjection field. (E, F) Magnified view and illustration of microinjection using *D. pulex* egg. (G) Schematic illustration of embryo incubation after injection.

2.2. RNAi machinery in daphnid species

The well-known natural role of RNAi in organisms is the innate immune system against viruses and transposable elements [40]. Using this phenomenon, RNAi induction has been developed as an innovative method for gene functional analysis by exogenous application of dsRNA in *Caenorhabditis elegans* [41]. The dsRNA is first recognized by Dicer protein and cut off into short 21–24 nucleotides referred to as short interfering RNAs (siRNA). These are then invariably incorporated into large Argonaute-containing effector complexes known as RNA-induced silencing complexes (RISCs), after which one-side strand of the dsRNA is cleaved and degraded. Finally, the active Argonaute-containing RISC cleaves the target RNA sequence with the complementary sequence to siRNA [40, 42]. In addition to this core machinery of the RNAi pathway, many eukaryotes have the potential to amplify an amount of siRNA by a host-encoded RNA-dependent RNA polymerase (RdRp). However, RdRp orthologs have not been identified from the Arthropoda genome including *D. pulex* except for the tick genome [42].

In the *D. pulex* and *D. magna* genomes, there are three Dicer and Argonaute paralogs, but the *D. magna* genome contains two copies of Dicer. Previous studies have shown that Dicer paralogs are categorized into two clusters corresponding to the microRNA (miRNA) pathway (Dicer-1) and the siRNA pathway (Dicer-2) [42, 43]. The miRNA is also a short 21–25 RNA, which is generated from a hairpin in pre-mRNA, and plays an important role in translational repression associated with RISC [44]. Phylogenetic analyses found that all Dicer paralogs of *D. pulex* and *D. magna* were classed into the Dicer-1 group [45] (Figure 5A). Moreover, we successfully recruited three Argonaute paralogs from the genome of each daphnid and constructed a phylogenetic tree (Figure 5B). Previous studies revealed that Argonaute family members are key components in different RNA silencing pathways and are categorized into two subfamilies, Argonaute and PIWI (P-element induced wimpy testis). Argonaute subfamily members have been found in widespread taxa, including yeast, plants, and animals and have been identified as Argonaute-1 and Argonaute-2, which are involved in miRNA and siRNA pathways, respectively. In contrast, the PIWI subfamily has been identified only in animals as Argonaute-3, which is involved in PIWI-interacting RNA (piRNA) pathways [42, 46, 47]. Four Argonaute family members were found from *D. pulex* and *D. magna* genome sequences in this study, although only two paralogs have already been previously reported [43]. Phylogenetic analysis revealed that both paralogs fall into the Arogonaute-1 clade of the Argonaute subfamily, whereas each one paralog was categorized into Argonaute-3 and PIWI clades of the PIWI subfamily (Figure 5B). The number of Argonaute family members found in daphnids seems to be as conserved as in insect species [42, 48], although no Argonaute-2 paralogs have yet been identified. Taken together, our results suggest that the genomes of daphnids might lack the Dicer-2 and Argonaute-2 orthologs, which are factors responsible for regulating the siRNA-inducing transcriptional gene-silencing pathway. In other words, our data imply that the RNAi machinery of daphnid species might be distinct from the equivalent well-studied mechanism in insects. To understand the full picture of the RNAi machinery of daphnid species, further studies that examine domain sequence similarity and gene functional analysis will be required.

http://dx.doi.org/10.5772/61485

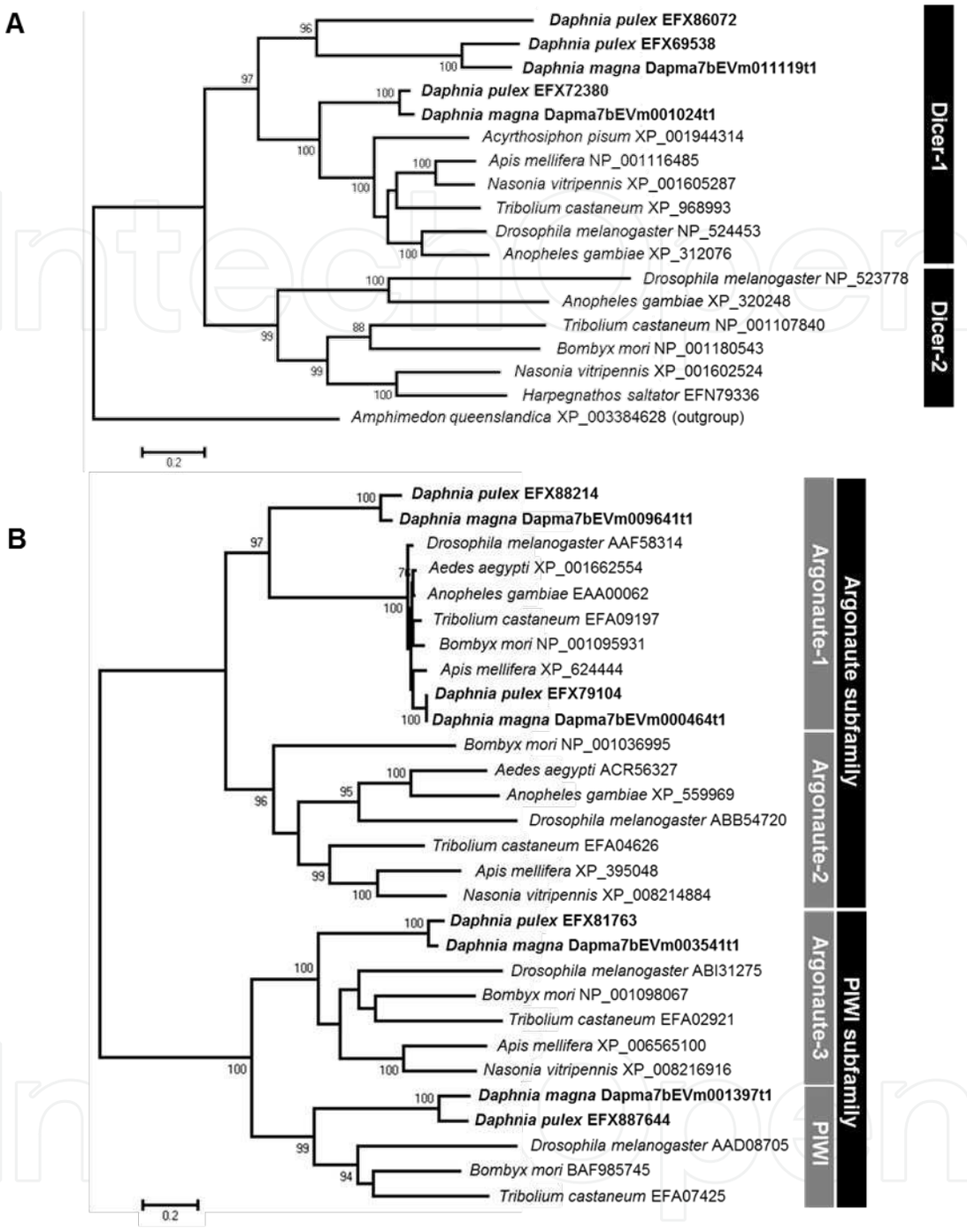

Figure 5. Phylogenetic trees of Dicer (A) and Argonaute (B). Amino acid sequences were aligned by ClustalW, and the maximum likelihood trees were constructed from these alignments using the JTT model with bootstrap analyses of 1000 replicates along with complete deletion options (818 and 597 amino acid positions were used, respectively) by MEGA6 [49]. Branches with bootstrap support >70% are indicated by numbers at nodes. Both *D. pulex* and *D. magna* are indicated in bold. To obtain the predicted sequences encoding the *D. magna* orthologs of RNAi-related genes, protein sequences of *D. pulex* were used in BLAST searches querying *D. magna* Genome BLAST (http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/BLAST/). The SID protein sequences of *D. pulex* were retrieved from wFleaBase (http://wfleabase.org/). Phylogenetic trees were constructed based on data from McTaggart et al. [43] with some modifications.

2.3. Future challenges for development of high-throughput RNAi method in daphnids

Despite the availability of a microinjection-based RNAi method in daphnids, as demonstrated by knocking down genes responsible for morphogenesis (*distal-less* and *eyeless*) [13, 14, 50], sexual differentiation (*doublesex1*) [51], endocrine system (*ecdysteroid-phosphate phosphatase*) [52], and neurogenesis (*single-minded homolog*) [53], this method has several experimental limitations. For example, microinjection can only be performed using an early stage (1-cell stage) egg, suggesting that this system cannot be used to perform a functional analysis of genes that act during the adult stage and is unsuitable for large-scale experiments. Moreover, specialized and skillful technical training is necessary to master the microinjection technique in daphnids. To overcome those technical hurdles, we introduce two potential ideas to establish more high-throughput and user-friendly methods for the study of daphnids. One is electroporation, which is a physical transfection method that uses an electrical pulse to increase the permeability of cell membranes, allowing nucleic acids and/or chemicals to be introduced into cells. Recent innovation of the electroporation system has enabled the establishment of rapid functional analysis in various organisms [54–56]. Indeed, our group has successfully developed an *in vivo* electroporation system for the introduction of foreign DNA into neonatal *D. magna* within 6 hours after release from the mother's brood chamber [57]. Therefore, it might be possible to apply this system for RNAi using various stages of daphnids.

The second method is a feeding (oral delivery) RNAi system, which was first developed in *C. elegans* [58]. The feeding RNAi system is the most convenient and inexpensive method for high-throughput screening since bacteria produce the designed dsRNA that are fed to host animals. This system has so far been applied in various insects [59] and decapod crustaceans [60–62]. Unlike the time-consuming and troublesome microinjection method that can only be performed in the early egg stage in daphnids, the alternative feeding RNAi method may potentially be applied for manipulating a wide range of genes in many individuals at the same time.

3. Extended microinjection-based applications

The microinjection system can be widely applied for the development of not only RNAi but also other attractive methods for gene functional analysis. Indeed, several microinjection-based functional methods have been developed in daphnid species. First, a transient overexpression system for arbitrary genes or reporter constructs was established by microinjection of synthesized mRNA bearing the 5′ cap structure and the 3′ poly(A) tail [51] and a DNA reporter construct [63]. These methods allow for a gain-of-function analysis, although only one example has shown that the phenotype induced by transient overexpression was only observed in the first instar juvenile [51]. However, the aforementioned overexpression and RNAi system in daphndis suffer from several drawbacks such as incomplete gain- or loss-of-function, transient effect, and limited analyzable stages.

To overcome these limitations, a transgenic *D. magna* line was established by using microinjected GFP or DsRED reporter plasmid, although the success rate was quite low (0.67%) [64,

65]. Furthermore, genome editing with engineered endonucleases is rapidly growing as a stable experimental method for generating heritable mutations in not only well-known in model organisms but also in nonmodel organisms. There are three representative methods: zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) systems [66]. In order to perform targeted mutagenesis in the *D. pulex* and *D. magna* genomes, TALEN and CRISPR/Cas9 systems have been established by microinjection of these engineered nucleases [50, 65, 67]. Taken together, these genome-editing techniques will enable scientists to accurately define arbitrary gene functions in daphnid species in future studies.

4. Conclusion

Recent growing innovations in high-throughput omics technologies (e.g., genomics, transcriptomics, proteomics, and metabolomics) enable us to obtain comprehensive profiles of a huge amount of candidate factors responsible for unique life history features of daphnids [12, 34, 68]. In order to investigate an arbitrary gene function, the establishment of an experimental method for gene functional analysis has been enthusiastically addressed by researchers using nonmodel organisms, even in the postgenomic era. In this chapter, we summarized (1) the experimental procedure with several tips for a microinjection system in *D. pulex* and *D. magna*, (2) information about genes responsible for their RNAi machinery, (3) potential concepts for novel user-friendly high-throughput RNAi systems in daphnids, and (4) other microinjection-based applications in daphnids. Further studies involved in the development of novel experimental methods and investigation of a wide range of gene functions can lead to a better understanding of the overview of the attractive environmental stimuli-dependent phenomenon in daphnids.

Acknowledgements

We thank members of the Iguchi laboratory for helpful advice and comments. Our work benefits from, and contributes to, the *Daphnia* Genomics Consortium. This work was supported by a JSPS Research Fellowship for Young Scientists to KT (No. 12J05579), a Sasakawa Scientific Research Grant from the Japan Science Society to KT, a Saito Ho-on Kai Scientific Research Grant from the Saito Gratitude Foundation to KT, grants from the Ministry of Education, Culture, Sports, Science, and Technology (TI), the Ministry of the Environment of Japan (TI), a Grant from the National Institute for Basic Biology (TI), and the Research Council of Norway (project 221455), Adverse Outcome Pathways for Endocrine Disruption in *Daphnia magna*, a conceptual approach for mechanistically based risk assessment (TI).

Author details

Kenji Toyota, Shinichi Miyagawa, Yukiko Ogino and Taisen Iguchi*

*Address all correspondence to: taisen@nibb.ac.jp

Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, and National Institutes of Natural Sciences, Higashiyama, Myodaiji, Okazaki, Aichi, Japan

References

[1] Dieter E. Ecology, Epidemiology, and Evolution of Parasitism in *Daphnia*. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information.; 2005.

[2] Iguchi T., Watanabe H., Katsu Y. Toxicogenomics and ecotoxicogenomics for studying endocrine disruption and basic biology. General and Comparative Endocrinology. 2007;153:25–29. DOI:10.1016/j.ygcen.2007.01.013

[3] Grosvenor G. H., Smith G. The life-cycle of *Moina rectirostris*. Quarterly Journal of Microscopical Science. 1913;2(58):511–522.

[4] Smith G. The life-cycle of Cladocera, with remarks on the physiology of growth and reproduction in crustacea. Proceedings of the Royal Society of London. Series B: Biological Sciences. 1915;88(605):418–435.

[5] Tollrian R. Predator-induced morphological defenses: costs, life history shifts, and maternal effects in *Daphnia pulex*. Ecology. 1995;76(6):1691–1705. DOI: 10.2307/1940703

[6] Wesenberg-Lund C. Von dem Abhiingigkeitsverhaltnis zwischen dem Bau der. Planktonorganismen nnd dem spezifischen Gewicht des Siifiwassers. Biologisches Zentralblatt. 1900;20:644–656.

[7] Banta A. M., Brown L. A. Control of sex in Cladocera. II. The unstable nature of the excretory products involved in male production. Physiological Zoology. 1929;2(1): 93–98.

[8] Hobæk A., Larsson P. Sex determination in *Daphnia magna*. Ecology. 1990;71(6):2255–2268. DOI: 10.2307/1938637

[9] Kleiven O. T., Larsson P., Hobæk A. Sexual reproduction in *Daphnia magna* requires three stimuli. Oikos. 1992;65(2):197–206. DOI: 10.2307/3545010

[10] Eads B. D., Andrews J., Colbourne J. K. Ecological genomics in *Daphnia*: stress responses and environmental sex determination. Heredity. 2008;100:184–190. DOI: 10.1038/sj.hdy.6800999

[11] Gilbert S. F., Epel D. Ecological Developmental Biology: Integrating Epigenetics, Medicine, and Evolution. Sinauer Associates, USA; 2009. DOI: 10.1093/icb/icp106

[12] Colbourne J. K., Pfrender M. E., Gilbert D., Thomas W. K., Tucker A., Oakley T. H., et al. The ecoresponsive genome of *Daphnia pulex*. Science. 2011;331(6017):555–561. DOI: 10.1126/science.1197761

[13] Kato Y., Shiga Y., Kobayashi K., Tokishita S., Yamagata H., Iguchi T., et al. Development of an RNA interference method in the cladoceran crustacean *Daphnia magna*. Development Genes and Evolution. 2011;220:337–345. DOI: 10.1007/s00427-011-0353-9

[14] Hiruta C., Toyota K., Miyakawa H., Ogino Y., Miyagawa S., Tatarazako N., et al. Development of a microinjection system for RNA interference in the water flea *Daphnia pulex*. BMC Biotechnology. 2013;13:96. DOI: 10.1186/1472-6750-13-96

[15] Gliwicz M., Slusarczyk A., Slusarczyk M. Life history synchronization in a long-life-span single-cohort *Daphnia* population in a fishless alpine lake. Oecologia. 2001;128:368–378. DOI: 10.1007/s004420100673

[16] Pijanowska J., Stolpe G. Summer diapause in *Daphnia* as a reaction to the presence of fish. Journal of Plankton Research. 1996;18(8):1407–1412. DOI: 10.1093/plankt/18.8.1407

[17] Toyota K., Miyakawa H., Hiruta C., Furuta K., Ogino Y., Shinoda T., et al. Methyl farnesoate synthesis is necessary for the environmental sex determination in the water flea *Daphnia pulex*. Journal of Insect Physiology. 2015;80:22–30. DOI: 10.1016/j.jinsphys.2015.02.002

[18] Caceres C. E. Interspecific variation in the abundance, production, and emergence of *Daphnia* diapuasing eggs. Ecology. 1998;79(5):1699–1710. DOI: 10.2307/176789

[19] Oakley T. H., Wolfe J. M., Lindgren A. R., Zaharoff A. K. Phylotranscriptomics to bring the understudied into the fold: monophyletic ostracoda, fossil placement, and pancrustacean phylogeny. Molecular Biology and Evolution. 2013;30(1):215–233. DOI: 10.1093/molbev/mss216

[20] Miyakawa H., Toyota K., Sumiya E., Iguchi T. Comparison of JH signaling in insects and crustaceans. Current Opinion in Insect Science. 2014;1:81–87. DOI: 10.1016/j.cois.2014.04.006

[21] Olmstead A. W., LeBlanc G. A. Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna*. Journal of Experimental Zoology. 2002;293:736–739. DOI: 10.1002/jez.10162

[22] Tatarazako N., Oda S., Watanabe H., Morita M., Iguchi T. Juvenile hormone agonists affect the occurrence of male *Daphnia*. Chemosphere. 2003;53:827–833. DOI: 10.1016/S0045-6535(03)00761-6

[23] Hiruta C., Nishida C., Tochinai S. Abortive meiosis in the oogenesis of parthenogenetic *Daphnia pulex*. Chromosome Research. 2010;18:833–840. DOI: 10.1007/s10577-010-9159-2

[24] Miyakawa H., Sugimoto N., Kohyama T. I., Iguchi T., Miura T. Intra-specific variations in reaction norms of predator-induced polyphenism in the water flea *Daphnia pulex*. Ecological Research. 2015;30(4):705–713. DOI: 10.1007/s11284-015-1272-4

[25] Threlkeld S. T. Estimating cladoceran birth rates: the importance egg mortality and the egg age distribution. Limnology and Oceanography. 1979;24(4):601–611. DOI: 10.1023/A:1003915431746

[26] Hiruta C., Toyota K., MIyakawa H., Sumiya E., Iguchi T. Sexual reproduction is a key element in the life history strategy of water fleas, *Daphnia magna* and *Daphnia pulex*: casting a spotlight on male induction and its morphology. In: *Daphnia*: Biology and Mathematics Perspectives. Nova; 2014. p. 261–278.

[27] Sagawa K., Yamagata H., Shiga Y. Exploring embryonic germ line development in the water flea, *Daphnia magna*, by zinc-finger-containing VASA as a marker. Gene Expression Patterns. 2005;5:669–678. DOI: 10.1016/j.modgep.2005.02.007

[28] Tsuchiya D., Eads B. D., Zolan M. E. Methods for meiotic chromosome preparation, immunofluorescence, and fluorescence in situ hybridization in *Daphnia pulex*. Methods in Molecular Biology. 2009;558:235–249. DOI: 10.1007/978-1-60761-103-5_14

[29] Watanabe H., Tatarazako N., Oda S., Nishide H., Uchiyama I., Morita M., et al. Analysis of expressed sequence tags of the water flea *Daphnia magna*. Genome. 2005;48(4):606–609. DOI: 10.1139/G05-038

[30] Cristescu M. E. A., Colbourne J. K., Radivojac J., Lynch M. A microsatellite-based genetic linkage map of the waterflea, *Daphnia pulex*: On the prospect of crustacean genomics. Genomics. 2006;88:415–430. DOI: 10.1016/j.ygeno.2006.03.007

[31] Routtu J., Jansen B., Colson I., Meester L. D., Ebert D. The first-generation *Daphnia magna* linkage map. BMC Genomics. 2010;11:508. DOI: 10.1186/1471-2164-11-508

[32] Xu S., Ackerman M. S., Long H., Bright L., Spitze K., Ramsdell J. S., et al. A male-specific genetic map of the microcrustacean *Daphnia pulex* based on single sperm whole-genome sequencing. Genetics. 2015;201:31–38. DOI: 10.1534/genetics.115.179028

[33] Eads B. D., Colbourne J.K., Bohuski E., Andrews J. Profiling sex-biased gene expression during parthenogenetic reproduction in *Daphnia pulex*. BMC Genomics. 2007;8:464. DOI: :10.1186/1471-2164-8-464

[34] Toyota K., Miyakawa H., Yamaguchi K., Shigenobu S., Ogino Y., Tatarazako N., et al. NMDA receptor activation upstream of methyl farnesoate signaling for short day-induced male offspring production in the water flea, *Daphnia pulex*. BMC Genomics. 2015;16:186. DOI: :10.1186/s12864-015-1392-9

[35] Toyota K., Kato Y., Miyakawa H., Yatsu R., Mizutani T., Ogino Y., et al. Molecular impact of juvenile hormone agonists on neonatal *Daphnia magna*. Journal of Applied Toxicology. 2014;34:537–544. DOI: 10.1002/jat.2922

[36] Fröhlich T., Arnold G. J., Fritsch R., Mayr T., Laforsch C. LC-MS/MS-based proteome profiling in *Daphnia pulex* and *Daphnia longicephala*: the *Daphnia pulex* genome database as a key for high throughput proteomics in *Daphnia*. BMC Genomics. 2009;10:171. DOI: 10.1186/1471-2164-10-171

[37] Poynton H. C., Taylor N. S., Hicks J., Scanlan L., Loguinov A. V., Vulpe C., et al. Metabolomics of microliter hemolymph samples enables an improved understanding of the combined metabolic and transcriptional responses of *Daphnia magna* to cadmium. Environmental Science and Technology. 2011;45:3710–3717. DOI: : 10.1021/es1037222

[38] Zaffagnini F. Reproduction in *Daphnia*. Memorie dell' Istituto Italiano di Idrobiologia. 1987;45:245–284.

[39] Elendt B. P., Bias W. R. Trace nutrient deficiency in *Daphnia magna* cultured in standard medium for toxicity testing: effects of the optimization of culture conditions on life history parameters of *D. magna*. Water Research. 1990;24(9):1157–1167. DOI: 10.1016/0043-1354(90)90180-E

[40] Ding S. W., Voinnet O. Antiviral immunity directed by small RNAs. Cell. 2007;130:413–426. DOI: 10.1016/j.cell.2007.07.039

[41] Fire A., Xu S. Q., Montgomery M. K., Kostas S. A., Driver S. E., Mello C. C. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 1998;391:806–811. DOI:10.1038/35888

[42] Obbard D. J., Gordon K. H. J., Buck A. H., Jiggins F. M. The evolution of RNAi as a defence against viruses and transposable elements. Philosophical Transactions of the Royal Society of London B. 2009;364:99–115. DOI: 10.1098/rstb.2008.0168

[43] McTaggart S. J., Conlon C., Colbourne J. K., Blaxter M. L., Little T, J. The components of the *Daphnia pulex* immune system as revealed by complete genome sequencing. BMC Genomics. 2009;10:175. DOI: 10.1186/1471-2164-10-175

[44] He L., Hannon G. J. MicroRNAs: small RNAs with a big role in gene regulation. Nature Reviews Genetics. 2004;5:522–531. DOI: 10.1038/nrg1379

[45] Gao Z., Wang M., Blair D., Zheng Y., Dou Y. Phylogenetic analysis of the endoribonuclease dicer family. PLoS One. 2014;9(4):e95350. DOI: 10.1371/journal.pone.0095350

[46] Zhou R., Hotta I., Denli A. M., Hong R., Perrimon N., Hannon G. J. Comparative analysis of argonaute-dependent small RNA pathways in *Drosophila*. Molecular Cell. 2008;32:592–599. DOI: 10.1016/j.molcel.2008.10.018

[47] Jinek M., Doudna J. A. A three-dimensional view of the molecular machinery of RNA interference. Nature. 2009;457(22):405–412. DOI: 10.1038/nature07755

[48] Wang G. H., Jiang L., Zhu L., Cheng T. C. Niu W. H. Yan Y. F., et al. Characterization of Argonaute family members in the silkworm, *Bombyx mori*. Insect Science. 2013;20:78–91. DOI: 10.1111/j.1744-7917.2012.01555

[49] Tamura K., Stecher G., Peterson D., Filipski A., Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution. 2013;30(12): 2725–2729. DOI: 10.1093/molbev/mst197

[50] Nakanishi T., Kato Y., Matsuura T., Watanabe H. CRISPR/Cas-mediated targeted mutagenesis in *Daphnia magna*. PLoS One. 2014;9(5):e98363. DOI: 10.1371/journal.pone.0098363

[51] Kato Y., Kobayashi K., Watanabe H., Iguchi T. Environmental sex determination in the branchiopod crustacean *Daphnia magna*: deep conservation of a *Doublesex* gene in the sex-determining pathway. PLoS Genetics. 2011;7(3):e1001345. DOI: 10.1371/journal.pgen.1001345

[52] Asada M., Kato Y., Matsuura T., Watanabe H. Early embryonic expression of a putative ecdysteroid-phosphate phosphatase in the water flea, *Daphnia magna* (Cladocera: Daphniidae). Journal of Insect Science. 2014;14(181):ieu043. DOI: 10.1093/jisesa/ieu043

[53] Morita S., Shiga Y., Tokishita S., Ohta T. Analysis of spatiotemporal expression and function of the single-minded homolog in the branchiopod crustacean *Daphnia magna*. Gene. 2015;555:335–345. DOI: 10.1016/j.gene.2014.11.028

[54] Ando T., Fujiwara H. Electroporation-mediated somatic transgenesis for rapid functional analysis in insects. Development. 2013;140(2):454–458. DOI: 10.1242/dev.085241

[55] Hashimoto M., Takemoto T. Electroporation enables the efficient mRNA delivery into the mouse zygotes and facilitates CRISPR/Cas9-based genome editing. Scientific Reports. 2015;5:11315. DOI: 10.1038/srep11315

[56] Nomura T., Yamashita W., Gotoh H., Ono K. Genetic manipulation of reptilian embryos: toward an understanding of cortical development and evolution. Frontiers in Neuroscience. 2015;9(45). DOI: 10.3389/fnins.2015.00045

[57] Kato Y., Kobayashi K., Watanabe H., Iguchi T. Introduction of foreign DNA into the water flea, *Daphnia magna*, by electroporation. Ecotoxicology. 2010;19:589–592. DOI: 10.1007/s10646-010-0460-9

[58] Timmons L., Fire A. Specific interference by ingested dsRNA. Nature. 1998;395:854. DOI: 10.1038/27579

[59] Huvenne H., Smagghe G. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. Journal of Insect Physiology. 2010;56:227–235. DOI: 10.1016/j.jinsphys.2009.10.004

[60] Sun P. S., Venson Jr. N. C., Calderon F. R. O., Esaki D. M. Evaluation of methods for DNA delivery into shrimp zygotes of *Penaeus* (*Litopenaeus*) *vannamei*. Aquaculture. 2005;243(1–4):19–26. DOI: 10.1016/j.aquaculture.2004.09.037

[61] Sarathi M., Simon M. C., Venkatesan C., Hameed A. S. S. Oral administration of bacterially expressed VP28dsRNA to protect *Penaeus monodon* from white spot syndrome virus. Marine Biotechnology. 2008;10(3):242–249. DOI: 10.1007/s10126-007-9057-6

[62] Treerattrakool S., Chartthai C., Phromma-in N., Panyim S., Udomkit A. Silencing of gonad-inhibiting hormone gene expression in *Penaeus monodon* by feeding with GIH dsRNA-enriched *Artemia*. Aquaculture. 2013;404–405:116–121. DOI: 10.1016/j.aquaculture.2013.04.024

[63] Asada M., Kato Y., Matsuura T., Watanabe H. Visualization of ecdysteroid activity using a reporter gene in the crustacean, *Daphnia*. Marine Environmental Research. 2014;93:118–122. DOI: 10.1016/j.marenvres.2013.11.005

[64] Kato Y., Matsuura T., Watanabe H. Genomic integration and germline transmission of plasmid injected into crustacean *Daphnia magna* eggs. PLoS One. 7;9:e45318. DOI: 10.1371/journal.pone.0045318

[65] Naitou A., Kato Y., Nakanishi T., Matsuura T., Watanabe H. Heterodimeric TALENs induce targeted heritable mutations in the crustacean *Daphnia magna*. Biology Open. 2015. DOI: 10.1242/bio.20149738

[66] Sakuma T., Woltjen K. Nuclease-mediated genome editing: at the front-line of functional genomics technology. Development Growth and Differentiation. 2014;56:2–13. DOI: 10.1111/dgd.12111

[67] Hiruta C., Ogino Y., Sakuma T., Toyota K., Miyagawa S., Yamamoto T., et al. Targeted gene disruption by use of transcription activator-like effector nuclease (TALEN) in the water flea *Daphnia pulex*. BMC Biotechnology. 2014;14:95. DOI: 10.1186/s12896-014-0095-7

[68] Miyakawa H., Sato M., Colbourne J. K., Iguchi T. Ionotropic glutamate receptors mediate inducible defense in the water flea Daphnia pulex. PLoS One. 10;3:e0121324. DOI: 10.1371/journal.pone.0121324

IntechOpen