Department for Farm Animals and Veterinary Public Health
University of Veterinary Medicine Vienna

Clinic for Poultry and Fish Medicine
Clinical Unit of Fish Medicine
(Head: Univ.-Prof. Dr.rer.nat. Mansour El-Matbouli)

Recent applications of gene editing in aquatic medicine

Diploma Thesis

University of Veterinary Medicine Vienna

Submitted by
Anikó Gutási

Vienna, May 2022

Contents

1^{st}supervisor

Priv.-Doz. Dr. Mona Saleh

2^{nd} supervisor

Priv.-Doz. Dr.rer.nat. Sabina Hammer

Dedicated to my family

1. Introduction

1.1. Fish industry

Global fish production became the fastest growing food technology in the major food yield in the past decades. Aquaculture produces more fish biomass than the production of the whole beef biomass around the world, and more biomass than capture fisheries (included with the amount of non-edible species) (Edwards and et al., 2019).

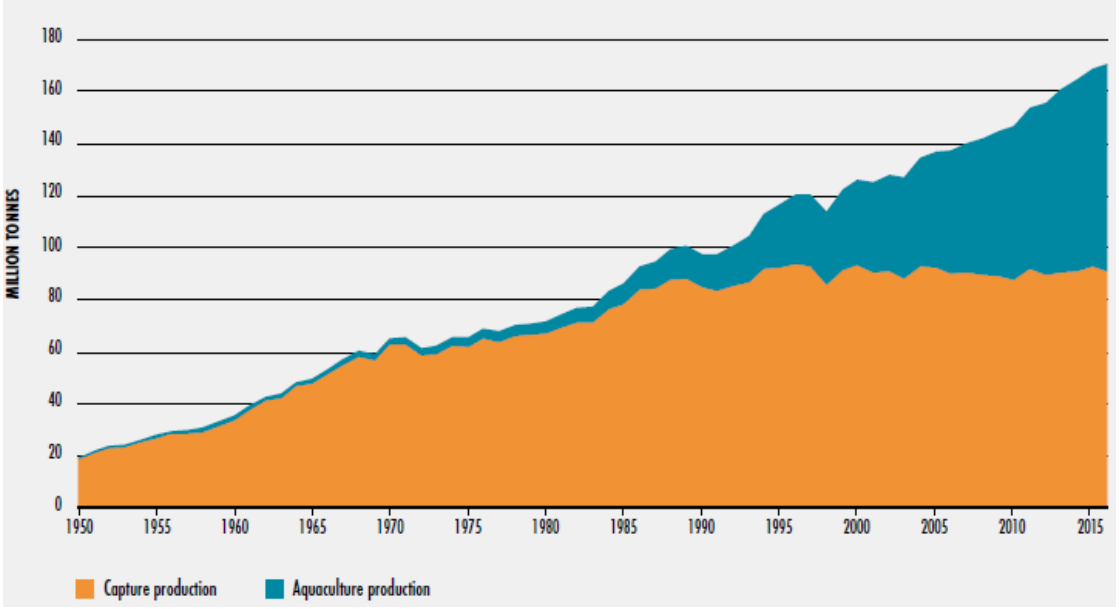

Figure 1: World capture fisheries and aquaculture production (FAO, 2018).

Since the middle of the twentieth century the enlargement in aquaculture and fisheries production is notable. Particularly the consumer society to diverse and nutritious food in the last twenty years has changed (FAO, 2018).

The world aquaculture production increased quickly from 5 million to 63 million tons. In addition, capture fisheries production increased from 69 million to 93 million tons over the last three decades (Food and Agriculture Organization (FAO), 2014) (Figure 1).
In 2016, the continuous growing of aquaculture to the global production of capture fisheries and aquaculture reached 46.8 percent, up from 25,7 percent in the year of 2000 (FAO, 2018).

Between 1961 and 2015, the average growth in total food fish consumption (3.2 percent) outran the population accession (1.6 percent) and transcended that of meat from collective terrestrial animals over 55 years, between 1961 and 2016. Food fish consumption grew around 1.5 percent yearly, from 9.0 kg to 20.2 kg per capita (Figure 2) (FAO, 2018).

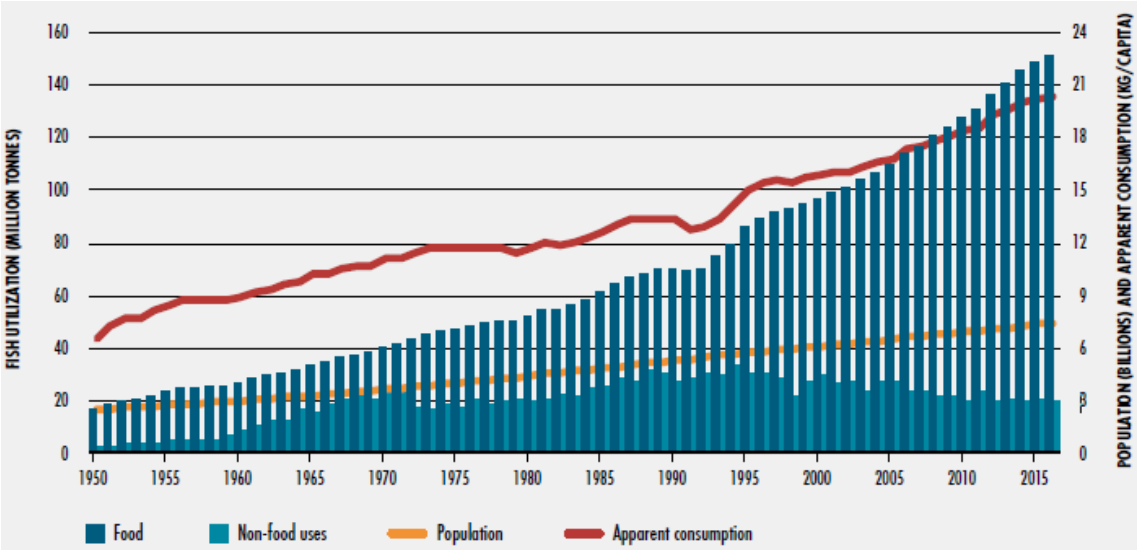

Figure 2: World fish utilization and apparent consumption (FAO, 2018).

Global aquaculture production included 80.0 million tonnes of food fish in the year of 2016. Farmed food fish produced 54.1 million tonnes of finfish, 17.1 million tonnes of molluscs, 7.9 million tonnes of crustaceans and 938 500 tonnes of other aquatic animals like turtles, sea cucumbers or edible jellyfish (FAO, 2018).

A significant part of the society is working in aquaculture sectors and in fisheries all around the world. Asia has been in first place with 89 percent of the global aquaculture production over the last twenty years. The Americas and Africa have raised their shares in production, whereas Oceania and Europe have decreased production in the same period. The data from 2016 shows that the employment was 85 percent of the global population in these work areas in Asia. In second place was Africa with 10 percent, followed by Latin America and the Caribbean with 4 percent.

The most dominant producer of farmed food fish was China in 2016, and they have produced more than the rest of the world combined every year since 1991. The other major producing countries were India, Indonesia, Viet Nam, Egypt, Bangladesh and Norway in the same year (FAO, 2018). In keeping with the model results, China will produce 37 percent of total fish (capture production and aquaculture production) worldwide, and is expected to account for 38 percent of the global consumption of food fish in 2030 (World Bank, 2013).

The consumption in Asia was the most remarkable; it was more than two-thirds of the global total consumption (149 million tonnes) in 2015. In the same year, Europe, Japan and the United States of America together represented only around 20 percent of the total food fish consumption on the world, although it was 47 percent in 1961 (FAO, 2018).

In 2050, the earth's population will probably reach 9 billion. Accordingly, the world food sector must secure nutriment and food for the increasing population. The food production sector needs to be more effective in utilizing productive resources; resources are scarcer in the more crowded world. Fish can be globally advantageous in feeding and nutritional security among the poor and vulnerable society (World Bank, 2013).
Fish is an excellent nutrition source; it has several positive values. It provides not only high-value protein but also it is low in saturated fats, carbohydrates, and cholesterol, containing several vitamins, minerals and polyunsaturated omega-3 fatty acids (FAO, 2012).
In 2016, the frequently most favoured form is live, fresh or chilled fish (45 percent) for the direct human consumption, followed by frozen fish (with 31 percent) (FAO, 2018).

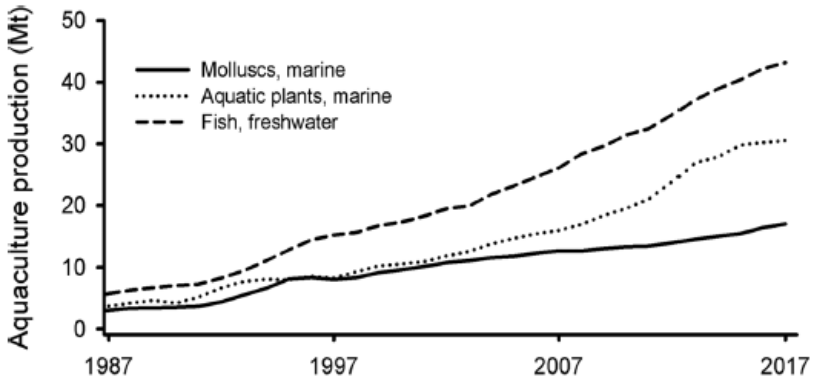

Figure 3: Global aquaculture production (m.t.), the x-axis shows the year (Food and Agriculture Organization, 2019).

Around 84 percent of the aquaculture consists of aquatic plants, molluscs and freshwater fish as reported by the United Nations Food and Agriculture Organization (Figure 3) (Food and Agriculture Organization, 2019). Aquatic plants and molluscs create approximately half of the total aquaculture volume. The key features of these species groups continuous growth are that they do not require inputs to farm nor do they need to be fed. (Boyd et al., 2020).
The farming of fed aquatic animal species has outpaced the farming of unfed species. The production of unfed animals has decreased constantly to 30.5 percent between 2000 and 2016. Despite this, both of type of the farming system expanded continuously, but the volume of fed species grew faster than the non-fed species.
On the list of the major produced species in the world aquaculture, finfish hold the first place in the year of 2016. The most produced finfish species was grass carp (*Ctenopharyngodon idellus)*. Fewer other species, such as species of molluscs, crustaceans or other animals like frogs or sea cucumber, were farmed.
The complete unfed species production increased to 24.4 million tonnes in 2016. It included 15.6 million tonnes of aquatic invertebrates, such as marine bivalve molluscs from the seas, lagoons and coastal ponds together with 8.8 million tonnes of filter feeding finfish in inland aquaculture, where the most significant species were bighead carp *(Hypophthalmichthys nobilis)* and silver carp (*Hypophthalmichthys molitrix)* (FAO, 2018).

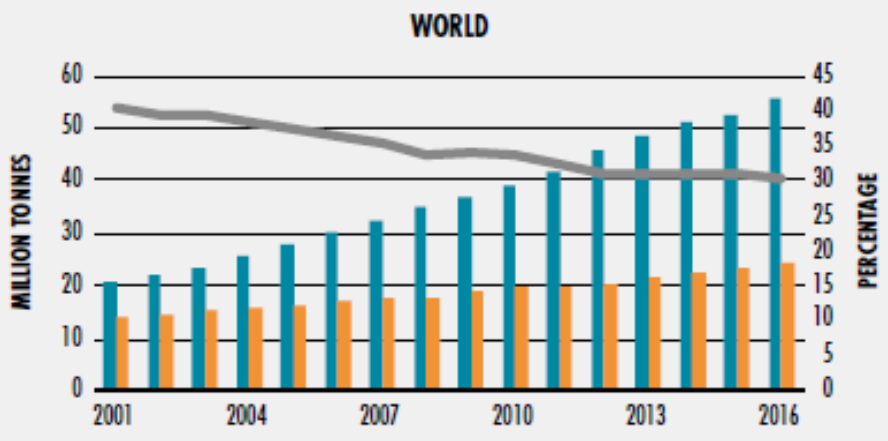

Figure 4: Fed and non-fed food fish aquaculture production, the blue bar graphs show fed species, the orange bar graphs show unfed species and the grey graph the unfed species share (%) from 2001–2016 (FAO, 2018).

During ten years, from 2006 to 2016, the totally commercially farmed species items climbed to 598, listed by FAO (Food and Agriculture Organization*)* and by producing countries. There was notable growth with 26.7 percent, from 472 in 2006 to 598 in 2016 (Figure 4). A species item contains a single species, an interspecific hybrid or a group of species. Different animals represent "species items ", the largest and most diverse part registers 369 finfishes (including 5 hybrids). In addition, it consists of 109 molluscs, 64 crustaceans, 40 aquatic algae, 9 aquatic invertebrates, 7 amphibians and reptiles (excluding alligators, caimans or crocodiles) (FAO, 2018).

It appears that the production of food fish has increased monumentally, making the fish and seafood globally more popular and reducing the price of fish. Therefore, greater investment is needed in new technologies in the industry (World Bank, 2013). Climate change presents the most serious challenge to growing level of sustainable global aquaculture. Definitive climate induced changes in physical and biological condition may require us to modify management practices in the future (Boyd et al., 2020).

Genetically modified aquatic animals are fundamentally needed for the quickly increased aquaculture production, to feed the growing human population globally and for curing inherited diseases in the aquaculture (FAO, 2018; H.K. Bartman, 2019.)

In the course of a literature search, an update on the current state of knowledge with regard to gene editing in aquaculture is compiled from various already published works.

This diploma thesis is created with the help of literature management programs as well as various abstract databases.

1.2. Gene editing

Gene engineering techniques have been employed by scientists and researchers to answer some combined questions in biology. In the late 1980s and early 1990s, attempts have been made to precisely modify the complex genome. Thanks to the developed techniques, it was possible to decipher of DNA structure, its replication, transcription and translation of genomics. Genetic engineering authorized the functional recombinant proteins, high-yielding transgenic plants and animals, thereby identifying the molecular signatures of genetic and pathogenic disease.

Furthermore, new genetic engineering techniques have revolutionized the genetic manipulation creating a significant impact on modern medicine, principally gene therapy (Cathomen und Keith Joung, 2008; Malik et al., 2019).

Gene editing or genome engineering is a technique, in which the DNA or nucleotide sequences are inserted, deleted or replaced at the specific place in the genome of living organisms or cells using specific set of engineered nucleases as molecular scissors. Diverse genome editing techniques have been developed for manipulating the DNA sequence of the organism (Malik et al., 2019).
Gene editing has been very effective and has provided new prospects for biological discovery in the last few decades (Gratacap et al., 2019).
It targets modification of the genome to introduce precise, favourable changes on site specific location. It is possible to induce favourable changes, for example fixing alleles at existing trait loci, or introducing alleles from different strains or species (Gratacap et al., 2019).
Although, the process causes breaks of the gene, later it can be repaired via activation of DNA repair mechanism, and hence parts of the DNA can be positioned into site-specific regions in the genome of interest (Karre, 2020).
The genotypic and phenotypic characteristics of an organism can also be modified by creating exact and special changes in the genome (Malik et al., 2019).

Thanks to the gene editing tools and technologies, diverse fields and departments have been covered, which can help researchers to develop new experiments. It can be applied in the areas of the agricultural science, in biomedical science, in the development of environmental science and in various areas of medicine, like veterinary medicine or human medicine (Karre, 2020).

Genome engineering can definitely be applied in the treatment of genetic diseases, different chronic health diseases, cancer and management of diseases. It offers the extraordinary ability to introduce any targeted modification into genome and takes this method to a practical reality (Malik et al., 2019).

The most often used methods in gene editing, which are widely successful, utilize sequence-specific programmable nucleases (Bibikova et al., 2003; Moscou und Bogdanove, 2009). These molecular scissors precisely cut the DNA at a special localisation. Consequently, the DNA can be modified and specific sequence inserted (Egelie et al., 2016).

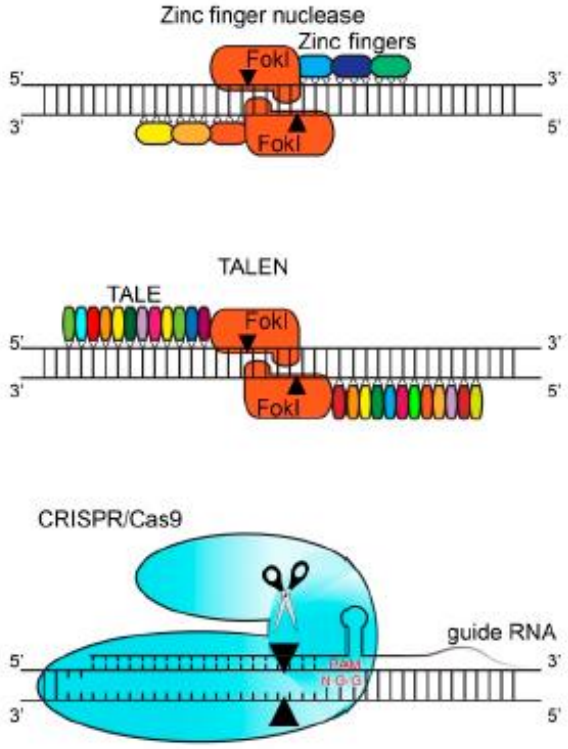

Figure 5: Overview of the diverse gene editing nucleases (ZFN, TALEN, CRISPR/Cas9). The black arrows illustrate the cleavage site of the DNA (Xu et al., 2020).

These specific include two different types. Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) are both protein guided, whereas the two component CRISPR/Cas9 systems contain RNA-guided endonucleases (RGENs) (Figure 5) (Malik et al., 2019; Xu et al., 2020.). Table 1 presents the most significant characteristics of these gene editing tools.

Table 1: Comparison of three significant genome editing tools- ZFNs, TALENs and CRISPR (Malik et al., 2019).

Feature	**Zinc finger nucleases (ZFNs)**	**Transcription activator-like effector nucleases (TALENs)**	**Clustered regularly-interspaced short palindromic repeats (CRISPR)**
Structure	Fusion of zinc finger DNA-binding domain (DBD) with DNA-cleavage domain of Fok I endonuclease	Fusion of Transcription activator-like effector DNA (TALE) repeats with DNA-cleavage domain of Fok I endonuclease	Cas9 endonuclease and guide RNA (gRNA)
Size of recognition site	9-18 bases in DNA	30-36 bases in DNA	23 DNA bases in DNA
Ease of designing	Difficult than TALENs and CRISPR	Easier than ZFNs	Easier than other two
Multiplexing	No	No	Yes
Off target	Same as that of TALENs	Same as that of ZFNs	More than other two
Ease of redesigning/ adaptability to target new site	Difficult, require recording of large DNA segments (500-1000 bp)		Easy, requires only change in 20-bp protospacer of gRNA
Viral delivery	Using Lenti und adenoviruses. Needs cotransduction with two lentiviral vectors each encoding a monomer to form functional heterodimer	Using adenovirus	Difficult than other two because it requires polyadenylation signal and promoter
Efficacy	+ +	+ +	+ + +
Application	Indels, obligate ligation-gated recombination (ObLiGaRe). Can insert a 15-kb inducible gene expression cassette at a defined locus in human cell lines, Tag ligation.		Indels
Cost	Higher than CRISPR	Higher than CRISPR	Less than other two

All of these methods can generate targeted double-strand DNA breaks (DSBs) in the DNA. The generated double-strand breaks can lead to a loss of large chromosomal regions, which may cause the most hazardous type of DNA damage.

The two major types of the endogenous cellular DNA repair pathways are non-homologous end joining (NHEJ) and homologous recombinational (HR) repair or HDR in eukaryotic cells (Takata et al., 1998; Barnes, 2001; van den Bosch, M., Lohman, P.H., 2002; Lieber, 2010; Chang et al., 2017) (Figure 6).

During the sub pathway of NHEJ, the break ends are identified, resected, polymerized and ligated by proteins in flexible mode (Chang et al., 2017).

The break ends are directly ligated, it does not require homologous template for the repair. This method is an error-prone progress, which often comes with imprecise repairs, like

loss/gain of some nucleotides. Therefore, the result is variable, namely deletions and insertions of nucleotide or nucleotide substitutions occurs in the broken region (Swiech et al., 2015; Malik et al., 2019).
The mechanism comprises individual and sequential steps: (1) Identification of DNA end, assembly and stabilisation the NHEJ complex at the place of DNA double-strand break; (2) Bridging of the DNA ends and support of break end stability; (3) Processing of DNA end; (4) Ligation of the DNA broken ends and dissolution of the NHEJ complex (Davis und Chen, 2013).

The homology directed repair (HDR) mechanism can be exploited by the cells when there is homologous DNA as a template to restore a DSBs (Chu et al., 2015). Following the introduction of a DSB into the genome, proteins are enlisted to the exposed the ends of DNA to start repair the break (Liu und Huang, 2016). The result of this type of repair is precise and controllable. Therefore, this pathway is effective used to precisely edit genomic sequence, to induce specific deletions, insertions or designer mutations as well as to insert exogenous sequence (Chu et al., 2015). It occurs low in post mitotic and differentiated cells. The effectiveness of HDR is highly determined by the target locus of the genome, the template itself as well as the cell type and stage of life (Saleh-Gohari und Helleday, 2004).

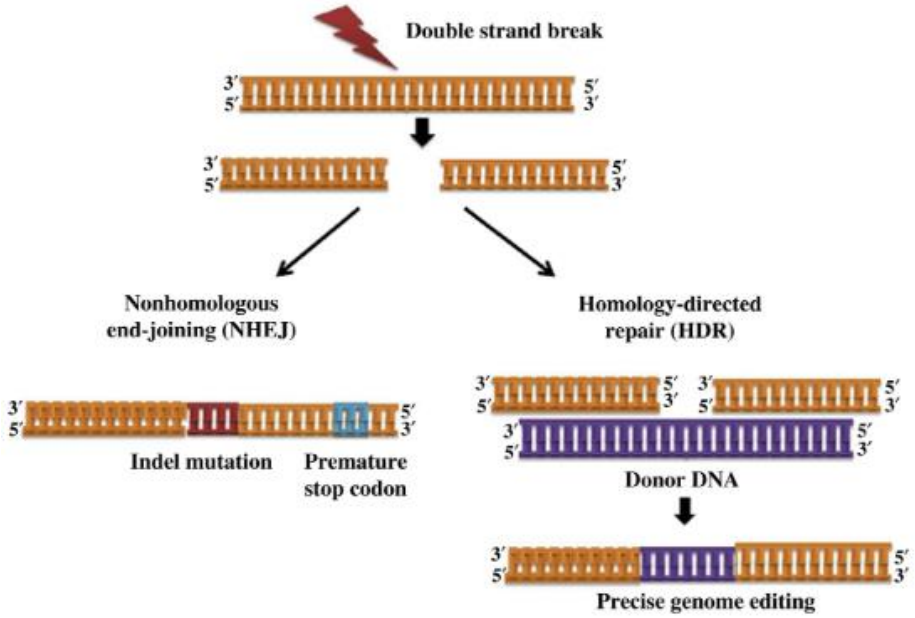

Figure 6: Various strategies of DNA double-strand breaks into genomic loci, which are repaired by non-homologous end-joining (NHEJ) or homology-directed repair (HDR) pathway (Bharati et al., 2019).

There are several methods of gene editing technology that have motivated researchers editing the genome in specific genes in order to allow targeted alterations. Tools using

CRISPR-Cas9, TALEN and ZFN have become a powerful new methodology, which promoted connected the gene editing technology (Karre, 2020).

1.2.1. Zinc finger nucleases (ZFNs)

Zinc finger nucleases are artificially engineered hybrid proteins, which were discovered in 1994. As the first modulated gene editing tools, they have a prominent place in the field of the genome engineering (Choo et al., 1994).
Its principle is that different zinc fingers identify different sets of nucleotide triplets. This hybrid protein consists of specific DNA binding domains, who fused with the endonuclease *Fok* I, created to targeted specific genome sequences. (The original source of restrictive endonuclease Fok I is *Flavobacterium okeanokoites)* (Choo et al., 1994; Kim et al., 1996; Urnov et al., 2010; Tang et al., 2015b).
Zinc Finger Proteins (ZFPs) have a unique ability to recognize and bind to specific DNA sequences and ZFN enzymes can cut the DNA in the targeted sequences (Tang et al., 2015b). In addition ZFN can create a DNA double-strand break (DSB) at preselected sites (Cathomen und Keith Joung, 2008). The significant concern for ZFNs is off-target cleavage, contrarily with many natural endonucleases (Carroll, 2014).
ZFNs and ZFPs are classified in three major subtypes (C2H2, C4, and C6), in which C2H2 is, due to its simplicity, the most broadly used in engineered ZFNs (MacPherson et al., 2006; Tang et al., 2015b).
The major advance of this technique compared to standard gene therapy is the potential to conserve temporal and tissue-specific gene expression (Cathomen und Keith Joung, 2008).

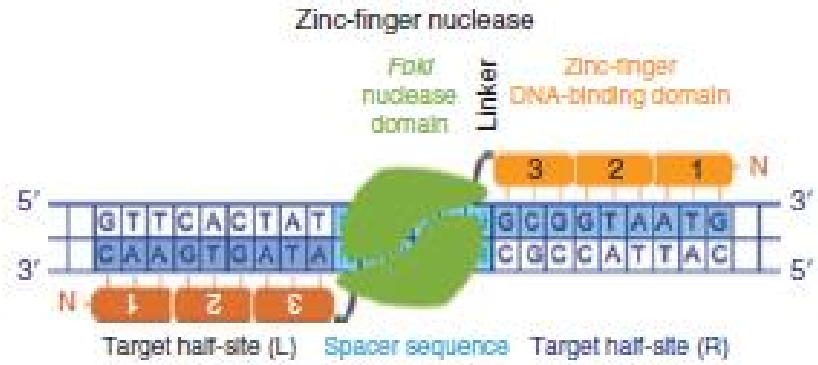

Figure 7: Zinc-finger nuclease (ZFN)-mediated genome editing (Cathomen und Keith Joung, 2008).

Figure 7 shows the architecture of ZFNs. The two monomer subunits of the multimerized ZFNs bind to the target locus of the DNA sequence. Each subunit contains three zinc-fingers (orange, 1-2-3), which identify 9 base pairs within the full target site, and the Fok I endonuclease domain (green). The two short linkers (grey) associate with two domains. After the dimerization of the two subunits is activated, and they cut the DNA in the spacer

sequence. ZFN creates a double-strand break (DSB) and separates the two target half sites (L) and (R) (Cathomen und Keith Joung, 2008).
After the break occurs, primarily, the error-prone NHEJ process, also the DNA repair takes place (Malik et al., 2019).
The ZFNs can contain individually between three and six zinc finger domains that each recognize and bind between 9 and 18 base pairs target site (Liu et al., 1997). The 3 zinc finger motifs monomer is the minimal requirement, and it was also reported that the strings with 3 to 4 zinc finger motifs have the highest binding ability (Tang et al., 2015b).

In summary, the endonuclease activity together with special nucleotide sequence binding particularities of ZFs takes a part in the genome engineering via targeted DSB formation. Several successful endonuclease-mediated gene editing attempts had been applied in different species, thanks to the high conservation of DNA-repair mechanism (Palpant und Dudzinski, 2013). This application has been used to manipulate the genome, for example, of zebrafish (Meng et al., 2008), rodents (Geurts et al., 2009), *Drosophila melanogaster* (Beumer et al., 2006) and of numerous human cells including primary somatic cells (Urnov et al., 2005) and embryonic stem cells (Hockemeyer et al., 2009).

1.2.2. Transcriptional activator-like effector nucleases (TALENs)

TALENs consist of special effector proteins, which contain the DNA binding domain and Fok 1 nuclease domain. These domains work in pairs as dimers, binding to the opposite strand DNA and induce DSB. TALENs are applied for genome editing and introducing targeted DSBs into specific DNA sites of interest, as an alternative to ZFNs (Joung und Sander, 2013; Malik et al., 2019). These unique nucleases are secreted by the pathogenic bacteria *Xanthomonas,* which infect the cytoplasm of plant cells (Boch et al., 2009).

Each of these nuclease platforms has a central domain for the special DNA binding and distinct N- and C-termini architectures for localization and activation (Boch und Bonas 2010, Miller et al. 2011, Joung und Sander 2013, Lamb et al. 2013). The DNA binding domain comprises monomers with 10 to 30 repeats and each of them binds with one nucleotide of the target DNA sequence (Moscou und Bogdanove 2009, Lamb et al. 2013). Besides, they comprise a non-specific FokI catalytic nuclease domain combined to a customizable DNA binding domain.
TALENs bind as dimers to target sites in the nucleus with FokI domains is located at the c-termini and cleavage occurring in the „spacer" sequence (Li et al., 2011; Mahfouz et al., 2011; Joung und Sander, 2013).
Thereafter, the repair of DNA breaks, which occurs primarily in the same way as ZFNs, the error-prone NHEJ way (Malik et al., 2019).

TALENS can be very easily and rapidly designed. Their high rates of cleavage activity and their relevant limitless targeting array make them appropriate for non-specialist researchers (Figure 8) (Joung und Sander, 2013).

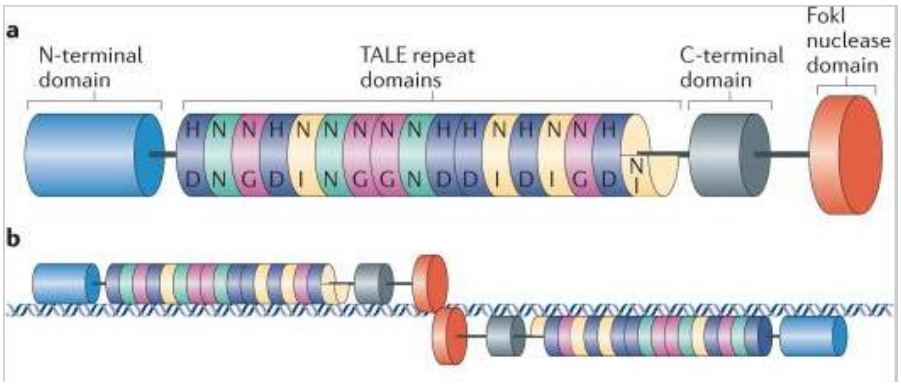

Figure 8: a) Schematic diagram of a TALEN, b) TALENs bind and cleave as dimers on a target DNA site and cause DSBs in the spacer sequence (Joung und Sander, 2013).

Taken together, the TALEN-based method requires engineering a pair of large repetitive sequence encoding domains for site-specific DNA identification and cleavage in the genome (Malik et al., 2019).
With the use of TALEN, efficient introduction of targeted modifications has been achieved in numerous model organisms (Joung und Sander, 2013). Genes from several species have been manipulated including zebrafish (Huang et al., 2011; Sander et al., 2011; Bedell et al., 2012), rat (Tesson et al., 2011) and pig (Carlson et al., 2012).
These nucleases, thanks to their capacity of targeted gene modification, can also be used in treatment of a wide range of diseases and genetic disorders (Joung und Sander, 2013).
However, their highly repetitive sequences make long TALE repeats frequently inefficient, labour consuming and expensive to create. For this reason, there was demand to develop new simpler, more rapid, robust, more efficient and cost- effective techniques for the gene editing in the biomedical field (Malik et al., 2019).

1.2.3. Clustered Regularly Interspaced Short Palindromic Repeats – Cas-9 system

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) gene engineering technique is one of the latest trends in the genome editing toolbox. This most recent gene editing method was discovered in 2012. It is a significant technical jump forward to biomedical applications and research, as well as one of the fastest to progress to the use for precise gene modification in different organisms (Jinek et al., 2012; Crudele und Chamberlain, 2018; Malik et al., 2019).
It has various advantages over the above-mentioned (ZFNs- and TALENs- based) processes. It is more effective, much simpler to accomplish and appropriate for high-performance and multiple gene editing in many living organisms and cell lines (Malik et al. 2019). Due to its simplicity, speed and low cost to devise, it is widely adopted and is now the technology of choice (Lucas und Southgate, 2013; Pankaj, 2014; Malik et al., 2019).
It works in simple as well as more complex cells (Mali et al., 2013).
This system was utilized to develop RNA-guided endonucleases that enable targeted genome editing (Malik et al., 2019). Originally, it was naturally present in prokaryotic cells, namely in bacteria and archaea (Grissa et al., 2007).
There are at least 11 diverse CRISPR/Cas systems, which have been categorized into three groups according to the attribute of Cas protein: type I, type II and type III. The type II system uses only one Cas protein to identify and cleave targeted DNA sites, while different type I and type III systems expect a set of Cas proteins (Brouns et al., 2008; Makarova et al., 2011; Wiedenheft et al., 2011).
Because of the simpleness of the type II system, which is also known as CRISPR/Cas9, it has been considered a potent programmable mechanism to specific modifications in the genome (Malik et al., 2019) (Figure 9).
CRISPR/Cas9 system comprises a Cas9 endonuclease, and a modified single guide RNA (sgRNA/gRNA), which comprises a targeting CRISPR RNA (crRNA), and trans-activating crRNA (tracrRNA) (Jinek et al. 2012). Hence, the two essential components are the Cas9 protein and sgRNA (Jinek M., Jiang, F., Taylor, D.W., Sternberg,S.H., Kaya E. 2014). The Cas9 nuclease is directed to its target sequence by a precisely designed guide RNA of about 20 base pairs (Gersbach 2014). Thus, one of the great advantages of this system is that it requires simply the change of 20 nucleotide sgRNA spacer sequences, which is easier to manipulate, and not the large repetitive complex design of DNA binding arrays for each novel genomic target site, as in ZFN - and TALEN systems (Joung und Sander 2013, Malik et al. 2019).
Another important part is the protospacer-adjacent motif (PAM) of the target sequence which binds with Cas9. PAM is a short specific sequence (NGG trinucleotide sequence) following the target DNA sequence, presenting a downstream of the crRNA binding site and necessary for Cas nuclease-mediated break.
The cleavage of the DNA is carried out by the Cas9 enzyme at position 3-4 nucleotides upstream of PAM (Jinek et al., 2012; Malik et al., 2019).

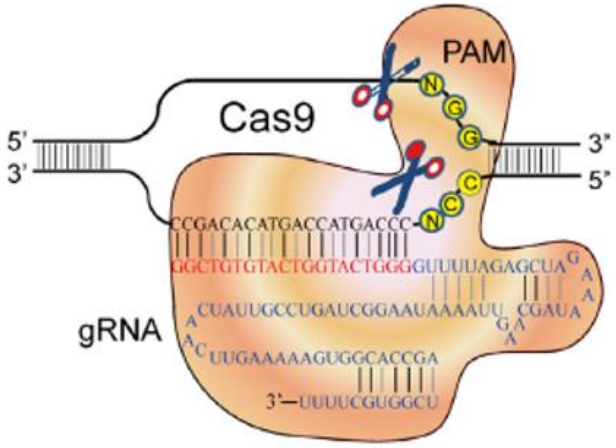

Figure 9: Genome editing with CRISPR/Cas9 (Li und Wang, 2017).

Summarily, the Cas9 endonuclease precisely cuts the target DNA sequence and introduces a DSB under the control of sgRNA. Accordingly, researchers can add or delete sequences of the genetic material or switching an existing segment with an altered sequence of the DNA to create modifications. A DBS can be repaired either via NHEJ or HDR (Liang et al., 1998; Lees-Miller und Meek, 2003; Malik et al., 2019).
This system opened several innovative chances in addition to applications for genome editing techniques of both in vivo and in vitro systems (Xu et al., 2020).

1.3. Gene silencing

Gene silencing also known as RNA interference (RNAi) has reformed the genetics. Earlier, it looked clear that DNA creates messenger RNA (mRNA) and that mRNA makes proteins (Hood, 2004). But with time, it has become clear for researchers through intensive studies and research, that RNAi has a central role in the regulation of diverse processes in animals and in plants (Indra Pratap Singh, Sara Hasan, 2019).
In the cell, genes would be expressed under normal situations but they can be switched off by certain apparatus in the cell (Daneholt, 2006).
RNAi occurs in all eukaryotes organisms. It is a mechanism for silencing gene expression, namely it inhibits the translation of RNA (Indra Pratap Singh, Sara Hasan, 2019).
This new, reliable method transforms experimental biology from single-celled protozoa to mammals. RNAi has several advantages over other nucleic-acid-based methodologies and as a result, it is recently the most broadly applied gene silencing technique in the functional genomics (Estrada et al., 2008).
The mechanism requires an endonuclease enzyme called dicer. Dicer is a cytoplasmic RNAse III enzyme with endonuclease activity that cuts the long double stranded RNA (dsRNA) or hairpin RNA (hpRNA) into short fragments of 20-25 base pair nucleotide. These generated short fragments are called small interfering RNA (siRNA), which are duplexes after the cleavage but then are unwound into two single strands. One of the two strands is degraded in the cytoplasm by subsequent cellular proceedings. This strand called passenger strand. The other strand, also known as the guide strand, incorporates with Argonaute (Ago) and with other proteins to form an RNA-induced silencing complex (RISC) (Sen und Blau, 2006). The other three enzymes in this multiprotein complex are specifically helicase, nuclease-ribonuclease and RNA-dependent RNA polymerase (RdRp). Each enzyme has a specific function; Helicase unwinds the double stranded siRNA. Whease, nuclease-ribonuclease cuts mRNA and RdRp extends the silencing signal. Ago protein, the catalytic component of the RISC, cleaves the target mRNA strand. The guide siRNA of the siRNA/RISC complex leads the gene silencing, namely to target mRNA, thus, results the degradation of the target transcript or inhibition of translation. Consequently, the protein synthesis is interrupted (Figure 10). The elements of siRNA/mRNA complex can be reused. RISC or siRNA duplexes will be generate and amplified by the act of RdRp (Borges und Martienssen 2015, Singh et al. 2016, Indra Pratap Singh, Sara Hasan 2019).

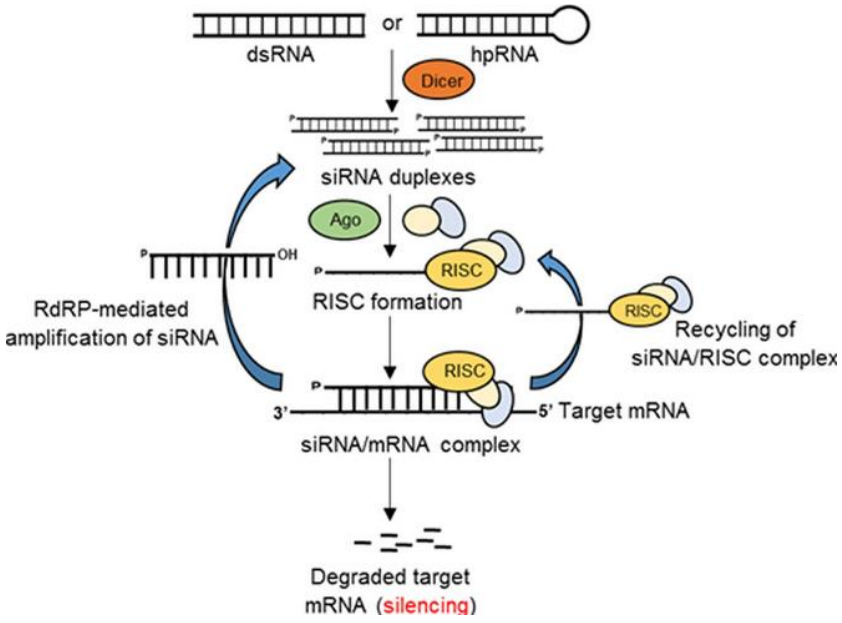

Figure 10: Graphic of the mechanism of the RNAi in eukaryotic cells (Majumdar et al., 2017).

Gene silencing has two types, which regulate the endogenous genes at: transcriptional level and posttranscriptional level (P. Parveen, K. Deepti Brundavani, K. Mahathi 2019).

In transcriptional gene silencing, histones are modified, generating an environment of heterochromatin around a gene. Thus, the process of transcription is not possible because the gene is inaccessible to the transcriptional procedure after the modification (Walsh et al., 2011; P. Parveen, K. Deepti Brundavani, K. Mahathi, 2019).

In the posttranscriptional gene silencing, the mRNA will be inhibited accordingly, preventing translation. Furthermore, it will initiate the degradation of mRNA (Keum et al., 2011; Indra Pratap Singh, Sara Hasan, 2019). The RNAi mechanism belongs to posttranscriptional gene silencing (Keum et al., 2011).

The RNAi mechanism has two main types, with small differences. They are mediated by either siRNA (with 21-23 nucleotides) or dsRNA that is longer and may create a great population of siRNA (Tirasophon et al., 2005; Tiu und Chan, 2007). The dsRNA created more varied pool of efficient siRNA combined into RISC complexes than the shorter siRNA (Naito et al., 2004).

The processes of gene silencing protect the genome from invading viruses and transposons. It is probably a part of an ancient immune system protecting the genetic material from infectious gene elements (GL, 2002). In addition, it executes cellular functions to survival, health and development (Hood, 2004).

RNAi is a powerful technology to study gene function, and to explore the gene expression regulatory mechanisms. In addition, it can supply an innovation for gene therapy (Wang et al., 2007).
In summary, the use of this pathway is a promising tool in biotechnology and in medicine (Hammond, 2005).

2. Applications (Targeted gene modification in animals)

Marine and aquaculture industries belong to significant sectors of global trade and food production. Regrettably, there are loads of infectious pathogens, which have a negative effect on the fish food industry. These infectious pathogens should be detected and characterized, and treatment strategies with modern and up to date techniques should be developed against them to outpace great disease outbreaks (Gotesman et al., 2018). Furthermore, the effectiveness, production, efficiency and wellbeing of the cultured fish could be improved with enhanced disease resistance transgenic fish (Lucas und Southgate, 2013).

Fish are a potential model, with several advantages as bioreactors in comparison to mammals. They have a short generation interval, easy and low cost of maintenance, enormous numbers of individuals and high density culture. In addition, mammalian viruses and prions are not found in fish populations. Some examples are now available representing the potential of fish as bioreactors for medical products. Besides, various developed complexes can be applied in fish spawning (Lucas und Southgate, 2013).

This chapter summarizes the above mentioned different applications of gene modifications in fish medicine.

2.1. Gene editing in fish medicine using CRISPR/Cas9

Gene editing can help to create physiological changes in phenotypic results. Researchers use biological scissors (specific nucleases) to cut the DNA at specific sites and join the fragments, thus modifying it. These are widespread practises in the gene engineering (Karre, 2020). Gene editing can be used to modify cells, tissues and organs of animals in order to handle cure abnormality and dysfunctions in patient (Perota et al., 2016). The new molecular biology tools make targeted changes in the genetic material. Over 70 aquatic fishes' genome have been deciphered during the last decades (Okoli et al., 2021).

Some products in the aquaculture, which created by CRISP/Cas9 will one day be appraised for commercialization. Notable advances being developed in several fish species such as sterility, disease resistance, pigmentation and improved growth (Table 2.). Gene editing methods have the ability to provide far-reaching keys to challenges the fish aquaculture (Okoli et al., 2021).

Table 2: Effective applications of CRISPR/Cas9 genome editing in different aquatic species (Gratacap et al., 2019).

Species	**Target gene**[x]	**Trait of interest**	**Notable features**
Atlantic salmon, *Salmo salar*	*tyr/slc45a2*	Pigmentation	
	dnd	Sterility	
	elov-2	Omega-3 metabolism	
Tilapia, *Oreochromis niloticus*	*dmrt1/nanaos2-3/foxl2*	Reproduction	Germline transmission
	gsdf	Reproduction	
	aldh1a2/cyp26a1	Reproduction	
	sf-1	Reproduction	Germline transmission
	dmrt6	Reproduction	
	amhy	Reproduction	
	wt1a/wt1b	Reproduction	
Sea bream, *Sparus aurata*	*mstn*	Growth	
Channel catfish, *Ictalarus punctatus*	*mstn*	Growth	Germline transmission
	ticam1/rbl	Immunity	
	LH	Sterility	
Southern catfish, *Silurus meridionalis*	*cyp26a1*	Germ cell development	
Common carp, *Cyprinus carpio*	*sp7a/sp7b/mstn(ba)*	Muscle development	
Rohu carp, *Labeo rohita*	*TLR22*	Immunity	Homology-directed repair
Grass carp, *Ctenopharyngodon idella*	*gcjam-a*	Disease resistance	In vitro
Northern Chinese lamprey, *Lethenteron morii*	*slc24a5/kctd10/wee1/soxe2/wnt7b*	Pigmentation/development	
Rainbow trout, *Oncorhynchus mykiss*	*igfbp-2b1/2b2*	Growth	
Pacific oyster, *Crassostrea gigas*	*mstn*	Growth	

[x]Full gene names: *aldh1a2*, aldehyde dehydrogenase family 1, subfamily A2; *amhy*, anti-Mullerian hormone; *cyp26a1*, cytochrome P450, family 26, subfamily a, polypeptide 1; *dmrt1*, doublesex and mab-3 related transcription factor 1; *dmrt6*, doublesex and mab-3 related transcription factor 6; *dnd*, dead end; *elovl-2*,

ELOVL fatty acid elongase 2; *foxl2*, forkhead box L2; *gcjam-a*, grass carp junctional adhesion molecule-A; gsdf, gonadal somatic cell derived factor; *igfbp-2b1/2b2*, IGF binding protein 2b1/2b2; *kctd10*, potassium channel tetramerisation domain containing 10; *LH*, luteinizing hormone; *mstn*, myostatin; *nanos2*, nanos C2HC-type zinc finger 2; *nanos3*, nanos C2HC-type zinc finger 3; *rbl*, rhamnose binding lectin; *sf-1*, steroidogenic factor 1; *slc45a2*, solute carrier family 45 members 2; *soxe2* SRY-box transcription factor E2; *sp7a/sp7b*, transcription factor Sp7-like; *ticam1*, toll-like receptor adaptor molecule 1; *TLR22*, toll-like receptor 22; *Tyr*, tyrosinase; *wee1*, WEE1 G2 checkpoint kinase; *wnt7b*, wingless-type MMTV integration site family, member 7B; *wt1a/b*, Wilms tumour 1 transcription factor a/b (Gratacap et al. 2019).

2.1.1. CRISPR/Cas9 in fishery science

Zebrafish is widely used as a model organism to study and investigate genetic modifications. It is an excellent model of vertebrate diseases and development because of its fast growth, transparent embryos and its comparatively facile forward genetics. The researchers have used gene-editing tools in zebrafish to get answers for important problems in fish genetics, reproduction, toxicology, drug-receptor and host-pathogen interaction with favourable results. The most effective and promising gene-editing tool for studying several biological mechanism is the CRISPR/Cas9 technology. It has been successfully used in the development of gene modification in diverse fish species, like Atlantic salmon, medaka, zebrafish and tilapia (Lieschke und Currie, 2007; Doyon et al., 2008; Auer et al., 2014; Edvardsen et al., 2014; Qiu et al., 2014; Wang et al., 2015).

Li et al.,(2014) presented the effective targeted and heritable gene editing method using CRISPR/Cas9 in Nile tilapia (*Oreochromis niloticus*). The mutation in two genes (*foxl2* and *dmrt1*), induced by CRISPR/Cas9 were successfully transmitted through the germline to the F_1 generation. Moreover, this study shows the usefulness of the CRISPR/Cas9 technique with high efficiency in non-model species like genetically engineered tilapia and other aquaculture fish.

2.1.2. CRISPR/Cas9 in mono-sex population

Gene editing tools propose various, and nature-friendly ways to produce a mono-sex population. The sexual dimorphism is a traditional detail. There also is systematic difference in plenty of fish species, which is presented in body growth. As an example, male tilapia grow faster than females, while female rainbow trout (*Oncorhynchus mykiss*) and Indian major carps grow faster than their male partners do. The difference in growth rates can be evaded with the production of mono-sex population, which could rise the yield rates per unit of area. Additionally, it can reduce the threat of unwanted reproduction of prolific fishes in wild through the production of a mono-sex population. With targeted nucleases, it became possible to produce mono-sex and sex-reserved fishes by a direct route disrupting the sex-determining genes without provoking any significant influence on biodiversity. Knockout the genes in tilapia which determine the sex of the female (with XX sex-determining chromosome), such as *fox12*, *sf-1* or *cyp19a1a,* were disturbed via targeted nucleases in testicular development. (Another way to process the sex reversal is made with organisation of androgen or gynogen hormones; however, this way leads to massive issues like bioaccumulation, biomagnification and other problems with water quality and biodiversity.) (Li und Wang, 2017; Malik et al., 2019).

Medaka fish (*Oryzias latipes*) is particularly useful in the studies about reproduction because of the availability of its genetic manipulations and the property of information on the regulation of its reproduction (Matsuda et al., 2002; Karigo et al., 2012; 2014). In a study (Takahashi et al., 2016) TALEN technique to generate target gene knockout (KO) for gene *gnrh1* (hypophysiotropic GnRH) (Cattanach et al. 1977), *lhb*, and *fshb* (vital subunit for LH and FSH hormone, separately) was used in medaka (*Oryzias latipes*). The study reported that TALENs successfully cut the targeted sites of corresponding genes. TALEN-induced disruption of the *gnrh1* guide to female infertility due to anovulation besides all male KO medaka were fertile, and their testes reached normally the maturity. The infertility of the *gnrh1* KO female medaka clearly verified that GNRH1 has an important role in the regulation of reproduction in females.

2.1.3. CRISPR/Cas9 in sterility of fishes

The sterility in fish could easily be tackled with targeted nucleases. Controlling unwanted fish reproduction in predatory and weed species, as well control over establishment of exotic and transgenic fishes in the wild if they accidental escape from separated milieu like ponds and flow-through systems. It is feasible with the production of sterile fishes. For example, sterile channel catfishes (*Ictalurus punctatus*) are created by using ZFN technology, and by disrupting the β subunit gene of pituitary luteinizing hormone. The sterile catfishes reduced the potential environmental and ecologic hazards; therefore, the catfish industry could profit. This was the first sterilisation using ZFN mechanism in aquaculture as well as the first effective gene editing of channel catfish (Qin et al., 2016; Malik et al., 2019).

A major problem of fish farming are the escaped Atlantic salmon (*Salmo salar* L.), as it is the cultured fish that are carried in open sea cages during the growth period (Taranger et al., 2015). Sterile fish, namely germ cell-free salmon, could reduce this problem by stopping the introgression, the gene flow between domesticated salmon into wild stocks (Glover et al., 2012). In a study, Wargelius et al. (2016) produced germ cell-free salmon in F0 by using CRISPR-Cas9 to knockout *dead end (dnd)* gene. *Dnd* allows the survival of germ cells. The knockout of the *dnd* gene in mammals leads to an all-male offspring (Youngren et al., 2005). CRISPR/Cas9 also can be used to knockout the pigmentation in salmon, namely the targeted knockout of the *slc45a2* (*alb*) pigmentation gene leads to completely albino phenotype (Edvardsen et al., 2014) (Figure 11.). Hence, in the study *dnd/alb* KO mutant Atlantic salmons were produced through double allelic mutations, with the use of CRISPR/Cas9. As a result, the fish were completely lacking pigmentation and devoid of germ cells in F0. The study showed that the biallelic KO allows with high probability also in long-life-cycle-species, which prohibits the generation of F2. This study demonstrated for the first time that CRISPR/Cas9-mediated KO of *dnd* leads to complete loss of germ cells in F0 generation in any fish species. Besides, the germ cells are not required for female sex differentiation but may be required for establishing a normal structure in the ovarian in Atlantic salmon.

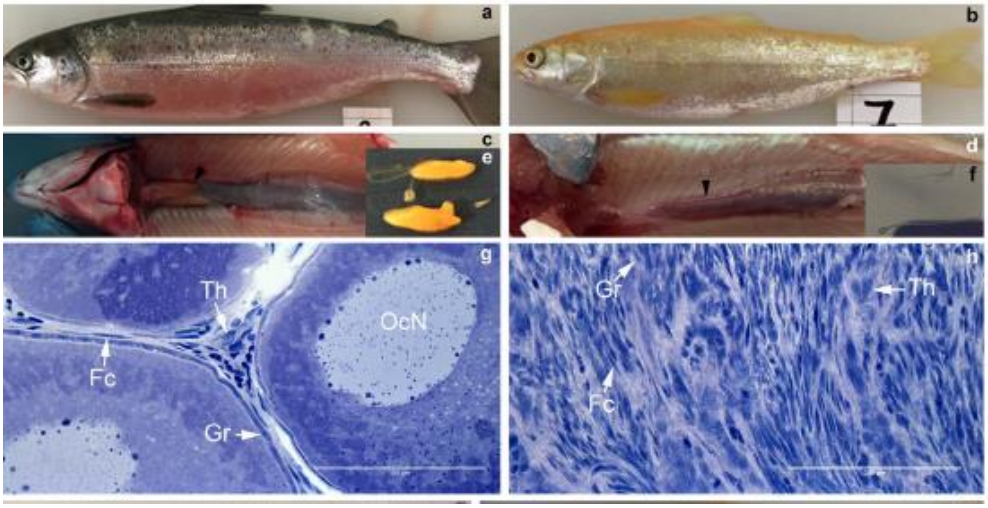

Figure 11: Morphology and histology of one-year-old dnd/alb KO and control Atlantic salmon. Control fish presented on the left side (a, c, e, g). Dnd/alb KO fish presented on the right side (b, d, f, h). Fish b is a female dnd/alb KO fish, d and f show the gross morphology of the female dnd/alb KO in comparison to the gross morphology of the control female (a, c, e): the lack of the ovarian bulb in comparison to control (e), g and h show the histology of the female gonad in dnd/alb KO fish (h) in comparison to control ovary (g). Abbreviations: Th – theca cell; OcN – oocyte nucleus; Gr – granulosa cell; Fc – fibrocyte. (Wargelius et al., 2016).

2.1.4. CRISPR/Cas9 in reproduction

Kiss1/Gpr54 system (kisspeptin-encoding gene - *Kiss1* (Dungan et al., 2006)*;* its G protein-coupled receptor 54 - *GPR54* (Lee et al. 1999)*)* has a central role in the regulation of reproduction in most vertebrates (Popa et al., 2008; Roa et al., 2008; Oakley et al., 2009). These systems have also been identified as multiple *kiss1*/*gpr54* paralogous genes (*kiss/kissr*) in non-mammalian vertebrates, which is different from the mammals. During a study (Tang et al., 2015a) zebrafish *kiss1*-/-, *kiss2*-/- and *kiss1*-/-; *kiss2*-/- mutant lines together with *kissr1*-/-, *kissr2*-/- and *kissr1*-/-;*kissr2*-/- mutant lines were generated using optimized TALEN restriction enzyme. The result clearly showed that the spermatogenesis, folliculogenesis and a reproductive potential are not damaged in all of these mutant lines. Fish were normal and fertile in both sexes. Furthermore, the data indicated that *kiss/kissr* systems are not required for zebrafish reproduction, signifying that the *kiss/kissr* systems represent unnecessary roles for reproduction in definite non-mammalian vertebrates. It is also showed that mammals and fish have developed different strategies for neuroendocrine control of reproduction.

2.1.5. CRISPR/Cas9 in fast-growing fishes

Several endemic cold-water fish species have a slow growth rate because of their genetic nature, physiology and environmental limitations of their surroundings. But this cold-water species has an excellent virtue: they can live in stagnant water (ponds) while other species demand continuous clean and well-aerated water. With the help of targeted nucleases, the expression of growth-promoting genes could be increased. Furthermore the gene inhibiting the skeletal muscle growth could be knocked out (Malik et al., 2019). In the study from (Zhong et al., 2016), the gene coding from myostatin (suppressor of muscle growth) in common carp (*Cyprinus carpio)* was disrupted by CRISPR/Cas9. As a result, the mutated fishes have grown considerably more muscle cells, and shown larger phenotypes in F_0 generation, so the carp genes were successfully targeted.
Analogous methods have been used to increase the production of slow-growing cold-water fishes like snow trout (Malik et al., 2019).

2.1.6. CRISPR/Cas9 in ornamental fishes

The production of ornamental fishes with desired colour and pigmentation can also be realized by targeted genome editing tools. Thanks to ZFN, TALEN and CRISPR/Cas9 techniques, the mutation of golden genes resulted in the making of light-coloured eyes that are inheritable up to F1 generation (Doyon et al., 2008; Dahlem et al., 2012; Jao et al., 2013).

Somatic and germline disruption of genes in zebrafish *(Danio rerio)* was accomplished with the use of zinc-finger nucleases (ZFNs). Designed ZFNs targeted the *golden* and *no tail/ Brachyury (ntl)* genes of the zebrafish. Thanks to the injection of ZFN-encoding mRNA into the one-cell embryos, a significant percentage of the animals had different mutations at the ZFN-specified locus in the fish and were presented with corresponding awaited loss-of-function phenotypes. The results of this study confirm that ZFN technology is applicable to precisely and professionally produce heritable mutant alleles at loci of interest. This study also suggests that this method may be essential in severe organisms that allow mRNA delivery into the fertilized eggs (Doyon et al., 2008).

CRISPR/Cas9 nuclease system also represents a highly effective gene knockout method in zebrafish. The study from Jao et al. (2013) reported that with custom guide RNAs and a zebrafish codon-optimized Cas9 enzyme were efficiently targeted the correspondent transgene *Tg(-5.1mnx1:egfp*). Furthermore, four endogenous loci were also successfully

targeted (*tyr, golden, mitfa, and ddx19).* The high rate of the mutagenesis proves that most cells contained biallelic mutations. In four of the five target cases recessive null-like phenotypes were observed, denoting the high level of the biallelic gene disruption. Additionally, effective germ-line transmission of the Cas9-induces mutation was noticed. The result of this research also indicates that five genomic locations can be targeted together, at the same time, with outcomes in multiple loss-of-function phenotypes in the same vaccinated zebrafish.

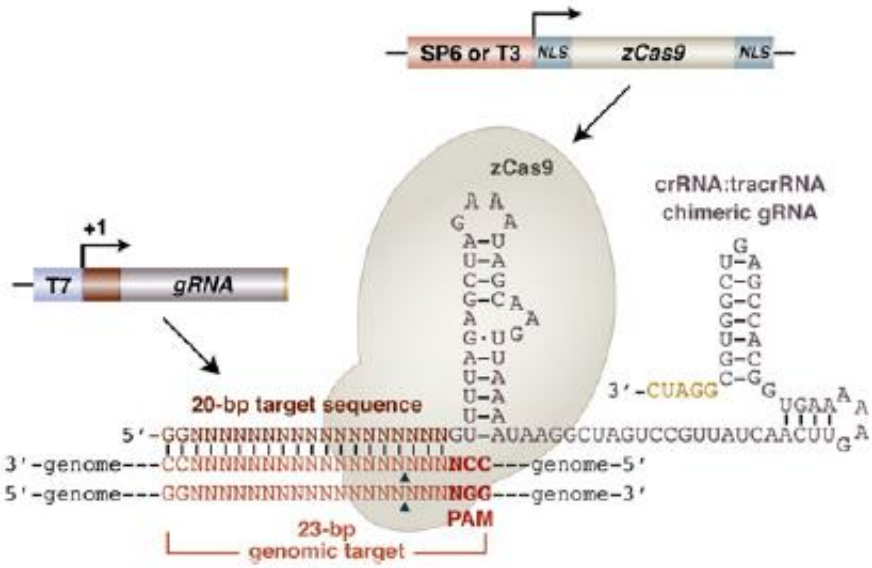

Figure 12: Genome editing with CRISPR/Cas9 nuclease system in zebrafish (Danio rerio) (Jao et al., 2013).

Figure shows the mechanism of the CRISPR/Cas9 system in the above-mentioned study: the dual NLS-tagged zebrafish codon-optimized Cas9 protein with a single crRNA:tracrRNA chimeric gRNA create the nuclease system. The mix was injected into one-cell–stage embryos to induce RNA-guided targeted DNA DSB through the Cas9 enzyme. Both components together comprised a 20-bp target sequence (dark red) to a PAM site of NGG and first produced as RNAs by in vitro transcription from the SP6 or T3 (for Cas9) or T7 (for gRNA) promoter (Jao et al., 2013).

2.1.7. CRISPR/Cas9 in pigmentation

The study from (Ma et al., 2015) represented a successful gene editing process with the help of TALEN in the teleost fish, namely in the cavefish *(Astyanax mexicanus*). This fish species is a brilliant organism for studying the genetic basis of evolution. The study used designed TALEN to target two genes in the cavefish (*oculocutaneous albinism 2 (oca2) and melanocortin 1 receptor (mc1r*)), that contain coding changes and are responsible for reduced pigmentation. The result shows that the genes of cavefish can be mutated using this technique and that the change on the fish is noticeable. Specifically, the induced mutations in *oca2* result in the mosaic loss of melanin pigmentation, namely the lack of melanin-producing melanophores in the regions that were lighter in appearance under the microscope. They appear as albino patches in F0 founder fish, signifying biallelic gene mutations in F0s as well permitting us to evaluate the role of this gene in pigmentation. Apparent differences in phenotype were not observed in the pigmentation of *mclr*-TALEN injected fish compared to uninjected familial fish. This process demonstrates that TALEN has a potential to create mutations at specific locations in *Astyanax mexicanus.* This organism has become a dominant model system for researching the genetic basis of evolution in an extreme location, the cave. This study also shows that TALEN has an advantage over CRISPRs for this type of experiment. TALEN can be targeted at approximately any site in the genome. However, only limited sites can be targeted through CRISPRs as that they need a PAM sequence (Blackburn et al., 2013).

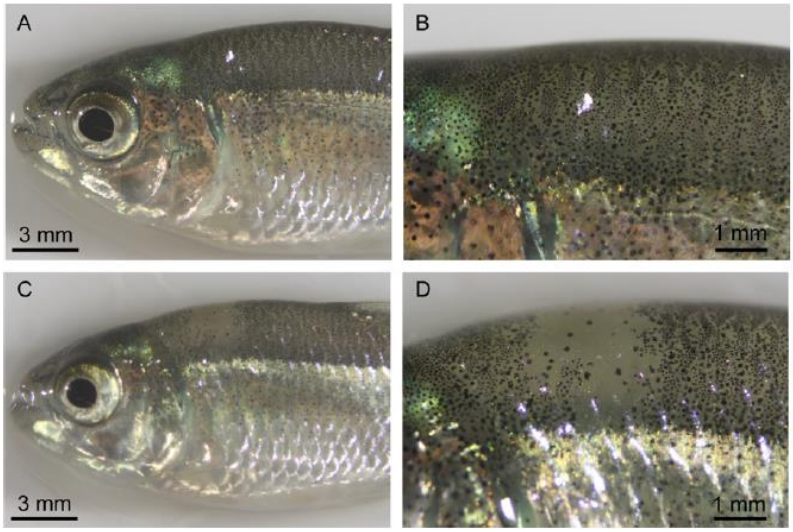

Figure 13: Interpretation of pigmentation in surface fish in oca2-injected F0s (Ma et al., 2015).

The panels present the following analysis (Figure 13): A: Pigmentation in an uninjected fish. B: Close up of the dorsal region of uninjected surface fish from picture A. C: Pigmentation in a 400 pg *oca2* exon 9 injected F0 surface fish. D: Close up of pigmentation patch lacking the melanin pigmentation from picture C.

A recent study (Adi Segev-Hadar, Tatiana Slosman, Ada Rozen, Amir Sherman, Avner Cnaani, 2021) describes the generation of stable and heritable red tilapia phenotype through induced loss-of-function mutations in the *slc45a2* gene of Nile tilapia *(Oreochromis niloticus).* The solute carrier family 45 member 2 (*slc45a2*) is a membrane-associated transporter protein that mediates melanin biosynthesis and is evolutionarily conserved from fish to humans. To achieve this purpose, the *slc45a2* gene in the fish was identified and highly specific gRNAs (gRNA2 and gRNA3) were designed against this gene. Tilapia zygotes at the single-stage cell got multiple microinjection of slc45a2-specific ribonucleoproteins (RNPs). As a result, the microinjection induced up to 97–99 % albinism, which generated a solid-red phenotype, including loss of melanin in the eye. Mutant alleles were carried in all injected fish with variable mutagenesis efficiencies, presented by the next-generation sequencing of the injected zygotes. The sequencing analysis of gDNA from the F0 albino mutant and its heterozygous F1 offspring demonstrated to us that the new *slc45a2* mutant alleles with a red phenotype in Nile tilapia are stable, trackable and heritable. CRISPCas9 system has applicative potential in *O. niloticus* culture.

The below mentioned figure (Figure) presented the phenotypic analysis of tilapias. Panel B: at 1-month post fertilization, the embryo revealed a normal gray-black pattern with dark eyes. C: with *slc45a2*-RNPs, injected mutant fish showed 97–99 % loss of melanin in the skin and no melanin in the eye. D: post sexual maturation, F0 mutant displayed a complete loss of melanin.

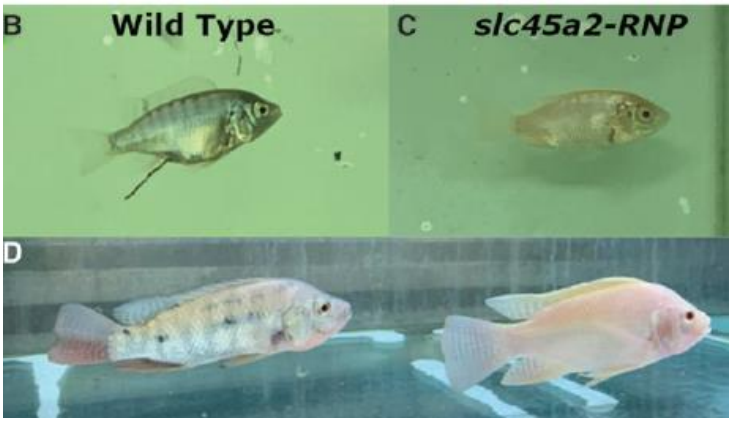

Figure 14: Different phenotypes between wild adult and slc45a2 mutant adult Nile tilapia (Adi Segev-Hadar, Tatiana Slosman, Ada Rozen, Amir Sherman, Avner Cnaani, 2021).

2.1.8. CRISPR/Cas9 in growth

The most work has concentrated on the transfer of GH (Growth Hormone) genes. Enhancement of growth (size and rate) has jumped from 0 % to an incredible 300 % under some circumstances (Lucas und Southgate, 2013).

The myostatin (MSTN) gene is a regulator of skeletal muscle growth in all vertebrates and controls myoblast differentiation in vitro (Souza et al., 2008). Modifying the myostatin via gene knockout or overexpression of inhibitors increases muscle mass in particular (McPherron et al., 1997; Lee und McPherron, 2001). In the study from (Khalil et al., 2017) a successful targeting of the muscle suppressor gene MSTN in channel fish (*Ictalurus punctatus)* through CRISPR/Cas9 system was presented. CRISPR zygote microinjection was used to knockout the MSTN gene and determine the effects of this knockout on growth. In the target protein-encoding site of MSTN high rates of mutagenesis were induced. Mutated fry had more muscle cells than the controls group, and their average body weight increased by 29.7 % 40 days after the microinjection. A large percentage of the embryos were mutated within the target sites and it was not detected any mutations nearby and outside the target site.
The results of this study exhibit that with the CRISR/Cas9 tool can edit the channel fish genomes very efficiently and that with this technique it will be possible to ease the genetic improvement and functional genomics of channel catfish. Maybe, thanks to this approach, growth-enhanced channel catfish will be produced and this will increase the productivity.

2.1.9. CRISPR/Cas9 in body configuration

The transgenic modification of the nutritional characteristics of fish is already probable via transgenesis, which could be advantageous for customers. Zebrafish transfected with *B-actin*-salmon desaturase genes have increased their meat the levels of omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Another analogous result was verified with the same transgene transfer to common carp and channel catfish. The expression of these transgenes was certified (Lucas und Southgate, 2013).

2.1.10. CRISPR/Cas9 in oomycetes

Oomycetes *Aphanomyces invadans* causes Epizootic ulcerative syndrome (EUS) in many fish species. It leads to mass mortality in cultured and wild fish worldwide and generate huge economic impact (Fallis et al., 1998; „OIE (World Organisation for Animal Health) Listed Diseases“, 2017). Extracellular proteases produced by this oomycete, these initiate the EUS disease process (Yike, 2011). The study from (Majeed et al., 2017) identified the secreted proteases from *A. Invadans* utilizing SDS-PAGE and mass spectometery followed by BLASTp analysis. Three prominent protein bands were shown through SDS-PAGE and identified via spectometer. The proteolytic activity of these proteases was assessed on casein and fish immunoglobulin M (IgM) of rainbow trout (*Oncorhynchus mykiss*) and giant gourami (*Osphronemus goramy*). The secreted proteases were able to degrade the casein and IgM in both of fish species. The activity of the antiprotease of the fish serum was also explored. The findings presented that inhibition of secreted proteases using several protease inhibitors to reduce the proteolytic activity. Furthermore, the results suggested that the extracellular proteases could be potentially affect in *A. invadans* as virulence factor. This study offers further functional investigations on the role of identified proteases in EUS pathogenesis and using genome editing tools, such as CRISPR/Cas9 nuclease for development the drug against this disease.

Majeed et al. (2018) designed three single guide-RNAs (gRNA) to target oomycete *A. invadans* serine protease gene. This oomycete is a group of parasites and primary causal factor for epizootic ulcerative syndrome (EUS). The zoospores develop in the sporangium in fish tissues and create dermal lesions coming out as deeper ulcers, red spots or blackish burn-like marks or (ROBERTS et al. 1993, Pathiratne et al. 1994, Lilley und Roberts 1997, Vishwanath et al. 1998). Protease secret, especially genes from serine protease secret from *A. invadans*, which have already been identified, have been used as targeted gene for gene editing (Majeed et al., 2017). CRISPR/Cas9 system was used to select these genes in a test to investigate its function in EUS. Three dwarf gourami (*Trichogaster lalius*) groups were intramusculary injected with three different suspensions to examine the effect of edited genes on the virulence of the oomycete. One group get non-treated *A. invadans* zoospores, another group RNP-treated *A. invadans* zoospores and the third group inoculated with autoclaved pond water as negative control. During the 30 days of *in vivo* experiment, the group with the RNP- treated zoospores and the control group did not present any clinical signs, the PCR did not extend the DNA of *A. invadans*. Histologically was also not found any infiltration or necrosis of the muscles tissue in these two groups. On the contrary, the positive control group showed characteristic symptoms, like ulcers in skin and muscles, reddering, swelling and presented *A. invadans* hyphae.

Summarily, this study established a successfully gene editing via CRISP/Cas9 which prevented the production of serine protease. With this promising tool and a great opportunity it is practicable to study of oomycetes and secreted proteases, to control of EUS and might it helps in the development of drugs against this pathogen.

2.2. Gene silencing in fish medicine

The RNAi tool has been commonly utilized to understand and examine the gene function in aquatic diseases. Additionally, this technique can be investigated the RNA-based viruses (Biacchesi, 2011; Reshi et al., 2014). It is also appropriate for the development of therapies for viral diseases in livestock and aquatic creatures. In addition, it represents one of the newest and most promising method in antiviral medicines and therapeutics (Lima et al., 2013). Almost all the studies applying RNAi tools in fish have been successfully done in zebrafish (*Danio rerio*), which is a valuable fish model organism for aquaculture and biomedicine applications (Carpio und Estrada, 2006; Estrada et al., 2008) (Table 3.).

Table 3: Effective applications of RNAi technique in fish (Estrada et al., 2008).

Type of molecule	**Genes targeted**	**RNAi response**		**Organism**
		Specific	**Nonspecific**	
Long dsRNA	*ntl, fl h, pax2.1, LacZ*	x	x	Zebrafish embryos[a]
Long dsRNA	*Gfp, Zf-T, pax6.1*	x		Zebrafish embryos
Long dsRNA	*Tbx16/spt, LacZ*		x	Zebrafish embryos
Long dsRNA	*pouII-1, gfp, terra*		x	Zebrafish embryos
Long dsRNA	*RanBP1*		x	Zebrafish embryos
Long dsRNA	*M2mAchR*	x		Zebrafish embryos
siRNA	*gfp, tyrA*	x		Rainbow trout embryos[b]
siRNA	*dmd*	x		Zebrafish embryos
esiRNA	*ntl*		x	Zebrafish embryos
siRNA	*ntl*	x		Zebrafish embryos
siRNA	*laminA* and *B2, Eg5, GL2, gfp*	x		ZFL, SJD and ZF4[c]
siRNA	*laminA, GL2, gfp*		x	Zebrafish embryos
Long dsRNA	*myostatin*	x		Zebrafish embryos

T7RPshRNA	*ntl, gfp*	x		Zebrafish embryos

dsRNA: double-stranded RNA; Long dsRNA: double-stranded RNA >30 nt; siRNA: small interfering RNA
(21–25nt); esiRNA: endoribonuclease digestion-derived siRNA; T7RP-shRNA: short-hairpin RNA (shRNA)
expression system, based on T7 RNA polymerase (T7RP)-directed transcription machinery.
a *Danio rerio*.
b *Oncorhynchus mykiss*.
c Cell lines derived from adult and embryonic zebrafish (*Danio rerio*).

2.2.1. Gene silencing in viral disease of fish medicine

With RNAi based therapies for viral disease, invertebrate, vertebrate and human pathogens can also be treated (Hammond, 2006).

The inhibition of gene transcription and the study of viral replication were completed in the study from (Gotesman et al., 2015): SiRNA molecules targeted the nucleoprotein "N" and phosphoprotein "P" transcripts to inhibit in vitro replication of spring viremia of carp virus, (SVCV) and they were tested in a cell line from *epithelioma papulosum cyprini* (EPC). (This virus belongs to *Rhabdoviridae* family of viruses and causes severe loss in carp farms.) The study shows that using siRNA to inhibit the of SVCV-N and SVCV-P genes expression reduced SVCV replication.

In another study, the in vitro viral replication of cyprinid herpesvirus-3 (CyHV-3) was inhibited by (si)RNA in common carp brain cells (CCB cells). (This virus causes high mortality rates both in common and koi carp *Cyprinus carpio* L.) The siRNAs were meant to target either thymidine kinase (TK) or DNA polymerase (DP) genes, which are the codes of transcripts in DNA replication. The treatment with siRNA shows that TK or DP genes reduced the release of viral elements from contaminated CCB cells, that is siRNA inhibited the viral replication (Gotesman et al., 2014).
CyHV-3 is most successfully inhibited via RNAi-mediated gene silencing technique when multiple viral genes are targeted (Adamek et al., 2014; Gotesman et al., 2014).

2.2.2. Gene silencing in parasitic disease of fish medicine

The treatment of parasitic diseases with RNAi mechanism has shown promising results as well.

The study of Saleh and co-workers demonstrated that siRNA could be used to knock down the expression of specific genes of *Heterosporis saurida*, a parasite of the lizardfish (*Saurida undosquamis*) (Saleh et al.,2016).

SiRNAs were designed to inhibit the ATP/ ADP antiporter 1 and methionine aminopeptidase II genes and tested in vitro cultivation model. This study came to the conclusion that siRNA reduced the targeted gene transcription and spore counts of H. saurida and also concluded that this process is an advance development for inhibiting this microsporidian parasite.

Salmon whirling diseases are caused by the cnidarian myxozoan parasite (*Myxobolus cerebralis*), whose one alternative host is an invertebrate oligochaete*, Tubifex tubifex* (B., 1903; Sarker et al., 2015). (Sarker und El-Matbouli, 2015) used targeted siRNA mediated gene silencing for MyxSP-1 serine protease in vivo in *M. cerebralis*-infected oligochaetes, providing intervention strategy in salmonid whirling disease. Under the research, *T. tubifex* was soaked in a special solution with fluorescently labelled siRNA, and as a result it was observed that siRNA was taken up from *T. tubifex*. The fluorescence was detected in the body of oligochaetes. In addition, the researchers observed knockdown in MyxSP-1 mRNA expression.

Another study from (Sarker et al., 2017) demonstrated that *T. tubifex* soaked in solution holding dsRNA targeting the *MyxSP-1* of the *Myxobolus cerebralis* injected the cnidarian myxozoan parasite from contaminating the rainbow trout (*Oncorhynchus mykiss*) fry host. The specific-pathogen-free rainbow trout fry were immersed in water inhabited by live siRNA-treated *T. tubifex*. The siRNA treatment with *MyxSP-1* presented maximum significant knockdown, and salmonids did not generate salmonid whirling diseases.
These results show the proof of RNA-based therapy in vivo against this parasitic infection in salmons.

2.2.3. Gene silencing for gene function studies in fish medicine

The successful inhibition of zebrafish gene expression via a short hairpin RNA (shRNA)-mediated process was presented in a study from De Rienzo et al. (2012). ShRNAs originate from longer double-stranded (ds) precursors, and they can be used for gene silencing because they can post-transcriptionally prevent the expression of complementary RNA (Bartel, 2009). Two genes (*wnt5b* and *zDisc1*) were used for the test, each with a similar phenotype in both genetic mutants and morphants. The results show that shRNAs inhibited *wnt5b* expression and targeted *zDisc1* effectively and specifically. In sum, shRNAs decrease endogenous RNA levels in zebrafish gene expression.

Wang et al. (2007) studied the knock down of green fluorescent protein (*gfp*) and no tail (*ntl*) gene expression by in vivo-transcribed short-hairpin RNA (shRNA) with T7 plasmid

system in zebrafish (*Danio rerio*) embryos. The T7RP expression vector and the T7shRNA vectors target these two genes, respectively.
The study was based on the specific identification of the T7RP to T7 promoter, and the transgenic zebrafish line stably expressing T7RP was recognised. Additionally, shRNA vectors which targeted foreign *gfp* gene and endogenous *ntl* gene were created (Figure 15.). Ultimately the shRNA constructs (pT7Bmp2b) were injected into the F3 embryos of the pCMVT7R transgenic line. The results revealed that the T7 transcription system could function to drive the expression of shRNA in zebrafish embryos and eventuate the gene knock down effect (Figure 16).

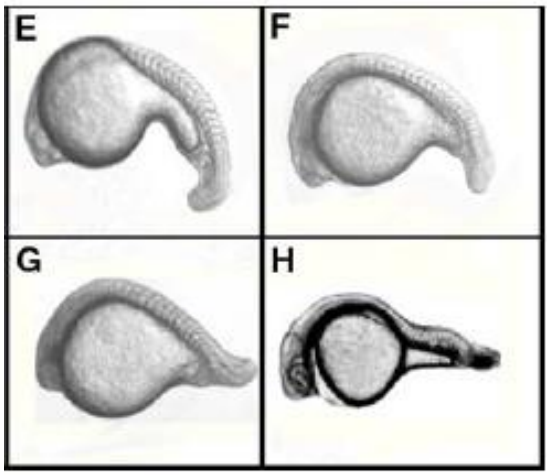

Figure 15: E–H shows the phenotype that appearing at the 25-somite stage of zebrafish embryos. The similar ntl phenotype was also detected in 14 % (11/77) embryos (F–G). E illustrates a wild type zebrafish embryo and H presents the ntl mutant (Wang et al., 2007).

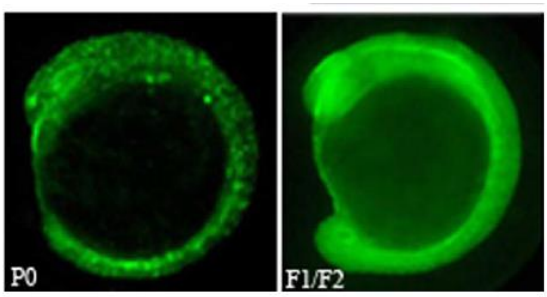

Figure 16: Gfp gene expression in pCMVT7R transgenic zebrafish embryo of mid-somite stage. P0 embryo expressed the gfp gene mosaically, F1/F2 one expressed the gfp gene uniformly in the whole embryo (Wang et al., 2007).

Another study (Gruber et al., 2005) used two different siRNA techniques to demonstrate a highly efficient gene knock down method in three different zebrafish lines, ZFL, SJD and ZF4 cell lines, which was derived from adult and embryonic zebrafish (*Danio rerio).* Different zebrafish genes, *lamin A, lamin B2,* kinesin related motor protein *Eg5* and exogenous GFP (*eGFP*) were chosen as target genes to be silenced. Knockdown of the target genes with specific phenotypes was noted from previously studies for homologous siRNA in mammalian cells.
By contrast, injection of *lamin A, GL2* (control) and *eGFP* siRNAs into zebrafish embryos influenced the morphology and led to morphological defects, abnormal development and the early death of most embryos.
This study presented for the first time that the cellular RNA interference mechanism works in *Danio rerio* cell lines. Moreover, it demonstrated that the active RNAi machinery of specific gene in cell lines is possible.

2.2.4. Gene silencing in oomycetes

The first application of gene silencing in a relevant aquaculture pathogenic oomycete, *Saprolegnia parasitica* was described in the study from Saraiva et al. (2014). The gene of tyrosinase, *SpTyr* is neccesary to the melanin biosynthetis of this fish pathogen. It is involved in pigment formation and the decrease in gene expression can cause detectable changes in the phenotype. Different *S.parasitica* lines were treated with SpTyr-dsRNA. After tyrosinase gene silencing the melanin production was reduced, tyrosinase activity decreased between 38 % and 60 %. The SpTyr-silenced lines exhibited less pigmentation in developing sporangia, sporadically a modified, abnormal morphology, and also a less electron dense cell wall. This work demonstrated that gene silencing via RNAi is a suitable method to functionally identify genes in *S.parasitica*.

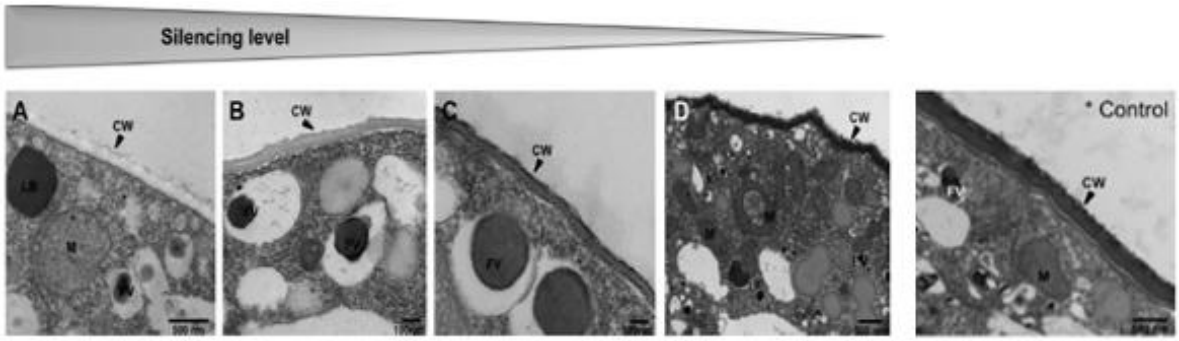

Figure 17: Effect of silencing of SpTyr gene on cell wall of Saprolegnia parasitica (Saraiva et al., 2014).

Fehler! Verweisquelle konnte nicht gefunden werden. Figure describes the gene expression level of SpTyr-silenced lines using TEM. This method exposes an electron dense layer in the cell wall (CW) of sporangia of control lines (*) and a non-silenced line (D). The pictures (A-C) show the decreased electron dense layer in the cell wall of the sporangium with decreased levels of SpTyr-expression.

2.3. Gene silencing in crustaceans

The limited information regarding the gene content of crustaceans and the absence of tools for genetic manipulation has made it challenging to follow the mechanistic basis for dsRNA in crustaceans. Growing our knowledge about genomics and proteomics in crustaceans should supply the key to solving the molecular mechanism in this new occurrence. Presently, few studies have explained the RNAi method and recognized its practical use in the study of gene function in crustaceans (Estrada et al., 2008). Another study declares that the RNAi method is widely utilized as a technique to examine gene function and develop antiviral agents to fight viral infections in invertebrate animals (Capodici et al., 2002).

The first metazoan in which the gene silencing process was registered was a nematode *Caenorhabditis elegans* (Fire et al., 1998). The nature of RNAi presented in this animal the capability of cells to notice and interiorize extracellular dsRNA to initiate intracellular procedures of gene silencing, in vivo (Winston et al., 2002; Feinberg und Hunter, 2003).
Gene silencing can be generated in different way, for instance via feeding, injection or transgenic expression of dsRNA molecules (Grishok, 2005).
The following table (Table 4) shows a summary about the RNAi method in crustaceans from a study (Estrada et al., 2008).

Table 4: Effective applications of RNAi technique in crustaceans (Estrada et al., 2008).

RNA	Target	Genes	RNAi response	Organism
dsRNA	Endogenous[A]	spalt	Pleiotropic effects	*Artemia franciscana*
dsRNA		chh	Decrease in glucose levels	*Litopenaeus schmitti*
dsRNA		ALF	Protection against WSSV	*Pacifastacus leniusculus*
dsRNA		pmYRP65	Inhibition of YHV cell entry	*Penaeus monodon*[c]
dsRNA		proPO	Increased bacterial [D] growth	*Pacifastacus leniusculus*
dsRNA		pacifastin	Decreased bacterial[D] growth	*Pacifastacus leniusculus*
dsRNA		Mih-B	Reduction of vitellogenin gene	*Metapenaeus ensis*
dsRNA		Pem-GIH	Decrease in *Pem-GIH* transcripts and reduction of vitellogenin gene	*Penaeus monodon*
dsRNA	Virals[B] and (unrelated)	hel, pol, pro, gp116, gp64	Inhibition of YHV replication	*Penaeus monodon*[C]
dsRNA		(gfp)	Non-specific antiviral immunity	
dsRNA		vp28, vp15	Non-specific antiviral immunity and lower viral protection	*Penaeus monodon*
dsRNA		pro	Inhibition of YHV replication	*Penaeus monodon*
dsRNA		(gfp, TSV pol)	Partial inhibition of YHV replication	
siRNA		(duck *u)*	Non-specific antiviral immunity	*Litopenaeus vannamei*
siRNA		vp28	Non-specific antiviral immunity	*Penaeus japonicus*

A Produced sequence-specific response.
B Produced both non-sequence-specific and sequence-specific antiviral immune reactions.
C Primary culture of lymphoid 'Oka' cells.
D *Aeromonas hydrophila*

2.3.1. Gene silencing in viral disease of crustaceans

Recently, three unrelated virus diseases in shrimps have been target inhibited with the dsRNA technique: white spot syndrome virus (WSSV), yellow head virus (YHV) and Taura syndrome virus (TSV). This chapter describes the successful studies that address them.

In a study of Tiransophon and co-workers, primary cultures of black tiger shrimp (*Penaeus monodon*) lymphoid 'Oka' cells were used to verify the inhibition in the viral replication of YHV through RNAi mediated gene silencing (Tirasophon et al., 2005). In vitro transcribed dsRNA of YHV helicase (*hel*), protease (*pro*), polymerase (*pol*), and structural viral genes *gp116* and *gp64* were transfected into a shrimp Oka cells culture, and the morphological change was investigated under a microscope. As a result, it was found to inhibit YHV replication. DsRNA was more effectively targeted to the non-structural genes (protease, polymerase, and helicase) of YHV than the structural genes in suppressing the viral replication. The targeted structural genes (gp64 and gp116) had the least inhibitory effect on viral replication.
This study demonstrated that dsRNA controlled the primary cell culture of *Penaeus monodon* protect against YHV infection. In addition, it shows the first proof that RNAi mediated gene silencing is also working in shrimp cells.

The YHV shrimp virus causes significant economic damage and production losses in farmed penaeid shrimp (Flegel, 1997; Lightner et al., 1998). A study (Assavalapsakul et al. 2006) used dsRNA-mediated RNA interference silencing to specifically downregulate the pmYRP65 message. The 65-kDa receptor protein by YHV therefore inhibited the whole virus entry in the *Penaeus monodon* cells. A primary cell culture from the lymphoid (Oka) organ of *P. monodon* was then applied, to target of these virus infections.
This report marks the first identification of an intervertebrate *Nidovirus* receptor, namely pmYRP65. The antibodies against this protein, and the down regulation of the pmYRP65 message via RNAi, are in a position to inhibit the entry of yellow head virus into Oka cells, recommend that the protein identified is certainly a YHV receptor protein, the 65-kDa protein. In the absence of the message, the lymphoid organ cells were shown to be refractory to infection with this virus, proving that pmYRP65 acts equally a receptor protein for YHV.

White spot syndrome virus (WSSV) gives rise to mortality and causes serious losses in commercial shrimp farms worldwide because of the current intensity of aquaculture practices.
An alternative and effective methodology to prevent this infection in shrimp could be the utilisation of RNA interference. Shorter 21-nucleotide siRNAs with homology were investigated to the WSSV either *vp15, vp28* or *gfp* genes to give a sequence- specific interference and response in the shrimp *Penaeus monodon* in a study from (Westenberg et

al. 2005). *Vp15* is a basic DNA binding protein of WSSV (Witteveldt et al., 2005); *vp28* is a main WSSV cover protein besides probable participates in systemic virus infection (Van Hulten et al. 2001); *gfp* siRNAs are useful for nonspecific control for siRNA effects. The intramuscularly injection of the *vp28* and *vp15* siRNAs resulted an important reduction in shrimp mortality upon WSSV infection but no such specific different in the reduction when they gave control *gfp* siRNA the injection.
Consequently, both shrimp injected siRNAs and large dsRNA molecules induce a sequence-independent anti-viral immunity.

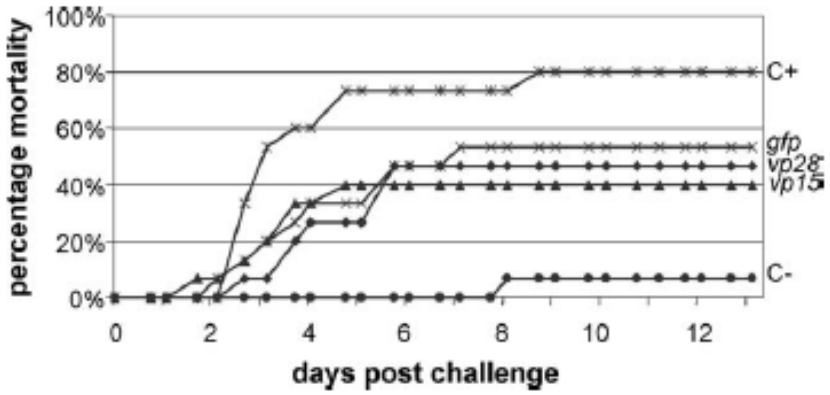

Figure 18: Time-mortality graph of shrimps (*Penaeus monodon*) injected with siRNAs (Westenberg et al., 2005).

The Figure presented the above mentioned study (Westenberg et al. 2005). Each shrimp was vaccinated with 10 µM siRNAs or buffer (C+ and C-). After twenty-four hours the injection they were challenged with WSSV or injected with buffer (C-). Their collective mortality rate is shown against a day after challenge (n = 15).

A study of Liu and co-workers used freshwater crayfish (*Pacifastacus leniusculus)* to experimentally infect with the white spot syndrome virus (WSSV) (Liu et al., 2006). Numerous differentially expressed genes were recognised and characterized in this study. The protein, namely antilipopolysaccharide factor (ALF) was picked out because its transcript levels increased upon viral infection. Quantitative PCR represented in cell culture of hematopoietic tissue from freshwater crayfish that knockdown of ALF via RNAi caused about 10-fold higher WSSV levels than those treated with control dsRNA.
Accordingly, RNA interference experiments with ALF in the animals and in cell cultures indicated the protection of ALF against WSSV infection in crayfish as the knockdown of ALF through RNAi leads to higher rates of viral replication. In other words, the function of ALF protein in viral propagation is interesting since its removal via RNAi results in an important improvement of viral replication.
Consequently, the report showed that ALF disturbs WSSV dissemination applying RNAi both in vivo and in vitro. It was the first study to describe RNAi in vitro with a crustacean. It was also the first to identify an endogenous factor interfering with WSSV dissemination in

crustacean. ALF probably has a prominent place in the immune protection against viral infection of crayfish.

2.3.2. Gene silencing in bacterial disease of crustaceans

A study from Liu et al. (2007) indicates that phenoloxidase (PO) is a significant element of the protection against a highly pathogenic bacterium, *Aeromonas hydrophila,* in the infection in the freshwater crayfish, *Pacifastacus leniusculus.* Pheoloxidase is the terminal enzyme in the melanisation cascade, and it takes part in the recognition of and immune defence toward microbial infection in invertebrates. Gene silencing using dsRNA-mediated RNA interference transcript depletion of crayfish prophenoloxidase (proPO) caused several changes: increasing bacterial growth, lower phagocytosis, decreased phenoloxidase activity, lower nodule formation, and higher mortality rate when infected with this bacterium. Contrarily, if the inhibitory domain of the crayfish prophenoloxidase activation cascades, namely the pacifastin gene is modified with dsRNAi, opposite of the above-mentioned processes occurs. Specifically, it results in lower bacterial growth, increased phagocytosis, increased nodule formation, higher phenoloxidase activity, and delayed mortality. In conclusion, the data from this study elucidate that PO is necessary in the freshwater crayfish defence against pathogenic bacterial infection by *A. hydrophila.*

2.3.3. Gene silencing in decrease glucose level by crustaceans

The crustacean hyperglycemic hormone (CHH) is essentially responsible for the regulation of hemolymph glucose levels, growth, molting, and reproduction (Soyez et al., 1990). A study (Lugo et al., 2006) examined the facility of dsRNA to inhibit the function of this hormone in an Atlantic Ocean shrimp, *Litopenaeus schmitti,* in vivo. CHH gene silencing was implemented through the injection of CHH dsRNA into the abdominal hemolymph sinuses of the shrimps. After 24 hours the undetectable CHH mRNA levels, the suppression of CHH gene function, and an analogous decrease in hemolymph glucose levels in adult shrimps, demonstrated that effective gene silencing had occurred. This study demonstrates the first time that the dsRNA process is working in adult shrimps in vivo, and that it can be used to study the gene function in crustaceans.

2.3.4. Gene silencing in pleiotropic effect by crustaceans

A recent study of Copf and co-workers demonstrated dsRNA generated the knockdown of the expression of *spalt* genes in the branchiopod crustacean *Artemia franciscana* (Copf et al., 2006). *Spalt* genes have a central effect in development and their function has been nearly joined with the function of *Hox* genes in different contexts. This study examined the role of *spalt* genes in *Artemia* and found that *spalt* is expressed in the presegmental 'growth zone' and in a series of stripes in each of the trunk segments as they appear from the growth zone. The reduced effects of *spalt* function in *Artemia* were studied via the RNAI method. Due to the knocking down of *spalt* gene expression, it created pleitropic effects. These effects represented several homeotic transformations in phenotypes, thoracic to genital (T→G), genital to thoracic (G→T) and post-genital to thoracic (PG→T) that are combined with a stochastic depression of *Hox* genes in the analogous segments of RNAi-treated animals. The most common phenotype was the growth of rudimentary or malformed appendages (Figure 19). In summary, it appears that *spalt* genes maybe have a possible role in the maintenance of *Hox* gene repression in *Artemia* and in other species. In addition, this result would be advantageous in unravelling the genetic ways that underline specific volutionary process in *Artemia franciscana.*

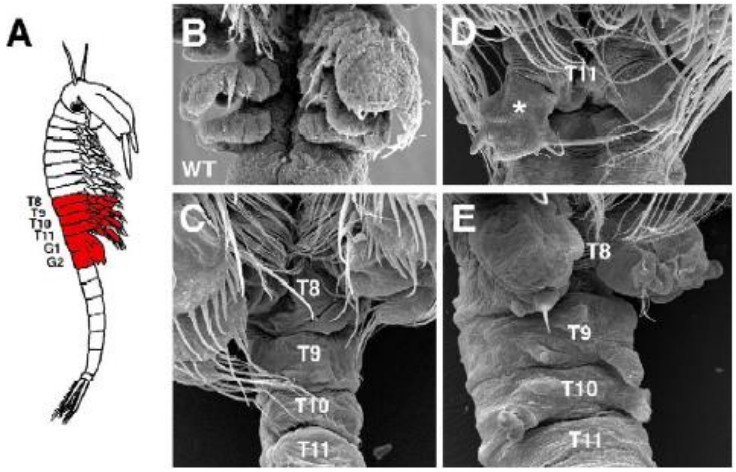

Figure 19: Artemia franciscana spalt RNAi modified phenotypes: malformed, rudimentary and missing appendages of different regions the body.
Panel (A) highlighting the region of the body where malformed, rudimentary or missing appendages happen, together with posterior thoracic (T8–T11) and genital (G1 and G2) segments. (B) Scanning electron microscopy (SEM) of thoracic appendages in a normal individual, during mid-larval stages, presenting the characteristic morphology of juvenile, growing phyllopodous appendages. (C, D, E) SEM of dsRNA- treated individuals in late larval stages: (C) Individual with missing appendages in segments T9-T11; (D) individual with malformed appendage (marked by asterisk) in T11 segment, presenting clear abnormalities compared to normal phyllopodous appendages or to juvenile appendages (compare to panel B); (E) individual with rudimentary and malformed (in T9 and T10) or missing (in T11) thoracic appendages. Anterior is up in all panels (Copf et al., 2006).

2.3.5. Gene silencing in reproduction of crustaceans

A report from a study by Treerattrakool and co-workers demonstrates that the use of double-stranded RNA elucidate the function of gonad-inhibiting hormones (GIH) in black tiger shrimp (*Penaeus monodon*) (Treerattrakool et al., 2008). GIH is an essential peptide hormone that regulates reproduction in crustaceans and modulates ovarian maturation by inhibiting the synthesis of vitellogetin (Vg), the precursor of yolk proteins. This study inquired into the cDNA encoding GIH (Pem-GIH) from shrimp and its probable role in vitellogenesis. CDNA encoding a GIH from the eyestalk of *P. monodon* was cloned via RT-PCR and RACE methods. The Pem-GIH transcript was detected in eyestalk, brain, thoracic and abdominal nerve cords of adult shrimps. With the help of the RNA interference technique the gonad-inhibiting activity of Pem-GIH was investigated. DsRNA can activate a

decrease in Pem-GIH transcript levels both in eyestalk ganglia and abdominal nerve cord explant cultures and in female *P.monodon* bloodstock. Functional-knockdown study of Pem-GIH through dsRNA was used to exhibit the negative influence on Vg mRNA expression in the ovary of previtellogenic adult female for the first time, and thus it provides proof for its role as a gonad-inhibiting hormone in this shrimp species. The study characterized and recognised the Pem-GIH cDNA of *P. monodon* in both biological and molecular viewpoints. As a result, this study proved that dsRNA-mediated gene silencing is a potent tool for functional study of the genes in crustaceans.

Neurosecretory structures in crustaceans' eyestalks are produced neuropeptides, namely the crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH) and gonad-inhibiting hormone (GIH) of the CHH/MIH/ GIH gene family. These can regulate several processes, such as molting and reproduction (Keller, 1992; De Kleijn und Van Herp, 1995; Chan et al., 2003). A study (Tiu und Chan, 2007) described the production of recombinant protein and dsRNA for the eyestalk neuropeptide gene and an RNA interference methodology to study the reproductive function of the molt-inhibiting hormone (MeMIH-B) in female sand shrimp, *Metapenaeus ensis*. Ovary and hetapopancreas explants were cultured in mediums including recombinant MeMIH-B and the vitellogenin gene (*MeVg1*) expression level was upregulated in a dose-dependent way. In this way the maximum of *MeVg1* transcript level in the hepatopancreas explants treated with 0.3 nm recombinant MeMIH-B reached. Moreover, an increase in the *MeVg1* expression in the hepatopancreas was detected when shrimp with recombinant MeMIH-B were injected. In addition, the vitellogenin-like immunoreactive protein showed a corresponding increase in the gonad and hemolymph of these female shrimps.
Female shrimps were injected with MeMIH-B dsRNA and a significant reduction in MeMIH-B transcript level in thoracic ganglion and eyestalk was observed. There was also a significant drop of *MeVg1* expression in the hepatopancreas and ovary, and the vitellogenin level in hemolymph was also reduced. This study demonstrated that the combined used of recombinant protein and RNAi tools can stimulate the function of MeMIH-B in vitellogenesis in *M. ensis.*

3. Ethical issues and future of gene editing

Ethically, the benefits must be greater than the risks. The use of CRISPR-Cas9 technique can be risky and harmful since it may produce off-target mutations (Yang et al., 2013). Several ethical and bioethical issues are connected to the implementation of gene-editing methods that can affect the use of gene-editing technology. The process is complex and it includes numerous ethical and technical issues that can influence the performance of genome editing. Such tools and technologies have resulted in the development of mutations that can cause several side effects when they are administered without appropriate protocol (Karre, 2020). This technique can also lead to cell death or transformation through the cutting of unintended sequences created mutations (Fu et al., 2013). To reduce the off-target mutations efforts via new variants of Cas9 enzyme have been made (eSpCas9, hypaCas9, Cas9HF-1), but these need further improvements, such as accurate modifications for therapeutic interference (Cong et al., 2013; Hsu et al., 2013). The CRISPR method was previously a technical 'disruptor' and we should contemplate how it can be turned into a 'health disruptor' (Ledford, 2015; Capps et al., 2017). The main disadvantage is the cost of the tools and techniques, besides the reagents, that are applied in the procedures (Karre, 2020).

On the other hand, there are risks. The unplanned release of the genetically modified (GM) experimental organisms in the natural world and caused extinction of the whole population due to targeted gene drive. Consequently, radical outcomes to the natural balance in the ecosystem form (Oye et al., 2014; Malik et al., 2019). Gene drive is a process of biased inheritance of genetic variants in a population in non-Mendelian way (Collins, 2018). The conversion efficiencies of the CRISPR-Cas9 editors of targeted gene drive mechanisms have been described as higher than 98 % (Gantz und Bier, 2015). The off-target mutations can amplify in each generation, and it is risky to transfer genes and modified sequences to other species. The negative characteristics can be transmitted to related organisms far and wide. Therefore, the dispersion of the gene drive trait may be difficult to control (Esvelt et al., 2014). Furthermore, it makes more difficult to identify the GM organism outside the lab, thanks to precise genetic modifications through the effective CRISPR/Cas9 method. There are additional aspects which play serious roles: the health effects of an allergic reaction to GM products, and the environmental effects of the uncontrolled release of transgenes. Additionally, the diversity of natural genomes is reduced. This shows up the sociocultural aspect of "playing God" (Hackett et al., 2014).

The emergent gene editing platform is a switch from the slow but commonly accessible tools to novel and sharp gene editing ones (Capps et al., 2017). Genomic selection is on the threshold of becoming a reality, and is making affected impressions in the genetic development of livestock. The betterment of the genes of aquatic species is a continuing process. Innovations are being reported together with quick advancement technology of targeted gene editing. In the future, the best genotypes for aquaculture applications will be

developed via traditional selective breeding together with new biotechnologies and molecular/genomic methods. To grow aquaculture production, more management tools will be required, especially the genetic enhancement, which has strong potential to efficiently and sustainably enhance production. Genetic improvement can be revolutionizing with a non-transgenic method, with highly effective gene editing tools. Genetic enhancement in aquatic creatures develops quickly, and the food production, competence and potential environmental impressions using genetic improvement appears promising for the future (Lucas und Southgate, 2013).

Transgenic salmon were lately accepted for public consumption. If there is a public acceptance of transgenic fish flesh in the marketplace, then genetic enhancement of aquacultured organisms will dramatically increase. (Lucas und Southgate, 2013).

In addition, CRISPR/Cas9 can revolutionize gene and cell substitution therapies. It can be used for identification of new drug targets and it has made the making of disease models easier. The CRISPR-based genomics screens allow the identification of mutations that confer drug resistance (Shalem et al., 2014).

The publicity could not tolerable the rapid expanding CRISPR zoo. The regulation of patents and economic interests create more remarkable issues. Patents make it possible to have the biotechnological companies' excessive power and benefit on the other hand support to regulate the field. In addition, through the practice of patenting there are probably initiate litigations and frictions between researchers and biotechnological companies (Malik et al., 2019).

Thanks to the genome editing technology there are several important advancements in biomedical research however; it is presented with various challenges (Malik et al., 2019).

4. Conclusion

In modern times the marine industries are essential sectors of the food production and global trade. On the grounds of the biological advantages of fish models, numerous novel protocols have accomplished gene modification in different fish species over the last few years.

These studies demonstrate that gene editing is a very effective and widely used method in extensive range of fish species. It is applied from species with special adaptations (e.g., cavefish) to evolutionarily primitive species (e.g., lamprey), as well as from large species with economic relevance (e.g., Atlantic salmon) to model organisms (e.g., zebrafish) and cell lines (e.g. ZFL, SJD, ZF4) (Zhu und Ge, 2018).

Targeted insertion, deletion or replacement of specific base sequences can create mutations of the specific gene in different fish species. These modifications in genomic DNA may bring radical changes in aquaculture production in the future. This makes it possible to improve characteristics in aquaculture, like disease resistance, growth or reproduction.

RNAi play a crucial role in the silencing of gene expression. With this new method over eco-friendly molecular device, it makes the RNAi-mediated gene knockdown of a target gene possible. It can also influence the development of functional genomics and therapeutic applications in fish species and crustaceans.

In summary, the creation of mutant animals in aquaculture through specific gene modification methods is the reality.

5. Zusammenfassung

Gene Editing und Gen Silencing Techniken haben das Potenzial, unser Wissen über Biologie und Pathogenität von Fischen und anderen Wassertierarten zu revolutionieren. Jene Techniken können den Phänotyp verändern, und dabei Anomalien und Dysfunktionen bei Lebewesen behandeln. Des Weiteren wird Gene Editing derzeit in weiten Bereichen der Aquakultur untersucht, beispielweise beim Wachstum, bei der kontrollierten Fortpflanzung, bei der Sterilität oder der Krankheitsresistenz. Zinkfingernuklease, TALENs und CRISPR/Cas9-basierte Gene-Editing-Methoden schneiden gezielt die DNA und führen zu erwünschten Veränderungen an den spezifischen Stellen. Das CRISPR/Cas9-system ist aufgrund seiner Einfachheit, Schnelligkeit und der geringen Kosten das leistungsfähigste Instrumentarium. Gene Silencing kann dabei verwendet werden, um die Übersetzung von RNA zu hemmen, bzw. die Genexpression zu regulieren. Zudem führt Gene Silencing zur Inaktivierung von Zielgenen oder Chromosomenregionen und verhindert dadurch einige Infektionen in der Aquakultur. Diese Methode wird von Forschern häufig zur Untersuchung von Genen mit verschiedenen Störungen eingesetzt.

Aus ethischer Sicht erschweren diese präzisen genetischen Veränderungen die Erkennung gentechnisch veränderter Organismen in der Natur und können durch die erzeugten Mutationen verschiedene Nebenwirkungen verursachen. Zusammenfassend lässt sich sagen, dass die Veränderung der genomischen DNA die Aquakulturmedizin in Zukunft umfassend beeinflussen kann.

6. Summary

Gene editing und gene silencing techniques have the potential to revolutionize our knowledge in biology and pathogenicity of fish and other aquatic animals. It can change the phenotype, handles cure abnormalities and dysfunctions in creatures. Gene editing is currently experimental in wide fields of the aquaculture include growth, controlled reproduction, sterility or disease resistance. Zink finger nuclease, TALENs and CRISPR/Cas9 based gene editing methods targeted cleavage the DNA, consequently induce favourable changes to site specific locations. CRISPR/Cas9 is the most powerful toolbox because of its simplicity, speed and low-cost.

Gene silencing can be used to inhibit the translation of RNA, namely to regulate the gene expression. It leads to inactivation of targeted genes or chromosome regions therefore prevents some infections in the aquaculture. This methodology is widely used by researchers to investigate genes with different disorders.

On the ethical side, these precise genetic modifications make us more complicated to recognise GM organism in the natural world and can cause several side effects through created mutations. To put in concisely, the modification of genomic DNA can comprehensive influence the aquaculture medicine in the future.

7. Acknowledgement

I would like to express my gratitude to Priv.-Doz. Dr. Mona Saleh for her supervision and guidance and for giving me the chance to write this work.

My deepest thanks to my family for all their love, support and opportunities they have given me and their believe in me throughout my studies at the University of Veterinary Medicine Vienna.

8. List of figures

9. List of tables

10. Abbreviations

ZFN - Zinc finger nuclease

TALEN - Transcriptional activator-like effector nuclease

HDR - homology-directed repair

NHEJ - non-homologous end joining

DSB - Double-strand break

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

sgRNA/gRNA - single guide RNA

crRNA - CRISPR RNA

tracrRNA - trans-activating crRNA

PAM- Protospacer adjacent motif

RNAi - RNA interference

dsRNA - double stranded RNA

siRNA- small interfering RNA

hpRNA - hairpin RNA

shRNA - short-hairpin RNA

Ago - Argonaute protein

RISC - RNA induced silencing complex

RdRp - RNA-dependent RNA polymerase

SVCV- Spring viraemia of carp virus

EPC - epithelioma papulosum cyprinid

CyHV-3 - cyprinid herpesvirus-3

TK - thymidine kinase

DP - DNA polymerase

shRNA - short hairpin RNA

MSTN - Myostatin

KO - knockout

GM - genetically modified

GH - Growth Hormone

DHA - docosahexaenoic acid

EPA - eicosapentaenoic acid

CCB - common carp brain

ALF - antilipopolysaccharide factor

WSSV - white spot syndrome virus

CHH - crustacean hyperglycemic hormone

PO - phenoloxidase

proPO - prophenoloxidase

SEM - scanning electron microscopy

WSSV - white spot syndrome virus

YHV - yellow head virus

TSV - Taura syndrome virus

GIH - gonad-inhibiting hormone

Vg - vitellogetin

CHH- crustacean hyperglycemic hormone

MIH - molt-inhibiting hormone

GIH - gonad-inhibiting hormone

eGFP - exogenous GFP

11. Literature

Adamek M, Rauch G, Brogden G, Steinhagen D. 2014. Small interfering RNA treatment can inhibit Cyprinid herpesvirus 3 associated cell death in vitro. Polish Journal of Veterinary Sciences, 17(4).

Adi Segev-Hadar, Tatiana Slosman, Ada Rozen, Amir Sherman, Avner Cnaani and JB. 2021. Genome Editing Using the CRISPR-Cas9 System to Generate a Solid-Red Germline of Nile Tilapia (Oreochromis niloticus). The CRISPR Journal, 4.

Assavalapsakul W, Smith DR, Panyim S. 2006. Identification and Characterization of a Penaeus monodon Lymphoid Cell-Expressed Receptor for the Yellow Head Virus . Journal of Virology, 80(1).

Auer TO, Duroure K, De Cian A, Concordet JP, Del Bene F. 2014. Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. Genome Research, 24(1).

B. H. 1903. Über die Drehkrankheit der Regenbogenforelle. Allg Fisch-Ztg.

Barnes DE. 2001. Non-homologous end joining as a mechanism of DNA repair. Current Biology, 11(12).

Bartel DP. 2009. MicroRNAs: Target Recognition and Regulatory Functions. Cell, 136(2).

Bedell VM, Wang Y, Campbell JM, Poshusta TL, Starker CG, Krug RG, Tan W, Penheiter SG, Ma AC, Leung AYH, et al. 2012. In vivo genome editing using a high-efficiency TALEN system. Nature, 491(7422).

Beumer K, Bhattacharyya G, Bibikova M, Trautman JK, Carroll D. 2006. Efficient gene targeting in Drosophila with zinc-finger nucleases. Genetics, 172(4).

Bharati J, Punetha M, Sai Kumar BAA, Vidyalakshmi GM, Sarkar M, D'Occhio MJ, Singh RK. 2019. Genome editing in animals: An overview. Genomics and Biotechnological Advances in Veterinary, Poultry, and Fisheries.

Biacchesi S. 2011. The reverse genetics applied to fish RNA viruses. Veterinary Research, 42(1).

Bibikova M, Beumer K, Trautman JK, Carroll D. 2003. Enhancing gene targeting with designed zinc finger nucleases. Science, 300(5620).

Blackburn PR, Campbell JM, Clark KJ, Ekker SC. 2013. The CRISPR system - Keeping zebrafish gene targeting fresh. Zebrafish, 10(1).

Boch J, Bonas U. 2010. Xanthomonas AvrBs3 family-type III effectors: Discovery and function. Annual Review of Phytopathology, 48.

Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, Lahaye T, Nickstadt A, Bonas U. 2009. Breaking the code of DNA binding specificity of TAL-type III effectors.

Science, 326(5959).

Borges F, Martienssen RA. 2015. The expanding world of small RNAs in plants. Nature Reviews Molecular Cell Biology, 16(12).

van den Bosch, M., Lohman, P.H. P. 2002. DNA double-strand break repair by homologous recombination. 873–892.

Boyd CE, D'Abramo LR, Glencross BD, Huyben DC, Juarez LM, Lockwood GS, McNevin AA, Tacon AGJ, Teletchea F, Tomasso JR, et al. 2020. Achieving sustainable aquaculture: Historical and current perspectives and future needs and challenges. Journal of the World Aquaculture Society, 51(3).

Brouns SJJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJH, Snijders APL, Dickman MJ, Makarova KS, Koonin E V., Van Der Oost J. 2008. Small CRISPR RNAs guide antiviral defense in prokaryotes. Science, 321(5891).

Capodici J, Karikó K, Weissman D. 2002. Inhibition of HIV-1 Infection by Small Interfering RNA-Mediated RNA Interference. The Journal of Immunology, 169(9).

Capps B, Chadwick R, Joly Y, Mulvihill JJ, Lysaght T, Zwart H. 2017. Falling giants and the rise of gene editing: Ethics, private interests and the public good Ruth Chadwick. Human Genomics, 11(1).

Carlson DF, Tan W, Lillico SG, Stverakova D, Proudfoot C, Christian M, Voytas DF, Long CR, Whitelaw CBA, Fahrenkrug SC. 2012. Efficient TALEN-mediated gene knockout in livestock. Proceedings of the National Academy of Sciences of the United States of America, 109(43).

Carpio Y, Estrada MP. 2006. Zebrafish as a genetic model organism. Biotecnologia Aplicada, 23(4).

Carroll D. 2014. Genome engineering with targetable nucleases. Annual Review of Biochemistry, 83.

Cathomen T, Keith Joung J. 2008. Zinc-finger nucleases: The next generation emerges. Molecular Therapy, 16(7).

Cattanach BM, Iddon CA, Charlton HM, Chiappa SA, Fink G. 1977. Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism. Nature, 269(5626).

Chan SM, Gu PL, Chu KH, Tobe SS. 2003. Crustacean neuropeptide genes of the CHH/MIH/GIH family: Implications from molecular studies. General and Comparative Endocrinology, 134(3).

Chang HHY, Pannunzio NR, Adachi N, Lieber MR. 2017. Non-homologous DNA end joining and alternative pathways to double-strand break repair. Nature Reviews Molecular Cell Biology, 18(8).

Choo Y, Sánchez-García I, Klug A. 1994. In vivo repression by a site-specific DNA-binding

protein designed against an oncogenic sequence. Nature, 372(6507):642–645.

Chu VT, Weber T, Wefers B, Wurst W, Sander S, Rajewsky K, Kühn R. 2015. Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. Nature Biotechnology, 33(5).

Collins JP. 2018. Gene drives in our future: Challenges of and opportunities for using a self-sustaining technology in pest and vector management. BMC Proceedings, 12.

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, et al. 2013. Multiplex genome engineering using CRISPR/Cas systems. Science, 339(6121).

Copf T, Rabet N, Averof M. 2006. Knockdown of spalt function by RNAi causes de-repression of Hox genes and homeotic transformations in the crustacean Artemia franciscana. Developmental Biology, 298(1).

Crudele JM, Chamberlain JS. 2018. Cas9 immunity creates challenges for CRISPR gene editing therapies. Nature Communications, 9(1).

Dahlem TJ, Hoshijima K, Jurynec MJ, Gunther D, Starker CG, Locke AS, Weis AM, Voytas DF, Grunwald DJ. 2012. Simple Methods for Generating and Detecting Locus-Specific Mutations Induced with TALENs in the Zebrafish Genome. PLoS Genetics, 8(8).

Daneholt B. 2006. Advanced information on the nobel prize in physiology or medicine 2006. The Nobel Assembly at Karolinska Institute.

Davis AJ, Chen DJ. 2013. DNA double strand break repair via non-homologous end-joining. Translational Cancer Research, 2(3).

Doyon Y, McCammon JM, Miller JC, Faraji F, Ngo C, Katibah GE, Amora R, Hocking TD, Zhang L, Rebar EJ, et al. 2008. Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. Nature Biotechnology, 26(6).

Dungan HM, Clifton DK, Steiner RA. 2006. Minireview: Kisspeptin neurons as central processors in the regulation of gonadotropin-releasing hormone secretion. Endocrinology, 147(3).

Edvardsen RB, Leininger S, Kleppe L, Skaftnesmo KO, Wargelius A. 2014. Targeted mutagenesis in atlantic salmon (Salmo salar L.) using the CRISPR/Cas9 system induces complete knockout individuals in the F0 Generation. PLoS ONE, 9(9).

Edwards P, Zhang W, Belton B, Little DC. 2019. Misunderstandings, myths and mantras in aquaculture: Its contribution to world food supplies has been systematically over reported. Marine Policy, 106.

Egelie KJ, Graff GD, Strand SP, Johansen B. 2016. The emerging patent landscape of CRISPR-Cas gene editing technology. Nature Biotechnology, 34(10).

Estrada MP, Lugo JM, Carpio Y. 2008. RNAi in fish and crustaceans. RNA Interference:

Methods for Plants and Animals.

Esvelt KM, Smidler AL, Catteruccia F, Church GM. 2014. Concerning RNA-guided gene drives for the alteration of wild populations. eLife, 3(July2014):1–21.

Fallis A., Lilley JH, Callinan RB, Chinabut S, Kanchanakhan S, MacRae IH, Phillips MJ. 1998. Epizootic ulcerative syndrome (EUS) technical handbook.

FAO. 2012. The State of World Fisheries and Aquaculture 2012. FAO Fisheries technical paper.

FAO. 2018. The State of Fisheries and Aquaculture in the world 2018.

Feinberg EH, Hunter CP. 2003. Transport of dsRNA into cells by the transmembrane protein SID-1. Science, 301(5639).

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. 1998. Potent and specific genetic interference by double-stranded RNA in caenorhabditis elegans. Nature, 391(6669).

Flegel TW. 1997. Major viral diseases of the black tiger prawn (Penaeus monodon) in Thailand. World Journal of Microbiology and Biotechnology, 13(4).

Food and Agriculture Organization. 2019. No Fishery and aquaculture statistics. Global aquaculture production 1950–2017 (Fishstat).

Food and Agriculture Organization (FAO). 2014. FAO: Fisheries and Aquaculture Statistics.

Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, Sander JD. 2013. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nature Biotechnology, 31(9).

Gantz VM, Bier E. 2015. The mutagenic chain reaction: A method for converting heterozygous to homozygous mutations. Science, 348(6233).

Gersbach CA. 2014. Genome engineering: The next genomic revolution. Nature Methods, 11(10).

Geurts AM, Cost GJ, Freyvert Y, Zeitler B, Miller JC, Choi VM, Jenkins SS, Wood A, Cui X, Meng X, et al. 2009. Knockout rats via embryo microinjection of zinc-finger nucleases. Science, 325(5939).

GL H. 2002. RNA interference. Nature.

Glover KA, Quintela M, Wennevik V, Besnier F, Sørvik AGE, Skaala Ø. 2012. Three decades of farmed escapees in the wild: A spatio-temporal analysis of atlantic salmon population genetic structure throughout norway. PLoS ONE, 7(8).

Gotesman M, Soliman H, Besch R, El-Matbouli M. 2014. In vitro inhibition of Cyprinid herpesvirus-3 replication by RNAi. Journal of Virological Methods, 206.

Gotesman M, Soliman H, Besch R, El-Matbouli M. 2015. Inhibition of spring viraemia of carp virus replication in an Epithelioma papulosum cyprini cell line by RNAi. Journal

of Fish Diseases, 38(2).

Gotesman M, Menanteau-Ledouble S, Saleh M, Bergmann SM, El-Matbouli M. 2018. A new age in AquaMedicine: Unconventional approach in studying aquatic diseases. BMC Veterinary Research, 14(1).

Gratacap RL, Wargelius A, Edvardsen RB, Houston RD. 2019. Potential of Genome Editing to Improve Aquaculture Breeding and Production. Trends in Genetics, 35(9).

Grishok A. 2005. RNAi mechanisms in Caenorhabditis elegans. FEBS Letters, 579(26).

Grissa I, Vergnaud G, Pourcel C. 2007. CRISPRFinder: A web tool to identify clustered regularly interspaced short palindromic repeats. Nucleic Acids Research, 35(SUPPL.2).

Gruber J, Manninga H, Tuschl T, Osborn M, Weber K. 2005. Specific RNAi mediated gene knockdown in zebrafish cell lines. RNA Biology, 2(3).

Hackett PB, Fahrenkrug SC, Carlson DF. 2014. The Promises and Challenges of Precision Gene Editing in Animals of Agricultural Importance.

Hammond SM. 2005. Dicing and slicing: The core machinery of the RNA interference pathway. FEBS Letters, 579(26).

Hammond SM. 2006. MicroRNA therapeutics: A new niche for antisense nucleic acids. Trends in Molecular Medicine, 12(3).

H. K. Barman*, Kiran D. Rasal and Subhajit Mondal, 2019: Status and prospects of gene editing and transgenic in fishes

Hockemeyer D, Soldner F, Beard C, Gao Q, Mitalipova M, Dekelver RC, Katibah GE, Amora R, Boydston EA, Zeitler B, et al. 2009. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. Nature Biotechnology, 27(9).

Hood E. 2004. RNAi: What's all the noise about gene silencing? Environmental Health Perspectives, 112(4).

Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, et al. 2013. DNA targeting specificity of RNA-guided Cas9 nucleases. Nature Biotechnology, 31(9).

Huang P, Xiao A, Zhou M, Zhu Z, Lin S, Zhang B. 2011. Heritable gene targeting in zebrafish using customized TALENs. Nature Biotechnology, 29(8).

Van Hulten MCW, Witteveldt J, Snippe M, Vlak JM. 2001. White spot syndrome virus envelope protein VP28 is involved in the systemic infection of shrimp. Virology, 285(2).

Indra Pratap Singh, Sara Hasan PS. 2019. RNAi: Gene Silencing. International Journal of Research in Engineering, Science and Management, 2(9).

Jao LE, Wente SR, Chen W. 2013. Efficient multiplex biallelic zebrafish genome editing

using a CRISPR nuclease system. Proceedings of the National Academy of Sciences of the United States of America, 110(34).

Jinek M., Jiang, F., Taylor, D.W., Sternberg,S.H., Kaya E. M. 2014. Structures of Cas9 endonukleases reveal RNA-mediated conformational activation.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science, 337(6096).

Joung JK, Sander JD. 2013. TALENs: A widely applicable technology for targeted genome editing. Nature Reviews Molecular Cell Biology, 14(1).

Karigo T, Kanda S, Takahashi A, Abe H, Okubo K, Oka Y. 2012. Time-of-day-dependent changes in GnRH1 neuronal activities and gonadotropin mRNA expression in a daily spawning fish, medaka. Endocrinology, 153(7):3394–3404.

Karigo T, Aikawa M, Kondo C, Abe H, Kanda S, Oka Y. 2014. Whole brain-pituitary in vitro preparation of the transgenic medaka (oryzias latipes) as a tool for analyzing the differential regulatory mechanisms of LH and FSH release. Endocrinology, 155(2):536–547.

Karre A. 2020. GENE EDITING TECHNOLOGY.

Keller R. 1992. Crustacean neuropeptides: Structures, functions and comparative aspects. Experientia, 48(5).

Keum JW, Ahn JH, Bermudez H. 2011. Design, assembly, and activity of antisense DNA nanostructures. Small, 7(24).

Khalil K, Elayat M, Khalifa E, Daghash S, Elaswad A, Miller M, Abdelrahman H, Ye Z, Odin R, Drescher D, et al. 2017. Generation of Myostatin Gene-Edited Channel Catfish (Ictalurus punctatus) via Zygote Injection of CRISPR/Cas9 System. Scientific Reports, 7(1).

Kim YG, Cha J, Chandrasegaran S. 1996. Hybrid restriction enzymes: Zinc finger fusions to Fok I cleavage domain. Proceedings of the National Academy of Sciences of the United States of America, 93(3).

De Kleijn DPV, Van Herp F. 1995. Molecular biology of neurohormone precursors in the eyestalk of Crustacea. Comparative Biochemistry and Physiology -- Part B: Biochemistry and, 112(4).

Lamb BM, Mercer AC, Barbas CF. 2013. Directed evolution of the TALE N-terminal domain for recognitioLamb BM, Mercer AC, Barbas CF. 2013. Directed evolution of the TALE N-terminal domain for recognition of all 50 bases. Nucleic Acids Research, 41(21). n of all 50 bases. Nucleic Acids Research, 41(21).

Ledford H. 2015. CRISPR, the disruptor. Nature, 522(7554).

Lee DK, Nguyen T, O'Neill GP, Cheng R, Liu Y, Howard AD, Coulombe N, Tan CP, Tang-

Nguyen AT, George SR, et al. 1999. Discovery of a receptor related to the galanin receptors. FEBS Letters, 446(1).

Lee SJ, McPherron AC. 2001. Regulation of myostatin activity and muscle growth. Proceedings of the National Academy of Sciences of the United States of America, 98(16).

Lees-Miller SP, Meek K. 2003. Repair of DNA double strand breaks by non-homologous end joining. Biochimie, 85(11).

Li M, Wang D. 2017. Gene editing nuclease and its application in tilapia. Science Bulletin, 62(3).

Li M, Yang H, Zhao J, Fang L, Shi H, Li M, Sun Y, Zhang X, Jiang D, Zhou L, et al. 2014. Efficient and heritable gene targeting in tilapia by CRISPR/Cas9. Genetics, 197(2).

Li T, Huang S, Zhao X, Wright DA, Carpenter S, Spalding MH, Weeks DP, Yang B. 2011. Modularly assembled designer TAL effector nucleases for targeted gene knockout and gene replacement in eukaryotes. Nucleic Acids Research, 39(14).

Liang F, Han M, Romanienko PJ, Jasin M. 1998. Homology-directed repair is a major double-strand break repair pathway in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America, 95(9).

Lieber MR. 2010. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annual Review of Biochemistry, 79.

Lieschke GJ, Currie PD. 2007. Animal models of human disease: Zebrafish swim into view. Nature Reviews Genetics, 8(5).

Lightner D V., Hasson KW, White BL, Redman RM. 1998. Experimental infection of western hemisphere penaeid shrimp with asian white spot syndrome virus and asian yellow head virus. Journal of Aquatic Animal Health, 10(3).

Lilley JH, Roberts RJ. 1997. Pathogenicity and culture studies comparing the Aphanomyces involved in epizootic ulcerative syndrome (EUS) with other similar fungi. Journal of Fish Diseases, 20(2).

Lima PC, Harris JO, Cook M. 2013. Exploring RNAi as a therapeutic strategy for controlling disease in aquaculture. Fish & shellfish immunology, 34(3).

Liu H, Jiravanichpaisal P, Söderhäll I, Cerenius L, Söderhäll K. 2006. Antilipopolysaccharide Factor Interferes with White Spot Syndrome Virus Replication In Vitro and In Vivo in the Crayfish Pacifastacus leniusculus . Journal of Virology, 80(21).

Liu H, Jiravanichpaisal P, Cerenius L, Bok LL, Söderhäll I, Söderhäll K. 2007. Phenoloxidase is an important component of the defense against Aeromonas hydrophila infection in a crustacean, Pacifastacus leniusculus. Journal of Biological Chemistry, 282(46).

Liu Q, Segal DJ, Ghiara JB, Barbas CF. 1997. Design of polydactyl zinc-finger proteins for unique addressing within complex genomes. Proceedings of the National Academy of Sciences of the United States of America, 94(11):5525–5530.

Liu T, Huang J. 2016. DNA End Resection: Facts and Mechanisms. Genomics, Proteomics and Bioinformatics, 14(3).

Lucas JS, Southgate PC. 2013. Aquaculture: Farming Aquatic Animals and Plants: Second Edition.

Lugo JM, Morera Y, Rodríguez T, Huberman A, Ramos L, Estrada MP. 2006. Molecular cloning and characterization of the crustacean hyperglycemic hormone cDNA from Litopenaeus schmitti: Functional analysis by double-stranded RNA interference technique. FEBS Journal, 273(24):5669–5677.

Ma L, Jeffery WR, Essner JJ, Kowalko JE. 2015. Genome editing using TALENs in blind Mexican cavefish, Astyanax mexicanus. PLoS ONE, 10(3).

MacPherson S, Larochelle M, Turcotte B. 2006. A Fungal Family of Transcriptional Regulators: the Zinc Cluster Proteins. Microbiology and Molecular Biology Reviews, 70(3).

Mahfouz MM, Li L, Shamimuzzaman M, Wibowo A, Fang X, Zhu JK. 2011. De novo-engineered transcription activator-like effector (TALE) hybrid nuclease with novel DNA binding specificity creates double-strand breaks. Proceedings of the National Academy of Sciences of the United States of America, 108(6).

Majeed M, Kumar G, Schlosser S, El-Matbouli M, Saleh M. 2017. In vitro investigations on extracellular proteins secreted by Aphanomyces invadans, the causative agent of epizootic ulcerative syndrome. Acta Veterinaria Scandinavica, 59(1).

Majeed M, Soliman H, Kumar G, El-Matbouli M, Saleh M. 2018. Editing the genome of Aphanomyces invadans using CRISPR/Cas9. Parasites & Vectors, 11(1).

Majumdar R, Rajasekaran K, Cary JW. 2017. RNA interference (RNAi) as a potential tool for control of mycotoxin contamination in crop plants: Concepts and considerations. Frontiers in Plant Science, 8.

Makarova KS, Haft DH, Barrangou R, Brouns SJJ, Charpentier E, Horvath P, Moineau S, Mojica FJM, Wolf YI, Yakunin AF, et al. 2011. Evolution and classification of the CRISPR-Cas systems. Nature Reviews Microbiology, 9(6).

Mali P, Esvelt KM, Church GM. 2013. Cas9 as a versatile tool for engineering biology. Nature Methods, 10(10).

Malik YS, Barh D, Azevedo V, Paul Khurana SM. 2019. Genomics and biotechnological advances in veterinary, poultry, and fisheries.

Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey CE, Shibata N, Asakawa S, Shimizu N, et al. 2002. DMY is a Y-specific DM-domain gene required for male development in the medaka fish. Nature, 417(6888):559–563.

McPherron a C, Lawler a M, Lee SJ. 1997. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member [In Process Citation]. Nature, 387(May).

Meng X, Noyes MB, Zhu LJ, Lawson ND, Wolfe SA. 2008. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. Nature Biotechnology, 26(6).

Miller JC, Tan S, Qiao G, Barlow KA, Wang J, Xia DF, Meng X, Paschon DE, Leung E, Hinkley SJ, et al. 2011. A TALE nuclease architecture for efficient genome editing. Nature Biotechnology, 29(2).

Moscou MJ, Bogdanove AJ. 2009. A simple cipher governs DNA recognition by TAL effectors. Science, 326(5959).

Naito Y, Yamada T, Ui-Tei K, Morishita S, Saigo K. 2004. siDirect: Highly effective, target-specific siRNA design software for mammalian RNA interference. Nucleic Acids Research, 32(WEB SERVER ISS.).

Oakley AE, Clifton DK, Steiner RA. 2009. Kisspeptin signaling in the brain. Endocrine Reviews, 30(6).

OIE (World Organisation for Animal Health) Listed Diseases. 2017.

Okoli AS, Blix T, Myhr AI, Xu W, Xu X. 2021. Sustainable use of CRISPR/Cas in fish aquaculture: the biosafety perspective. Transgenic Research.

Oye KA, Esvelt K, Appleton E, Catteruccia F, Church G, Kuiken T, Lightfoot SBY, McNamara J, Smidler A, Collins JP. 2014. Regulating gene drives. Science, 345(6197).

P. Parveen, K. Deepti Brundavani, K. Mahathi MSB and SSS. 2019. Gene Silencing and DNA Methylation. American Journal of Phytomedicine and Clinical Therapeutics.

Palpant NJ, Dudzinski D. 2013. Zinc finger nucleases: Looking toward translation. Gene Therapy, 20(2).

Pankaj CY and. 2014. Cell Stem Cell. Scientific Reports, 331–335.

Pathiratne A, Widanapathirana GS, Chandrakanthi WHS. 1994. Association of Aeromonas hydrophila with epizootic ulcerative syndrome (EUS) of freshwater fish in Sri Lanka. Journal of Applied Ichthyology, 10(2–3).

Perota A, Lagutina I, Quadalti C, Lazzari G, Cozzi E, Galli C. 2016. The Applications of Genome Editing in Xenotransplantation. Journal of Genetics and Genomics, 43(5).

Popa SM, Clifton DK, Steiner RA. 2008. The role of kisspeptins and GPR54 in the neuroendocrine regulation of reproduction. Annual Review of Physiology, 70.

Qin Z, Li Y, Su B, Cheng Q, Ye Z, Perera DA, Fobes M, Shang M, Dunham RA. 2016. Editing of the Luteinizing Hormone Gene to Sterilize Channel Catfish, Ictalurus punctatus, Using a Modified Zinc Finger Nuclease Technology with Electroporation. Marine Biotechnology, 18(2).

Qiu C, Cheng B, Zhang Y, Huang R, Liao L, Li Y, Luo D, Hu W, Wang Y. 2014. Efficient

Knockout of Transplanted Green Fluorescent Protein Gene in Medaka Using TALENs. Marine Biotechnology, 16(6).

Reshi ML, Wu JL, Wang HV, Hong JR. 2014. RNA interference technology used for the study of aquatic virus infections. Fish and Shellfish Immunology, 40(1).

De Rienzo G, Gutzman JH, Sive H. 2012. Efficient shRNA-mediated inhibition of gene expression in zebrafish. Zebrafish, 9(3).

Roa J, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M. 2008. New frontiers in kisspeptin/GPR54 physiology as fundamental gatekeepers of reproductive function. Frontiers in Neuroendocrinology, 29(1).

ROBERTS RJ, WILLOUGHBY LG, CHINABUT S. 1993. Mycotic aspects of epizootic ulcerative syndrome (EUS) of Asian fishes. Journal of Fish Diseases, 16(3).

Saleh-Gohari N, Helleday T. 2004. Conservative homologous recombination preferentially repairs DNA double-strand breaks in the S phase of the cell cycle un human cells. Nucleic Acids Research, 32(12).

Saleh M, Kumar G, Abdel-Baki AA, Dkhil MA, El-Matbouli M, Al-Quraishy S. 2016. In Vitro Gene Silencing of the Fish Microsporidian Heterosporis saurida by RNA Interference. Nucleic Acid Therapeutics, 26(4).

Sander JD, Cade L, Khayter C, Reyon D, Peterson RT, Joung JK, Yeh JRJ. 2011. Targeted gene disruption in somatic zebrafish cells using engineered TALENs. Nature Biotechnology, 29(8).

Saraiva M, de Bruijn I, Grenville-Briggs L, McLaggan D, Willems A, Bulone V, van West P. 2014. Functional characterization of a tyrosinase gene from the oomycete Saprolegnia parasitica by RNAi silencing. Fungal Biology, 118(7).

Sarker S, El-Matbouli M. 2015. Can RNAi Target Salmonid Whirling Disease in Vivo? Nucleic Acid Therapeutics, 25(5).

Sarker S, Kallert DM, Hedrick RP, El-Matbouli M. 2015. Whirling disease revisited: Pathogenesis, parasite biology and disease intervention. Diseases of Aquatic Organisms, 114(2).

Sarker S, Menanteau-Ledouble S, Kotob MH, El-Matbouli M. 2017. A RNAi-based therapeutic proof of concept targets salmonid whirling disease in vivo. PLoS ONE, 12(6).

Sen GL, Blau HM. 2006. A brief history of RNAi: the silence of the genes. The FASEB Journal, 20(9).

Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelsen TS, Heckl D, Ebert BL, Root DE, Doench JG, et al. 2014. Genome-scale CRISPR-Cas9 knockout screening in human cells. Science, 343(6166).

Singh D, Chaudhary S, Kumar R, Sirohi P, Mehla K, Sirohi A, Kumar S, Chand P, Singh

PK. 2016. RNA Interference Technology — Applications and Limitations. RNA Interference.

Souza TA, Chen X, Guo Y, Sava P, Zhang J, Hill JJ, Yaworsky PJ, Qiu Y. 2008. Proteomic identification and functional validation of activins and bone morphogenetic protein 11 as candidate novel muscle mass regulators. Molecular Endocrinology, 22(12).

Soyez D, Noel PY, Van Deijnen JE, Martin M, Morel A, Payen GG. 1990. Neuropeptides from the sinus gland of the lobster Homarus americanus: Characterization of hyperglycemic peptides. General and Comparative Endocrinology, 79(2).

Swiech L, Heidenreich M, Banerjee A, Habib N, Li Y, Trombetta J, Sur M, Zhang F. 2015. In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. Nature Biotechnology, 33(1).

Takahashi A, Kanda S, Abe T, Oka Y. 2016. Evolution of the hypothalamic-pituitary-gonadal axis regulation in vertebrates revealed by knockout medaka. Endocrinology, 157(10).

Takata M, Sasaki MS, Sonoda E, Morrison C, Hashimoto M, Utsumi H, Yamaguchi-Iwai Y, Shinohara A, Takeda S. 1998. Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. EMBO Journal, 17(18).

Tang H, Liu Y, Luo D, Ogawa S, Yin Y, Li S, Zhang Y, Hu W, Parhar IS, Lin H, et al. 2015a. The kiss/kissr systems are dispensable for zebrafish reproduction: Evidence from gene knockout studies. Endocrinology, 156(2).

Tang L-M, Zhou C-L, Guo Z-F, Xiao L, Chüeh AC. 2015b. Advances in Zinc Finger Nuclease and Its Applications. Gene and Gene Editing, 1(1).

Taranger GL, Karlsen Ø, Bannister RJ, Glover KA, Husa V, Karlsbakk E, Kvamme BO, Boxaspen KK, Bjørn PA, Finstad B, et al. 2015. Risk assessment of the environmental impact of Norwegian Atlantic salmon farming. ICES Journal of Marine Science, 72(3).

Tesson L, Usal C, Meq́noret S, Leung E, Niles BJ, Remy S, Santiago Y, Vincent AI, Meng X, Zhang L, et al. 2011. Knockout rats generated by embryo microinjection of TALENs. Nature Biotechnology, 29(8).

Tirasophon W, Roshorm Y, Panyim S. 2005. Silencing of yellow head virus replication in penaeid shrimp cells by dsRNA. Biochemical and Biophysical Research Communications, 334(1).

Tiu SHK, Chan SM. 2007. The use of recombinant protein and RNA interference approaches to study the reproductive functions of a gonad-stimulating hormone from the shrimp Metapenaeus ensis. FEBS Journal, 274(17).

Treerattrakool S, Panyim S, Chan SM, Withyachumnarnkul B, Udomkit A. 2008. Molecular characterization of gonad-inhibiting hormone of Penaeus monodon and elucidation of its inhibitory role in vitellogenin expression by RNA interference. FEBS Journal, 275(5).

Urnov FD, Miller JC, Lee YL, Beausejour CM, Rock JM, Augustus S, Jamieson AC, Porteus MH, Gregory PD, Holmes MC. 2005. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. Nature, 435(7042).

Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. 2010. Genome editing with engineered zinc finger nucleases. Nature Reviews Genetics, 11(9).

Vishwanath TS, Mohan C V., Shankar KM. 1998. Epizootic Ulcerative Syndrome (EUS), associated with a fungal pathogen, in Indian fishes: Histopathology „a cause for invasiveness“. Aquaculture, 165(1–2).

Walsh AS, Yin H, Erben CM, Wood MJA, Turberfield AJ. 2011. DNA cage delivery to mammalian cells. ACS Nano.

Wang F, Shi Z, Cui Y, Guo X, Shi YB, Chen Y. 2015. Targeted gene disruption in Xenopus laevis using CRISPR/Cas9. Cell and Bioscience, 5(1).

Wang N, Sun YH, Liu J, Wu G, Su JG, Wang YP, Zhu ZY. 2007. Knock down of gfp and no tail expression in zebrafish embryo by in vivo-transcribed short hairpin RNA with T7 plasmid system. Journal of Biomedical Science, 14(6).

Wargelius A, Leininger S, Skaftnesmo KO, Kleppe L, Andersson E, Taranger GL, Schulz RW, Edvardsen RB. 2016. Dnd knockout ablates germ cells and demonstrates germ cell independent sex differentiation in Atlantic salmon. Scientific Reports, 6.

Westenberg M, Heinhuis B, Zuidema D, Vlak JM. 2005. siRNA injection induces sequence-independent protection in Penaeus monodon against white spot syndrome virus. Virus Research, 114(1–2).

Wiedenheft B, Lander GC, Zhou K, Jore MM, Brouns SJJ, Van Der Oost J, Doudna JA, Nogales E. 2011. Structures of the RNA-guided surveillance complex from a bacterial immune system. Nature, 477(7365).

Winston WM, Molodowitch C, Hunter CP. 2002. Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1. Science, 295(5564).

Witteveldt J, Vermeesch AMG, Langenhof M, De Lang A, Vlak JM, Van Hulten MCW. 2005. Nucleocapsid protein VP15 is the basic DNA binding protein of white spot syndrome virus of shrimp. Archives of Virology, 150(6).

World Bank. 2013. Fish to 2030: Prospects for fisheries and aquaculture - World Bank Report Number 83177-GLB. Agriculture and environmental services discussion paper, 3(83177).

Xu X, Hulshoff MS, Tan X, Zeisberg M, Zeisberg EM. 2020. Crispr/cas derivatives as novel gene modulating tools: Possibilities and in vivo applications. International Journal of Molecular Sciences, 21(9).

Yang L, Guell M, Byrne S, Yang JL, De Los Angeles A, Mali P, Aach J, Kim-Kiselak C, Briggs AW, Rios X, et al. 2013. Optimization of scarless human stem cell genome editing. Nucleic Acids Research, 41(19).

Yike I. 2011. Fungal Proteases and Their Pathophysiological Effects. Mycopathologia, 171(5).

Youngren KK, Coveney D, Peng X, Bhattacharya C, Schmidt LS, Nickerson ML, Lamb BT, Deng JM, Behringer RR, Capel B, et al. 2005. The Ter mutation in the dead end gene causes germ cell loss and testicular germ cell tumours. Nature, 435(7040).

Zhong Z, Niu P, Wang M, Huang G, Xu S, Sun Y, Xu X, Hou Y, Sun X, Yan Y, et al. 2016. Targeted disruption of sp7 and myostatin with CRISPR-Cas9 results in severe bone defects and more muscular cells in common carp. Scientific Reports, 6.

Zhu B, Ge W. 2018. Genome editing in fishes and their applications. General and Comparative Endocrinology, 257.