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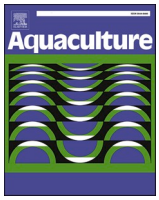

Short communication

CRISPR-Cas9/Cas12a-based genome editing in Atlantic cod (*Gadus morhua*)

Adrián López-Porras [a], Ragnhild Stenberg Berg [b], Erik Burgerhout [b], Øyvind J. Hansen [b], Ádám Györkei [a], Shuo-Wang Qiao [c], Finn-Eirik Johansen [a,*]

[a] *Section for Physiology and Cell Biology (FYSCELL), Department of Biosciences, University of Oslo, Oslo, Norway*
[b] *NOFIMA AS, Muninbakken 9-13, Tromsø 9019, Norway*
[c] *Department of Immunology, Institute of Clinical Medicine, University of Oslo, Oslo, Norway*

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ABSTRACT

Aquaculture is the fastest-growing food sector worldwide but faces sustainability challenges that need to be addressed in many ways, including genetic enhancement. Atlantic cod has re-emerged as an aquaculture species and tools for genetic manipulation are needed. Thus, we compared five formats of CRISPR to determine which was most efficient to generate knock outs in Atlantic cod. Cas9 protein was presented in preformed ribonucleoprotein (RNP) complexes with single guide or with duplex guide RNAs or an mRNA encoding Cas9 was used with the same two formats of guide RNAs. Cas12a was tested as RNP complexes with single guide RNAs. We found Cas9 mRNA with single guide RNA to be the most efficient format to knock out both alleles of the *slc45a2* gene, which resulted in an albino-like phenotype in up to 75% of surviving larvae. DNA analysis of individual larvae revealed mosaic genotypes with variable indel mutations. The mortality of injected eggs was high, resulting in low overall efficiency. Nevertheless, this study lays the foundation for further genetic and functional research using the CRISPR/Cas9 genome editing system in Atlantic cod.

1. Introduction

Aquaculture is the fastest growing food sector worldwide as reported by the Food and Agriculture Organization (FAO) of the United Nations (FAO, 2020). This demand is expected to continue to grow, and farmed aquatic species are predicted to be the main source of fish destined for human consumption by 2030 (FAO, 2020). However, the global rise of fish farming faces challenges threatening its sustainability, including disease outbreaks, suboptimal feed conversion, early sexual maturation, environmental issues, and lack of breeding programs for fish. (Puvanendran et al., 2022; Sonesson et al., 2023).

Aquaculture is the second most important economic activity in Norway, with Atlantic salmon as its flagship. Nevertheless, Atlantic cod (*Gadus morhua*) has re-emerged in recent years as a promising farmed species. It has had an important historical and cultural impact on societies of the north Atlantic. With its farming starting in the 1980s, Atlantic cod production rose during the next two decades reaching a maximum production of 21,000 tons in 2010 and collapsing in the period of 2011–2014 (Nardi et al., 2021). One of the factors that contributed to this collapse was the emergence of infectious diseases such as Francisellosis, which caused devastating outbreaks (Nylund et al., 2006; Ottem et al., 2008). A steady recovery of the Atlantic cod farming in Norway has been reported in the past years with a production of about 5000 tons in 2022 and more than 8500 tons first nine months of 2023 (*The Norwegian Directorate of Fisheries*, 2023).

Infectious diseases are one of the biggest threats to aquaculture sustainability. Vaccination is an efficient prophylactic method against diseases thereby improving animal health and reduction in the use of antimicrobial agents. The Atlantic cod possess a unique immune system when compared to mammalian and other vertebrates including other fish species like Atlantic salmon and rainbow trout. The absence of major histocompatibility complex class II (MHC II), invariant chain, and the CD4 molecules (Star et al., 2011) in cod is highly relevant to consider for the development of vaccination strategies. The Atlantic cod has a poor or null antibody response after vaccination even when protection was conferred (Magnadottir, 2014). Hence, understanding how the cod immune system works is crucial to secure effective vaccination strategies. Therefore, genome editing needs to be developed as a research tool in this species.

Genetic improvement has been shown to be a bulwark in the sustainability of several aquaculture species. This improvement has relied on methods including selective breeding, hybridization, chromosome

* Corresponding author.
E-mail address: f.e.johansen@ibv.uio.no (F.-E. Johansen).

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manipulation, genomic selection, transgenics, and genome editing technologies (Bentsen et al., 1998; Ponzoni et al., 2011; Tonelli et al., 2017). Genome-editing approaches can help to understand the basic functional aspects of the Atlantic cod immune system, enabling the enhancement of immunological competence, the increase of disease resistance, and the improvement of other productivity parameters. In the last years, the use of CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9) has gained popularity as a genome-editing tool because it is relatively simple to implement and cost-effective compared to other methods. This system provides a powerful tool for targeted gene editing, which enables scientists to selectively delete, modify, or insert genes in an organism's genome (Doudna and Charpentier, 2014).

The CRISPR-associated (Cas) nucleases, Cas9 and Cas12a, also known as Cpf1, have become instrumental for genome editing. Their activity is directed to specific sites in the genome by guide RNA sequences complementary to the target sequence (Swarts and Jinek, 2018). The Protospacer Adjacent Motif (PAM) sequence, necessary for target DNA recognition, differs between Cas9 and Cas12a. Cas9 typically requires an NGG PAM sequence while Cas12a requires a TTTV (V = A, G, or C) PAM sequence (Schubert et al., 2021). To increase the flexibility of target site choice, Cas enzymes with relaxed PAM sequence requirements have been developed (Hu et al., 2018; Kleinstiver et al., 2015; Walton et al., 2020). Originally, Cas9 was derived from *Streptococcus pyogenes* (SpCas9), while Cas12a was derived from *Acidaminococcus* sp. (AsCas12a) and *Lachnospiraceae bacterium* (LbCas12a) (Hillary and Ceasar, 2023). Cas12a has a more stringent PAM requirement compared to Cas9, which can limit its targeting range. However, this characteristic potentially increases its specificity and leads to lower off-target editing than SpCas9 (Zhou et al., 2022). Cas9 utilizes a dual RNA structure (crRNA and tracrRNA), although it can also use a single-guide RNA (sgRNA), while Cas12a requires a single crRNA, which simplifies the RNA preparation process (Swarts and Jinek, 2018). It has been reported that LbCas12a is more efficient at lower temperatures compared to AsCas12a and SpCas9, which can be highly beneficial for editing the genomes of ectothermic animals such as fish (Moreno-Mateos et al., 2017). CRISPR technology has been rapidly developed and is now widely applied across numerous organisms. This includes fish species utilized in both the biomedical field and aquaculture (Roy et al., 2022). Researchers have used CRISPR/Cas9 to generate disease-resistant strains of fish by knocking out or modifying genes associated with pathogenic infections (Simora et al., 2020); improve food intake (Kishimoto et al., 2018); and it has also been used to study and control the sex of fish by disrupting or manipulating the genes involved in sex determination and differentiation (Lau et al., 2016; Li et al., 2014; Wargelius et al., 2016).

Although the use of this technology has been increasing in the last years, differences among fishes' biology intrinsically pose challenges in the implementation of this technology for some species. Thus, standardization and establishment of protocols need to be addressed according to each species' needs and characteristics.

The goal of this paper is to compare, describe, and establish a CRISPR-based genome editing method for Atlantic cod. This tool can be used to further investigate functional aspects of this species' physiology and immunology, with the potential to enhance production-related traits.

2. Materials and methods

2.1. Ethics statement

Experiments were conducted according to the guidelines of the Regulation on the Use of Animals in Experiments of Norway.

2.2. Plasmids

The pT3TS-nCas9n plasmid (Addgene plasmid # 46757) was used to produce Cas9 mRNA (Jao et al., 2013). This plasmid encoding a zebrafish codon-optimized Cas9 protein was used to create the pT3TS-EGFPn (enhanced green fluorescent protein) by standard molecular biology methods. Briefly, the vector was prepared by partial digestion of pT3TS-nCas9n with *Bgl*II and complete digestion with *Nco*I (New England Biolabs; NEB) to excise the Cas9 gene and purified by gel electrophoresis (QIAquick Gel Extraction Kit; Qiagen, 28,704). The gene encoding EGFP was PCR amplified with Pfu polymerase (NEB) from pEGFP-1 (Clontech Laboratories) with primers EGFP-NcoI.for: GTACCATGGTGAGCAAGGGCGA and EGFP-BglII.rev: GCGAGATCTTCCCTTGTACAGCTCGTCCAT (recognition sites for NcoI and BglII underlined). The PCR product was digested with NcoI and BglII, gel purified (QIAquick Gel Extraction Kit) and inserted by DNA ligation into the pT3TS vector. Plasmid construction was verified by DNA sequencing (Eurofins Genomics).

2.3. Design of gRNAs

The *slc45a2* gene was selected for knockout as loss of both alleles of this gene causes an easily identifiable albino phenotype (Edvardsen et al., 2014). Four gRNAs were designed and selected with the web-based Benchling's CRISPR analysis tool (https://benchling.com) and CRISPOR tool (http://crispor.org; Concordet and Haeussler, 2018) with the gadMor3.0 Atlantic cod genome as template. For the targets of Cas9, the website tools returned several target sites in exons 1 and 6. Three gRNA target site sequences with high cutting frequency determination (CFD) scores (CRISPOR) and with no potential off-target and one sequence homologous to the site previously described to produce knockouts in Atlantic salmon (Edvardsen et al., 2014) were selected (Table 1). For each of the four Cas9 gRNAs, the same gRNA sequence in two different formats were used: duplex guide RNA (crRNA:tracrRNA, henceforth called dg) and single guide RNA (henceforth called sg). For the Cas12a, due to the more restrictive PAM sequence, fewer targets were retrieved and no targets within exon 1 were obtained. Thus, targets for Cas12a found were one in exon 2, one in exon 4, and two in exon 6 (Table 1). Since Cas12a requires a single-component guide RNA, only the single-guide crRNA (henceforth called cr) format was used. All gRNAs were purchased from Integrated DNA technologies (IDT).

2.4. In vitro transcription of Cas9 and EGFP mRNA

The pT3TS-nCas9n and pT3TS-EGFPn ("n" denotes nuclear localization signal) plasmids were linearized by *Xba*I (NEB) digestion and purified with QIAprep Spin columns (Qiagen) prior to in vitro transcription with mMessage mMachine T3 kit (Ambion). Cas9 and EGFP mRNAs were purified with the RNeasy MiniKit spin columns (QIAGEN) and the integrity of the mRNA was evaluated by gel electrophoresis according to the method described by Aranda et al. (2012).

2.5. Production of Cas9/Cas12a-guide RNA ribonucleoprotein complexes

Target-specific Alt-R crRNA and common Alt-R tracrRNA were used to prepare the crRNA:tracrRNA duplex according to manufacturer's instructions. Cas9 protein (Alt-R® S.p. Cas9 nuclease, v.3,) and Cas12a (Alt-R™ A.s. Cas12a (Cpf1) V3) were purchased from IDT and used to prepare duplex or single guide RNP complexes according to manufacturer's protocol. Prior to microinjection, the RNP complex solutions were incubated at 37 °C for 5 min and then placed at room temperature.

2.6. Fertilization of Atlantic cod and zebrafish eggs

Atlantic cod eggs and sperm obtained from the breeding program at NOFIMA (Tromsø, Norway) were freshly collected and transported to

Table 1
List of guide RNAs (gRNA) used for CRISPR/Cas9 and CRISPR/Cas12a genome editing. The table displays the oligo names, the targeted exon of the *slc45a2* gene, the corresponding gRNA sequences, and the nuclease used for each oligonucleotide.

gRNA name	Target exon	gRNA Sequence (5′-3′)	Nuclease	CFD score	Off-targets for 0–1–2-3-4 mismatches
xg1–1	#1	CGGCCGCGAGTTCTGCTACG	Cas9	97	0-0- 0-2- 31
xg1–2	#1	CCACCGCGTAGCAGAACTCG	Cas9	97	0-0- 1-4- 19
xg1–3	#1	ACGCCGGTGCTGCTGAGCGT	Cas9	97	0-0- 1-1- 40
xg6–1	#6	GGGGAAGAGGCCGATGAGGC	Cas9	85	0-0- 2-17- 270
cr2–1	#2	ACTTTGCGGCCGACTTCATTG	Cas12a	5	0-0- 0-0- 1
cr4–1	#4	TCTGCAACATGCTCTTCTTCA	Cas12a	22	0-0- 0-0- 8
cr6–1	#6	TGTTCGGCCTGGGCACCGGCC	Cas12a	5	0-0- 0-0- 2
cr6–2	#6	GCGTGATGTCGAGCACGCTGT	Cas12a	12	0-0- 0-0- 2

Note: the structure of the oligo names is as follows: xg indicates sequence for single-guide (sg)RNA or duplex-guide (dg)RNA for Cas9, and cr indicates CRISPR RNA for Cas12a. The number following the prefix refers to the exon targeted by the guide. The number following the hyphen indicates the specific guide variant for that exon. Scores are based on the CFD specificity score (a value of 0 indicates no predicted off-target activity whereas a value of 100 indicates a perfect match, higher scores indicate a higher likelihood of successful and specific DNA targeting) obtained from the CRISPOR analysis (http://crispor.org; Concordet and Haeussler, 2018). Based on this same tool, in the last column, for each number of mismatches, the number of off-targets is indicated. As an example: 1-3-20-50-60 means 1 off-target with 0 mismatches, 3 off-targets with 1 mismatch, 20 off-targets with 2 mismatches, etc.

the NOFIMA laboratory. Eggs were fertilized with sperm in a petri dish by swirling, adding filtered (0.2 μM mesh) natural seawater and kept for 4 min at 6 °C. Afterwards, the eggs were rinsed with filtered natural seawater and incubated for 15–30 min before microinjection.

Adult zebrafish (*Danio rerio*) stocks (Centre for Molecular Medicine Norway, Oslo, Norway) were kept at standard aquaculture conditions (i.e., 28.5 °C). Fertilized eggs were collected via natural spawning. Zebrafish embryos were reared under constant light conditions in embryo medium (1.5 mM HEPES, pH 7.6, 17.4 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4$, 0.18 mM $Ca(NO_3)_2$, and 0.6 μM methylene blue). All embryos and larvae were kept in an incubator, at 28.5 °C.

2.7. Microinjection procedures and screening of albino-like larvae

Glass capillaries (Narishige, G-100) were pulled using a micropipette puller (Narishige, PC-100) and subsequently beveled with a micropipette beveler (Narishige, EG-400) to make microneedles. To standardize the microinjection protocol, 1-cell stage zebrafish and fertilized Atlantic cod eggs were microinjected with 1.5 nL of EGFP mRNA (150 pg). The zebrafish embryos were kept at 28 °C and Atlantic cod embryos at 6 ± 2 °C until hatching. Cas9 mRNA (300 ng/μL) and either duplex or single gRNA(s) (100 ng/μL) mixtures, or 20 μM preformed Cas9 (or Cas12a): gRNA RNP complex solutions were injected into the fertilized cod eggs using the FemtoJet® 4i microinjector (Eppendorf) under a stereomicroscope (Motic, SMZ168). The injected volume was approximately 1.5 nL. After microinjection, Atlantic cod eggs were kept at 6 ± 2 °C through the hatching period until larvae were harvested shortly prior to the onset of the feeding stage. Zebrafish and cod larvae were screened for fluorescence and images were taken using a Leica M205 FA stereomicroscope. The Atlantic cod larvae microinjected with the different treatments were screened for albino-like phenotype under a stereomicroscope and images were taken with an Euromex 4 K Sony Ultra HD camera and analyzed using the Euromex ImageFocus Alpha. Selected larvae were euthanized with an overdose of MS-222, preserved in RNAlater® (Invitrogen™) and stored at −80 °C until further analysis.

2.8. Larvae DNA extraction and sequencing

DNA was extracted from individual larvae that exhibited an albino-like phenotype and from control larvae. The larvae were rinsed with PBS to remove excess RNAlater and DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen, 69,506) and eluted in 30 μL. PCRs to amplify the targeted exons were performed with 2.5 μL of the extracted DNA as template in a 50 μL-reaction with Phusion® High-Fidelity PCR Master Mix with HF Buffer (NEB, M0531S) and 2.5 μL of each primer (10 μM) listed in Table 2 with the following PCR settings: initial denaturation at 98 °C for 30 s; 35 cycles of denaturation at 98 °C for 15 s, annealing at 68 °C for 30 s, and extension at 72 °C for 15 s; final extension at 72 °C for 5 min; hold at 4 °C. Resultant amplicons were purified by gel electrophoresis with the QIAquick Gel Extraction Kit (Qiagen, 28,704) following the manufacturer's instructions. The forward primer from each unique PCR primer set was used to sequence the PCR amplicons. Sanger sequencing for each sample was conducted using the TubeSeq service provided by Eurofins Genomics, in accordance with the provider's instructions for sample preparation.

2.9. Assessment of CRISPR edits

The Sanger sequencing output files (.ab1 extension) for the forward strand of each selected larva were uploaded to Synthego's ICE (https://ice.synthego.com; Conant et al., 2022) and DECODR (https://decodr.org/analyze; Bloh et al., 2021) web-based tools for potential insertion and deletion (indel) analysis. Sequences derived from control group larvae were included as a wild-type template for comparison. The sequences obtained from wild type larvae were validated by aligning them to sequences retrieved from the Atlantic cod genome available in the NCBI database.

3. Results

3.1. Microinjection of zebrafish and Atlantic cod eggs with mRNA encoding enhanced green fluorescent protein

In order to evaluate the efficacy of the translation of injected mRNAs

Table 2
List of PCR primers used for genomic amplification of targeted regions.

Exon	Forward primer	Reverse primer	Product size
Exon 1	TGCTAGTGGAAGACCAGTCC	GTTGAGGAAGAGGCTGATGC	446 bp
Exon 2	TGTGTGTCTTGTCTTGCAGC	TGTGTGTCTTGTCTTGCAGC	304 bp
Exon 4	CATGCCTGGTGTTGTCTCCA	ACATACGTGCACGGAACTCG	597 bp
Exon 6	CTCATTAGCTCACGGTTCGC	GGCGAAGGAGACCACTCCAA	405 bp

in the fertilized egg as well as the microinjection skills and technical set up, in vitro transcribed EGFP mRNA was microinjected in zebrafish at the 1–2 cell stage and Atlantic cod eggs at the same stage or earlier and followed up until they were about to hatch. All injected zebrafish eggs expressed EGFP in the animal pole of the eggs five- and 48-h post fertilization (Fig. 1A&1B). For zebrafish, there was no appreciable loss of individuals during the follow-up period, which was until before the required feeding stage of larva. Microinjection of Atlantic cod eggs at 1–2 cell stage was difficult due to the hardness of the chorion. To circumvent this, we began the microinjection process prior to the appearance of the first cell. Furthermore, we noted that survival was poor compared with microinjected zebrafish eggs by the same researcher. Nevertheless, 22% of the survivors from the injected Atlantic cod eggs clearly expressed EGFP stably up to 20 days post-fertilization (Fig. 1C&1D).

3.2. Microinjection of Atlantic cod eggs with Cas9/Cas12a and guide RNAs

Four different gRNAs for both Cas9 and Cas12a were tested individually, or all combined in a single mix. For Cas9, both single guide (sg) RNAs and duplex crRNA:tracrRNA (dg) guides with identical target specificities were tested, i.e., 10 combinations of gRNAs were used. Furthermore, Cas9 gRNAs were tested by co-injection with mRNA encoding Cas9 protein as well as injection of preformed RNP complexes made from Cas9 protein and gRNAs. For preformed RNP complexes, we tested all sg RNAs as well as dg1–1 RNAs and the combination with all four tracers (dgAll). Cas12a guides were only tested as preformed RNP complexes with Cas12a, i.e., five combinations in total (Table 1).

A total of 8356 fertilized Atlantic cod eggs were microinjected in this study. Very high mortalities were recorded in all the microinjected groups. The overall mortality was 96.3% and some groups showed 100% mortality (Table 3). Mortality in the (non-injected) control groups of fertilized eggs was 18.4%, so high mortality must be related to the microinjection process itself. Additionally, given that fertilized Atlantic cod eggs were injected prior to the 1–2 cell stage, some injected eggs were likely not fertilized. This observation is based on the fact that 16% of the eggs from the non-microinjected control groups did not appear to be fertilized (data not shown).

3.3. Albino-like phenotypes and knockouts

Among the 305 microinjected eggs that survived until the end of the experiment, 15 specimens demonstrated an evident albino-like phenotype (Table 3; Fig. 2A&2B). Of the 22 treatment groups, only four produced albino-like larvae. Of these four groups, three were injected with

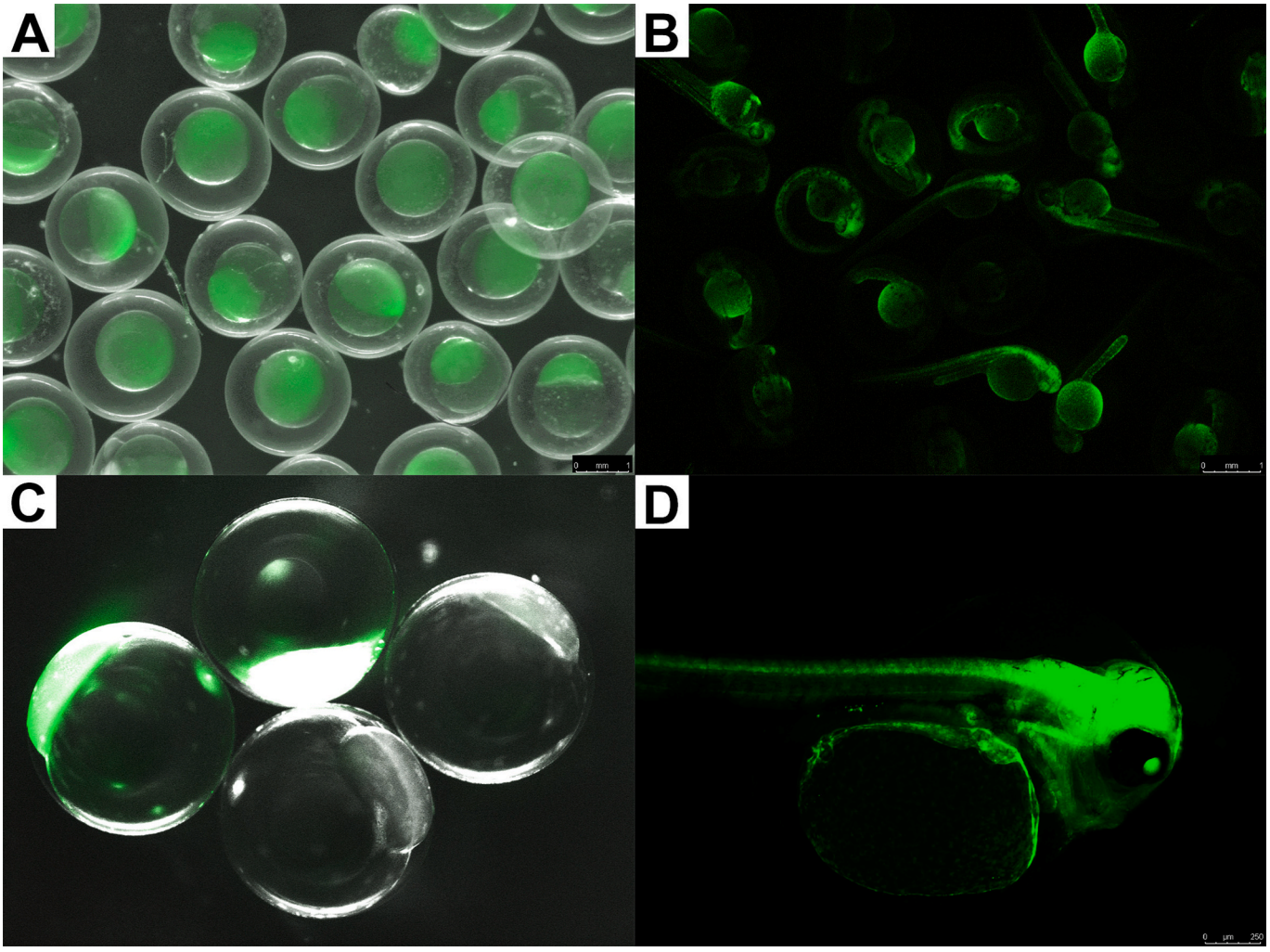

Fig. 1. Microinjection of EGFP mRNA into zebrafish and Atlantic cod fertilized eggs. A) Zebrafish embryos EGFP positive 5 h post-fertilization (hpf). B) Zebrafish embryos EGFP positive 48 hpf. C) Atlantic cod embryos, 36 hpf. Arrows indicate EGFP positive embryos. D) Atlantic cod larva, 20 days post-fertilization. Images in panels A and C are merged bright-field and green fluorescence channels. Images in panels B and D were taken only with green fluorescence channel. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Treatments used and treatment outcome in the present study. Treartments: "M-" denotes Cas9 presented as mRNA and "P-" denotes Cas9 presented as preformed ribonucleoprotein complexes (RNP) with either duplex guide (dg)RNA or single guide (sg)RNA. "12a" denotes Cas12a injected as preformed RNP with crRNA guides. "Total" denotes sum of all injected groups. See Table 1 for details of the guide RNAs. Treatment conditions that resulted in phenotypically albino-like larvae are highlighted in bold. * Eggs in control group were not microinjected. NA, Not Applicable.

Treatments	Microinjected eggs	Survivors	Albino-like	Survival	Albino-like survivors	Overall efficiency
Control	1549*	1264	NA	81.6%	NA	NA
M–dg1–1	261	1	0	0.4%	0%	0.00%
M-dg1–2	**289**	**30**	**1**	**10.4%**	**3%**	**0.35%**
M-dg1–3	291	2	0	0.7%	0%	0.00%
M-dg6–1	223	14	0	6.3%	0%	0.00%
M-dgAll	**660**	**8**	**6**	**1.2%**	**75%**	**0.91%**
M-sg1–1	**312**	**18**	**6**	**5.8%**	**33%**	**1.92%**
M-sg1–2	211	2	0	0.9%	0%	0.00%
M-sg1–3	190	3	0	1.6%	0%	0.00%
M-sg6–1	195	33	0	16.9%	0%	0.00%
M-sgAll	546	0	NA	0.0%	NA	NA
P-dg1–1	415	0	NA	0.0%	NA	NA
P-dgAll	265	5	0	1.9%	0%	0.00%
P-sg1–1	241	4	0	1.7%	0%	0.00%
P-sg1–2	680	9	0	1.3%	0%	0.00%
P-sg1–3	513	25	0	4.9%	0%	0.00%
P-sg6–1	461	4	0	0.9%	0%	0.00%
P-sgAll	732	76	0	10.4%	0%	0.00%
12a-cr6–1	378	18	0	4.8%	0%	0.00%
12a-cr6–2	325	5	0	1.5%	0%	0.00%
12a-cr2–1	403	8	0	2.0%	0%	0.00%
12a-cr4–1	78	25	0	32.1%	0%	0.00%
12a-All	**687**	**15**	**2**	**2.2%**	**13%**	**0.29%**
Total	***8356***	***305***	***15***	***3.7%***	***5%***	***0.18%***

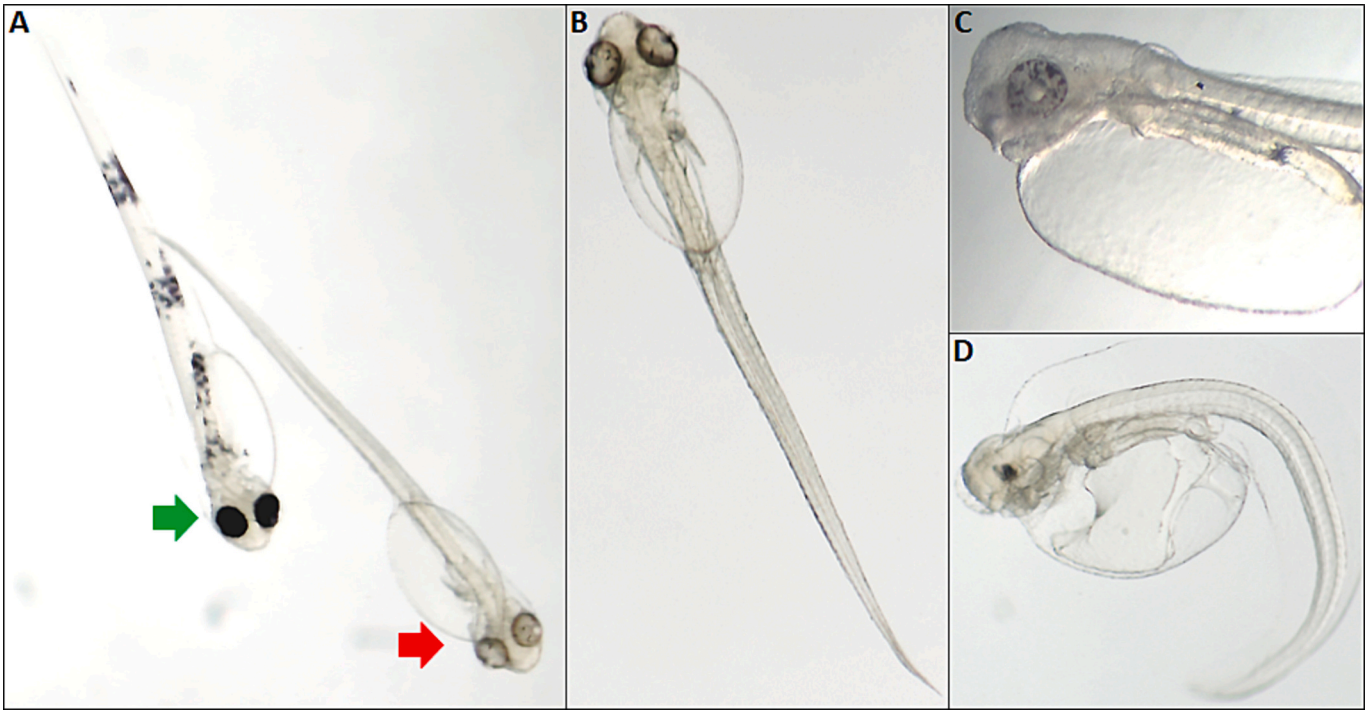

Fig. 2. Variations in phenotypes of albino-like Atlantic cod larvae. A) Atlantic cod larvae exhibit the normal pigmentation phenotype (green arrow) and the albino-like phenotype (red arrow). B) Close-up of a different Atlantic cod larva with the albino-like phenotype. C) Albino-like Atlantic cod larva featuring mosaicism with scattered pigmented cells in both the eye and the body. D) Albino-like Atlantic cod larva exhibiting deformities. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Cas9 mRNA and gRNAs, and one with Cas12a protein and gRNAs. No pigmentation changes were observed in embryos injected with pre-formed Cas9 gRNA RNP complexes. The most albino-like individuals were found in treatment groups "Cas9 mRNA-dgAll" and "Cas9 mRNA-sg1–1", both yielding 6 individuals with this phenotype. In the former group, 6 of 8 surviving larvae were albino-like, while in the latter group 6 of 18 had this phenotype. Thus, 75% and 33% of surviving larvae in these groups, respectively, showed an albino-like phenotype. However, 660 eggs were injected in group "Cas9 mRNA-dgAll" and 312 eggs in group "Cas9 mRNA-sg1–1", so the overall efficiency of albino-like phenotype obtained per egg injected was only 0.9% and 1.9%, respectively (Table 3).

To assess the CRISPR edits at the DNA level, we extracted DNA from all larvae with albino-like phenotype and some control larvae. PCR amplified the region of interest and subjected it to Sanger sequencing (Fig. 3). While mosaicism is a typical occurrence in the F0 generation and may manifest genetically rather than phenotypically, we opted to sequence only the specimens with reduced pigmentation, instead of sequencing all surviving microinjected embryos from the different treatments. All larvae that were selected for displaying albino-like phenotype showed DNA mosaicism at the target site in the *slc45a2* gene with a notable variation in indel frequencies among larvae within the same group and between distinct groups. Embryos from the "Cas9 mRNA-sg1–1" group displayed the highest indel percentage based on analyses with the ICE tool, ranging from 59% to 95% (Fig. 3; Table 4). The CRISPR edits from the "Cas9 mRNA-dgAll" group displayed lower frequencies of indels in exon 6 as well as in exon 1. The albino-like larvae treated with Cas12a and all four guide RNAs (Cas12a-crAll) showed a high level of indels. These larvae were analyzed with the DECODR website tool since the ICE website tool does not support analysis of sequences targeted with Cas12a.

4. Discussion

In this study, we established a protocol for CRISPR/Cas9 and CRISPR/Cas12a genome-editing systems in Atlantic cod. We first validated the effective production and viability of *in vitro*-transcribed mRNA and microinjection settings using zebrafish and Atlantic cod embryos. This *in vitro*-transcribed EGFP mRNA yielded EGFP protein expressed shortly after injections and present for a relatively long period. Fluorescence was still evident at the time the experiment was terminated, before the feeding stage, and at both high (28.5 °C, zebrafish) and low temperatures (6 °C, Atlantic cod). This gave us the opportunity to standardize the microinjection settings as well as to evaluate the procedure itself before using the actual CRISPR/Cas9 and CRISPR/Cas12a systems in Atlantic cod fertilized eggs. These conditions are particularly important to establish in advance to decrease the negative impact that technical challenges intrinsic to the targeted species can have on the efficacy of gene editing. For instance, Atlantic cod breeding is seasonal, and the quality of the eggs and sperm varies over the season, impacting the efficiency of fertilization throughout the season. Therefore, these are limiting factors in the sense that embryos cannot be obtained year-round and with efficiency outcomes varying through the season. Following fertilization, zebrafish eggs were microinjected at 1–2 cell stages, a procedure that is very well established in this species. In contrast, in Atlantic cod, due to the rapid initiation of chorion hardness, eggs were microinjected right after fertilization and before they reached the 1-cell stage.

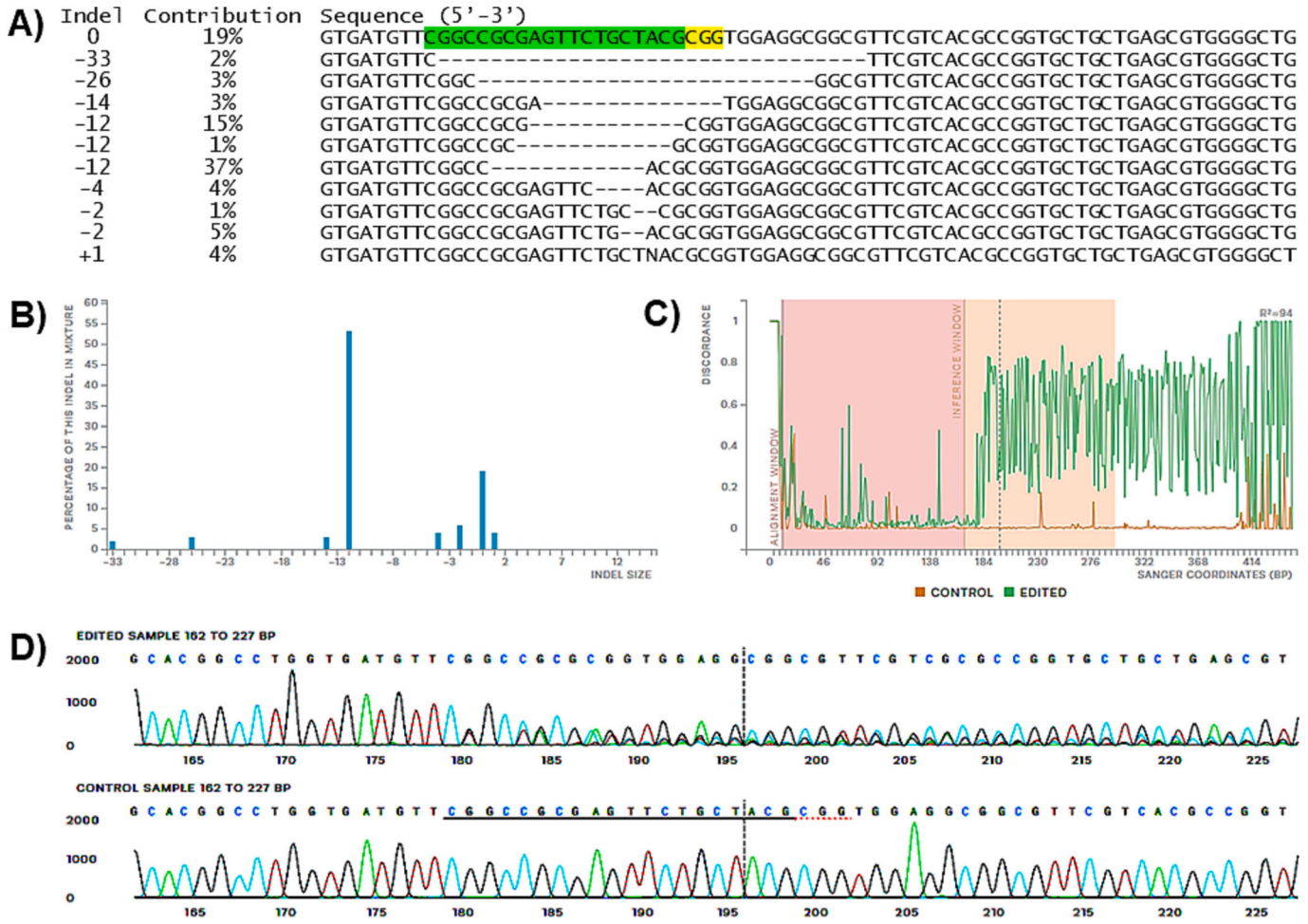

Fig. 3. A representation of the outputs from the ICE (Inference of CRISPR Edits) software for a guide RNA targeting the *slc45a2* gene in an Atlantic cod larva. (A) Sanger sequencing revealed indels in the Atlantic cod larva (ID = A1) treated with Cas9 mRNA-sg1–1. The target region is shown in green and the PAM site in yellow. (B) The inferred distribution and percentage of indels within the target region. (C) Discordance between the edited (in green) and control (in orange) trace files, illustrating the location and prevalence of editing. (D) Sanger sequencing traces after PCR of both edited and wild-type (control) Atlantic cod larvae in the region surrounding the guide sequence. The guide sequence is highlighted with a black underline, the PAM site with a red dotted underline, and the expected cut site is indicated by a vertical dotted line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Indel percentages (percentage of recovered genotypes from whole larvae with indel mutation) at different *slc45a2* exons in larvae subjected to different CRISPR/Cas9 and CRISPR/Cas12a treatments. "Larva ID" is the unique identifier for each individual larva within a treatment group. Percentage of indels (insertions or deletions) in the respective exon of the *slc45a2* gene based on analysis performed with the ICE website tool. (–) data were not applicable for the respective exon in the given larva. (*) Specimens were analyzed with DECODR because ICE is not suitable to analyze sequences targeted by Cas12a.

Treatment	Larva ID	Exon 1 indel %	Exon 2 indel %	Exon 4 indel %	Exon 6 indel %
Cas9 mRNA-sg1–1	A1	75	–	–	–
	B1	93	–	–	–
	C1	86	–	–	–
	C2	32	–	–	–
	D1	95	–	–	–
	E1	59	–	–	–
Cas9 mRNA-dg1–2	G11	3	–	–	–
Cas9 mRNA-dgAll	A3	3	–	–	28
	B3	0	–	–	28
	C3	2	–	–	28
	F3	2	–	–	35
	G3	0	–	–	38
	H3	1	–	–	31
Cas12a-All	G12*	–	0	98	0
	H12*	–	0	98	22

To our knowledge, in this study we report the first targeted gene knockout in Atlantic cod using the CRISPR technology. Thus Atlantic cod joins an expanding list of fish species successfully edited with this technology, which includes zebrafish (*Danio rerio*) (Hwang et al., 2013), medaka (*Oryzias latipes*) (Ansai and Kinoshita, 2014), tilapia (*Oreochromis niloticus*) (Li et al., 2014), Atlantic salmon (*Salmo salar*) (Edvardsen et al., 2014), channel catfish (*Ictalurus punctatus*) (Coogan et al., 2022), Goldfish (*Carassius auratus*) (Kuang et al., 2023), and common carp (*Cyprinus carpio*) (Zhong et al., 2016). Our approach entailed microinjection of single or duplex guide RNAs with endonuclease protein (Cas9 or Cas12a) or mRNA encoding Cas9, in each instance targeting the *slc45a2* gene, into Atlantic cod fertilized eggs. This approach generated 15 phenotypically apparent *slc45a2* knockout F0 larvae, which were mutated at different frequencies by insertions and deletions resulting in frameshifts. A known correlation exists between the embryonic development stage and genome editing outcomes (Liang et al., 2023; Zhang et al., 2023). Mosaicism is associated with stochastic editing at different embryonic stages resulting in individuals where not all cells carry the intended edit. Editing at the zygote stage may yield uniform edits across cells, while later-stage editing may lead to pronounced mosaicism. Although we injected Atlantic cod eggs shortly after fertilization, we observed considerable mosaicism, perhaps related to slower action of gRNA-Cas9 at lower temperature. Mortality of injected eggs was the main obstacle to obtaining a high overall efficiency, which was only 0.9% and 1.9% in the two best groups. However, the efficiency of gRNA-mediated DNA targeting was much higher in both groups as 75% and 33% of surviving larvae displayed an albino-like phenotype. The knockout efficiency (percentage of survivors with the desired gene modification) was therefore similar to that reported for other fish species such as zebrafish (0–100%; Hwang et al., 2013), medaka (44.8–100%; Ansai and Kinoshita, 2014), tilapia (24–50%; Li et al., 2014), Atlantic salmon (22–40%; Edvardsen et al., 2014), and common carp (16.6–55%; Shahi et al., 2022). Further efforts should emphasize the optimization of factors related to the high mortality including chorion softening, timing of the microinjection after fertilization, chemical, or antimicrobial saltwater treatments. Increasing the concentration of the Cas9/Cas12a and gRNA has been shown to increase the frequency of both specific targeting and off-target effects (Elaswad et al., 2018). We based the injected doses on standard protocols from zebrafish, accounting for the larger size of Atlantic cod eggs and did not explore the effect of concentration across multiple treatments. Furthermore, we targeted the *slc42a* gene as proof-of-principle and not for functional experiments with targeted fish and did not analyze off-target effects as these depend heavily on the sequence of the gRNA itself. Improving each of these variables might increase the survival and ultimately the overall efficiency of this system.

An important difference between the groups microinjected with Cas9 protein and Cas9 mRNA was observed. While the groups treated with Cas9 protein did not effectively produce any albino-like Atlantic cod larvae, those treated with Cas9 mRNA yielded the highest number of albino-like specimens. In general, each delivery format comes with its own set of advantages and disadvantages in terms of overall effectiveness and present unique challenges for successful delivery (Glass et al., 2018). It is important to note that Cas9 protein is immediately active from the time it is delivered. However, its activity might be more short-lived compared to Cas9 produced from mRNA, which has to be translated into protein but may nevertheless result in a longer window of Cas9 activity. This prolonged activity could increase the chances of successful gene editing, which may explain the higher number of albino-like specimens in the Cas9 mRNA group.

Targeting the first exon of the *slc45a2* gene with Cas9 mRNA-sg1–1 resulted in the highest indel levels in larvae with albino-like phenotype. Interestingly, although the Cas9 mRNA-dgAll combination resulted in a higher proportion of albino-like survivors than Cas9 mRNA-sg1–1 (75% vs. 33%), the percentage of indels in the former group was lower than that of the latter. While these findings are not conclusive in determining which method is superior due to the small sample size, both approaches can be utilized. However, it is important to consider that using fewer gRNAs can help decrease off-target effects.

While most of the larvae processed for DNA sequencing exhibited an albino-like phenotype, there were also larvae showing varying degrees of mosaicism, as well as a few with deformities that were not genotyped (Fig. 2C&2D). In this sense, our study took a more conservative approach by focusing solely on those exhibiting the albino-like phenotype. Considering that both alleles of *slc45a2* must be non-functional to obtain the albino-like phenotype, it is possible that other larvae not displaying this phenotype carried mutations that could have been passed on to their offspring to produce albino-like phenotypes, as has been described in previous studies (Wu et al., 2018). Future efforts to establish a genetic line by selecting F0 specimens should expand the screening to include all surviving specimens. Ideally, attempts should be made to obtain and screen F1 progeny. Unfortunately, the challenge lies in the fact that Atlantic cod only reaches sexual maturity at around 3 years of age, making it impractical for this purpose. In this sense, the CRISPR genome editing system provides an efficient strategy for gene knockout in F0, enhancing productivity and efficiency by reducing the time and resources required to breed mutant alleles to homozygosity for experimental purposes. This method proves to be particularly beneficial for animal models with extended periods to sexual maturity, seasonal breeding, or short breeding season, where breeding to homozygosity is unfeasible or challenging.

5. Conclusion

As aquaculture rapidly emerges as the primary source of fish for human consumption, it also presents new challenges that need to be addressed by different approaches. The increasing accessibility and affordability of CRISPR genome-editing technologies makes it one of the more important tools to address some of these challenges in an efficient and effective fashion. For a species of considerable commercial and ecological importance in the North Atlantic such as Atlantic cod, despite the modest efficiency shown in this study, the outcomes here described represent a significant milestone in applying new technologies to study this species. Our results contribute to the existing and expanding body of knowledge on applying genome-editing technologies in non-model organisms and open new avenues for future comparative studies to optimize CRISPR/Cas9 efficiency in Atlantic cod and other fish species. This

technology opens up an avenue to investigate physiology, immunology, and genetics of Atlantic cod at the cellular and molecular level.

CRediT authorship contribution statement

Adrián López-Porras: Conceptualization, Methodology, Writing – original draft, Visualization, Investigation. **Ragnhild Stenberg Berg:** Investigation, Visualization. **Erik Burgerhout:** Resources, Funding acquisition. **Øyvind J. Hansen:** Funding acquisition. **Ádám Györkei:** Conceptualization. **Shuo-Wang Qiao:** Conceptualization, Writing – review & editing. **Finn-Eirik Johansen:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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