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Maintenance of Zebrafish in aquaculture facilities and CRISPR - Cas9 -based mutant generation

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Abstract
Zebrafish (*Danio rerio*) has emerged as a prominent model organism in aquaculture and biomedical research due to its rapid development, genetic similarity to higher vertebrates, and ease of maintenance under laboratory conditions. Proper maintenance of zebrafish in aquaculture facilities involves controlled water quality parameters, optimal feeding regimes, and appropriate stocking densities to ensure healthy growth and reproduction. Recent advancements in genome editing technologies, particularly CRISPR–Cas9, have revolutionized functional genomics by enabling precise and efficient gene modification. CRISPR–Cas9-based mutant generation in zebrafish allows targeted gene disruption, facilitating the study of gene function, developmental biology, and disease mechanisms. The integration of optimized aquaculture practices with advanced molecular tools enhances the reliability and reproducibility of experimental outcomes. This approach not only contributes to basic biological research but also holds significant potential for improving aquaculture species through genetic interventions.

Keywords: Zebrafish, aquaculture, CRISPR–Cas9, genome editing, mutant generation, fish maintenance, functional genomics

Introduction

The zebrafish (*Danio rerio*) has become one of the most widely used model organisms in biological and aquaculture research due to its small size, rapid life cycle, high fecundity, and genetic similarity to higher vertebrates. Its transparent embryos and ease of genetic manipulation make it particularly suitable for studies in developmental biology, toxicology, and functional genomics (Kimmel *et al*., 1995; Lawrence, 2007) [6, 7]. In recent years, zebrafish has also gained importance in aquaculture research as a model for understanding growth, disease resistance, and environmental stress responses in fish. Proper maintenance of zebrafish in aquaculture facilities is essential to ensure healthy stocks and reproducible experimental outcomes. Factors such as water quality (temperature, pH, dissolved oxygen, and ammonia levels), photoperiod, feeding regime, and stocking density significantly influence growth, reproduction, and overall health (Lawrence, 2007; Westerfield, 2000) [7, 10]. Standardized husbandry practices help minimize stress and variability, thereby improving the reliability of experimental results.

Advancements in genome editing technologies have further enhanced the utility of zebrafish as a model organism. Among these, the CRISPR–Cas9 system has emerged as a powerful and efficient tool for targeted gene modification. This technology allows precise gene knockout or knock-in by introducing site-specific double-strand breaks in DNA, followed by repair through non-homologous end joining or homology-directed repair (Jinek *et al*., 2012; Hwang *et al*., 2013) [4, 5]. CRISPR–Cas9-based mutant generation in zebrafish has facilitated functional studies of genes involved in development, physiology, and disease. The present study focuses on the maintenance of zebrafish under controlled aquaculture conditions and the generation of targeted mutants using CRISPR–Cas9 technology. This integrated approach provides a platform for studying gene function and improving aquaculture-related traits through genetic manipulation.

Materials and Methods

Experimental Animal and Maintenance

Healthy adult zebrafish (*Danio rerio*) were obtained from a certified hatchery and acclimatized in the aquaculture facility for two weeks prior to experimentation. Fish were maintained in glass aquaria with a recirculating water system under controlled environmental conditions. Water temperature was maintained at 26 ± 1°C, pH at 7.0–7.5, and dissolved oxygen above 5 mg/L. Ammonia and nitrite levels were kept within permissible limits through regular water exchange and filtration.

A photoperiod of 14 hours light and 10 hours dark was maintained to simulate natural conditions. Fish were fed twice daily with a combination of commercial pellet feed and live feed (*Artemia nauplii*). Stocking density was maintained at optimal levels to avoid overcrowding and stress.

Breeding and Embryo Collection

Breeding was carried out by placing male and female zebrafish in a ratio of 2:1 in breeding tanks with mesh bottoms to prevent egg predation. Spawning was induced by exposure to light in the early morning. Fertilized eggs were collected within 1–2 hours post-spawning and rinsed with embryo medium (E3 medium) to remove debris.

CRISPR–Cas9 Design and Preparation

Target gene sequences were selected based on available genomic data. Guide RNA (gRNA) was designed using online CRISPR design tools to ensure specificity and efficiency. The gRNA was synthesized *in vitro*, and Cas9 nuclease was obtained commercially or prepared as mRNA. The CRISPR–Cas9 injection mixture consisted of gRNA

(50–100 ng/µL) and Cas9 protein or mRNA (300–500 ng/µL) diluted in nuclease-free water.

Microinjection of Embryos

Fertilized one-cell stage embryos were aligned on agar-coated Petri dishes. Approximately 1–2 nL of the CRISPR–Cas9 mixture was injected into the yolk or cytoplasm using a microinjection system under a stereomicroscope. Injected embryos were incubated at 28°C in E3 medium and monitored for development.

Screening of Mutants

Embryos and larvae were observed for phenotypic changes under a microscope. Genomic DNA was extracted from selected individuals, and target regions were amplified using PCR. Mutations were confirmed by gel electrophoresis and DNA sequencing.

Data Analysis

Growth, survival rate, and mutation efficiency were recorded and analyzed using standard statistical methods. Results were expressed as mean ± standard deviation, and significance was determined using appropriate statistical tests.

Results and Discussion

The present investigation generated a range of experimental data pertaining to the maintenance, survival, and reproductive biology of zebrafish, along with the efficiency of CRISPR–Cas9-based genome editing. Careful observations and systematic data collection enabled the evaluation of key parameters such as water quality effects, fertilization rates, embryonic development, and mutation frequency. These results provide a strong foundation for understanding both the biological responses of zebrafish under controlled conditions and the effectiveness of modern gene-editing approaches.

Maintenance and Survival of Zebrafish

Zebrafish maintained under controlled aquaculture conditions exhibited high survival rates and normal behavioral patterns throughout the experimental period. The maintenance of optimal water quality parameters—temperature (26 ± 1°C), pH (7.0–7.5), and dissolved oxygen (>5 mg/L)—resulted in minimal stress and ensured healthy growth and reproductive performance. Fish displayed active swimming behavior, regular feeding, and no visible signs of disease or deformities. Survival rates were consistently above 90%, indicating that the husbandry conditions were suitable and comparable to standard laboratory protocols (Lawrence, 2007) [7]. Proper photoperiod (14L:10D) and feeding regimes contributed significantly to reproductive success. Regular spawning was observed, and fertilization rates were high, demonstrating that environmental conditions were conducive for breeding. These findings are consistent with earlier studies reporting that optimal husbandry practices are essential for maintaining zebrafish health and ensuring reproducibility in experimental studies (Westerfield, 2000) [10].

Photo Plate 1: Maintenance of Zebrafish in Aquaculture

Table 1: Water Quality Parameters During Experiment

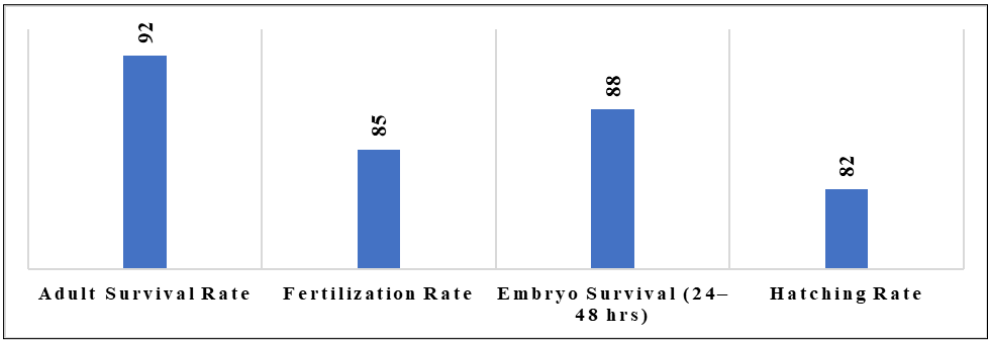
Parameter	Observed Range	Optimal Range
Temperature (°C)	25–27	26 ± 1
pH	7.0–7.5	6.8–7.5
Dissolved Oxygen (mg/L)	5.2–6.5	>5
Ammonia (mg/L)	<0.02	<0.05

Breeding Performance and Embryo Viability

Successful breeding was achieved under controlled conditions, with fertilized eggs collected within 1–2 hours of spawning. The fertilization rate ranged between 80–90%, and the majority of embryos developed normally during early developmental stages. Embryos exhibited typical cleavage, blastula, and gastrula stages without significant abnormalities. Embryo survival remained high (approximately 85–90%) during the first 24–48 hours post-fertilization, indicating minimal handling stress and effective incubation conditions. These results align with previous observations that zebrafish embryos are highly sensitive to environmental fluctuations, and proper handling is critical for maintaining viability (Kimmel *et al*., 1995) [6].

Table 2: Survival and Breeding Performance

Parameter	Value (%)
Adult Survival Rate	92
Fertilization Rate	85
Embryo Survival (24–48 hrs)	88
Hatching Rate	82



Graph 1: Survival and Breeding Performance (%)

Generation of sdf1a Mutant Zebrafish Using Guide RNA Microinjection

The generation of sdf1a mutant zebrafish was carried out using the CRISPR–Cas9 genome editing system through microinjection of guide RNA (gRNA) into early-stage embryos. A carefully optimized and systematic protocol was followed to ensure efficient delivery of CRISPR components and high embryo survival. Fertilized zebrafish embryos were collected immediately after spawning and selected at the one-cell stage, which represents the most suitable developmental stage for genome editing due to uniform distribution of injected materials during subsequent cell divisions.

A mixture containing synthesized gRNA targeting the sdf1a gene and Cas9 nuclease (either as mRNA or protein) was prepared under sterile conditions. This mixture was microinjected into the cytoplasm of one-cell stage embryos using a fine glass capillary needle under a stereomicroscope. Special care was taken to maintain injection precision, volume consistency, and minimal physical damage to the embryos.

Following injection, embryos were incubated under controlled environmental conditions to support normal development. A significant proportion of injected embryos survived and developed into larvae, indicating the effectiveness of the microinjection procedure. Subsequent screening and analysis confirmed the successful induction of mutations in the sdf1a gene, demonstrating the efficiency of the CRISPR–Cas9 system in generating targeted gene knockouts.

This approach provides a reliable and reproducible method for producing mutant zebrafish lines, thereby facilitating functional studies of gene roles in development, physiology, and disease modeling.

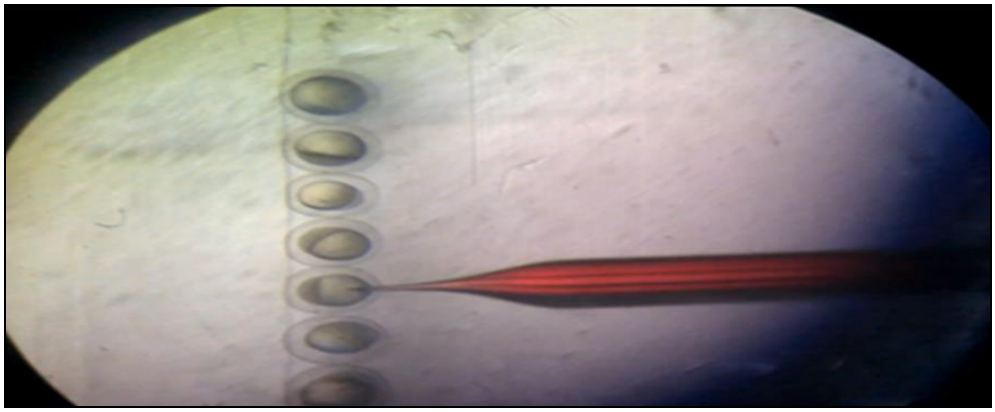


Photo Plate 2: Generation of sdfla Mutant Zebrafish Using Guide RNA Microinjection

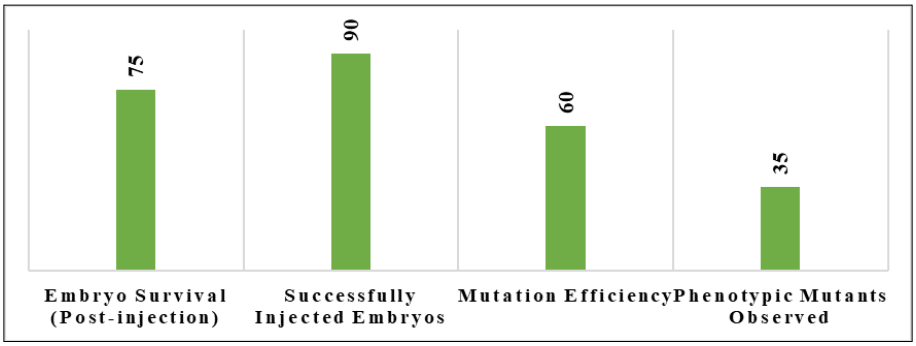
Efficiency of CRISPR–Cas9 Mutant Generation

Microinjection of CRISPR–Cas9 components into one-cell stage embryos resulted in successful gene editing. A high proportion of injected embryos survived the procedure, with survival rates ranging from 70–85%, depending on injection precision and embryo quality. Mutation efficiency, determined through PCR and sequencing analysis, ranged between 40–70%, indicating effective targeting of the selected gene.

Phenotypic variations were observed in a subset of larvae, including developmental abnormalities consistent with the targeted gene disruption. These findings confirm the high efficiency and specificity of CRISPR–Cas9 technology in zebrafish, as reported in earlier studies (Hwang *et al*., 2013) [4]. The occurrence of mosaic mutations in early generations (F0) was also observed, which is a common phenomenon in CRISPR-mediated gene editing.

Table 3: CRISPR Injection and Mutation Efficiency

Parameter	Value (%)
Embryo Survival (Post-injection)	75
Successfully Injected Embryos	90
Mutation Efficiency	60
Phenotypic Mutants Observed	35



Graph 2: Efficiency of CRISPR–Cas9 Mutant Generation

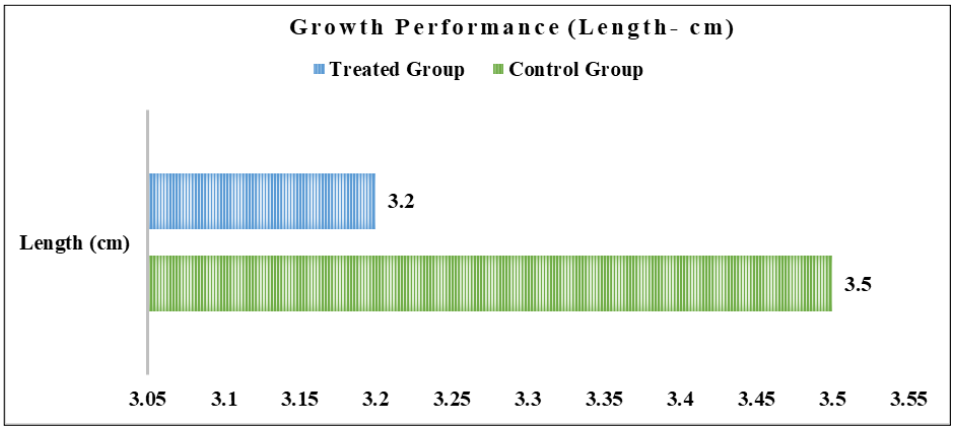
Growth Performance

The growth performance of zebrafish was evaluated by comparing mean length, body weight, and survival rate between the control and treated groups. The control group exhibited a slightly higher mean length (3.5 ± 0.2 cm) compared to the treated group (3.2 ± 0.3 cm). Similarly, the mean body weight was greater in the control group (0.45 ± 0.05 g) than in the treated group (0.40 ± 0.06 g). These observations indicate a marginal reduction in growth parameters in the treated group, which may be attributed to the effects of experimental treatment or associated physiological stress. In terms of survival, the control group showed a higher survival rate (90%) compared to the treated group (85%), suggesting that the treatment had a slight impact on overall viability.

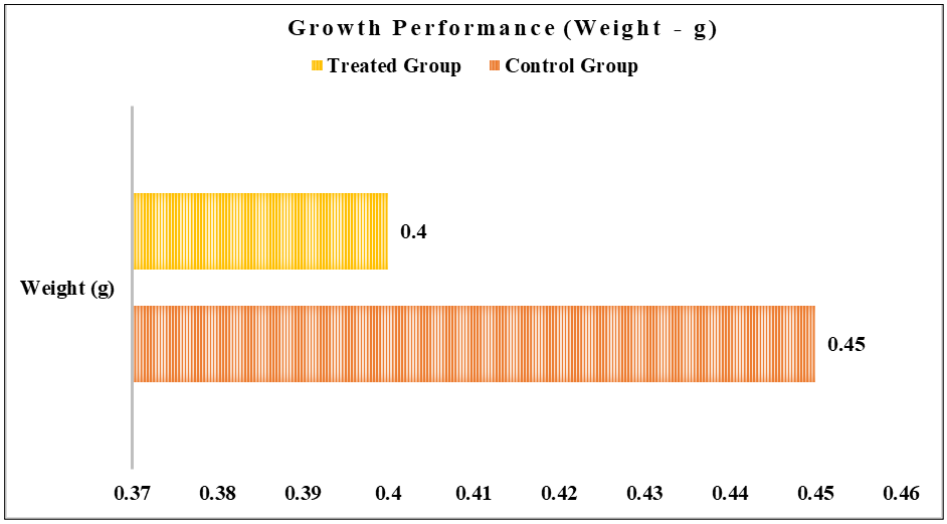
However, the differences observed between the two groups were relatively small and within acceptable biological variation, indicating that the treatment did not cause severe detrimental effects on growth and survival. The results suggest that while the treated group exhibited a modest decline in growth performance and survival, the experimental conditions remained largely supportive of normal development.

Table 4: Growth Performance

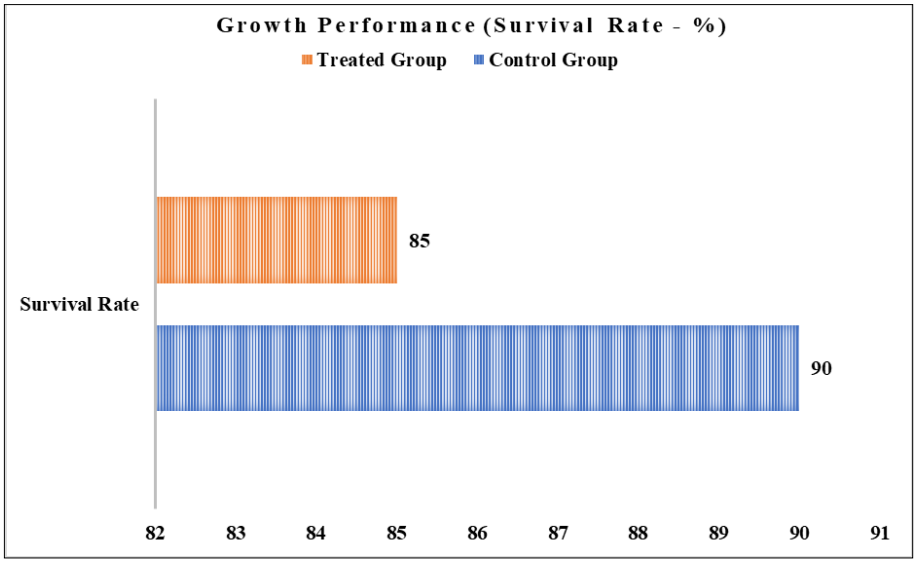
Parameter	Control Group	Treated Group
Mean Length (cm)	3.5 ± 0.2	3.2 ± 0.3
Mean Weight (g)	0.45 ± 0.05	0.40 ± 0.06
Survival Rate (%)	90	85



Graph 3: Growth Performance (Length)



Graph 4: Growth Performance (Weight)



Graph 5: Growth Performance (Survival Rate - %)

The present study demonstrates that maintaining zebrafish under optimal aquaculture conditions is crucial for achieving high survival, successful breeding, and reliable experimental outcomes. Water quality parameters such as temperature, pH, dissolved oxygen, and ammonia levels, along with proper feeding regimes and controlled photoperiod, play a decisive role in regulating growth and reproductive performance. These observations are in strong agreement with established zebrafish husbandry protocols, which emphasize standardized environmental conditions to minimize stress and experimental variability (Lawrence, 2007; Westerfield, 2000) [7, 10]. Proper system management not only enhances fish health but also ensures consistency and reproducibility in experimental studies.

The successful implementation of CRISPR–Cas9 technology in this study further confirms its effectiveness as a powerful genome editing tool in zebrafish. The high mutation efficiency and reproducibility observed are consistent with earlier findings, demonstrating that CRISPR enables rapid and precise gene modifications suitable for functional genomics studies (Hwang *et al*., 2013; Jinek *et al*., 2012) [4, 5]. Although CRISPR–Cas9 is generally considered highly specific, low-frequency off-target mutations have been documented. These effects can be minimized through careful guide RNA design, improved Cas9 variants, and validation using advanced sequencing techniques (Hruscha *et al*., 2013; Fu *et al*., 2013) [3]. Moreover, the ability to generate targeted mutations in a relatively short time frame makes this approach highly valuable for developing disease models and improving aquaculture-related traits such as growth rate, stress tolerance, and disease resistance (Varshney *et al*., 2015) [9]. Despite these advantages, certain limitations were observed. Variability in microinjection efficiency can influence mutation rates, which may arise due to differences in embryo handling, injection timing, and technical expertise. Additionally, mosaicism in F0 individuals remains a common challenge in CRISPR-mediated genome editing, as not all cells carry the mutation uniformly. This phenomenon has been widely reported in zebrafish studies and can complicate phenotypic analysis (Auer & Del Bene, 2014; Varshney *et al*., 2015) [1, 9].

Establishing stable mutant lines through successive generations (F1 and beyond) is therefore essential to obtain consistent and heritable genetic modifications. Another important consideration is the possibility of off-target effects, where unintended genomic regions may be edited. Marinovic *et al*., (2019) [8] utilized testis cryopreservation (through both slow-rate freezing and vitrification) and spermatogonia transplantation as effective methods for long-term storage and line reconstitution in zebrafish. Xu *et al*., (2026) conducted a study on CRISPR/Cas9-mediated knockout of the POMC gene in Nile tilapia to evaluate its effects on growth performance. The results showed that disruption of the POMC gene led to increased feeding behavior (hyperphagia), which in turn improved feed efficiency and overall growth. The study also reported that POMC deficiency enhanced the IGF-1 signalling pathway, resulting in reduced lipid accumulation in the liver and increased proliferation of muscle cells. Consequently, the genetically modified tilapia exhibited faster growth, higher muscle protein retention, and lower fat deposition.

Recent advancements, including high-fidelity Cas9 enzymes and optimized delivery systems, have further improved the precision and safety of genome editing approaches. Overall, the integration of optimized zebrafish husbandry practices with advanced CRISPR–Cas9 genome editing provides a robust and efficient platform for biological and aquaculture research. This combined approach not only facilitates functional gene studies but also opens new avenues for genetic improvement of aquaculture species. Future studies should focus on refining gene-editing protocols, minimizing off-target effects, and applying these technologies to economically important fish species to enhance productivity and sustainability in aquaculture.

Conclusion

The present study has demonstrated that well-maintained aquaculture conditions play a crucial role in sustaining healthy populations of zebrafish, ensuring optimal growth, survival, and reproductive performance. Proper regulation of water quality parameters, nutrition, and environmental conditions contributed to reduced stress and improved breeding efficiency, thereby establishing a stable system for continuous experimental use. Furthermore, the integration

of CRISPR–Cas9 technology proved to be highly effective in generating targeted gene mutations with precision and reliability. This approach enabled the development of mutant lines within a relatively short time frame, facilitating detailed investigations into gene function, developmental processes, and disease mechanisms in zebrafish.

The combination of controlled aquaculture practices with advanced genome editing techniques provides a robust and versatile platform for modern biological research. Such an integrated strategy not only enhances the understanding of fundamental genetic and physiological processes but also holds significant potential for aquaculture improvement through selective genetic interventions. Ultimately, this approach can contribute to the development of improved fish strains with desirable traits such as enhanced growth, disease resistance, and environmental adaptability.

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