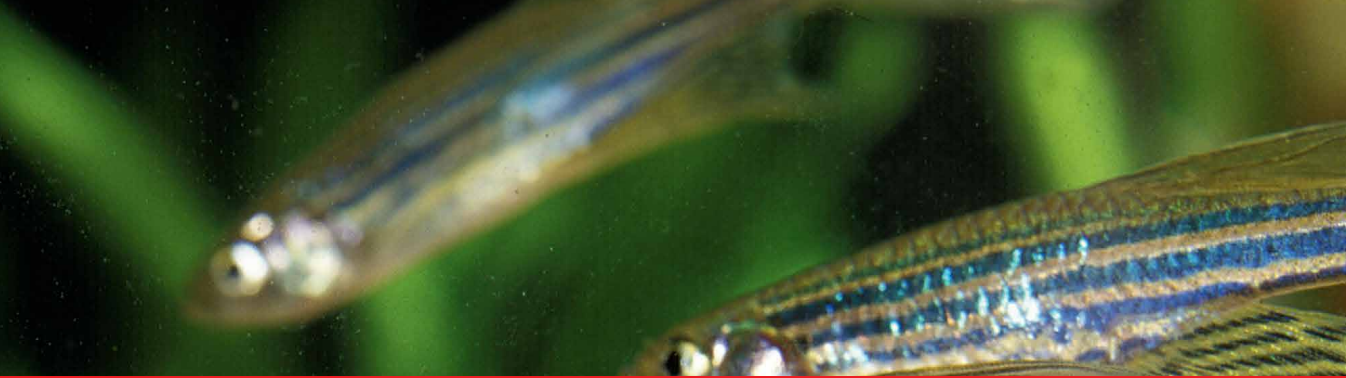




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Zebrafish Research

An Ever-Expanding Experimental Model

Edited by Geonildo Rodrigo Disner



Zebrafish Research - An Ever-Expanding Experimental Model

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Meet the editor



Geonildo Rodrigo Disner is a Brazilian biologist with a Ph.D. in Genetics and a postdoc in Applied Toxinology and Systems Toxicology. He collaborates with the Center of Toxins, Immune-Response and Cell Signaling (CeTICS/FAPESP) and the Butantan Institute's Zebrafish Platform, both in Brazil. In animal and alternative models, he has experience in the toxicity screening of chemicals of emerging concern, such as nanomaterials and pesticides, as well as drugs under development. In addition to researching the Natterin family of proteins and the anti-inflammatory peptide *TnP* discovered in the venom of the fish *Thalassophryne nattereri*, Dr. Disner studies the role of Aryl hydrocarbon receptor (AHR) in zebrafish immunological processes and immune response. He also works with (eco)toxicology at the Immunoregulation Unit of the Applied Toxinology Laboratory, Butantan Institute, São Paulo, Brazil.

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Preface

Throughout my research career, I have witnessed the prosperity of the *Danio rerio* (zebrafish) in science. My first direct contact with the zebrafish as an experimental model was around 2015 when I was working in an environmental mutagenesis lab and some peers used it for toxicity screening. I have been working with fish since I was an undergraduate biology student, not surprisingly, since fishing was something I remember doing with my father and family at an early age. Since I joined the Butantan Institute's Zebrafish Platform in Brazil, I have been fully dedicated to increasing the knowledge and use of zebrafish in toxicology, pharmacology, immunology, and biotechnology.

In recent years, zebrafish have been in the spotlight, driven by the utmost need for alternative animal models in science. The number of publications and research groups using zebrafish as the primary experimental model has increased considerably. That is why this book is so essential now; to celebrate and highlight zebrafish as a model that has come to stay.

Since I care a lot about the 3Rs of replacement, reduction, and refinement, I always advocate for science to push novel methodologies forward. I also seek laboratory testing and results to accurately foresee biological outcomes that produce safe predictions that could better translate to humans, improving health and well-being. This book is evidence of how advanced science can be, meeting experimental needs without losing ethical commitment. Moreover, the book presents cutting-edge research in different fields, portraying zebrafish as the ever-expanding model it is.

So, as zebrafish swim in schools, so do we; for such an endeavor, I could count on many incredible scientists who contributed to the success of this project. Since May 2023, when I was appointed academic editor, I have had the opportunity to access a set of significant chapter proposals that only confirmed how much this project is worth and is necessary.

The final version was organized to guide the reader through crucial topics such as laboratory animal care and management, which are fundamental for providing quality and healthy animals to be used in experiments. The first section is the introduction. In the second section of the book, the two chapters deal with the control of health status and disease control as well as colony management, especially when maintaining transgenic and mutant lineages.

The third section presents distinguished research seeking to provide up-to-date results that might be translated to humans, remarkably, to unveil novel mechanisms to treat human diseases or other biological phenomena shared among species.

Zebrafish not only render insights for medical research but also are a strong ally within the World Health Organization's One Health perspective. One Health can be

understood as an integrated approach to balancing and fine-tuning the health of people, animals, and the environment. That is why the book finishes with a notorious contribution of zebrafish to investigate environmental toxicology and, altogether, mounting a complete set of applications in which zebrafish can be applied.

This project would not have come to fruition without the chapter authors, whom I sincerely thank, and the IntechOpen publishing team, which worked very committedly, granting support to the academic editor and authors. Also, I want to thank the São Paulo Research Foundation for funding my research with this experimental model.

I genuinely hope that this book becomes a foundational reference for the zebrafish research community and that the readership can see the plethora of applications the zebrafish is able to yield, aware that they are comprehensive but not limited to the topics presented herein.

Finally, I hope this book inspires young researchers worldwide to continue pursuing high-quality science and stimulates them to consider zebrafish as the experimental model of choice. I wish you a pleasant read.

Kind regards,

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Butantan Institute,
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Section 1

Introduction

Chapter 1

Introductory Chapter: The Zebrafish Model is Pushing Science Forward

Geonildo Rodrigo Disner

1. Introduction

The use of *Danio rerio* (zebrafish) in science has a long track. It started in the 1970s and early 1980s, driven mainly by George Streisinger's (1927–1984) work. Streisinger was the founding father of zebrafish research while based at the University of Oregon, where he established a zebrafish research colony and developed the first practices for mutagenesis and mutation screening, aiming to study the development of the nervous system through molecular analysis. His fish clones stood among the primary successful vertebrate clones generated; altogether, those were the first steps that led to many novel applications around the globe. Interestingly, zebrafish had such a notorious impact on science that it is one of the few fish species to be part of a space mission to assess the effects of microgravity on their development.

Against this background, and notwithstanding the under-development research techniques still to be explored, the purpose of the present book is to pay homage to the zebrafish as such an exquisite alternative experimental model, celebrating all the significant achievements it has provided to the scientific community and what is yet to come.

2. From South Asia to the world and beyond

The zebrafish is a small freshwater fish native to South Asia, where it is naturally found in India, Bangladesh, Pakistan, and Nepal. The reason it is an extensively studied vertebrate model organism is due to the fact it can be bred rapidly in large numbers, fertilized eggs are transparent, and the embryo develops rapidly outside of the uterus, with precursors to all major organs taking shape within 36 hours of fertilization, making it attractive to study vertebrate development, physiology, and diseases.

Besides, over the last two decades, the zebrafish has joined the outstanding list of model organisms for biomedical research since it is amenable to genetics with a full suite of tools and genomic resources [1]. Just recently, the tenth anniversary of the publication of the complete zebrafish genome was celebrated. The zebrafish genome project kicked off at the Wellcome Trust Sanger Institute in 2001 and was completed in 2013 [2]. The sequencing provided evidence of more than 26,000 protein-coding genes, the most extensive set of any vertebrate sequenced so far. Additionally, the project demonstrated the high homology between zebrafish and humans, which share 70% genetic homology; perhaps more importantly, regarding genes that cause human diseases, around 84% of the zebrafish genes have a human counterpart.

The deep knowledge of the zebrafish genome has made it a powerful and versatile model organism, boosting its use in various fields of research, and contributing significantly to our understanding of fundamental biological processes and human health. Such advancements allowed thousands of mutants, transgenic lines, and a wealth of know-how techniques for embryological manipulation, drug discovery, among others. Genome sequencing, for example, is crucial for designing and implementing genetic manipulations, such as gene knock-in and knockout through CRISPR/Cas9 technology [3]. Understanding the genomic context is essential for precision targeting and accurately interpreting the outcomes of these manipulations. Moreover, it contributes to comparative genomics, biotechnological applications, and drug discovery. Zebrafish's genome sequencing bridges the gap between basic research and clinical applications, facilitating translational research. It provides insights into the genetic basis of diseases, enabling the development of novel diagnostics, therapies, and interventions, helping identify potential drug targets, and understanding the genetic factors influencing drug response and toxicity.

The zebrafish research advancements and information have been shared worldwide through several platforms such as the Zebrafish Information Network (ZFIN). ZFIN serves as a comprehensive and centralized resource for the zebrafish research community by providing essential information and tools for researchers studying *Danio rerio*. Also, it offers a user-friendly database encompassing curated data on zebrafish genes, mutants, transgenic lines, antibodies, and other molecular and phenotypic information. Moreover, it is well-known that using zebrafish as a model organism has substantially increased scientific publications over the years. Its popularity across disciplines has contributed to a growing body of literature, which might continue to expand.

3. New insights and research prospects from the zebrafish

Taken as a successful ever-expanding experimental model, zebrafish has already been instrumental in numerous scientific research; current investigations have still yielded advances in many significant fields. In cancer research, zebrafish models have played a fundamental part in studying cancer biology, including tumor initiation, progression, and metastasis. Researchers can also use it in drug screening assays to identify compounds with therapeutic potential [4]. The high-throughput nature of these screens allows for the rapid evaluation of a large number of potential drug candidates, including screening for potential anti-cancer drugs. Other areas benefiting from the gathered knowledge are neurological disorders, but not exclusively, where zebrafish are employed to study various aspects of neurobiology, including neural development, in addition to cardiovascular research and regeneration.

Notably, zebrafish's regenerative abilities in the heart make it particularly interesting for cardiac regeneration studies, in conjunction with the fact that zebrafish can serve as a regeneration model for lateral line hair cells during early-life stages, photoreceptor cells and retinal neurons, and tail fins after injury. Tail fin amputations work as a protocol to analyze their regrowth to test for the local inflammatory response and mutations. It was also demonstrated that histone demethylation occurs at the amputation site, transitioning the cells to a so-called active, regenerative, stem cell-like state [5]. The screening of gene expression during regeneration additionally helped to identifying critical signaling pathways which take part in the process, such as *Wnt* signaling and fibroblast growth factor [6].

Looking ahead, considerable advancements in zebrafish research are driven by the substantial demand for alternative models in animal experimentation. Zebrafish emerges as a pivotal link between in vitro and in vivo models, maintaining the complex features of a complete vertebrate organism. Zebrafish contribution continues to expand, underscoring its significance in experimental biology and advancing our understanding of fundamental biological processes and human health, fostering a promising trajectory for future scientific endeavors.

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Section 2

Zebrafish Care and Management

Managing the Welfare of Zebrafish in Laboratory: Overview of Prevailing Diseases and Health Issues

Monica Lopes-Ferreira, João Gabriel dos Santos da Rosa, Geonildo Rodrigo Disner and Carla Lima

Abstract

The zebrafish (*Danio rerio*) is a freshwater species native to South Asia belonging to the Cyprinidae family. Due to its easy housing and breeding, high fecundity, and rapid development, it has become a popular laboratory animal. Furthermore, zebrafish is an increasingly successful and widely used vertebrate model organism in scientific research, *e.g.*, in drug discovery, particularly preclinical development, mainly because of its immune response and well-known genome. Nevertheless, zebrafish health in laboratory facilities is crucial. Both the water quality and pathogens control are directly related to the zebrafish welfare, which, under inappropriate conditions, may confound experimental findings, leading to irreproducible outcomes and invalid or misinterpreted results. Infections by *Aeromonas* and mycobacterium, for example, interfere with the results of experiments by altering physiological parameters. Likewise, infection of the nervous system by *Pseudoloma neurophilia* causes behavioral changes in zebrafish, leading to misinterpretation in behavioral studies. In this chapter, we seek to provide valuable contributions about zebrafish housing and husbandry conditions known to influence animal health, drawing attention to the most common diseases and pathogens that eventually may affect zebrafish in the laboratory.

Keywords: immune system, laboratory animal, opportunistic pathogens, aquatic system, behavior, freshwater fish, infection, Mycobacteriosis, *Pseudoloma neurophilia*

1. Introduction

Research in several areas using the vertebrate zebrafish (*Danio rerio*) confirms its relevance as an alternative model. Zebrafish are used in forefront science due to a large number of factors including, but not limited to, their similarities to humans, fast and external development, transparency of the embryos, versatility, and genome-editing capabilities. Recent breakthroughs allow researchers to analyze the specificities of the immune system at the cellular level and explore the interactions between

immune responses and pathogens, benefiting from the knowledge offered by the sequenced genome and conserved immune response [1].

Maintaining zebrafish health in experimental environments is vital, and providing adequate water and food following ideal standards will ensure stability, immunity, and well-being. In these conditions of well-being, the probability of infections being manifested will be low [2]. On the contrary, a low sanitary aspect in zebrafish housing can induce changes in physiology, behavior, and immune response, detectable through altered characteristics and clinical signs, such as lethargy, opercular enlargement, swallowing air at the water surface, loss of floatability control, eating less or not eating, and signs in their appearance like ulcer, injuries, hemorrhage, changes in color or hydropsy [3].

Given the increasing growth of zebrafish research facilities worldwide, these organisms are housed within a spectrum of settings ranging from modest setups involving a few tanks to large collaborative core facilities boasting thousands of aquaria. In these scenarios, the typical configuration consists of numerous compact aquaria integrated into a recirculation framework using commercially designed “racks”, encompassing approximately 30 tanks to large systems supporting hundreds of tanks. Irrespective of scale, the fundamental layout involves the recirculation of effluent water through both mechanical and biological filtration stages, succeeded by ultraviolet sterilization procedures. Supplementary water is commonly sourced from reverse osmosis outlets to which salts are introduced to control conductivity level.

Moreover, the technologies applied to create mutants or transgenic lines have boosted the use of zebrafish in research. More often than not, these lines might suffer from the same diseases that wild-type animals, although depending on the type of gene edition, it could impair their health vulnerability and needs.

Understanding the specific requirements of zebrafish and comprehending the nuances of their immune system is paramount for maintaining this species in laboratory environments that ensure their optimal well-being and health. Zebrafish, as a model organism, offer valuable insights into various biological processes, but their intricate needs, both in terms of habitat and immune responses, must be met to uphold their viability as research subjects.

Adequate knowledge of their demands, such as optimal water quality, appropriate diet, and suitable housing conditions, directly influences their behavior, growth rates, immunity, reproductive success, survival, and life expectancy. Moreover, delving into their immune system intricacies equips researchers with the tools to address potential disease outbreaks anticipatively, promote disease resistance, and reduce mortality. By refining our understanding of zebrafish requirements and immune dynamics, we fortify the foundation for productive research while upholding the ethical responsibility to ensure their comfort and health within laboratory settings.

Concisely, during early-life stages, zebrafish rely on their innate immune response, which involves innate molecules, barriers (skin, gills, gastrointestinal - GI tract), phagocytic cells, and soluble mediators, such as cytokines, pore-forming toxins, and lectins, which regulate immune cell interactions and activities [4–6]. On the other hand, juvenile and adult zebrafish possess a well-developed complement system and an adaptive immune response similar to other vertebrates. Surface barriers like skin, gills, and GI tract are coated by mucus, offering both physical and chemical protection against pathogen microorganisms or aggressors, besides promoting osmotic balance. Further, zebrafish surfaces are provided with phagocytic macrophages and neutrophils. Also, GI tract secretions produce adverse environments for exogenous microorganisms [7].

This book chapter intends to provide valuable contributions about zebrafish housing and husbandry conditions known to influence animal welfare, drawing attention to the most common diseases and pathogens that might affect zebrafish in laboratories.

2. Biotic and abiotic factors and the zebrafish health

Zebrafish are subject to water quality concerns similar to those of other fish reared in intensive recirculating systems. The zebrafish housing facility acts as a life support system, similar to natural habitats, and alterations or poor water quality compromise several biological processes in zebrafish, such as growth, metabolism, immune function, behavior, and stress [8]. Thus, although zebrafish are quite tolerant to water quality parameter fluctuations, the standardization of husbandry procedures must be pursued to optimize experimental techniques and reach reliable results [9].

Therefore, physical, chemical, and biological parameters must be fulfilled to achieve conditions, where the fish perform their best once inadequate water quality produces important subclinical and clinical manifestations with severe morbidity and mortality [10].

Zebrafish are naturally found in South Asia, inhabiting slow-flowing water bodies in different ranges of water parameters, temperatures varying from 10 to 40°C, pH between 6 and 10, and up to 60 cm of water column [9]. According to Schaefer & Ryan (2006), thermal tolerance varies between individuals, and for zebrafish, the optimum range is between 24 and 28°C [11]. Temperature also interferes with other phenomena like sex determination, where masculinization is related to high rearing temperatures (reviewed in [8]). Higher temperatures also decrease the saturation of dissolved oxygen (DO), which leads to an imbalance of total ammonia nitrogen (TAN) and enhances the prevalence of unionized ammonia (NH_3), increasing the NH_3 toxicity [12].

The TAN balance is also influenced by pH concentration. Unionized ammonia and ammonium ions (NH_3 and NH_4^+ , respectively) rely on pH and must be regulated in order to prevent disbalance. Ammonia is oxidized to nitrite (NO_2^-), which is also toxic by oxidizing hemoglobin and converting it to meta-hemoglobin. Thus, the respiratory process is affected, leading to potential asphyxiation and death. The conversion of nitrite to nitrate (NO_3^-) is essential once this molecule presents minor toxicity, and levels up to 200 $\text{mg}\cdot\text{L}^{-1}$ are considered safe for zebrafish [13].

Water quality diseases caused by ammonia toxicity generally lead to behavioral alterations, like hyperexcitability and anorexia, which can be observed in the aquarium, and lesions observed in additional exams, such as gill hyperplasia and hypertrophy (reviewed in [2]). Sporadically, mats of bacteria are associated with hyperplastic gill lesions, although this is also most often secondary to suboptimal water quality and not a primary bacterial problem [14].

pH values reflect the concentration of hydrogen ions present in the solution and characterize the water as acidic, alkaline, or neutral when around pH 7. The production of TAN in the creation system acidifies the water ($\text{pH} < 7$); thus, neutralization with buffer solutions is necessary [12]. The tolerable pH range for freshwater fish is between 6.5 and 9, and for zebrafish, it stands around 6.2 and 8.5, with no behavioral or physiological alterations (reviewed in [8]). Acidification or alkalization of water has effects on fish, resulting in several physiological alterations in gills that are in direct contact with water, but also in gas exchange and electrolyte balance [15].

Moreover, pH alterations cause massive mucus production, besides behavioral changes [16], glycemia, a decrease in red blood cells (RBC), and an increase in white

blood cells (WBC) [17]. Additionally, any disturbance in the osmotic state leads to physiological stress and disease (reviewed in [8]).

Biological processes like neuronal signaling, muscle contraction, and osmoregulation depend on essential ions—calcium, magnesium, and ferrous iron, among others—that are absorbed from rearing water; these ions' concentration is represented by water hardness [18]. According to Chen *et al.* (2003), hardness >75 ppm (parts per million) is adequate for freshwater fish [19].

The concentration of DO is affected by external factors like water temperature, feeding rates, and fish density in an inversely proportional manner. Oxygen concentration between 6 and 8 ppm is suitable for zebrafish culture [12]. Meanwhile, high concentrations of carbon dioxide affect fish by diminishing hemoglobin affinity to oxygen and acidifying blood. Thus, it is imperative to keep lower concentrations of carbon dioxide. Besides DO and carbon dioxide, water nitrogen levels interfere with fish physiology. When water nitrogen supersaturation occurs, fish develop a syndrome known as gas bubble disease (**Figure 1**).

In housing systems, leaks on suction water pumps or intensive air injection on water can cause water gas supersaturation. Also, abrupt temperature alters gas concentration stability. Supersaturated water produces emboli (free gas bubbles) in the vascular system that spread through other tissues, mainly subcutaneous tissue [8], which is particularly problematic. Local inflammation and behavioral abnormalities can occur in affected zebrafish. Also, air emboli in the vascular system may cause obstruction of blood vessels, leading to local ischemia and tissue damage, and the severity of the disease depends on the intensity of the formed emboli. Necrosis areas derived from disease can suffer from secondary bacterial infections. Eyeballs are frequently injured by tissue gas bubbles, and clinical signs, such as exophthalmos (**Figure 2**), eye inflammation, cataracts, and blindness may occur [14]. Chronically affected fish can present emaciation, buoyancy defects, and reduced activity.

Likewise, nephrocalcinosis is also triggered by rough water quality. A range of nephrocalcinosis lesions in zebrafish can be assessed by histology, most of which are diagnosed in clinically normal fish. Calcium deposits in renal tubules and ducts may occur as a consequence of high dissolved CO₂ [14].

Abnormal behaviors, such as frequent aggression, lethargy, and erratic swimming, are reliable indicators of welfare disruption, as well as specific clinical signs indicating punctual situations, such as opercular flaring (**Figure 3**), reflecting respiratory distress.

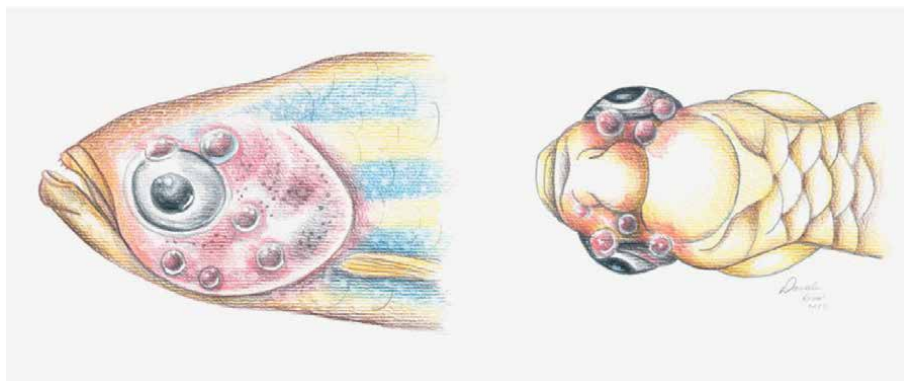


Figure 1.
Illustration of gas bubble disease; lateral view (left) and dorsal view (right).

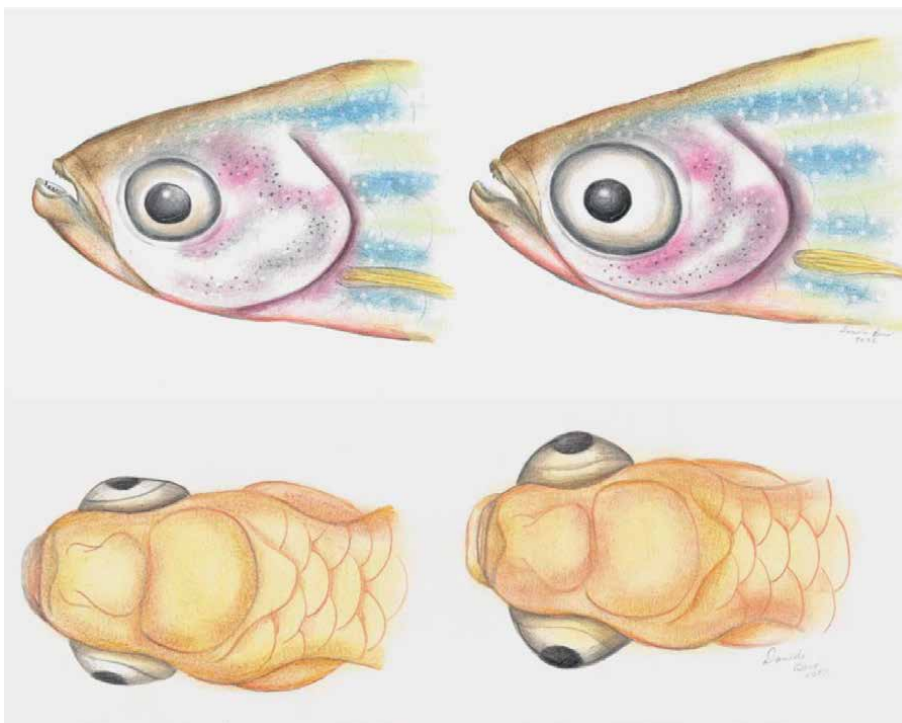


Figure 2.
Illustration exophthalmia; healthy (left) and sick (right) animals in lateral and dorsal view.

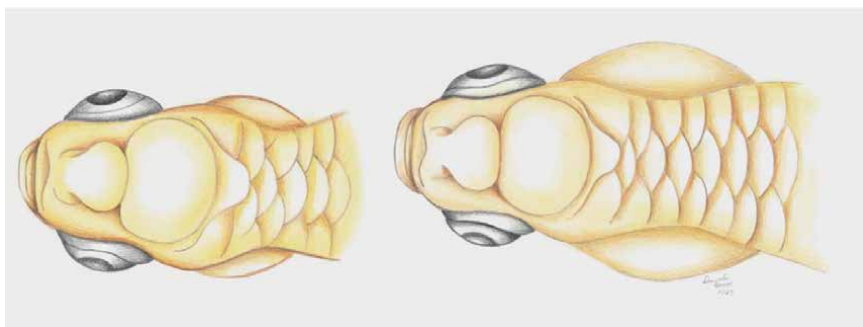


Figure 3.
Illustration of opercular flaring.

3. Stress and zebrafish

As highly evidenced, among all the critical modulating factors on health and equilibrium, stress has a lot to do with many biological responses as a state of threatened homeostasis. Stress is the consequence of external or internal factors (stressors) implicated in changes in biological equilibrium. It might initiate an adaptive response to restore baseline physiology, seen as a change in physiology, psychological state, or behavior.

In laboratory zebrafish, stress can induce alterations notably in four broad physiologic categories: behavior, the autonomic nervous system (ANS), the neuroendocrine

system, and immune function [20]. Consequent modifications in these functions can be assessed by evaluating behavioral patterns, *e.g.*, appetite, displacement, aggression, and, eventually, response to handling; physio-morphological alterations, *e.g.*, respiration intensity, weight loss, growth rate, color change, blood cell count and cell structure, cardiac output and blood flow; and biochemical alterations, such as through hormone levels; and reproductive performance impairment, which can manifest in zebrafish as decreased spermatozoa quality, reduced spermatozoa cell motility [21], accelerated ovulation, reduction in the number of embryos and their survival rates. Overall, threats to homeostasis activate a complex pattern of behavioral changes and responses in the central and autonomic nervous and endocrine systems; if the stress system is chronically activated, these effects may lead to severe pathology.

Lastly, zebrafish nutrition is an additional important factor for their health and proper development. Some diseases that affect zebrafish are caused by a nutritional deficiency. In this regard, for example, vitamin E or selenium deficiency might cause muscle degeneration, vitamin C deficiency can lead to scoliosis, and pantothenic acid deficiency is likely to cause gill disease.

4. Bacterial infections

Clinical or subclinical infections may distract experimental findings, leading to irreproducible outcomes and invalid or misinterpreted results [22]. In general, local lesions are noticeable and may indicate bacterial, fungal, or protozoal infections located on the skin and fins, with hemorrhagic areas or ulcerative lesions. These clinical signs vary in severity according to the intensity of infection and are the base for correct diagnosis, along with microorganism culture [23].

In fish, skin infections are caused by bacteria such as *Cytophaga*, *Flavobacterium*, and *Flexibacteria*, while *Aeromonas salmonicida*, *Pseudomonas spp.*, *Edwardsiella spp.*, *Streptococcus spp.*, and *Mycobacterium spp.* are mostly responsible for systemic infections (reviewed in [23]). Moreover, species like *Flavobacterium columnare* are prone to causing bacterial gill disease (**Figure 4**) or fin rot (**Figure 5**).

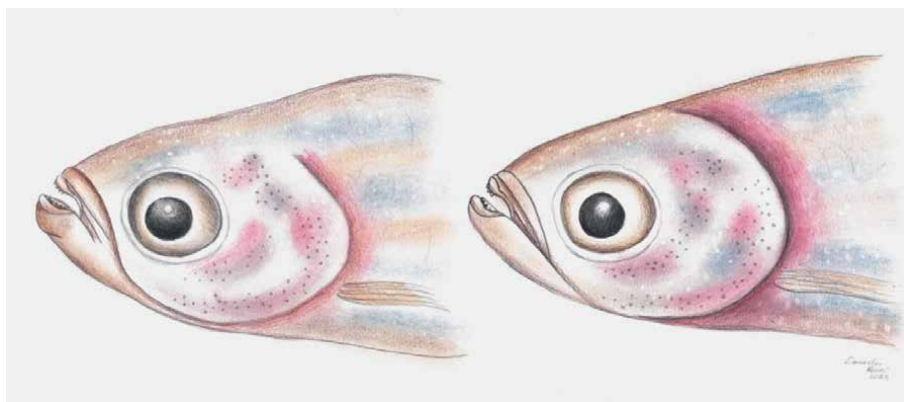


Figure 4.
Illustration of gill inflammation in zebrafish.

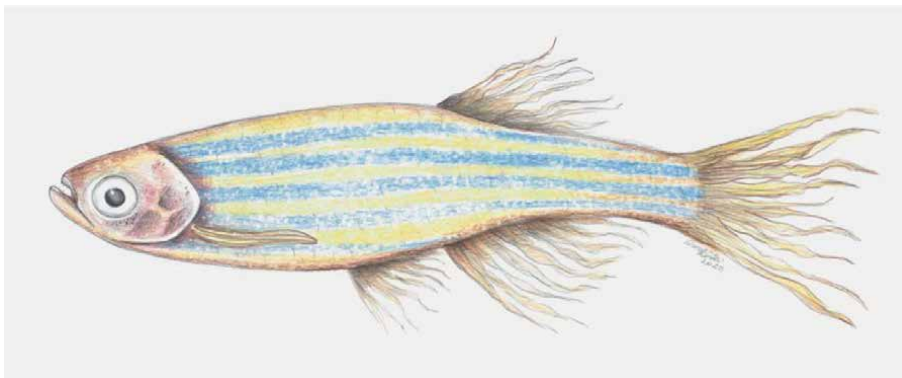


Figure 5.
Illustration of zebrafish fins and tail with frayed edges.

4.1 Columnariosis

The infection by the opportunistic bacteria *F. columnare* generates a disease called columnariosis. The disease can clinically manifest in different forms, including skin, gills, and fins, and has a high rate of morbidity and mortality. The infection lesions appear as white or grayish lesions, exposing muscles in the more eroded wounds [24]. Clinical signals of distress may occur, such as increased breathing rate and opercular flaring, and this is due to intensive infections of the gills.

Zebrafish with compromised immune system are especially vulnerable to *F. columnare* infection and can transmit the disease through direct contact between infected and healthy fish and also through contaminated water or equipment. Poor water quality and other stressors, such as overcrowding and inadequate handling, can contribute to the onset of the disease, while open wounds can serve as bacteria entry [25].

As an infectious disease, the affected fish must be isolated from healthy individuals and treated with antibiotics—oxytetracycline or florfenicol. Improving water quality and general handling, as well as quarantine adoption of new fishes, tend to be effective against the *F. columnare* infection.

4.2 Edwardsiellosis

The gram-negative bacterium *Edwardsiella ictaluri* is the etiologic agent causative of Edwardsiellosis and the enteric septicemia of catfish. It has been reported to infect other species and, regarding zebrafish, is a disease that must be monitored to prevent outbreaks between zebrafish colonies. The infection in zebrafish is uncommon; however, the disease has a high mortality rate [26]. Zebrafish liver and spleen are the most affected organs, while skin may be stricken with ulcerations, along with intestine, heart, and nervous tissue [27, 28]. Behavioral alteration can also appear before or during the intense infection.

Diffuse erythema on the skin and necrosis in intern organs are common clinical findings, and the olfactory neural pathway is also a target for the infection [29]. Quarantine is key to prevention, once infected fishes rapidly evolve the characteristic lesions, approximately two weeks, antibiotics are the usual treatment for infected fishes.

Besides local signs, infections in zebrafish also have a systemic character, with clinical manifestations such as peritonitis and generalized edema. The edematous condition of skin and abdominal distension is called dropsy (**Figure 6**) and is derived from fluid accumulation into the elomatic cavity caused by systemic infections [23, 30].

According to Pullium *et al.* (1999) [31], housing situations like high density, poor water quality, and inadequate husbandry (*i.e.*, stress, abrupt temperature changes) favor systemic infections, especially with *Aeromonas hydrophila* and *Streptococcus* sp. [2]. In these infections, symptoms such as lethargy and petechial hemorrhage are observed. These bacteria are gram-negative and usually found in the mucosa and internal organs of healthy fish. Thus, the infection is considered opportunistic [32, 33]. One of the differences between *A. hydrophila* infection and mycobacteriosis, for example, is that the mortality rate is acute.

4.3 Mycobacteriosis

Bacterial infections usually cause notable symptoms, provoking mortality or health decline in the zebrafish population. However, subclinical infections lead to uncontrollable biases in experimental protocols [34], so in laboratory conditions, the fish must be observed, and good practices should be taken to prevent these infections.

Mycobacteria are ubiquitous, naturally occurring gram-positive, acid-fast, and aerobic bacteria. They are found in both environmental and laboratory water besides soil, and several species have been described to infect zebrafish, like *Mycobacterium abscessus*, *M. chelonae*, *M. fortuitum*, *M. haemophilum*, *M. marinum*, and *M. peregrinum*

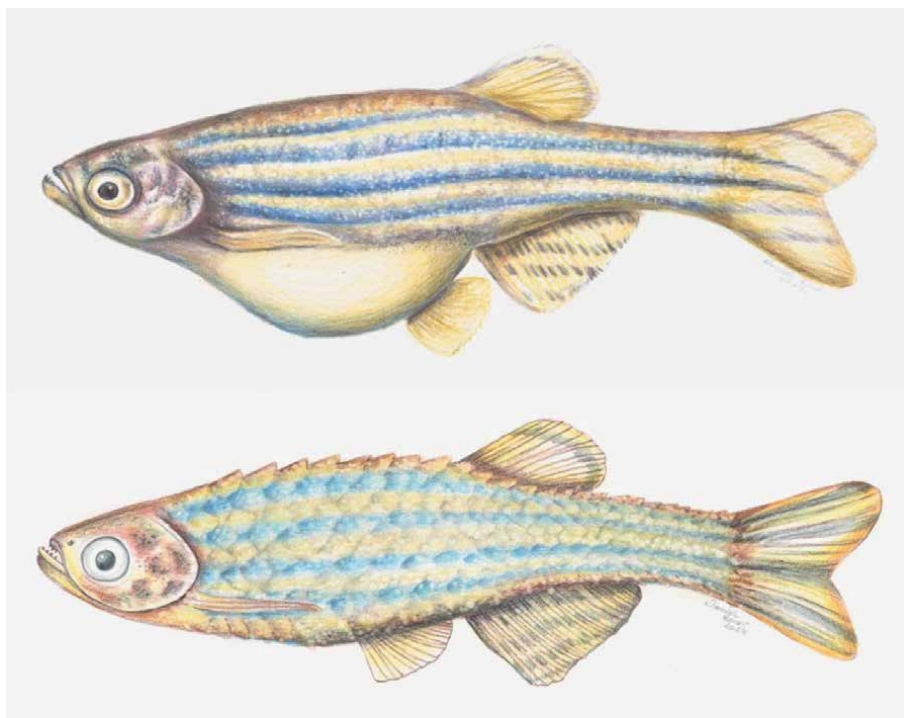


Figure 6.
Illustration of dropsy zebrafish with fluid accumulation in the coelomic cavity (top) and scales protrusion (bottom).

(reviewed in [2]), and approximately 40% of zebrafish facilities present mycobacterium occurrence [35]. According to Whipps *et al.* (2012), due to its large distribution and its environmental occurrence, mycobacteria may be considered pathogenic or opportunist in zebrafish [36].

Poor water quality and stressed animals are the pre-existing factors for this organism to establish itself. The clinical presentation varies greatly. Some lesions observed in the fish will be discoloration (**Figure 7**), skin ulcerations, or granulomas (nodules) in the abdominal cavity (**Figure 8**). However, lethargy may be another sign of infection. This is a chronic disease, and if not properly treated, it can lead to death. Mortality and morbidity of Mycobacteriosis vary according to species, where *M. marinum* and *M. haemophilum* are the most severe infections. Inflammatory granulomas in hematopoietic organs and occasional chronic inflammation are commonly described in infected zebrafish (reviewed in [37]).

Zebrafish facilities affected by Mycobacteriosis must adopt rigorous protocols to eliminate the contamination. Preventive measures, such as quarantine of newly introduced fish, embryo disinfection, effective sterilization of system water, and satisfactory sanitation of rearing tanks, must be taken in every zebrafish facility [36].

Mycobacteria are oligotrophic organisms and, thus, can survive and proliferate in a plethora of environments. In zebrafish aquaria, when sanitation is not efficient, biofilms are formed, which are adequate environments for the survival of mycobacteria despite the cleanness of water [37].

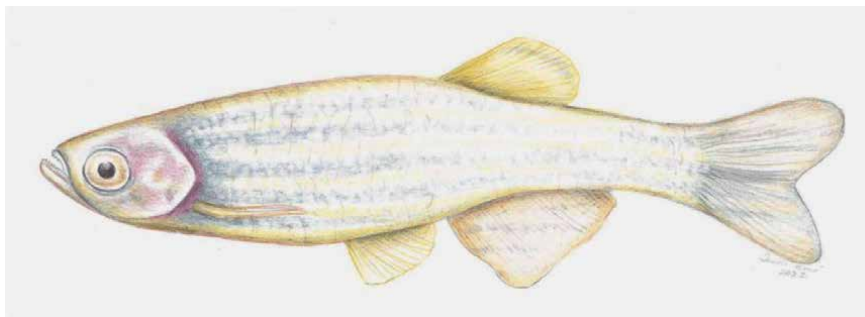


Figure 7.
Illustration of zebrafish showing epithelial discoloration.



Figure 8.
Illustration of zebrafish with skin ulcerations.

Chang, Lewis, and Whipps (2019) reported that *M. chelonae* can be transmitted by infected fishes to aquaria biofilms, suggesting that resident fishes could naturally acquire *M. chelonae* infections from tank biofilms. The authors also suggested that other species of mycobacteria could be transmitted in the same way and emphasized the importance of safety control in zebrafish facilities [37].

Research programs with zebrafish can be severely affected by bacterial outbreaks in the facilities. Generally, mycobacteria are opportunistic pathogens; however, two species are of special relevance: *M. marinum* and *M. haemophilum*. These species produce severe and persistent infections, and *M. marinum* is associated with high mortality rates (Reviewed in [38]).

The gastrointestinal system is also considered a route for mycobacteria infection. There have been reports of salmonids and medaka infected through feed, and mycobacteria were identified in the zebrafish intestinal tract (reviewed in [39]). Studies pointed out that transmission through other organisms could maximize contamination of Mycobacteriosis since Japanese medaka were more infected by mycobacteria-contaminated mosquitoes, and infected protozoan *Paramecium caudatum* transmitted the infection to zebrafish [40], as well as Chang, Benedict, & Whipps (2019) demonstrated the transmission of mycobacteria through live feed [39].

Mycobacteriosis is considered a zoonotic disease, and non-tuberculous mycobacteria (NTM)-induced cutaneous infections have been described in dermatological care clinics. Most of the cases were caused by *M. marinum*, although other species have also been identified in some cases. Thus, besides ensuring the zebrafish health, sanitation measures also prevent contamination of staff members (Reviewed in [38]).

5. Fungal infections

Fungal infections generally present a cotton or wool-like form that can derive from necrotic lesions, although species like *Aphanomyces* sp. produce primarily necrotic ulcerative lesions. The most common fungal infection in freshwater fish is Saprolegniasis, also referred to as molds or water molds, which can affect fish and their eggs. It is important not to overfeed or leave dead fish in your tanks, because *Saprolegnia* will establish itself and grow extremely fast on the animals or the excess food. *Lecythophora mutabilis* is another water mold that creates a biofilm around the fish head and then establishes itself in the biofilm. Then, the cause of death usually happens due to starvation and asphyxiation of the animals. The fungal identification is usually made by skin scraping, fungal hyphae and spores visualization, and posterior fungal culture to confirm [29].

5.1 Saprolegnia

Saprolegniasis is an infectious disease caused by the fungi of the Saprolegnia genus, notably *S. ferax*, *S. diclina*, and *S. parasitica*. The infection is known as “water mold” since the skin lesions produce cotton-like growths that start as small lesions and develop into more aggressive ones. A wide range of aquatic species, including zebrafish, can be affected by the fungus. As the genus Saprolegnia is usually found in aquatic environments, the disease generally occurs when the fish’s immune system is compromised, making them susceptible to infection [41].

The affected fish can present lesions on the skin, gills, and fins, showing a “fuzzy” appearance, and with the disease aggravation, a significant portion of the body is affected [42]. The affected surface area, which may be composed of large areas,

predicts the severity of the disease. Thus, destruction of the epidermis and respiratory collapse due to infection of the gills cause behavioral changes, with lethargy leading to a high risk of predation. The impairment of physiological processes such as osmoregulation and the eventual entrance of fungal hyphae in internal organs in advanced stages can lead to organ failure.

Pre-existent skin lesions and infection by other pathogens could represent a serious risk for contracting Saprolegnia infections. Moreover, physiological stressors such as poor water quality, high density, and sudden temperature change have an important impact on zebrafish immune homeostasis, increasing the susceptibility to opportunistic infections, such as Saprolegniosis. Improving water quality, avoiding stressing factors, quarantining newly introduced fish, and regularly cleaning tanks and equipment are key actions to prevent fungal occurrence [43].

Infected fishes must be isolated from the experimental aquaria, along with anti-fungal treatments in order to preserve sanitary conditions of the experimental set and prevent further infections. Also, proper monitoring of fish is essential to recognize and take immediate action for treatment.

6. Parasitic infections

6.1 Ichthyophthiriasis

Ichthyophthiriasis is a parasitic infection caused by the protozoan *Ichthyophthirius multifiliis*. This disease is commonly known as “white spot disease” due to the clinical signs of infected fish: small, white, raised spots in skin, fins, and gills. The protozoan *I. multifiliis* occurs in aquatic environments and is prevalent in both natural habitats and experimental sets, and its ability to spread rapidly is of concern to zebrafish laboratories [44].

The Ichthyophthiriasis characteristic white spots consist in the trophont stage of the parasite, which is the stage when the parasite adheres to fish skin. The skin infection leads to behavior alteration, and the fish starts to rub against objects’ surfaces, increasing mucus production. This behavior represents a visible irritation and discomfort of infected fishes, and the stressful situation provokes immunodepression, making them more susceptible to *I. multifiliis* and other opportunity pathogens, such as bacteria and fungus. In advanced stages, the disease can have a significant impact on the zebrafish population [45].

Containment measures like regular monitoring of fish behavior, avoiding high density, and keeping water quality seem to be effective in avoiding the parasite. Meanwhile, infected fish must be isolated for therapeutic intervention and to avoid the spread of the disease [46]. The treatment can be executed through two approaches: the interruption of the parasite’s life cycle, with a gradual elevation of salinity and temperature, or with chemical substances, such as formalin or copper-based medication.

6.2 Pseudoloma neurophilia

Microsporidia are obligate intracellular spore-forming parasites that can infect approximately 150 fish species, as well as terrestrial animals such as rodents, rabbits, and primates including humans [47]. Microsporidiosis is a common disease in laboratory fish that affects the central nervous system and can cause emaciation and spinal

deformity [48, 49]. Also, infection by microsporidia is characterized by xenomas, a cellular growth formed by hypertrophied, unorganized, and unstructured polyploid cells due to parasite proliferation [50].

In zebrafish, microsporidia infection was described by de Kinkelin (1980) [51], and the most common parasite was identified as *Pseudoloma neurophilia* [48]. This parasite is associated with multiple xenomas in hindbrain, spinal cord, and nerve roots, and free spores can be found within phagocytes in other areas, causing myositis, meningitis, and encephalitis, and can be transmitted vertically and horizontally. In laboratory zebrafish, *P. neurophilia* infection is asymptomatic, only detected when clinical signs turn perceptible, like spinal deformation (**Figure 9**) or emaciation (**Figure 10**) [48, 49].

The spinal cord is associated with locomotor ability and motor function, and areas of the hindbrain control neural pathways associated with behaviors like anxiety, fear, and learning. Thus, it has been suggested that lesions caused by *P. neurophilia* in these areas could alter zebrafish neural signaling, directly interfering with outcomes of experiments in several fields, such as neurobiology, toxicology, and pharmacology [52].

According to Spagnoli, Sanders, & Kent [53], zebrafish infected with *P. neurophilia* presented altered shoal behavior and startle response, as well as inflammation on the spinal cord, meninges, and muscles, which could be caused by spore stage [54]. As reviewed by Midttun *et al.* [52], several model organisms present altered behaviors and biological processes due to intra- and extracellular parasites, *e.g.*, *Drosophila spp.* colonies presenting altered circadian rhythm and rodents with activated cytokine signaling. Thus, behavioral phenotypes such as stress and anxiety-like behavior may be a result of *P. neurophilia* infection despite experimental treatment *per se* [53].



Figure 9.
Illustration of zebrafish with abnormal morphology, spinal deformity (top), and kyphosis (bottom).



Figure 10.
Illustration of zebrafish with emaciation.

7. Viral infections

Health management in zebrafish laboratories is mandatory since this model system becomes strikingly important to biomedical sciences in both larvae and adult stages [55]. Although several mammalian viral infections are modeled in zebrafish [56], naturally occurring viral infections are scarcely studied in zebrafish [57].

As the importance of zebrafish in science rises, aquatic laboratory facilities also start to emerge in research centers, and the utilization of shared systems exacerbates the possibilities for transmission of undiagnosed viral infections [58, 59]. The implementation of different mutant lines, such as immunocompromised fish, to studies in the immunology field contributes to the introduction of new diseases in the population, including viral infections (reviewed in [57]).

Bermúdez *et al.* (2018) described for the first time a natural occurrence of *Megalocytiavirus* infection in zebrafish research facilities [60]. The author reported the contagion by the infectious spleen and kidney necrosis virus (ISKNV), an iridovirus. *Iridoviridae* is a virus family that infects poikilothermic vertebrates, and the authors identified the virus by genetic alignment with known *Megalocytiavirus* sequences. The clinical observations included altered swimming and behavior, hyperemic gills, respiratory distress, and hemorrhage. The diagnosis was based on genetic correspondence and pathological findings once hypertrophied cells, besides necrotic and degenerative areas, were found primarily in the kidney and spleen.

Picornaviridae is a virus family known to infect vertebrates through cell surface receptors of intestinal, respiratory, or hepatic tissue. Altan *et al.* described a natural infection of a divergent picornavirus in zebrafish without marked clinical signs [61]. According to Kent, Harper, and Wolf [62], the occurrence of asymptomatic diseases impacts the results of research using zebrafish as they impose additional variants on experimental data.

8. Concluding remarks

Zebrafish have been considered an outstanding model for biomedical research. One of many advantages of the zebrafish is the possibility to screen small molecules to identify potential therapeutics with precision. Moreover, the conservation of immune mechanisms among vertebrates strengthens the translational character of zebrafish as a model system.

However, like other animal models, zebrafish are subject to suffering from disorders, including being contaminated and transmitting infectious diseases. *P. neurophilia* is a common infectious agent that produces important effects on zebrafish, leading to behavioral alterations, which cause misinterpretation of data, invalidating experimental protocols. Natural viral infections have been described in zebrafish, and although several studies model mammal viruses in zebrafish and explore unique insights, the occurrence of natural viruses includes unknown biases in protocols, generating uncertainties in the experiment.

The study of natural diseases that can occur in zebrafish, mainly in fish kept in a scientific experimentation environment, may provide the opportunity to directly visualize the pathogen-host dynamics and to carry out chemical screenings, which facilitates the development and testing of new therapies, ensuring animal welfare.

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Conflict of interest

The authors declare no conflict of interest.

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Colony Management at Zebrafish Facilities

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Abstract

Fish facilities often maintain tens to hundreds of zebrafish lines, among transgenic, mutant and wild types. The replicability and reproducibility of the research based on these animals relies on its quality. However, keeping fish lines healthy and genetically stable across generations is not trivial. Housing space is often limited, so each stock is frequently restricted to a few tens of animals and inbreeding depression can rapidly install, causing health issues, gender bias and decreased reproductive performance. To minimise this, it is fundamental to define colony management strategies adequate to each fish line and each facility. This chapter discusses common difficulties and approaches to overcome them, including line-specific mating schemes and complementary actions to keep lines hardy. In addition, strategies to prevent genetic cross contaminations and record keeping methods are also addressed as part of a complete colony management program.

Keywords: replicability, reproducibility, line maintenance, inbreeding depression, genetic variability, databases

1. Introduction

With the rapid evolution of efficient gene-editing tools, the number of new transgenic and mutant zebrafish lines has increased steeply, contributing to the acceleration of scientific knowledge on a wide range of research fields. In parallel, it also brought challenges to the management of fish colonies since the physical space in fish facilities is often limited, each fish line has specific features to account for and maintaining genetically stable lines to guarantee the quality of scientific outputs is not an easy task. To keep up with the increasingly demanding research, it is crucial that zebrafish facilities implement custom-designed colony management programmes, regardless of the number of fish and fish lines held.

The largest share of a colony management program is line maintenance, which refers to the actions required to ensure that a line, including its genomic and phenotypic traits, is preserved across generations. Those actions include selecting brood-stock and breeding schemes, defining criteria for offspring selection and determining the periodicity to launch a new generation. Mating schemes can be based on two main categories: inbreeding, which is the mating of individuals more closely related than the population average (also known as incross), and outbreeding, the mating of individuals less closely related than the population average (also known as outcross) [1].

In species such as mice and medaka, breeding plans are often based on inbreeding. Briefly, close relatives share many alleles for multiple genes, so when they are incrossed, at least some progeny will inherit the same alleles from both parents, increasing homozygosity at different loci and reducing the genetic diversity among the offspring. When a line reaches 98% homozygosity, typically after 20 consecutive generations of full sibling matting and thorough selection of the best breeders and traits of interest and against harmful phenotypes, it is defined as an ‘inbred strain’, often abbreviated to ‘strain’ [2]. At that point, the strain is considered genetically homogeneous with little or no phenotypic variance, and the individuals are practically clones of each other. Given the influence that the genome has on physiology and behaviour, the genetic stability of inbred strains is a great advantage, reducing uncontrollable variability and increasing the statistical power of experimental results and their interpretation, replicability and reproducibility [3]. Ultimately, allows a faster transition from basic to translational studies and eventually ‘real life’ applications.

As for zebrafish, there have been several attempts to create inbred strains such as the C32 [4], SJD [5], CG1/CG2 [6], IM12m and IM14m lines [7], some of which continue to be available at international resource centres. Nevertheless, consecutive filial incross in zebrafish can produce detrimental effects on fish development and reproduction that become incompatible with the continuity of the lines after just a few generations, a phenomenon known as inbreeding depression.

The absence of zebrafish inbred strains created a recognised problem for reliability of the outcomes from research based on this model. This is because fish from the same lines but maintained in different facilities are many times genetically distinct [8], making comparisons between experimental results questionable. An alternative to inbred strains could be the creation of genetically defined lines. Actually, some lines were created and extensively characterised at the genomic level at a defined generation, namely, SAT [2], NHGRI-1 [9] and T5D. However, opposite to inbred strains, the fish that compose these lines conserve a considerable degree of allelic variability that originate changes in the line genetic composition across generations due to the natural allele recombination that occur at meiosis. Thus, to become real assets, they would need to be under continuous genetic monitoring to keep the genetic map updated, which has not been the case mostly due to the high costs associated and lack of expertise on how to perform such monitoring [3].

While there is no consensus solution to the concern regarding the reliability of experimental data obtained from zebrafish, fish facilities put efforts on keeping their colonies healthy and reproductively active based on somewhat opposite strategies that hinge on allelic diversity preservation [2]. By doing so, there is also a reduction of the natural genetic divergence between fish from the same lines that are held at different facilities, which is another positive contribution to data reproducibility and replicability [10, 11]. In the next sections of this chapter, common difficulties and current approaches for the management of wild-type, transgenic and mutant colonies are described.

2. Inbreeding depression and genetic drift

The main reason why it is so difficult to generate and maintain zebrafish inbred lines is because inbreeding frequently produces deleterious phenotypes well before the 20th filial generation. The phenomenon, also known as inbreeding depression, is defined as the loss of fitness in a population submitted to increasing

homozygosity [12]. Signs of inbreeding depression include gender biases (typically more males); decrease in fertility, growth and survival; more frequent and severe developmental defects (e.g., small head, underdeveloped lower jaw and poor circulation) and shorter lifespan [1, 7, 13], and ultimately, it may even lead to fish lines' extinction. Inbreeding morphological abnormalities tend to become evident after six to eight generations [13]. Some malformations may become more pronounced and evident as fish age advances and may be associated with inbreeding, though not genetically confirmed so far. These include skeletal malformations, such as spinal curvatures in one or multiple sites (**Figure 1B**); caudal fin developmental deformities (**Figure 1C**) and craniofacial phenotypes that comprise shortening of the frontonasal area (**Figure 1D**), terminal infolding of the opercular apparatus (**Figure 1E**) and hypo- or hypertrophy of the jaw (**Figure 1F**) [14].

The origin of inbreeding depression stands on the mating of closely related fish, such as the cross of full siblings or parents with their progeny. When that happens,

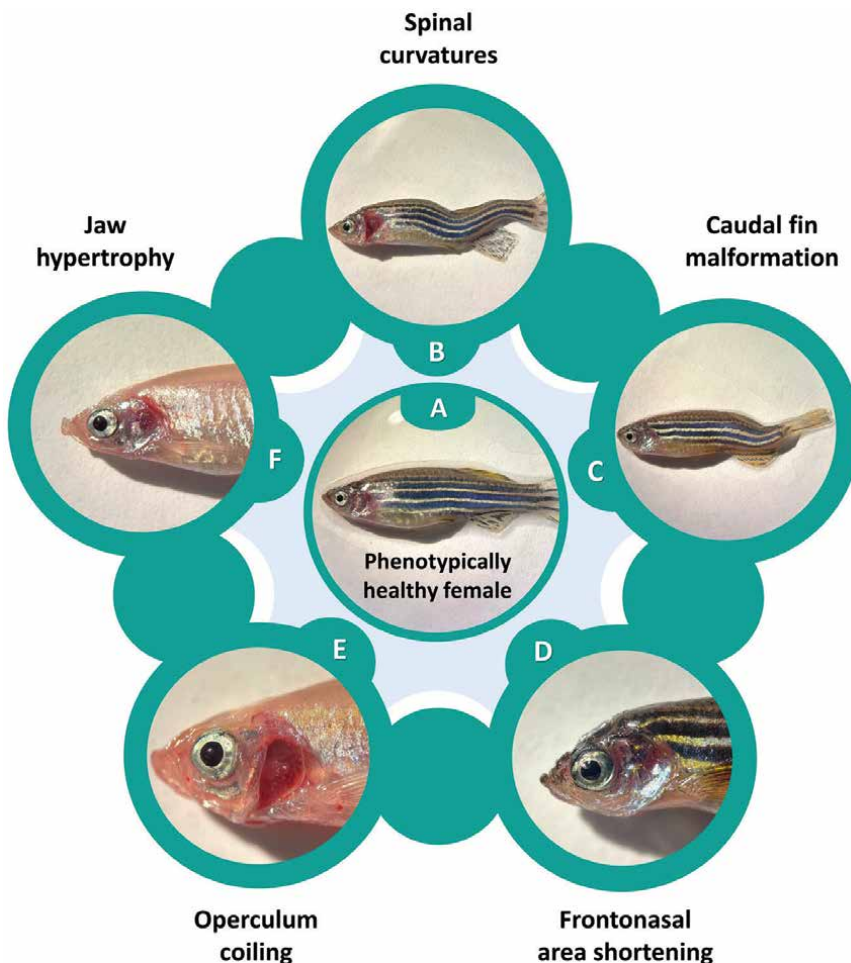


Figure 1.
 Phenotypes possibly associated with inbreeding depression in adult zebrafish. A – Control, phenotypically healthy female; B – Spinal curvatures in multiple sites; C – Caudal fin developmental malformation; D – Frontonasal area shortening; E – Operculum coiling; F – Jaw hypertrophy.

deleterious recessive alleles that are present as single copies on each parent can be paired and expressed in their offspring, being the accumulated expression of harmful alleles the cause behind inbreeding depression. In addition, there are studies that indicate that inbreeding depression can also occur as a consequence of some allele combinations being superior in terms of the benefits for the animal when they are expressed in heterozygosity, so the increase in homozygosity lowers population fitness. More studies are required, but if this is the case, inbred lines should always have inferior genetic fitness due to the loss of heterozygosity, which would explain the failure in creating zebrafish inbred strains [15].

Another natural factor that negatively affects the maintenance of healthy animal stocks is genetic drift, defined as random changes in gene frequency over several generations, leading to a decline in the population's genetic diversity and occasional irreversible loss of alleles [1]. It is considered a twin sister of inbreeding depression, since the latter accentuates the former. In fact, the primary source of genetic variation within a zebrafish line is often an extreme case of genetic drift designated as 'founder effect' that happens upon the establishment of a new colony from a few imported animals, or when a subset of fish is selected as spawners for the next generation. Briefly, it is the random allelic variation derived from the split of the original population into smaller groups that are not genetically representative of the first population [16]. Instead, each group holds a different subset of alleles and some get lost.

Inbreeding depression and genetic drift may affect not only the population's fitness but also the replicability and reproducibility of experimental results. Both happen more intensely in small populations; hence, the facility dimension and size of the colony for each line have to be considered from the earliest times in the design of a colony management programme.

3. Line maintenance strategies

The term 'colony management' is often used as a synonym for line maintenance. In our view, the former includes the latter as well as additional topics that are addressed in this chapter. Line maintenance commonly refers to the controlled cycle that ensures the continuity of a line across as many generations as intended or possible. It includes four main stages: the maintenance of adult fish, breeding, larvae selection and rearing and finally the replacement of the parent stock by the new generation (**Figure 2**). The frequency of this cycle is determined by the required replacement rate, defined by either the researcher's experimental needs or the age limit imposed by the facility (typically 15 to 18 months of age) as a preventive health measure, since older fish are more susceptible to disease and spawning efficiency is lower [17, 18].

Line maintenance strategies need to be fish line-specific; there is no 'one fits all' formula. This includes defining the breeding scheme and the criteria for breeder and offspring selection, which can have a direct effect on the extent of inbreeding depression and genetic drift per generation. In addition, it is wise to consider the possible need for special housing and husbandry of more delicate lines, such as, for example, immunodeficient or tumour carriers. By doing so, it is possible to prevent welfare and survival issues that could increase morbidity and mortality, causing indirect negative impact on the maintenance of future generations. All these decisions should take into consideration the type of line (wild type, transgenic or mutant), intended zygosity, available housing space, pedigree record keeping and experimental needs.



Figure 2.
 Line maintenance cycle main stages. (1) adults are housed and kept in holding tanks. Some can be used for experiments as long as there is still enough broodstock for line propagation. (2) fish are bred by predefined methods at scheduled intervals and embryos collected. (3) embryos are incubated at 28°C until 5 days post fertilisation and selected before entering a nursery holding system. (4) older fish are replaced by the next generation typically at 15 to 18 months of age, since they become more susceptible to disease.

3.1 Overview of zebrafish breeding in a laboratory setting

Understanding zebrafish breeding in the wild provides a good base for the development of breeding methods in captivity. Zebrafish breeding is influenced by intrinsic factors difficult to control such as mate choice, courtship and social behaviours, as well as by environmental aspects that can be adjusted in a facility, like water chemical and physical parameters, amount and type of food, vegetation and volume of water mass [19]. A number of decisions need to be made before setting a cross, depending on its purpose: the type of tank to use, number of fish, sex ratio and genetic aspects like fish pedigree and genotype of interest.

In terms of physical setup options, crosses can be set directly in the housing system, in large isolated spawning apparatuses that can be used in recirculating or static mode, or in static breeding tanks. The first two options hold larger broodstocks and provide better control over water quality stability, so they are preferred when fish are left to breed for more than one overnight. Zebrafish also seem to prefer larger volumes

of water for spawning [19]. There are several models of mass spawning tanks on the market with holding capacity up to 300 L and ability to hold up to some hundreds of fish [10, 19]. On the other hand, 0.7 to 2 L breeding tanks with static water freshly collected from the housing system are the most used option. They are available in different sizes and designs, are easy to set, disassemble and clean and their small size eases handling and embryo collection.

Most breeding tanks come with an inner insert tank with perforated bottom that allows embryo passage and their accumulation on the outer tank. This is because zebrafish show no parental care behaviour, so eggs need to be protected from being eaten by the adults. In nature, zebrafish are regularly found in shallow slow-flowing waters, so some inner tanks have a tilted bottom that creates low-depth areas. Many spawning systems also include a divider insert to keep males and females separated when a controlled and synchronous spawning is required. Physical enrichment is also encouraged to create spawning sites that meet zebrafish preference. This includes gravel or gravel-like images at the bottom of the tanks, non-toxic plastic shelters and plastic plants to provide hiding sites and decrease male aggression [20]. Crosses are typically set in the afternoon or early evening and left undisturbed until the following morning; then, fish spawning is stimulated by dawn [2]. Males tend to be territorial, which interferes with female oviposition [20], the reason why a female:male ratio of 2:1 is generally recommended.

3.2 Breeding and offspring selection plans

Good planning of the crossing strategy and progeny selection method for each fish line is the best insurance against inbreeding depression and genetic drift accumulation to deleterious levels.

Ideally, the starting point should be to define, for each fish line, the level of inbreeding and genetic drift one is willing to accept over a certain number of generations. Based on that, there are specific equations to estimate the number of breeders that are required on each generation to fulfil those genetic criteria. In an ideal population, all individuals equally contribute genes to the next generation. However, most real populations are far from ideal and individual contributions to the offspring vary considerably. Consequently, factors such as type of cross, broodstock number and sex ratio, spawning success ratio, offspring viability and selection strategy need to be included in the equation when calculating the number of breeders to use in order to preserve the genetic pool of the line [1, 21, 22]. Realistically, the vast majority of the zebrafish facilities do not follow the ideal approach. The equations are complex, not widely divulged or discussed in zebrafish meetings and require detailed information that not all facilities keep. On top of that, housing space per fish line is frequently limited and consequently broodstock number is insufficient for ideal line propagation plans. Fortunately, there are alternative strategies to preserve allele variability for several generations.

The first thing to do is to find how many females and males compose the broodstock of the line of interest. The larger the colony, the easier the maintenance of genetic variability across generations. Second, decide the crossing unit—single pair or bulk cross. And finally, establish how many mating units will contribute progeny to the next generation and how many crosses need to be set considering the expected spawning success. Different combinations of these variables is what defines the most common types of breeding schemes (**Table 1**). The specific breeding schemes implemented depend greatly on the fish line and colony size.

Type of cross	Single pair	Small bulk cross	Large bulk cross	Multiple paired crosses	Multiple units of small bulk crosses
Work effort	Low	Low	Moderate	High	High
Number of breeders	One pair	≤ 15	> 40	As many cross units as possible	As many cross units as possible
Female:male ratio	1:1	$\sim 2:1$	$\sim 2:1$	1:1	$\sim 2:1$
Offspring selection	All progeny are full siblings	No control over the number of effective spawners	No control over the number of effective spawners	Controlled mix of progeny from all spawning units	Controlled mix of progeny from all spawning units
Genetic diversity obtained	Very low	Low	High – Very high	High – Very high	High – Very high

Table 1.
Common types of breeding schemes according to the number of fish and number of crosses.

Single pair cross (**Figure 3A**) is the mating of one female with one male. The pair can produce hundreds of eggs in a single spawning event [10], yet genetic variability in the offspring is limited to the alleles present in the two parents. The method is useful for individual screening of adults based on their progeny’s phenotype or genotype. This is required during the establishment of a new fish line, to find founders with germline transmission of the feature, mutation or transgene of interest. In addition, it is helpful to identify intruders upon accidental mix of adult fish from lines that look alike at visual inspection but carry different transgenes or mutations that are expressed in larvae. In those cases, a cross is set between the candidate under screen and a fish with a known genotype (often a wild type), and the progeny is screened: if the phenotype or genotype of interest is found, then the parent under screen is positive for the feature of interest. Single pair mating is also the best choice for establishing new lines from single founders with unique expression patterns and genomic features [2]. Still, if a single pair is used to launch a new generation, there is a steep reduction in allelic diversity known as founder effect, which greatly increases the rate of genetic drift and inbreeding depression [1, 10].

Another breeding and offspring selection method is pedigree mating. It is a time-consuming strategy but effective in keeping even small populations fit for multiple generations as it meets the two fundamental criteria: the entire broodstock must be genetically represented in the following generation—to preserve the line genetic variability—and the mating fish cannot be closely related—to minimise inbreeding depression [1]. Respecting these criteria is not trivial given the difficulty in keeping track of the pedigree of zebrafish individuals, since they should not be housed alone due to their shoaling nature. On top of that, there is no zebrafish equivalent to mice ear punches for individual tracing within a group because the fins regenerate in a matter of days. Yet pedigree mating overcomes such obstacles. This method is depicted on **Figure 3A**. It starts with the setting of several single pair crosses, each composed of one female and one male that are not closely related (not full siblings or parent–progeny). The following day, after fish spawning, the progeny from each pair is collected into

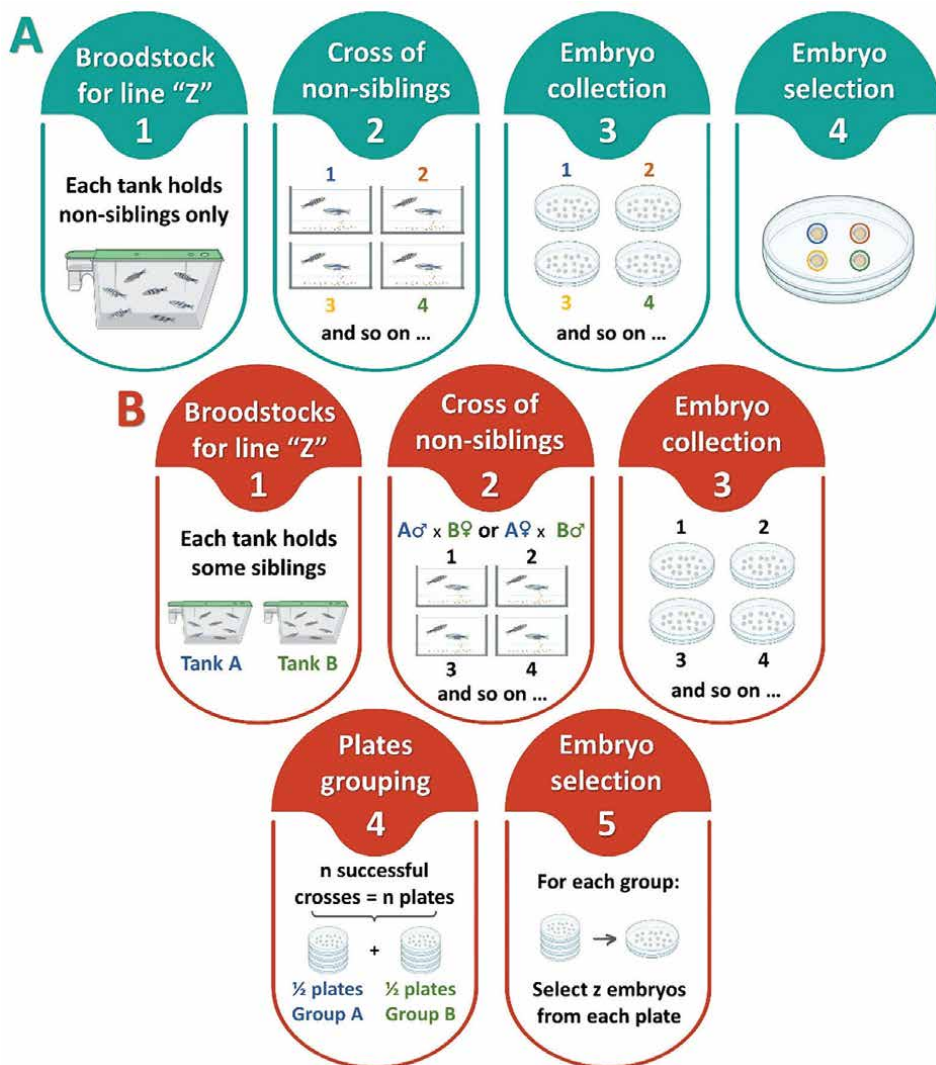


Figure 3.
Line propagation schemes based on pedigree mating and two progeny selection methods. (A) (1–2) pair-up (single pairs) or group-up (bulk crosses) fish from the same tank. (3) collect embryos from each mating unit separately, and (4) mix one embryo from each cross into one plate. Repeat to create as more non-sibling plates as needed for research. Raise the fish from each plate in separate tanks. Repeat the process every generation. Adapted from [23]. (B) (1–2) pair-up (single pairs) or group-up (bulk crosses) males from one tank with females from another tank, to guarantee they are not siblings. (3) collect the embryo clutch from each mating unit separately, and (4) assign a different subset of successful crosses (e.g., group A and group B) to each of the tanks that will hold the new generation of the fish line (e.g., tank A and tank B). (5) for each subset of petri dishes, mix 'z' embryos from each plate into a new one and then to a nursery tank, to raise. There will be some siblings on each tank. Future sibling mating is avoided by only crossing fish from different tanks.

separate petri dishes. Within the next few days, larvae are screened for the traits of interest and one individual from every cross is selected and pooled together into a new petri dish. Finally, the selected group is transferred from the petri dish into a holding tank and raised as the new generation of the fish line of interest [23]. If the number of fish to raise exceeds the capacity of one holding tank, additional tanks can be set up following the same mating and selection approach. The method ensures that

there are no full-siblings within each tank, so when they are adults and the line needs to be refreshed with a new generation, the fish within each tank can be paired up in mating tanks, restarting the breeding and selection cycle.

When the number of breeding pairs is lower than the number of fish that need to be raised, it is not possible to select just one embryo per crossing pair as previously described, yet control over pedigree is still recommended. In this case, a variation of the former method can be applied, as follows: multiple crosses of single pairs are set using females and males from non-sibling tanks, and the embryo clutches from the different breeding pairs are collected into separate petri dishes (**Figure 3B**, panels 1–3). Then, the colony manager defines the number of tanks, greater than one, where the larvae will be raised, and assigns a different subset of the collected clutches to each tank (**Figure 3B**, panel 4). Next, a few siblings per petri dish within each subset are selected, mixed and finally transferred to the corresponding holding tank, to be raised (**Figure 3B**, panel 1 and 5). The number ‘z’ of larvae selected per clutch depends on how many clutches are assigned to each tank, the size of the tanks and the intended fish holding density. As a result of this selection approach, each tank will hold a unique mix of siblings from different progenitors. Therefore, to avoid sibling mating when the next generation of the fish line needs to be established, all the new breeding pairs should include a female from one tank and a male from another.

Since zebrafish are shoaling fish known to naturally spawn in large groups, if we are not facing any broodstock number limitation, large bulk spawning in tanks of 40–300 L capacity is less time-consuming. Compared to pair crossing approaches, it increases fertilisation efficiency, provides better batches of viable embryos and enhances the population’s genetic variability [19]. This approach is more frequently carried out in large-scale facilities, since it requires large broodstocks. More than 10,000 embryos can be collected using mass spawning tanks, in just a 10-minute period [10]. For small fish stocks, the strategy can be downsized and small bulk crosses set holding around 5 to 15 fish with a ~ 2:1 female:male ratio, which takes less time to set and house than multiple units of single pairs. However, group crosses have some downsides: it is not possible to know the individual contribution of each brood fish to the progeny or how many fish actually spawned. Consequently, the offspring selection is random and there is no direct control over a population’s genetic diversity. The chance of selecting some siblings is real, especially in small bulk matings and when the number of offspring selected to propagate the line is low. To overcome that, it is possible to set multiple small bulk crosses and follow the same rationale for progeny selection as for multiple paired crosses (**Figure 3**). By doing so, the chance of spawning success on each mating unit is higher. In addition, if the progeny selection strategy includes the collection of more than one embryo per cross (**Figure 3B**), there is a possibility of selecting non-siblings from each crossing unit, further contributing for a more genetically diverse group of fish.

3.2.1 Wild-type lines

There are plenty of reasons to maintain wild-type lines with good fitness and high genetic variability. The wild-type zebrafish maintained in research facilities are the closest representations of the species in the wild. Keeping these lines robust and genetically diverse is crucial for reproducible experimental outcomes which, in turn, is key for validating scientific evidence [3]. Aside from direct experimental use, wild-type embryos are the most common base for the generation of transgenic and mutant lines upon delivery of the molecular components required for the intended genetic

modification into the embryos. Additionally, once established, genetically modified lines are often maintained in heterozygosity by crossing with a wild-type line as a means of avoiding inbreeding depression [2, 23].

Strategies for breeding wild types are typically adaptations of crosses described in Section 3.2. The decision depends greatly on the size of the stocks that each facility holds, but the goal should always be to preserve as many alleles as possible across generations. Only phenotypically healthy larvae should be selected.

3.2.2 Transgenic lines

Maintenance of transgenic lines requires the preservation of the transgene or transgenes of interest while keeping genetic diversity of the remaining genome. A simple yet effective strategy is to maintain the transgenic fish in heterozygosity through outcross with a wild-type line. At each line maintenance cycle, the wild-type fish used should have little or no relation to the wild-type individuals used to set the former transgenic generation, and offspring should be carefully screened. In addition, including cross units with transgenic males and wild-type females and other units with the opposite can avoid the loss of genetic or epigenetic information transmitted to the offspring by one of the sexes alone [24, 25].

Another approach is to incross the line for several consecutive generations using mating schemes that involve multiple crossing units. The progeny will include embryos that are homozygous for the transgene and by selecting only those embryos to raise, its expression gets stronger. This is an interesting option, for example, when transgene expression is detected by dim fluorescent reporters or when it silences fast (e.g., UAS reporters) and to increase confidence in phenotypic screens because homozygous are often easier to identify. Moreover, researchers may require the propagation of lines in homozygosity for experiments. The downside of successive incrosses is that to keep fish away from inbreeding depression, the transgenic lines need to maintain a broodstock larger than the capacity of many facilities, whether that is due to space limitations, budget constraints, 3Rs policies or a combination of factors. In those cases, a good option is to intercalate incross with outcross to a robust wild-type line every other generation, or at least every 1 to 3 generations of incross, for the introduction of new alleles (**Figure 4**) [23].

Once offspring are obtained, it is necessary to select the individuals to raise. Screening approaches vary with the transgene and also if discrimination of zygosity matters. When the transgene has a fluorescent reporter, it is possible to preserve the genotype across generations without genotyping, by raising only the fish with the brightest fluorescence and complete expression pattern. If the number of fish that respect those criteria is lower than the required, it is worth repeating the cross until reaching the target number, instead of selecting suboptimal fish. A similar rationale can be applied to transgenes with other traceable phenotypes.

In some transgene expression systems, such as Cre-loxP, Gal4-UAS and Q system, the promoter and the reporter are inserted into individual constructs that may be delivered to separate fish [26–28]. This increases the number of possible combinations between promoter and reporter upon outcrossing the lines with the elements of interest. Yet there is no direct way to identify the fish that carry each element alone, because the expression of the reporter only happens in fish that carry both components. A practical solution is to include in the construct of each element (promoter or reporter) an expression cassette for a fluorescence marker, such as a heart or retina marker that will be expressed in all the fish that carry that element [29, 30].

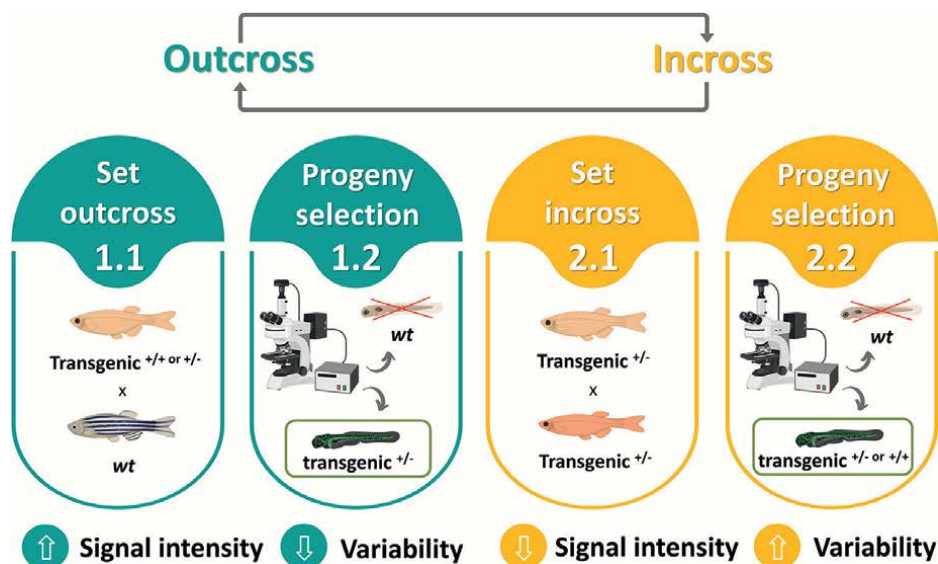


Figure 4.
 Example of a line maintenance approach for transgenic zebrafish. (Outcross, 1.1) transgenic fish are outcrossed with a robust wild-type line. (1.2) the heterozygous progeny is selected, raised and maintained as broodstock or for experiments. (Incross, 2.1) when a new generation is required, the adult heterozygous transgenics are incrossed and (2.2) the best offspring for the trait of interest are raised. It may be possible to distinguish phenotypically homozygous from heterozygous fish, otherwise they can be raised together and addressed to transgene positives. Incrosses should be alternated with outcross every other generation or at least every 1 to 3 generations of incross, to keep the colony hardy.

Alternatively, select embryos based on good health and screen them individually upon sexual maturity. For that, mate them in single pairs with fish that carry the complementary element of the transgene expression system and analyse the progeny: if at least some of the progeny express the expected phenotype, then the parent under screen is positive for the transgene.

Single paired crosses and progeny analysis can also be used to learn parental zygosity without the need to genotype by molecular methods, as long as the transgene of interest has a detectable phenotype. The mating pair needs to be negative for the transgene of interest and similar phenotypes. On top of that, if screening for one of the elements of a two-component transgene expression system, the mating partner also needs to be homozygous for the element complementary to that under screen. Then, if all the offspring exhibit the phenotype of interest, the parent under screen is homozygous for the transgene; if just part of the progeny expresses it, then the parent is heterozygous.

Finally, for transgenes not plausible of phenotypic screen, the solution is to collect a biological sample for total DNA extraction and processing for genotyping. The DNA source can be either whole embryos, larvae finfold, adult fin biopsies, skin swabs or the bathing water of larvae subject to microfluidic harmonic oscillation. The latter two are less-invasive so less likely to cause pain to the animals [31–34].

3.2.3 Mutant lines

Like for transgenics, preservation of mutant zebrafish across generations involves the conservation of the mutation at the same time that the genetic diversity of the

remaining genome is preserved. If a mutation is lethal in heterozygosity, it is not possible to raise a line, but lines with lethal recessive mutations can be maintained in heterozygosity by outcrossing with a different line (typically a wild type) using the breeding scheme that best meets the needs. The offspring is then screened to distinguish mutation carriers from fish that only have the wild alleles [10]. Screening relies on phenotyping or genotyping depending if there is a detectable phenotype or not. For non-lethal mutations, a similar outcross approach can be used to preserve the lines as long as there is not a research need for homozygosity. In fact, keeping mutations in heterozygosity is best for prevention of unexpected harmful phenotypes. The downside is that screens can become expensive if there is not a phenotype that is easy to trace, because it is necessary to run genotyping every time a new generation is raised.

To cut screening time and costs, or if research demands it, viable mutations can also be propagated by incross of the homozygous fish. In this case, all offspring are also homozygous for the mutation, so there is no need for screens other than selecting individuals that look robust. However, unless fish numbers are large, inbreeding escalates steeply. So, a compromise approach between reduction of screening costs and maintenance of line hardiness is to use a two-generation cycle that includes an additional and optional sidestep, as depicted in **Figure 5**.

It starts by outcrossing homozygous mutants with a wild type so that all offspring are heterozygous for the mutation (no need to screen) and genome diversity is refreshed by the alleles passed on by the wild-type parents. For the next generation, incross the heterozygous and screen the offspring for homozygous mutants. For cost reduction, screen for a low number of mutants of each gender. Then, the line propagation cycle restarts using multiple paired matings between each mutant and a wild type. This way, the reduction in allele diversity caused by mutant colony downsizing is compensated by the new alleles brought by the outcross. The sidestep is introduced

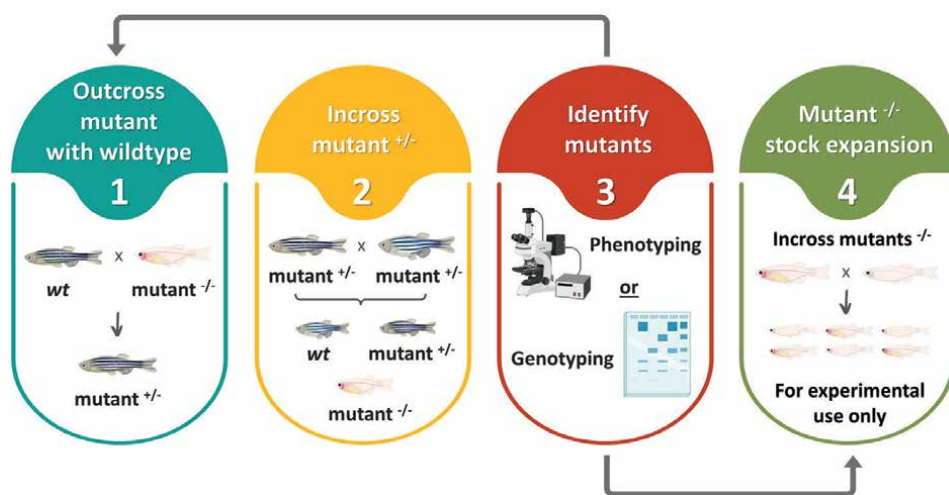


Figure 5.

Example of a cost-effective line maintenance approach for mutant zebrafish. (1) mutants (homozygous) are outcrossed with a robust wild-type line and the heterozygous progeny is raised and (2.1) incrossed when it is time to launch the next generation. (2.2) homozygous mutants are identified by phenotyping or by genotyping and are used to restart the cycle on step 1. In parallel, the screened and raised homozygous mutants can be (3) incrossed for direct mutant stock expansion for experimental use. These fish should not be included in the line maintenance cycle.

when there is a demand for larger numbers of homozygous mutants. In that case, the screened mutants are incrossed for line expansion as a sidestep. Importantly, this group is not used for line propagation to avoid faster build-up of the line inbreeding.

3.3 Alternative breeding strategies

Having clear mating and screening strategies is fundamental but not always enough to ensure the continuity of fish lines throughout time. Factors like advanced age, isolation, stress, nutrition and health issues can lead to the decline of fish fitness independently of the size of the fish stock [2]. If fish fail to breed naturally, *in vitro* fertilisation (IVF) may be a means to obtain embryos. Protocols are available in the literature [35, 36]. The basic concept is to collect gametes from anaesthetised and sexually mature fish, without letting the cells contact with water. Then, mix the sperm and oocytes in small dishes and add holding system water to activate fertilisation.

A more complex approach is to cryopreserve sperm in liquid nitrogen [35, 37, 38]. Cryopreservation responds to the challenge of long-term storage of an ever-increasing number of fish lines in the limited space of fish facilities, by saving the time and space it takes for fish housing and husbandry. In addition, it meets two of the three 3Rs principles: 'Reduction', by allowing a decrease in the number of fish raised while still preserving lines that may be useful in future research, and 'Replacement' of fish shipping with cells shipping for sharing of lines between institutions. It can also be a way of preventing the loss of lines due to natural catastrophes, the spread of infectious diseases or failures in fish breeding capacity [39]. Importantly, in the context of maintenance of lines away from inbreeding, colony management programmes can include the sperm cryopreservation as soon as lines are established or soon after they are imported as a means of preserving genetic diversity before inbreeding and genetic drift act. Some generations later, those sperm samples can be used to reintroduce lost alleles through IVF using freshly collected oocytes. Programmes for cryopreservation of whole embryos are not a reality yet, but there is promising work in progress [39].

3.4 Complementary approaches against inbreeding

No matter what breeding strategy is applied, inbreeding and genetic drift are inevitable. As such, colony managers may want to complement line maintenance with additional measures that help keeping populations hardy. These include (a) reduction of stock renewal frequency by extending the lifetime of each generation within the limits of reproductive capacity, (b) increase of the number of fish maintained per line, (c) outcross of genetically modified lines with a robust wild type at least every 1–3 generations and (d) import fish, especially wild types, from health-accredited centres or trusted facilities every 1–3 years (larger stocks and with longer generation time require less frequent import); upon fish import, either outcross the new fish with the in-house ones to introduce new alleles, or replace the former stock completely if its fitness is very poor [10, 15, 40].

4. Prevention of genetic cross contamination

There are no standard guidelines on how to manage the daily activities of a fish facility, but prevention of genetic contamination, or line mixing, should always be

Line maintenance step	Actions against genetic cross contamination
Crosses & housing	Bring one tank at a time to the working area. House it back before bringing the next one. Use one net per tank.
Embryo plates cleaning	Use one pipette per plate.
Surface disinfection	Ensure clear labelling of embryo containers to identify the corresponding fish lines.
Phenotypic screens	Use one pipette per plate. Check fluorescence on all emission spectra present in lines housed at the facility.
Genotypic screens	Identify tanks with the isolated fish. Ensure clear match between fish and genotyping samples.
Larvae entrance in the holding system	Ensure clear correspondence between each embryos plate and the tank where they will be housed.
Fish transfer to clean tank	Label the new tank before transferring the fish. Work one tank at a time. Use one net per tank.
At all times, keep all fish containers clearly identified and avoid distractions	

Table 2.

Preventive actions against genetic cross contamination of fish lines during line maintenance.

a matter of concern. In many cases, fish from different lines look alike under visual inspection, so accidental mixes will not be noticed unless genetic screens are made and can jeopardise the accuracy of experimental results.

Centralising colony management on the fish facility staff is ideal to avoid errors; nevertheless, human resources are often insufficient to do so. Providing extensive training to researchers is also helpful. Two effective practices to save time and keep the focus on the ongoing task are to have fish containers clearly identified at all times and to have the most frequently required information readily available on tanks labels. It is also convenient to register all crosses that are set so that the genealogical history of each batch can be traced as a way of finding sources of genetic contamination [23]. Moreover, a close and open communication with all the facility users helps in identifying cases of line mixing faster, as well as learning more about the quality of the experimental fish. Finally, there are additional and simple actions that can be implemented to prevent genetic cross contaminations at different steps of the line maintenance cycle, as listed on **Table 2**.

5. Record keeping and databases

A colony management program is not complete without accurate records of a multitude of details that characterise the lines and influence its husbandry and maintenance cycle. No matter how many tanks and fish lines a facility has, it is valuable to keep as many records as possible. Relevant information often includes:

- a. Date of crosses. Many facilities implement a minimal resting period of one to 2 weeks to allow the development of a new batch of gametes under no reproductive stress and to preserve the spawning capacity for longer since fish spawned more frequently have shorter reproductive span [2, 23].
- b. Spawning success and embryo quality. Both inform about the breeding performance and the fitness of the animals. A simple way of assessing them is through

code systems that indicate ranges of fertilised eggs (for spawning success) and ranges of survival rate at defined ages (for embryo quality).

- c. Pedigree for each stock of a line. This is to avoid matting of closely related individuals. At least keep record of the parents for each new batch that is raised. From here, it is possible to trace back family trees.
- d. Centralised line maintenance calendar. Examples of information to include for each line: line designation, propagation frequency, developmental stage for screening, screening method, what to screen for, month and year of birth of the latest generation and for launching the next one, lab that owns the line and person responsible for it and a comments section for relevant information.
- e. Age of fish on each tank. As fish get older, spawning success and gametes quality decrease and susceptibility to pathogens is higher. Renewal of the lines' stock is recommended in general after 1.5 years old [2, 23].
- f. Health monitoring and clinical cases.
- g. Survival rate at defined developmental stages (e.g., 30, 60, 90 days old).
- h. Number of fish and sex ratio on each tank.
- i. Information required for animal usage reports. Examples: number of animals used per experiment and per project, individual severity.
- j. Location of single stocks within a holding system, to save time.
- k. Line original lab and any paperwork related to fish imports or exports.

Notebooks, labels posted on the tanks' outer surface, worksheets and spreadsheets are helpful and inexpensive to keep all records but are not efficient to navigate through complex data collected over years and for many fish lines. For that reason, digital databases were developed to save comprehensive and detailed records of fish lines [23, 41–45]. Databases are expected to compile many of the information listed above or more, to have extensive search data filter options and to connect to printers for creation of tank labels with the pre-defined information, such as the line name, stock number, date of birth, parents and name of the person responsible. Some databases also generate statistics and plots for different parameters. Options available include ZeBase [42], DanioData [40], Zebabase [44], PyRAT Aquatic, Facile Fish, ZebraFish Database [43], ARMIS and FishNET [41]. Some are open-source software; others are commercially available at different price levels. The ones that can be opened on any device with a web browser allow for remote access. Regarding data storage, some databases include an option for storage on a web server running on a personal computer or in a dedicated server. Other software requires a full licence purchase, and others depend on variable dedicated computer storage capacities. Databases with good and fast technical assistance are always preferable.

6. Conclusions

Effective colony management programmes are complex yet necessary to maintain fish lines healthy and vigorous across many generations. Colony management programs should be designed and understood as part of the solution against the lack of genetically stable zebrafish lines, which is a recognised problem that limits the reliability of data produced from this animal model. Frequent genetic monitoring of fish lines could be the next best alternative, but its implementation as part of colony management programs is not standard yet and so was left out of this chapter. Breeding and embryo selection strategies are the heart of colony management plans and typically centred in the reduction of the deleterious effects of the inbreeding depression and genetic drift that inevitably accumulate as the number of generations of a fish line advance. Whatever the options implemented, line maintenance needs to take into account the interests of both the animals and the researchers. Fortunately, many times, the protocols that favour fish health and robustness are also the best for the production of solid experimental outcomes.

Fish facilities can house from a few units to thousands of different lines, many of which are phenotypically alike at visual inspection, which makes accidental mixes possible, no matter how careful people are. As such, it is important to assume that possibility and include in the colony management programme as many preventive actions as possible, as correction measures are much more time-consuming and possibly more expensive. As years go by and generations accumulate, the amount of relevant information also increases, including family trees that get more complex and observations on each fish line that may be relevant to adjust propagation protocols. Consequently, finding good record keeping methods to organise and save all that data is thus another topic to bring to the table while designing facility-specific colony management plans.

The methods and ideas present here are commonly applied as described, but the main goal of this chapter was to provide tools that can and should be adjusted according to the fish lines and conditions of each facility.

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Notes/thanks/other declarations

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Section 3

Zebrafish as an Outstanding Model for Translational Research

Zebrafish for the Study of Enhancer Activity in Human Traits and Disease

*Emily Morice, Caleb Calhoun, Robert Lalonde
and Megan Leask*

Abstract

Enhancers are genetically encoded elements that are critical for controlling gene expression. Despite the importance of enhancers in development, normal biological function, and disease, they have been historically overlooked and remain understudied. To understand how enhancers function, appropriate molecular tools are required that can capture the temporal and spatial function of enhancers within appropriate biological contexts. Zebrafish are an excellent *in vivo* model for the study of enhancer elements and the genetic variants that alter their function. Because zebrafish larvae are transparent, transgenes encoding enhancers tagged by fluorescent reporters can be visualized in the tissues and developmental stages where the enhancers function. Transgenesis of enhancers can be achieved using various plasmid technologies and transgene integration methods. Here, we describe the history and most recent developments in the zebrafish enhancer assay, from vector designs to various transgene integration techniques. We go on to describe how the application of these assays have been integral for our understanding of genetic variants found in humans and within enhancers that can contribute to both human Mendelian and complex polygenic disease.

Keywords: enhancer, non-coding, transgenesis, genome-wide association studies, disease

1. Introduction

Phenotypic diversity between the cells and tissues of an individual organism is immense, but differences in the protein coding sequences of genes do not fully explain this diversity. Instead, genomic regulatory elements, that reside outside of genes in the non-coding regions of the genome, are largely responsible for this diversity by controlling gene expression [1]. This regulation of gene expression typically involves two types of genomic elements: the gene promoter and the enhancer [2]. The term enhancer reflects its function; the ability to enhance or modify the transcription of a gene from its promoter [3]. Enhancers are defined as pieces of DNA, spanning 100 base pairs in length to a few kb in length, that encode instructions that transcription factors recognize to promote gene transcription (**Figure 1**) [4]. Importantly, this regulation of gene expression

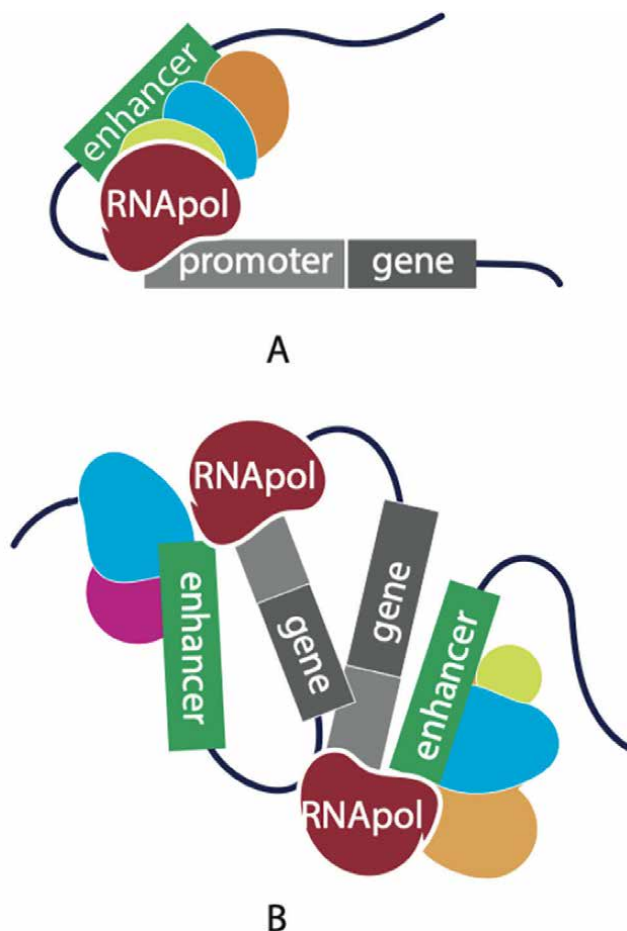


Figure 1. Enhancer mediated regulation of gene expression. A single (A) or multiple enhancer(s) (green) (B) loops to a gene promoter (gray) bound by transcription factors and/or chromatin binding proteins and recruits RNA polymerase to drive gene expression.

involves the coordination of chromatin looping events that bring the enhancer, bound by transcription factors, and gene promoters into close spatial proximity. The configuration of these physical interactions, such as single enhancer: gene pairs (**Figure 1A**), single enhancer: multiple genes, or multiple enhancers: multiple genes (**Figure 1B**) are important for the tissue specificity, developmental timing, and strength of the transcriptional control of genes [5]. This fine tuning of gene expression drives multicellular development, lineage commitment, and ultimately leads to the phenotypic differences that we observe between cells, tissues, individuals and animal species.

2. The study of enhancers for human health

The human genome is predicted to have 1 million enhancers, although this is likely an underestimate [6]. Given this, and their role in gene expression, it is not surprising that the disruption of enhancers has been implicated time and time again as the cause of genetic trait differences, Mendelian disease, and predisposition to common diseases.

The function of enhancers can be altered in a number of ways; deletions, insertions that separate the target gene from the influence of the enhancer, and single nucleotide changes in the enhancer that either ablate transcription factor binding, or introduce novel or additional binding sites. These alterations in the enhancer code can affect the regulation of gene expression, either by loss of function resulting in diminished gene expression, or gain of function resulting in overexpression of the target gene. A prime example of this, and commonly overlooked as being controlled by the non-coding genome, is the blue eyed human trait. The blue eye trait follows a predictable Mendelian inheritance pattern, which indicates that it is caused by a single genetic change. The causal genetic variant, found in roughly 18% of people with European ancestry, is located in the intron of *HERC2* [7]. This non-coding variant disrupts an enhancer that loops to the nearby gene promoter, driving *OCA2* gene expression (pigmentation gene), and results in pigments being deposited in a cell-specific manner in the eye. This genetic variant arose from a single shared ancestor, and was likely selected because it conferred an evolutionary advantage [8]. Over evolutionary time, this variant has dramatically increased in frequency in people of European ancestry.

In comparison to the blue eye genetic variant, only a small handful of genetic variants responsible for Mendelian disease that disrupt enhancers have been identified. This is likely due to the historical focus on protein-coding genes, and difficulty in unraveling molecular mechanisms of regulatory genetics. However, genetic variation within non-coding regulatory elements can have profound biological impacts that are equivalent to coding variants. For example, the duffy mutation that impacts susceptibility to malaria [9], and non-coding variants at *SLC2A9* that influence serum urate [10, 11].

The recent developments in sequencing technologies, and the reduction in cost, enables us to expand our search to the non-coding genome. The explosion of genome-wide association studies (GWAS), indicates that >90% of the genetic variants underpinning complex human diseases and traits fall in the non-coding genome and overlap significant regions of regulatory activity [10]. Researchers are now interrogating enhancers for discovery of disease and disease susceptibility genetic variants, particularly where variants in the protein-coding genes cannot be identified. Recent examples are emerging of therapeutics that target non-coding elements. For example, an erythroid-specific enhancer that was initially discovered via GWAS has been successfully targeted in gene therapy for sickle cell disease [12]. Importantly because regulatory control by enhancers is often tissue-specific, therapeutic targeting of these non-coding elements raises the possibility that there will be fewer side effects due to systemic off-target effects. Moreover, the sheer number of non-coding elements in the genome offers a wealth of drug targets to exploit. Therefore, identifying and characterizing non-coding disease-causing genetic variants has remarkable therapeutic promise for the treatment of Mendelian and complex disease.

The conserved characteristics of enhancers can be exploited for their discovery. Active enhancers have distinct hallmarks in the chromatin landscape. They are generally depleted of nucleosomes, and have characteristic histone modifications (*e.g.*, acetylation of H3K27 (H3K27ac)). These characteristics can be detected using DNase I hypersensitivity mapping (DHS) and chromatin immunoprecipitation, followed by sequencing (ChIP-seq) using antibodies for specific histone modifications. In an effort to comprehensively annotate the non-coding regions of the genome, international consortia have been developed to consolidate this regulatory information (*e.g.*, ChIP-seq and DHS data) that is being generated by scientists worldwide. Leading the way in this endeavor, is the ENCyclopedia of DNA Elements (ENCODE)) project, which was established in 2003 [6]. As an example of its utility, the publicly available ENCODE

data including ChIP-seq, transcription factor binding, and DHS data can be visualized using the UCSC browser, and overlapped with genetic variants of interest to identify putative causal enhancer variants (discussed in more detail in subsequent sections of this chapter). Another extremely valuable dataset that aids in the identification of putative enhancers containing genetic variants, is the Genotype-Tissue and Expression Project (GTEx). This database includes ~1000 individuals who donated their bodies to science and have been genome-wide genotyped, and had RNA-sequencing carried out in >50 tissues. This data allows scientists to connect genetic variants in the non-coding genome to gene expression (expression quantitative trait loci (eQTL)), identifying regions of the genome that control gene expression. This eQTL data can be overlaid with regulatory data from ENCODE to identify putative enhancers.

The wealth of information gleaned from these datasets and repositories indicate that our efforts should now be focussed on the functional validation of these computationally and biochemically predicted enhancer elements, and how the genetic variants within them alter their activity. The gold standard to date for functional validation has utilized reporter assays (*e.g.*, luciferase assays and massively parallel reporter assays). However, because enhancer activity is highly dependent on the availability of the right combination and stoichiometry of specific transcription factors [13], these cell-based assays cannot recapitulate the complexity of the developmental and tissue-specific activity of an enhancer [14]. To truly understand an enhancer's regulatory mechanisms, a reporter assay must utilize a whole organism (*in vivo*). *In vivo* reporter assays of enhancer function, with reporter-tagged non-coding DNA encoding potential enhancers, have been carried out since the late 90s, and were first conducted in *Drosophila* [15, 16]. These reporter assays, vector construction, and the various transgene insertion techniques have been successfully translated into the zebrafish (the general reporter assay strategy is outlined in **Figure 2**) over the past 25 years [17–23], and will be discussed in the following sections of this chapter.

3. Zebrafish as an ideal model for enhancer function and application of reporter assays

Zebrafish have been an important *in vivo* model for the study of enhancers and gene regulation, and studies consistently show that human regulatory elements can be effectively studied using this model [24–29]. Zebrafish are ideal for the study of enhancers for a number of reasons. For example, humans and zebrafish are only ~420 million years diverged [30]. This equates to >70% of protein-coding genes at the sequence level being conserved between humans and zebrafish. These genes include a large suite of transcriptional machinery, such as transcription factors, which via enhancers function to regulate gene expression [31, 32]. HNF4 α is an excellent example of the conservation of transcriptional machinery between humans and zebrafish. HNF4 α is a key transcriptional regulator that controls cell-specific gene expression in development, and is conserved between humans and zebrafish [33]. The function of this transcription factor in development is so tightly constrained by evolutionary mechanisms that there are no amino acid differences in the DNA binding domain between zebrafish and humans [34]. In both humans and zebrafish, the conserved DNA binding domain of HNF4 α functions to recognize the HNF4 α motif that is encoded in the DNA sequence. The ligand binding domain of HNF4 α responds to cell-specific cues to drive HNF4 α transcriptional regulation [34]. Therefore, zebrafish HNF4 α maintains the ability to recognize human HNF4 α motifs encoded by human DNA sequence [35, 36].

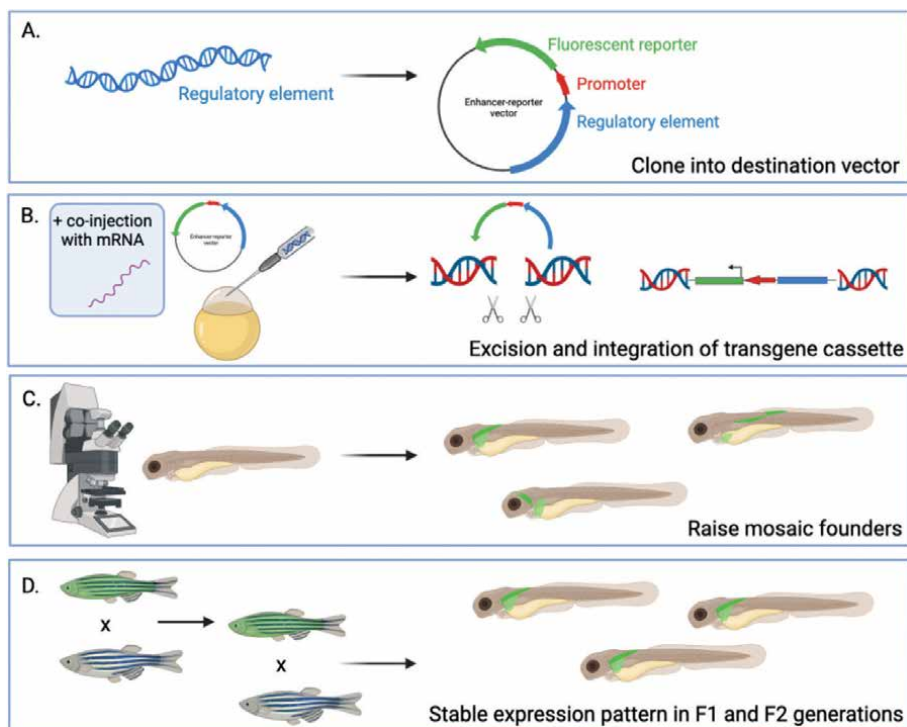


Figure 2.
 Reporter assay strategy in zebrafish. (A) a regulatory element is cloned into a destination vector. Vector design varies, but at minimum includes a promoter and a fluorescent reporter. (B) the reporter assay vector is injected into fertilized zebrafish eggs with, or without, the co-injection of additional integration technologies. The transgene is excised from the donor plasmid vector and integrated into the zebrafish genome. (C) Injected fish are screened for reporter fluorescence by confocal microscopy. Individual fish that express the reporter are raised. (D) Fo founders are outcrossed with wild-type fish to establish F1 and F2 transgenic lines, where stable reporter expression patterns emerge. Created using BioRender.com.

While a large proportion of the protein-coding genome is conserved, the zebrafish genome lacks many enhancers identifiable by sequence conservation in other species, including humans [37, 38]. However, because the transcriptional machinery involved in gene regulation is conserved, the genetic regulatory mechanisms also persist [39]. For example, enhancer elements within the RET locus exist in both humans and zebrafish, which act analogously to control RET expression. Although the enhancer elements have evolved so that the sequence is no longer orthologous, their function, recruitment of the same transcription factors, is preserved [40].

Additional features of zebrafish, including ease of transgenic manipulation [41] and larval transparency, make it possible to directly observe enhancer activity in vivo [42]. For example, layering imaging of regulatory element expression with fluorescently-labeled cell markers allows for the assessment of tissue-specific enhancer activity over developmental time. Additionally, by comparing tissues where the enhancer drives expression to those where a gene is expressed (e.g., in situ hybridisation), one can approximate the target gene of an enhancer if the enhancer and gene expression colocalizes [36, 43]. Zebrafish are also a cost-effective and relatively low-maintenance model system, particularly when compared with mice (for which reporter assays exist), which makes them suitable for the longer-term rearing and maintenance [18]. This allows for multiple reporter lines to be generated so that

qualitative comparisons can be made between different enhancer elements, or variants within the same enhancer.

In zebrafish, a typical *in vivo* reporter assay (outlined in **Figure 2**) involves the construction and insertion of a plasmid vector. This vector contains a reporter protein placed downstream of a minimal promoter and a predicted enhancer sequence to be tested (**Figure 2A**). Insertion of the transgene into the zebrafish genome is mediated via endonuclease, retrovirus, transposon, or recombinase-based integration (**Figure 2B**) [44, 45]. Expression of the reporter protein in zebrafish larvae is used as a readout of enhancer activity because the reporter gene should only be expressed if the minimal promoter is driven by regulatory activity in the vicinity of the enhancer element.

In the context of human health and disease, alterations to enhancer activity by genetic variants within them can also be assessed in zebrafish reporter assays [19]. Several consequences of variants can be tested. For example, a complete loss of expression in a tissue may indicate the disruption of a key transcription factor binding site within the enhancer, affecting the formation of a transcription factor complex required for regulatory activity [46]. Modification of the strength of expression similarly may indicate a change in transcription factor binding affinity [19]. A novel expression pattern may indicate ‘gain of function’, where the DNA sequence change is such that a transcription factor binds more tightly, or a new transcription factor binding site is introduced [19]. Finally, no change in fluorescence strength or location may indicate that the variant has no functional effect on the enhancer’s activity, or may play some other role that is not captured in the context of this assay, for example in the organization of chromatin in the nucleus [47].

Importantly, there are several factors to consider when interpreting the results of a reporter assay in zebrafish. First, transgenic F0 fish will display mosaic expression of the transgene. This is because not all of the cells of the injected embryos will receive the transgene. However, a proportion of embryos will incorporate the transgene into their gametes, and their F1 progeny (stable lines) will either have the transgene in all cells, or none at all. Additionally, a phenomenon termed ‘position effect’ exists. The position effect is caused by the transgene being integrated into various genomic positions, which can in turn be influenced by the genomic context with which it is inserted [48–50]. The phenomenon often causes fluorescent expression to be identified that is not driven by the non-coding sequence of interest, but by the random insertion of the transgene into a region where strong regulatory activity exists. This can result in variable ectopic transgene expression, silencing of the transgene, or mutagenic effects if it is inserted within a coding region of the genome. To reduce the potential for bias arising from the over-interpretation of inaccurate fluorescent patterns and overcome mosaicism, multiple independent stable lines (F1 or F2 generations) of zebrafish should be screened for reproducible expression patterns, which may indicate the most likely expression pattern of the putative enhancer’s activity.

Before the advent of superior transgenesis techniques in the early 2000s, the method employed to generate transgenic zebrafish involved the injection of vast quantities of plasmid DNA ($\sim 10^6$ plasmid copies) in the hope that some of this DNA would be transcribed and then integrated into the genome to be inherited in subsequent generations [51]. This approach has very low rates of integration and germline transmission, and many different integration techniques have subsequently been developed that have dramatically improved these rates. For example, I-SceI mediated integration, first described in 2002 in medaka fish [45]. This method involves the co-injection of the meganuclease I-SceI, a rare-cutting endonuclease isolated from yeast, alongside a vector containing a transgene flanked by corresponding I-SceI recognition

sites. The meganuclease excises the transgene and provides recombinogenic ends to the DNA that facilitate integration into the genome. In comparison to a non-mediated transgenesis approach, this method reduces the amount of mosaicism present in the F0 fish and increases germline transmission rates (**Table 1**) [45].

Technique	Method	Merits	Limitations
Large plasmid quantity	Inject large quantities of plasmid DNA containing a transgene of interest ($\sim 10^6$ plasmid copies) into zebrafish embryos, where some of this DNA is likely to be transcribed and then integrated into the genome.		Low rates of genomic integration Low rates of germline transmission High mosaicism present Position effects Tandem insertions leads to silencing
Endonuclease	Endonuclease mRNA is co-injected with a vector containing a transgene flanked by corresponding endonuclease cutting sites. Once excised from the vector, the transgene is left with recombinogenic ends that facilitate integration into the zebrafish genome.	Reduced F0 mosaicism Increased germline transmission	Position effects Tandem transgene insertions
Retrovirus	Vector is injected into zebrafish embryos that contain the transgene and a modified viral sequence that has removed proteins required to replicate the virus but retained those that promote its integration into the genome.	High integration rate High rate of germline transmission	Variable copy number integration Position effects Multicopy insertions
Transposase	Transposase mRNA is co-injected into single cell zebrafish embryo with a vector containing a transgene flanked by non-autonomous transposon sequences recognized by the transposase which catalyzes the transposition of the transgene into the genome.	High integration rate High rate of germline transmission Plenty of published vector technologies for use in reporter assays No tandem insertions	Position effects Multicopy insertions Transgenes can remobilize if you inject with Tol2
Recombinase	Transgenic fish are generated with recombinase 'landing sites'. Injected into embryos of the progeny of these fish is the recombinase mRNA and a vector containing a recombinase site. The enzyme initiates the recombination of the transgene into the genome at the 'landing site'.	Very high integration rate Very high rate of germline transmission Single-insertion Targeted integration limits position effects Reduced F0 mosaicism Transgene insertion can be permanent (can inject these with phiC31 + vector)	Application is not widespread in zebrafish Specialized zebrafish transgenic lines required Current lack of understanding of good genomic integration positions and stable lines with mapped landing sites

Table 1.
Integration methods for the insertion of transgenes in zebrafish embryos to study regulatory function of enhancer elements.

The use of a retrovirus-mediated system has also been explored within the zebrafish model for the detection of putative enhancer sequences [52]. A retroviral vector system contains viral sequences that promote reverse transcription from RNA to DNA and integration into the genome, but do not encode the viral proteins required to replicate and produce additional virus. Instead of viral proteins, reporter genes and non-coding sequences can be engineered within the vector [53]. The use of these engineered retroviral vectors in zebrafish reporter assays enables the integration of a transgene into the genome and its inheritance through the germline. However, variation in reporter expression is caused by variable copy number integration and position effects (**Table 1**).

Over the past two decades, two dominant approaches have emerged for the integration of transgenes into the zebrafish genome that aim to combat the issues described above. The first, transposons, mediate the random integration of a transgene into a genome while the second, recombinase enzymes, mediate site-directed integration. These two methods will be discussed further in sections 3.1 and 3.2 and are outlined in **Table 1**.

3.1 Transposon-based integration

Transposons are genetic elements that can move from one area in the genome to another. Some elements are autonomous and encode a functional transposase protein that can catalyze the transposition of DNA [54], and other elements are non-autonomous, and do not encode a functional transposase but maintain essential sequences that can be transposed in the presence of transposase [55, 56]. An autonomous Tol2 mobile element, belonging to the hAT transposon family [57], was discovered in the medaka fish [58]. In zebrafish, where this element is naturally absent, a two-component transposition system utilizing the Tol2 transposon to integrate a transgene of interest has been developed [59]. This system requires both a plasmid containing a nonautonomous Tol2 element lacking part of the transposase gene, and the mRNA of the Tol2 transposon. These components are co-injected into a single-cell zebrafish embryo, where the Tol2 transposon catalyzes the transposition of the nonautonomous element from the plasmid into the zebrafish genome (**Figure 2**) [60–62]. This technology has been adapted to study predicted enhancer elements by designing a vector with an enhancer-promoter-reporter cassette in-between two nonautonomous Tol2 elements [63]. Fish injected using this system are analyzed for their fluorescent expression patterns indicative of enhancer activity, and these fish are then raised to adulthood and used to create transgenic lines. The rate of transgene integration is very high, particularly when compared to simply injecting just plasmid DNA, and this technology has been successfully applied to study a range of human enhancers. However, a large caveat to transposon-mediated transgenesis is that the transgene is inserted randomly and predominantly into active genomic regions, which causes position effects [63, 64]. Many groups have utilized this Tol2 transposon-based system to functionally investigate enhancers in zebrafish through the use of specifically designed vectors.

Some vector designs containing Tol2 sequences are of a relatively simple design; a green fluorescent protein (GFP) reporter, a minimal promoter, and a Gateway cassette for easy recombination of DNA of interest [17]. While these designs are successfully used to generate transgenic fish, there are additional components that can be added for ease of use, and that have improved our ability to assess enhancer activity in zebrafish. For example, the more simple vector designs lack an internal positive

control, which makes it difficult to determine the success of a genome integration event or the passage of this integration down the germline. This is particularly challenging when the spatiotemporal nature of enhancer activity is considered, whereby an enhancer may only drive expression of the reporter at a specific developmental stage and/or a tissue that may not be visually obvious.

The Zebrafish Enhancer Detector (ZED) vector was designed to address the need for a positive control for transgenesis and additionally, inconsistent fluorescent expression caused by the position effect phenomenon (**Figure 3**) [65]. The ZED vector contains a positive control cassette which includes a Cardiac Actin promoter and red fluorescent protein (RFP). This cassette drives RFP expression in the somites of the fish and therefore, can be used as an obvious indicator of the success of vector integration into the genome. In comparison, the enhancer detection cassette contains the green fluorescent protein (GFP) as a reporter for activity so that visualization of the success of integration and potential enhancer activity can be visualized on different light channels. The ZED vector decreases position effects by flanking the reporter cassette with insulator sequences so that the cassette is shielded from position effects. Furthermore, this vector contains an improved minimal promoter to drive GFP which was selected for optimal enhancer activity detection, and two excision cassettes. The excision cassettes allow for the genetic deletion of the transgene, should a researcher want to confirm that the fluorescent activity is dependent specifically on the enhancer element.

The ZED vector's key improvements allow for the efficient and rapid generation of transgenic zebrafish that contain an internal transgenesis control and reduced

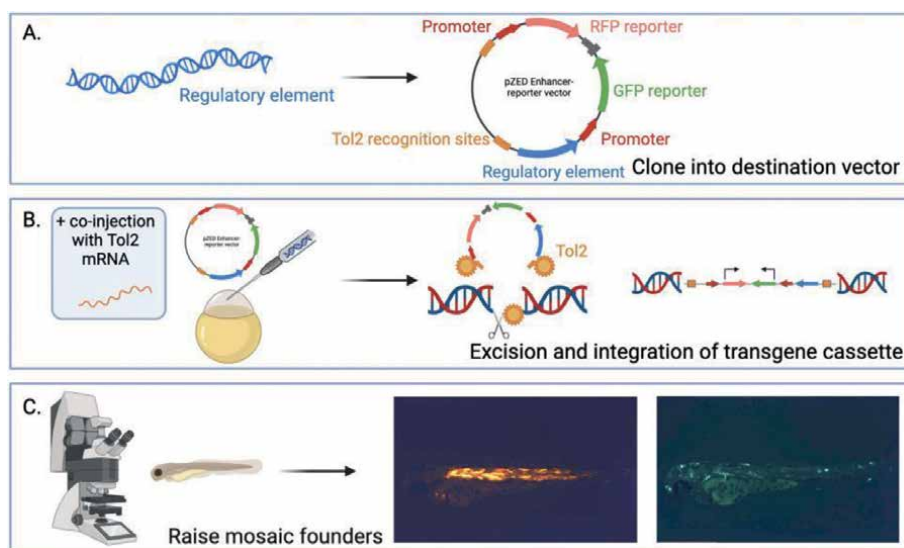


Figure 3. Zebrafish enhancer detection (ZED) vector system. (A) a regulatory element is cloned into the ZED vector which contains a positive control cassette, consisting of a cardiac actin promoter and RFP, and a reporter cassette, consisting of a gateway entry site upstream of a minimal promoter and GFP. (B) the vector is co-injected into single-cell fertilized zebrafish eggs alongside Tol2 mRNA. The Tol2 transposase is transcribed into an enzyme that binds to the recognition sites on the vector and excises the transgene, and then binds to a sequence in the genome where it mediates the transgene's integration. (C) Confocal microscopy is used to screen injected embryos for RFP (integration control), and GFP (regulatory element activity) expression. Zebrafish photos are original work. Created using BioRender.com.

position effects. However, limitations to this system remain. For example, while position effects have been reduced, they unfortunately still remain which can interfere with the interpretation of fluorescent signals. Another issue with this system, is while RFP expression in somites makes for an obvious and effective control, the expression of this protein can be so strong and so abundant throughout the fish that the RFP can bleed through other fluorescent light channels (as seen in **Figure 3C**) and it is hard to analyze the location of specific GFP expression in the fish. Despite the short-comings of this particular vector, various improved transgenesis reporter fluorescent markers continue to be designed and incorporated into reporter assays for example, cryaa: venus is a cerulean fluorescent marker of transgenesis expressed only in the lens on the fish [66].

Another example of an assay that applies the Tol2-mediated transgenesis is the dual-color reporter-based assay, used for the analysis of variants within enhancer elements in parallel (**Figure 4**) [19]. In this assay, two Tol2 constructs bearing the alternate alleles of a genetic variant within an enhancer are recombined with different fluorescent reporter cassettes in a multi-gateway reaction. These two plasmids are then co-injected with Tol2 mRNA into zebrafish embryos. Developing fish are then screened for reporter fluorescence and the positive fish are outcrossed to generate stable dual-reporter-expressing lines. Using confocal microscopy, tissues where the activity of two enhancers overlap are observed as yellow, while independent activities are green or red. This system allows for the direct comparison of the spatial and temporal activities of different enhancers, or different variants within the same enhancer, in a single zebrafish [19]. This assay significantly reduces the number of zebrafish normally required in a reporter assay. A limitation is that this system cannot

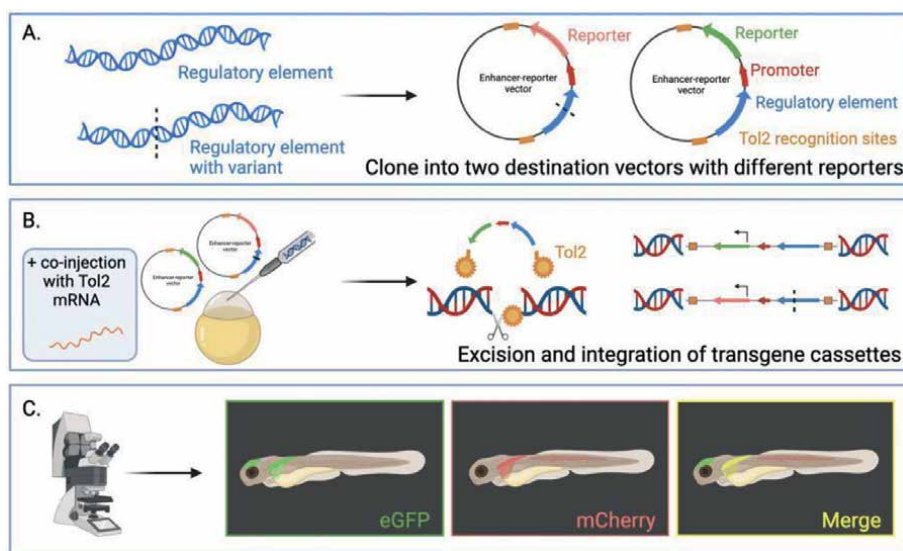


Figure 4. Dual-color zebrafish transgenesis strategy. (A) Two regulatory elements are cloned into vectors containing a minimal promoter and two different fluorescent reporters. (B) both vectors are co-injected into single-cell fertilized zebrafish eggs alongside Tol2 mRNA. The Tol2 transposase is transcribed into an enzyme that binds to the recognition sites on the vector and excises both transgenes, and then binds to a sequence in the genome where it mediates the transgene's integration. (C) Confocal microscopy is used to screen injected embryos for fluorescence where independent regulatory activity is observed as green or red, and overlapping activities are yellow. These mosaic F₀ founders are raised to generate transgenic lines. Created using BioRender.com.

control for the position of insertion sites, and the zebrafish tend to display variation in expression patterns due to positional effects. Additionally, the two enhancer sequences will integrate into the genome in variable numbers because they are within different plasmids, limiting the system for quantitative assessment of altered enhancer activity.

3.2 Recombinase-based integration

In comparison to the random integration methodology of transposons such as Tol2, site-specific recombinases are enzymes that catalyze recombination events at highly specific target sequences. This method has the potential to mitigate the variability caused by the random integration of transgenes and resulting positional effects by creating an efficient and reproducible site-directed integration system, where enhancer elements and their variation can be quantitatively compared. Owing to their specificity, pre-defined recombinase sites can be established within a model organism's genome, permitting the integration (**Figure 5B**) or excision (**Figure 5A/6B**) of transgenes at a defined position by recombinases [67–70]. Notable recombinases that have been employed within the zebrafish model system include Cre, derived from bacteriophage P1, Flp, from yeast, and phiC31 recombinase, from phage phiC31. In comparison to the recombinases Cre and Flp who target loxP and FRT sites respectively, phiC31 targets two heterotypic sites termed attB and attP, resulting in the directional and irreversible integration of DNA (**Figure 6D**) [71–73].

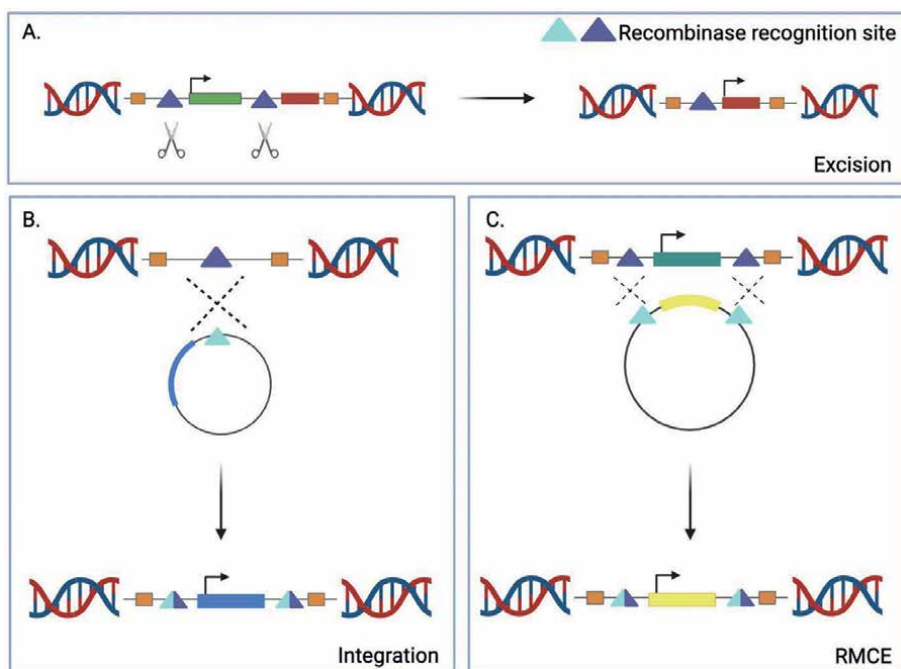


Figure 5. Recombinase-based transgene integration strategies. (A) Excision of a previously inserted cassette, typically employed to induce regulatory activity at a specific tissue or point in development. (B) Site-directed integration of an introduced transgene in the genome. (C) Recombination-mediated cassette exchange (RMCE) between a new and previously introduced transgene. The phiC31 integrase system is effective at controlling cassette excision, integration, as well as RMCE. Created using BioRender.com.

PhiC31's canonical role is the integration of a phage genome into a bacterial genome via recombination between attP (phage) and attB (bacteria) sequences. Utilizing the specificity of phiC31 recombinase to these sites, an exogenous plasmid containing a transgene with an attP (or attB) site can be injected alongside phiC31 encoding mRNA into a zebrafish embryo harboring a previously integrated attB (or attP) landing site in their genome (**Figure 6**). The phiC31 initiates the recombination of this transgene into the genome at these attB/attP sites [74–78]. The irreversible nature of this system caused by the completed attP/attB recombination, produces hybrid attL (left) and attR (right) sites which cannot be used by phiC31 as substrates. This lends itself to heightened efficiency because even in the presence of large quantities of plasmid, the recombination between the attP/attB sites cannot be reversed [71].

The use of phiC31 attP/attB recombination for the insertion of transgenes such as putative enhancer sequences, is routine in *Drosophila* work [68, 79] however, its translation into the zebrafish model has been largely limited to proof-of-principle studies. Two such studies describe a technique by which phiC31 catalyzes a recombination event in a part of a previously inserted transgene, causing the excision of a

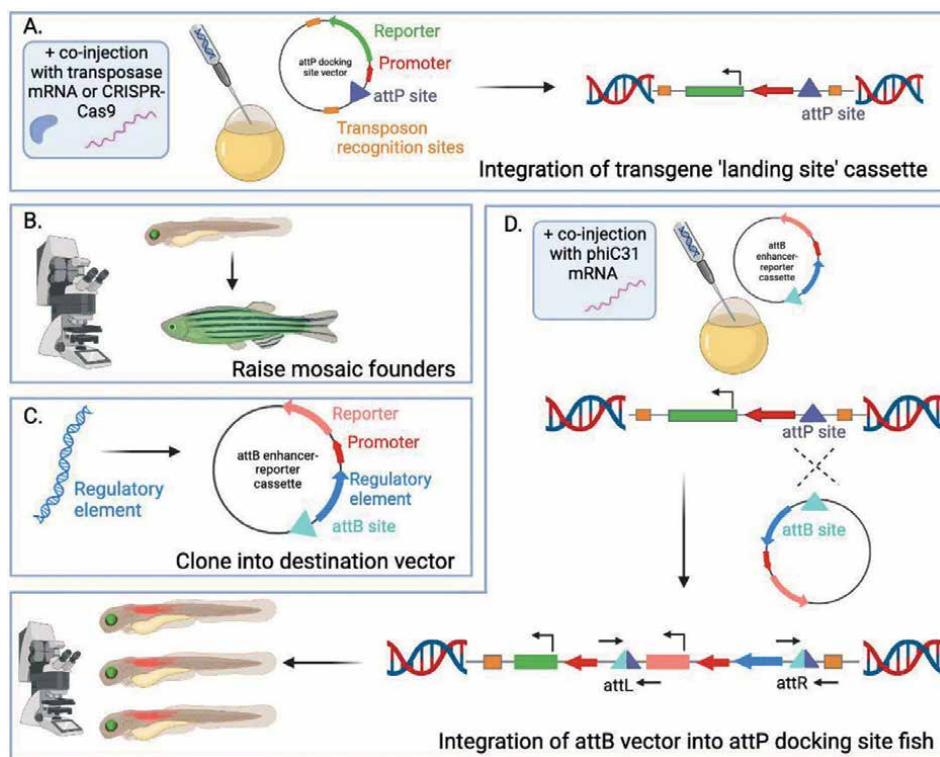


Figure 6.

Generation of attP/attB-recombinant zebrafish transgenic lines using phiC31 recombinase. (A) a vector containing GFP-tagged attP sites is co-injected with integration technologies, such as transposase mRNA, into fertilized single-cell zebrafish embryos, where the transgene is integrated into the genome. (B) Using confocal microscopy, larvae are screened for fluorescence, reflecting the integration of the landing site cassette into the zebrafish genome, and these fish are raised to generate transgenic lines. (C) a regulatory element is cloned into a vector containing an attB site and a fluorescence reporter. (D) the vector is co-injected with phiC31 mRNA into transgenic embryos containing an attP integration site. A recombination event occurs, mediated by phiC31, between the attB and attP sites, and the transgene is integrated at this landing site. Using confocal microscopy, larvae are screened for fluorescence expression. Created using BioRender.com.

control cassette and the activation of a second coding sequence (**Figure 5A**) [75, 76]. In comparison to the single phiC31-mediated recombination event, recombinase-mediated cassette exchange (RMCE) involves the phiC31 integrase excising and replacing a reporter-tagged integrated transgene with another reporter labeled transgene (**Figure 5C**) [80]. During RMCE, a transgene containing a fluorescent reporter protein flanked by attP (or attB) sites is integrated into the zebrafish genome via Tol2-mediated transgenesis. These zebrafish are screened for germline transmission, and the fluorescent-positive embryos co-injected with phiC31 mRNA and a vector containing an enhancer sequence tagged with a different fluorescent reporter, flanked by attB (or attP) sites. PhiC31 facilitates recombination between the attP/attB sites which excise the fluorescently labeled reporter cassette and integrates the other fluorescently labeled cassette. A limitation of this approach comes down to the initial random insertion of the transgene by Tol2, as the number and position of these insertions are not controlled. Therefore, zebrafish can have multiple independent insertions and many transmit both fluorescent markers following injection, reflecting that recombination did not occur at every attP/attB site [80]. To improve upon this system, stable transgenic zebrafish lines with well characterized attP/attB sequences that support efficient phiC31-mediated recombination are required. These lines would eliminate the time and labor required to generate new attP/attB-containing transgenic acceptor lines for every experiment and additionally, would eliminate the need to screen and map each transgene insertion site.

A large amount of time and labor is dedicated to the generation of stable single-insertion attP/attB transgenic zebrafish lines. A research group in 2013 reported to have generated two such lines [78]. Tol2-mediated transgenesis was employed to generate several independent attP landing site zebrafish lines containing a full-length attP acceptor site alongside a GFP-tagged promoter. These lines were assessed for a 50% Mendelian inheritance pattern in the F2 generation, reflective of a single transgene insertion. These lines were co-injected with phiC31 mRNA and a vector containing a GFP-tagged transgenesis marker flanked by full-length attB sites, and screened for fluorescence reflecting a successful integration of the transgene. Two landing site lines consistently demonstrated reproducible attP/attB recombination, and the integration position was mapped and characterized. Reproducible attP/attB recombination was determined through a variety of experiments. One such screen tested integration in mutant backgrounds, where attP landing-site lines were crossed with fish lacking a transcription factor essential for melanocyte development. The progeny were injected with phiC31 mRNA and a vector containing the missing transcription factor, and skin pigmentation was successfully rescued [78]. Ultimately, this group was able to successfully establish, characterize, and maintain functional attP landing site fish for the site-directed generation of transgenic lines. Multiple other teams of scientists have also been generating single landing-site zebrafish [42, 77], and have demonstrated that this technique successfully limits variability of transgene activity caused by positional effects, particularly when compared to lines generated with random Tol2-mediated transgene insertions [77].

While this technique has proven successful for the integration of transgenes at a single genomic position, our understanding of the genomic positions where the rate of this integration is most successful is lacking. The use of gene editing technologies such as CRISPR-Cas9, would enable a specific locus to be targeted for attP/attB-site insertion, removing the unpredictability caused by the initial Tol2 transposon-mediated insertion. Validated integration loci have been identified in mammalian systems at the Hipp11 (h11) locus [81–83], and work has been undertaken to identify similar

sites in zebrafish [84]. There is some evidence that zebrafish lines with stable transgene expression have integrated transgenes adjacent to regions marked with native histone marks, reflecting open and active chromatin [84]. In the future, we strongly encourage researchers to map the chromatin dynamics and the genomic coordinates of transgene integration sites produced by random integration. These insights will help identify features that support successful, reproducible, and inherited integration which can then be applied to generate landing site lines with targeted technologies, such as CRISPR-Cas9.

4. Application of reporter assays to understand human disease

Historically, the search for genetic variants contributing to human disease has been restricted to exons, representing only 1–2% of the human genome. In comparison, variants within non-coding regions and their contribution to disease remain poorly investigated. This is because variants within coding regions are much easier to interpret, as mutations that truncate the mRNA transcript or alter the structure of the protein intuitively result in aberrant protein function and disease phenotypes. Less clear are the potential effects of variants within non-coding enhancer elements. However, the impact of non-coding variants have become well recognized in the etiology of Mendelian and polygenic diseases, and the utilization of the zebrafish reporter assay has been an invaluable tool for our understanding of their contribution.

4.1 Mendelian disease

Complex phenotypes and diseases are influenced by thousands of genes and genetic variants that are common. Comparatively, Mendelian disorders are caused by rare and typically singular genetic variants that have predictable Mendelian inheritance patterns. For example, Cystic Fibrosis is an autosomal recessive disorder that is caused by disruptions, most commonly the p.Phe508del variant, in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene [85]. The search for genetic variants contributing to Mendelian disorders has largely been limited to exons. Historically, it was understood that there are enough redundancies between enhancers that regulate the same gene, such that any pathogenic impact of an enhancer mutation would be mitigated by this redundancy [86–88]. However, a large percentage of people with a disease that follows a Mendelian inheritance pattern do not have a genetic variant in the coding sequence [89], indicating that the causal genetic variant may instead reside in functional non-coding sequences, such as enhancers. The reduction in the costs of DNA sequencing has led to the more routine use of targeted or whole genome sequencing in the clinic, which in turn has led to singular pathogenic enhancer variants being uncovered. For example, several variants have been identified in an enhancer 25 kb upstream of *PTF1A* in patients with pancreatic agenesis [90]. These genetic variants display Mendelian inheritance patterns in affected families. The enhancer activates a developmental enhancer cluster, and loss-of-function variants prevent the hierarchical activation of other enhancer domains therefore, circumventing functional redundancy, and causing the disease phenotype [90]. This finding highlights the necessity of investigating the non-coding genome for regions such as enhancers that are disrupted by Mendelian genetic variants.

The zebrafish reporter assay can be employed to validate Mendelian genetic variants discovered in enhancers, furthering our understanding of their contribution to

disease. For example, a regulatory variant was identified in a person with the cardiac features of Holt Oram disease (congenital condition of the heart and limbs) that disrupts the expression of *TBX5* (a transcription factor with defined roles in cardiogenesis and forelimb development [91, 92]). In this study, the region surrounding *TBX5* was sequenced in an individual with the most common heart defect observed in Holt-Oram Syndrome, but who did not have limb defects. A reporter assay was employed in zebrafish (see **Figure 2**) to interrogate potential enhancer activity and the variant's effect. The enhancer sequence was PCR amplified from the individual with the variant and an individual without. These regions were Gateway cloned into an entry vector containing a GFP reporter and two Tol2 transposon recognition sites. Each construct was co-injected alongside Tol2 mRNA into one-cell zebrafish embryos, and these F0s were screened for GFP expression after 24 hrs. In fish injected without the variant-containing construct, 34% of fish displayed reproducible GFP expression in the heart. Comparatively, only 1.8% of fish injected with the variant-containing construct displayed weak GFP expression in the heart [26]. These data indicate that the enhancer sequence has regulatory activity that recapitulates *TBX5* expression in the heart but no other tissues associated with Holt-Oram syndrome (e.g., limb progenitors). The data also indicate that there is a functional consequence of the variant, virtually eliminating the enhancer's ability to drive gene expression in a cardiac-specific manner. Importantly, this information decoupled the molecular mechanisms underpinning the limb and cardiac malformations that are seen in Holt-Oram syndrome, broadening understanding of the disease. A caveat to this study is that they did not make transgenic stable zebrafish lines which would have reduced any position effect bias caused by the random transposon-mediated integration. However, the study provided a convincing example of the contribution of non-coding variants to Mendelian disease.

Another example of an enhancer that is causal of Mendelian disease, that was characterized in zebrafish, is found upstream of the *SOX9* gene and linked to the Mendelian disease Pierre Robin sequence (PRS) [93, 94]. *SOX9* is a transcription factor essential for chondrocyte differentiation, neural stem state determination, and male sexual development [95]. Pathogenic PRS variants have been identified within an enhancer ~1.5 Mb upstream of *SOX9* [93]. It was hypothesized that the enhancer has specific spatiotemporal activity, and that the disruption of this activity leads to misexpression of *SOX9*, and consequently the craniofacial abnormalities seen in people with PRS. A dual-color reporter-based assay in zebrafish (see **Figure 4**) [19] was employed to test the enhancer region, termed HCNE-F2 [93], for enhancer activity, and the ability of a variant identified in people with PRS to alter this activity [96]. The HCNE-F2 region of interest was PCR amplified from the DNA of a person with PRS carrying the variant of interest, and a control. These were cloned into a vector containing either a GFP or mCherry reporter respectively, flanked by Tol2 recombination sites. The vectors were co-injected alongside Tol2 mRNA into one-cell stage embryos and screened for fluorescence, and positive fish raised to adulthood. A number of stable lines were generated by in-crossing fish with stable germline transmission and their progeny were screened for dual-fluorescence from both transgenes. The HCNE-F2 region that did not contain the variant drove fluorescence in the developing pharyngeal arches and craniofacial cartilage, and this expression was lost in the HCNE-F2 region containing the variant [96]. The application of this dual-reporter assay enabled the direct comparison between control and variant-containing sequences of a putative enhancer region. The results indicate that the enhancer region has craniofacial regulatory activity, and the variant ablates this activity, potentially leading to the misexpression of *SOX9* and the craniofacial defects seen in people with PRS.

4.2 Complex polygenic disease

Complex polygenic conditions, such as type 2 diabetes and cardiovascular disease, are those where individual common genetic variants contribute only very small effects. To capture important regions of the genome that contribute to the development of polygenic disease, genome-wide association studies (GWAS) that consist of many hundreds of thousands of participants are the gold standard approach [97]. Crucially, GWAS find that <10% of associated regions of the genome have causal mechanisms implicating a protein-coding variant. Instead, the majority of the polygenic associations are outside of the coding regions, suggesting that altered gene regulation is a major contributor to complex disease. Integrated meta-analyses of genomic and epigenomic annotations have demonstrated that GWAS variants are significantly enriched in functional non-coding regions [98, 99], particularly in close proximity to enhancers [100, 101]. For example, ~60% of likely causal variants map to enhancer elements in an autoimmune disease GWAS [102]. These data suggest that a substantial proportion of genetic variants identified using GWAS contribute to disease by disrupting enhancer activity. However, studying the biological consequences of genetic variation at GWAS loci is challenging for a number of reasons. Identifying the causal gene(s) can be difficult, as candidate enhancers do not always affect nearby genes, but can have long-range interactions with promoters [103]. In addition, in most cases, the causal variant at a GWAS locus will also be associated with many other genetic variants due to linkage disequilibrium. Unfortunately, there has been very little progress in translating these loci into biology since the advent of GWAS in the early 2000s, and identifying causal variants and determining how and where non-coding variants affect target genes remains poorly understood [104].

For example, it took almost 10 years for the well-known obesity associated locus in intron 1 of *FTO*, first identified by GWAS in 2007 [105, 106], to be biologically linked to an enhancer that functions to regulate *IRX3* and *IRX5* [107, 108]. This example at *FTO* highlights the challenge of pinpointing both the causal gene(s) and causal genetic variant underpinning a genetic association with disease. It was initially hypothesized that *FTO* was the causal gene at this locus, however numerous studies using human cell lines, primary human adipocytes, and mouse models eventually showed that *IRX3* and *IRX5* were the causal genes underpinning the genetic association [108]. Additionally, more than 100 linked variants ($LD R^2 > 0.8$) associated with obesity were identified at *FTO*, over a 47 kb block of linkage disequilibrium [109], such that the causal variant(s) have been hard to define.

In an attempt to assign function to the *FTO* region of association, without having to systemically test the individual genetic variants within this LD block, a research group took a sledgehammer approach by applying Bacterial Artificial Chromosome (BAC) vectors [110–112] in an enhancer-reporter zebrafish assay. Importantly, this type of vector enables testing of very large portions of DNA for enhancer function using GFP reporter cassettes. Fluorescent-positive zebrafish, injected with modified BAC vectors and Tol2 mRNA, were identified, raised, and used to create stable transgenic lines. In this study they were able to show that 183 kb of human genomic DNA spanning ~30 kb upstream of the *FTO* promoter, and 150 kb into the *FTO* gene including exon 4, were able to drive expression of GFP in the brain of the developing zebrafish larvae. In addition, immunohistochemistry for GFP showed GFP expression indicative of enhancer activity within the hypothalamus of the adult zebrafish, recapitulating the brain expression of *IRX3*. Intriguingly, this zebrafish study is not well cited and largely ignored by researchers working on untangling the mechanisms

underpinning this genetic association at FTO. Crucially, the exact molecular mechanisms of the FTO genetic association are still unclear, complicated by multiple signals of association and population-specific effects [113]. A putative causal variant was identified in 2015, and was found to disrupt a conserved ARID5B motif [108], but it is not clear if the ARID5B disrupting variant is the only causal variant at this locus. This ambiguity highlights the potential for next generation zebrafish assays to substantially contribute to the scientific debate, and our understanding of the mechanisms surrounding this locus.

The BAC approach described above, where very large pieces of DNA are inserted, was particularly useful before we had better prediction methods for regulatory function for genetic variants. Now, with data made available by the ENCODE consortium [6] and GTEx, it is possible to overlay genetic variants with regulatory data and gene expression data (eQTL) to prioritize putative causal variants before testing in zebrafish assays. The success of this approach is highlighted by two studies [35, 36] that investigated loci with genetic associations of serum urate, a key metabolite involved in the development of the inflammatory arthritis, gout [114]. In these studies, transcription factor binding sites were overlaid with urate-associated genetic variants at the *PDZK1* and *MAFTRR* genes. At both loci, PAINTOR (a bioinformatic tool that statistically tests the likelihood a genetic variant is causal) identified that genetic variants that were within, or close to, HNF4A core consensus motifs had the highest PAINTOR probability scores. Using the ZED-vector based assay described in Section 3.1 (see **Figure 3**) [65], the regions encompassing these HNF4A binding sites, and the genetic variants associated with serum urate, were introduced into zebrafish embryos. GFP expression was observed in the kidney tubule of developing zebrafish and additionally, in the liver and intestine for the *PDZK1* enhancer [35]. Importantly, enhancer activity overlapped expression of zebrafish *HNF4A*, *PDZK1*, and *MAF*

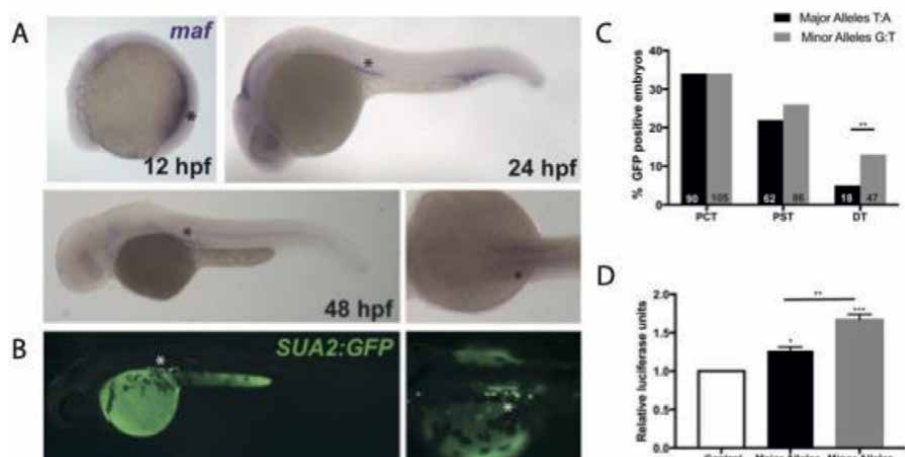


Figure 7. Zebrafish *maf* coincides with enhancer activity. A. *in situ* hybridization for zebrafish *maf* indicates expression in the pronephros at 12 hpf and the proximal tubules at 24 and 48 hpf. B. Enhancer expression (GFP) in the proximal tubules at 48 hpf. C. Percentage of transgene positive embryos (RFP) that present with GFP-positive cells in the PCT, PST, and DT. D. Luciferase reporter assay in HEK293 cells. A one-way ANOVA test resulted in a significant difference between the means of the control and the enhancer containing each variant. This figure has been adapted from Leask et al. 2019 with permission from the authors.

(the regulatory target of the lncRNA *MAFTRR*), causally linking the enhancers to target gene expression (**Figure 7A–B**).

For the enhancer at *MAFTRR*, differences in GFP expression between genetic variant alleles was semi-quantified in F0 fish (**Figure 7C**), showing that the urate raising alleles had a greater ability to drive expression in the proximal convoluted tubule [36]. The allelic differences in expression were confirmed by luciferase assay in kidney cell lines (**Figure 7D**). Importantly, this is another example that highlights the need for a zebrafish assay that can robustly quantify allelic differences at enhancers. Such an assay would dispense with the cell-based assays, such as luciferase assays and CRISPR activation screens, for allelic quantification, which require prior knowledge about the types of cells and tissues where the enhancer functions. The value of an in vivo assay was highlighted in the *PDZK1* example, as although GFP expression was observed in the kidney, liver and intestine of the zebrafish, only the liver cell lines selected for the luciferase assay have *HNF4A* expression [35]. Therefore, it would not have been possible to robustly test the allelic effects of the enhancer variants in kidney cell lines. In this context, the zebrafish assay circumvents this challenge because the transcriptional machinery required to drive gene expression is within the appropriate biological context.

5. Conclusion

This chapter has highlighted the role of the zebrafish report assay to test enhancer activity in vivo and the various tools available to researchers using this model. We describe how in zebrafish, reporter assays have been applied and improved over the years to test for regulatory activity of predicted enhancer elements. In the future, recombinase landing site zebrafish lines will reduce the intensive labor required to screen transgenic fish generated using random integration technologies. These lines will also be an essential tool for the accurate in vivo quantification that both Mendelian and complex-disease variants have on enhancer function. The advent of cost-effective whole genome sequencing and large-scale GWAS has enabled us to comprehensively discover non-coding Mendelian disease variants and common variants that contribute to complex disease. It is therefore more important than ever to develop tools that are scalable and can accurately recapitulate enhancer activity in the relevant biological context, to comprehensively understand the underlying molecular and disease mechanisms.

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Conflict of interest

The authors declare no conflict of interest.

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Zebrafish as an Experimental Model for Inherited Retinal Diseases

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Abstract

Zebrafish are becoming a popular experimental animal model for vision science and human-inherited retinal diseases. In this chapter, we describe application of zebrafish for the retinitis pigmentosa (RP) caused by digenic LDL receptor-related protein 5 (*LRP5*) and Eyes shut homolog (*EYS*). RP is the most common genetic disorder in inherited retinal diseases, and *EYS* is one of the major causes of RP. *EYS* orthologs are absent in rodents but present in zebrafish. Using this advantage, we generated and analyzed the digenic *eys*^{+/-}; low-density lipoprotein (LDL)-related receptor-5 (*lrp5*)^{+/-} zebrafish, the same form of gene defects emerged from a human case report as a candidate of RP. The analysis discovers that *retinol binding protein 1a* (*rbp1a*) gene is remarkably downregulated and that Lrp5 protein is a strong candidate for the receptor of all-*trans*-retinol in the visual cycle. Furthermore, in this review, we also discuss functional roles of *EYS* in vertebrates with an emphasis on its possible involvement in the retinal metabolism, the visual cycle, aiming at integrating our findings with recent advances in the research field.

Keywords: retinitis pigmentosa, Eys, Lrp5, Rbp1, visual cycle, familial exudative vitreoretinopathy

1. Introduction

Humans rely heavily on vision for a lot of information. Curing or slowing the progression of the vision loss contributes significantly to medicine, as vision loss impairs quality of life significantly. Vision is an intricate biological phenomenon that involves the retina and the visual field in the brain. Vision is initiated and processed by a network of retinal cells and molecular processes within the retina, which is located at the back of the eye. The first step of vision is to detect light, i.e., photons, and convert the information to chemical information in photoreceptors within the retina (for review, see [1, 2]).

In the photoreceptors, 11-*cis*-retinal (11-*cis* RAL) forms a Schiff's base with a lysine residue (K) in the protein opsin to form rhodopsin (or cone opsins) in darkness. 11-*cis* RAL is a derivative of vitamin A. Vitamin A, a fat-soluble natural compound, circulates in the body bound with retinol-binding protein 4 (RBP4) and enters the

retina from the retinal pigment epithelium (RPE) through the receptor, STRA6 [3, 4]. In the context of sustained vision, which is particularly important for mesopic to photopic vision, the retinoid cycle called the “visual cycle” is the fundamental process. The visual cycle is a series of biochemical reactions between the retina and the RPE and within the retina and is essential for maintaining a constant supply of 11-*cis* RAL, a crucial component for phototransduction.

Both environmental and genetic factors can cause blindness. In this review, we focus primarily on genetic factors (i.e., IRDs). Among IRDs, retinitis pigmentosa (RP) and familial exudative vitreoretinopathy (FEVR) are included. In developed countries, IRD is going up in ranking as the cause of visual impairment. Although mouse genetics using the knocked-out (KO) approach is powerful to study whether a gene of interest is causative of an IRD and/or elucidating its pathogenesis and molecular functions, there are cases where the human phenotype is not recapitulated well [5, 6] or a gene of interest does not exist in mice [7, 8].

Researchers have been using zebrafish (*Danio rerio*) as a valuable model organism. Findings and implications extend beyond adding new knowledge to fish biology, and insightful information can come from fish (e.g., [9]). More specifically, the study of zebrafish orthologous genes associated with human inherited retinal diseases has provided insightful knowledge for uncovering the molecular mechanisms underlying visual impairment and vision [10, 11]. The eyes shut homolog (*eys*) gene is one of them as *eys* possesses striking homology to the human *EYS* gene implicated in various forms of photoreceptor degeneration and blindness. On the other hand, we also have to be aware that there are some differences between humans and zebrafish. For example, in zebrafish, there are four types of cones expressed and red and green cones form unique shape called double cone.

In this chapter, we aim to insightfully bridge the current knowledge gap between fish and mammals and get insight into the role of EYS in human vision that is key to photoreceptor degeneration by closely reviewing the accumulating results of *eys* mutant zebrafish and medaka fish as well as studies human patients are employed. We particularly provide zebrafish *lrp5* and *eys* genetics, the visual cycle, and their implications for human vision. Through the integration of novel insights from the knockout zebrafish models and existing and novel literature, we aim to shed light on the major gene responsible for human inherited retinal diseases, bringing our current complex knowledge one step closer to unraveling the functional role(s) of EYS protein.

This review first focuses on the findings from the zebrafish low-density lipoprotein (LDL)-related receptor-5 (*Lrp5*) and *Eys* proteins and then possible EYS roles in vision. Recent advancements in genetic manipulation techniques, including CRISPR/Cas9, have enabled the creation of knockout zebrafish models more easily than before, offering a unique opportunity to investigate the functional consequences of disruption of gene(s) of interest. Although not the main topic of this review, we briefly introduce these techniques for readers who are not familiar with zebrafish.

Human *LRP5* gene (<https://www.ncbi.nlm.nih.gov/gene/4041>; its proteins is LRP5 protein) or *EYS* gene (<https://www.ncbi.nlm.nih.gov/gene/346007>; its proteins is EYS) have one copy of zebrafish ortholog; the *lrp5* gene (<https://www.ncbi.nlm.nih.gov/gene/568518>; its proteins is Lrp5 protein) and the *eys* gene (<https://www.ncbi.nlm.nih.gov/gene/557044>; its proteins is Eys protein), respectively. *EYS* gene is responsible for one of the major causes of retinitis pigmentosa in human inherited retinal diseases with significant implications for understanding its function. This research not only contributes to our fundamental understanding of the

retinoid and visual cycle but also holds promise for uncovering novel therapeutic targets to combat human inherited retinal diseases. As we delve into the intricacies of the visual cycle, it becomes apparent that Lrp5 and possibly Eys too play a pivotal role in these processes. Our exploration of knockout zebrafish models targeting the genes aims to decipher the repercussions of gene disruption on the visual cycle and, by extension, on visual function. This would contribute significantly to the understanding of the basis of pathogenesis of these diseases and, more broadly, photoreceptors/RPE biology.

2. Inherited retinal diseases (IRDs)

IRD is a group of diseases characterized by a progressive degeneration of the retina as time goes, which can lead to progressive severe vision loss or blindness. IRD primarily affects the structure and/or function of the retina and alters them [12]. This process could be during the developing of the retina (this can be syndromic that may also be critically important for normal development in tissues other than the retina) or after the completion of development of the retina (i.e., maintenance and therefore, typically nonsyndromic) depending on types of IRDs. Because phenotypes can be changed by the types of mutations in a same gene, and sometimes it is difficult to distinguish phenotypes by those typical names, it may be preferable to use the term IRD in some cases, which would include all types of blindness.

There are several different types of IRDs where photoreceptors are especially affected, among the six major retinal neuronal cell types [13]. Leber congenital amaurosis (LCA) is a congenital retinal disease that results in severe vision loss present at birth [14, 15]. LCA can be caused by mutations in genes involved in a wide variety of photoreceptor functions such as centrosomal protein 290 (*CEP290*), guanylate cyclase 2D, retinal (*GUCY2D*) and retinoid isomerohydrolase *RPE65* (*RPE65*). In RP, rod photoreceptors, dominant in the periphery in humans, are mainly affected. On the other hand, cone photoreceptors are affected in cone-rod-dystrophy (CRD). Although there is debate about environmental and genetic factors as causes of this disease [16], in age-related macular degeneration (AMD), central vision is affected. In humans, cones are dominant in the central region, macula, and its central region is called fovea. Therefore, the cones are considered to be mainly dysfunctional in AMD. The awareness of the symptoms usually starts during young adulthood in RP and CRD. Instead, AMD mostly occurs in people with the age over 50. Stargardt disease is a rare genetic disease caused by the accumulation of fatty substances in the macula [17], and, unlike AMD, onset of this disease usually occurs before age 20.

Ciliopathies are a group of diseases that involve dysfunction of the cilium. Because the photoreceptor outer segment is one of the cilia particularly specialized for absorbing light, mutations in genes important for formation and/or maintenance of cilia can cause IRDs frequently accompanying a wide range of syndromic phenotypes such as obesity. Among them, Bardet-Biedl syndrome (BBS) is well known [18], and Meckel-Gruber syndrome (MKS) and Alström syndrome (ALMS) are also known [19–21].

Adjacent supporting cells, the retinal pigment epithelium (RPE), are crucial for maintenance of photoreceptors. Therefore, dysfunction of RPE cells can kill photoreceptors, and there are known IRDs such as RP, AMD, and CRD.

FEVR is also a group of IRDs. This disease is characterized by abnormal retinal angiogenesis and abnormal vascularization of the peripheral retina with subsequent retinal ischemia. Abnormality can be both hypovascularization and

hypervascularization. Norrin cystine knot growth factor NDP (*NDP*) (OMIM 300658), frizzled class receptor 4 (*FZD4*) (OMIM 604579), *LRP5* (OMIM 603506), tetraspanin 12 (*TSPAN12*) (OMIM 613138), zinc finger protein 408 (*ZNF408*) (OMIM 616454), and kinesin family member 11 (*KIF11*) (OMIM 148760) are the common genes that cause FEVR [22, 23]. Most of them are known to be involved in the Wnt signaling.

One thing to note about FEVR is that the phenotype of mouse models of angiogenesis including knockout mice often does not recapitulate the symptoms of human patients; in other words, the phenotypes of humans and mice are often different [24], presumably due to the difference of capillary. In relation to zebrafish, confirmative studies have been conducted for *Znf408* and *Fzd4* [25, 26]. In general, in many cases, with some of those exceptions, IRD-related genes are conserved between zebrafish and humans, and vision scientists use zebrafish as an appropriate animal model.

Usually, mutation(s) in a single gene account for IRD. On the other hand, it could be caused by multiple genes. For example, it is well known that digenic mutations in peripherin 2 (*PRPH2*) and retinal outer segment membrane protein 1 (*ROM1*) cause RP [27]. Likewise, heterozygous cyclic nucleotide-gated channel subunit alpha 3 (*Cnga3*)-null mutation exacerbates the cone dystrophy phenotype in cyclic nucleotide-gated channel subunit beta 3 (*Cngb3*)^{R403Q/R403Q} mice [28]. CNAGA3 and CNGB3 proteins are known to form the tetrameric cyclic nucleotide-gated ion channel in cone photoreceptors. However, it is difficult to track multiple generations, and genetic test is limited particularly in humans.

3. Visual cycle

Visual cycle is a retinoid metabolism that regenerates photoisomerized retinal that allows rhodopsin or cone opsin can capture light multiple times for sustained vision [29, 30]. The visual cycle takes place mainly between photoreceptors and the RPE as well as between cones and Müller cells (called cone-specific visual cycle). In darkness, 11-*cis* RAL (a derivative of vitamin A) forms a Schiff's base with a lysine residue (K) in the protein opsin to form rhodopsin (or cone opsins).

Upon absorption of light, 11-*cis* RAL in rhodopsin or cone opsin is photoisomerized to all-*trans*-retinal (atRAL). atRAL is converted to all-*trans*-retinol (atROL) by ATP binding cassette subfamily A member 4 (ABCA4). atROL is released from photoreceptors, which is unique to vertebrate opsins and is incorporated into the RPE.

Inside the RPE, retinol binding protein 1 (RBP1) plays a role [31–33]. RBP1 binds and transports atROL to endoplasmic reticulum (ER). atROL is esterified within this RBP1-atROL complex by lecithin retinol acyltransferase (LRAT). This all-*trans*-retinyl ester is converted to 11-*cis*-retinol (11-*cis*-ROL) by the retinoid isomerohydrolase, RPE65. 11-*cis*-ROL is converted to 11-*cis* retinal (11-*cis*-RAL) by retinol dehydrogenase 5 (RDH5). The RPE turns 11-*cis*-RAL back to photoreceptors again by a transporter, retinaldehyde binding protein 1 (RLBP1), and inter-photoreceptor retinol binding protein (IRBP).

Retinoid metabolism crucial for sustained light absorption involves multicellular and multiple proteins. These proteins are essential and some of them show no phenotype in mice suggesting that visual cycle is conserved in vertebrates but there could be some variation among species. atROL could be carried by IRBP, however, how atROL is released from photoreceptors and is incorporated into the RPE has not been well known [34, 35].

4. LRP5 in humans and zebrafish and its involvement in IRDs

LRP5 protein is a member of the LDL receptor superfamily conserved in vertebrates. LRP5 protein was identified in 1998 in human and mouse cDNA libraries [36–39]. LRP5 is best known as a co-receptor for Wnt ligands involved in the wingless (Wnt) signaling pathway on cell membranes, which is essential for the development of vascular endothelial cells, Müller cells, and retinal interneurons [40]. LRP5 is also reported to be involved in cholesterol and glucose uptake [41, 42]. Furthermore, the possible involvement of LRP5 in cell types involved in vitamin A (i.e., atROL) is suggested based on its expression [43].

Domain structures of human LRP5 and zebrafish *Lrp5* are well conserved between humans and zebrafish. Human and zebrafish LRP5 proteins consist of a signal peptide sequence, low-density lipoprotein receptor YWTD (LY; also known as β -propeller), EGF, and LDLa domains in the N-terminal extracellular domains. These domains are followed by one transmembrane domain and low complexity region in the C-terminal intracellular region. LRP5 protein seems to act as a receptor of both protein and small molecules.

In addition to our group, there are at least two groups that use *lrp5* mutant zebrafish [44, 45], both of them study osteoporosis, a highly prevalent systemic skeletal disease characterized by decreased bone mass and decline of bone mineral density (BMD) resulting in compromised bone architecture, bone fragility and increased fracture risk. Cholesterol biosynthesis pathway is particularly changed in *lrp5*^{-/-} skulls suggesting conservation of *Lrp5* function between zebrafish and mammals.

In vision, *LRP5* is a well-known causative gene of FEVR [46–48]. Hypovascularization, where peripheral vasculogenesis is affected, accompanying leakage is the typical phenotype of *LRP5* mutations. This can be observed in both autosomal dominant (ad) and recessive (ar) forms [48–50]. It appears that it is an ad form (i.e., typically gain-of-function), particularly when the type of mutations is missense mutation. On the other hand, the ar form of the mutation (i.e., loss-of-function) in *LRP5* is found in the pedigrees of FEVR [47, 48], and hypovascularization is the retinal phenotype of loss-of-function of *Lrp5* by animal studies using mouse and rat *Lrp5* knockout models [51, 52]. So far, only one mutation has been suggested to cause RP, and this is a heterozygous digenic form together with mutation in *EYS* [53].

5. EYS in humans and zebrafish and its involvement in IRDs

Human eyes shut homolog (*EYS*) gene (Online Mendelian Inheritance in Man no. 612424), an ortholog of the *Drosophila* eyes shut/spacemaker (*eyes*, <https://www.ncbi.nlm.nih.gov/gene/3771890>) [7, 8], is a major causative gene of nonsyndromic autosomal recessive retinitis pigmentosa (RP) [7], and cone-rod dystrophy (CRD) [54, 55]. *EYS* is originally identified in *Drosophila* which is important for maintenance of photoreceptors. And several years later human ortholog, *EYS*, was found to be a causative gene of RP. And in 2010 and 2012, our group and others reported that this gene is a major cause of RP in the French [56] and Japanese population [57, 58], and therefore its importance in ophthalmology globally [59].

The predominant human *EYS* transcript, tv1, encodes 3144 amino acids and zebrafish *eyes* transcript encodes 2906 amino acids, and through the splices, the domain structure of the orthologous protein is conserved [60]. *Eys* is structurally related to Pikachurin and Agrin [61]. Characteristics of these proteins are that they consist of

multiple EGF and EGF-like domains in its 5' portion and 3' portion consists of multiple Laminin G domains [62]. Human and zebrafish EYS proteins consist of a signal peptide sequence for secretion, epidermal growth factor (EGF), EGF-like and Ca^{2+} EGF domains in the N-terminal half, and laminin A G-like (LamG) and EGF domains in the C-terminal half.

This protein is present in invertebrates as well. Eys was originally identified in *Drosophila* that is a secreted protein filling a space in photoreceptors [63, 64]. This is in accordance with the other related proteins such as Pikachurin and Agrin that are secreted and functions outside cells [65]. In *Drosophila*, Eys is believed to be important for open rhabdom and other invertebrates having this structure together with prominin 1 (Prom1) [66]. Based on localization studies in zebrafish and primates including humans, this protein, Eys, is shown to be localized to ciliary pocket in both rod and cone photoreceptors [67]. In addition, EYS is also localized to cone OSs and terminals [67]. In particular, it is interesting that EYS is localized unilaterally to the edge of cone OSs, ciliary axoneme, in primates, and it looks as “whip-like” signal [68].

Our group reported that this gene is most prevalent in the Japanese population [58], and five mutations that are most frequently observed in the Japanese population (JV1, c.4957dupA and p.(Ser1653Lysfs*2); JV2, c.8868C > A and p.(Tyr2956*); JV3, c.2528G > A and p.(Gly843Glu); JV4, c.6557G > A and p.(Gly2186Glu); JV5, c.6563 T > C and p.(Ile2188Thr)) [69]. These five mutations are located from N-terminal region to C-terminal region of EYS protein. Full-length Eys is highly expressed in zebrafish photoreceptors and this expression is specific or dominant [60], and full-length EYS is expressed in human photoreceptor-like cells derived from dermal fibroblasts (our unpublished observation). It would be important to note that the evidence suggests that full-length EYS protein is crucial for preventing photoreceptors from degeneration.

6. Available resources and genetic manipulation in zebrafish

In zebrafish, the eye starts to form around 20–24 hours post-fertilization (hpf) [70, 71]. Around this time point, zebrafish eyes are transparent allowing unique opportunities for eye formation or photoreceptor differentiation [72], which occurs around 48 hpf in the ventronasal patch [73].

We initially investigated zebrafish orthologs of human *EYS* and *LRP5* genes. As a result of whole-genome duplication, there are two orthologs corresponding to mammals for many genes [29, 74]. Recent advance in the database has made it dramatically easier to make connections of human, mouse, and zebrafish orthologs than decades ago. Orthologs of human *EYS* and *LRP5* genes are *ey*s and *lrp5*, respectively (see also Introduction for links to the National Center for Biotechnology Information (NCBI) websites).

One more important thing to note is that there are multiple strains actively used in zebrafish; from our experience using two different strains, AB and TL, we do not observe substantial differences in molecular cloning in both strains.

We used mutant zebrafish obtained from Zebrafish International Resource Center (ZIRC) where *N*-ethyl-*N*-nitrosourea (ENU)-induced mutant zebrafish are stocked. Zfin (<https://zfin.org/>) allows users to look up all available mutant zebrafish for each gene. Busch Lab website (<https://zmp.buschlab.org/>) is also helpful to look up mutations in more detail about the ENU-induced mutant zebrafish. Once desired mutants are found, they can be obtained through ZIRC (<https://zebrafish.org/home/guide.php>) or the European Zebrafish Resource Center (EZRC) (<https://www.ezrc.kit.edu/>).

We received 50–100 of 3–5 days post-fertilization (dpf) zebrafish embryos and raised the embryos to adults. We collected tails, extracted genomic DNAs, and genotyped to screen the obtained zebrafish and about 25% of them carried the described mutation in the *eys* gene in a heterozygous manner. In our experience with a total of several mutant lines from the ZIRC, 25–50% of the resulting zebrafish in any strain had the exact mutation described and were not particularly troubled.

To avoid off-target effect potentially arisen from ENU-treatment, it would be ideal to cross-obtained mutant zebrafish with wild-type zebrafish for several generations to reduce the possibility of unexpected ENU-induced mutations being introduced at other loci. In our experience, this is probably sufficient for practical purposes, as the zebrafish obtained from these resource centers are of F₂ generation, and ENU-induced mutations that induce point mutations are more random than CRISPR-induced off-target mutations.

Alternatively, because the CRISPR/Cas9 system has become accessible and much easier in zebrafish, researchers can generate knockout zebrafish using CRISPR/Cas9 in their own labs. We briefly explain our method here [75], which we believe is easiest. We obtain Cas9 protein (Alt-R® S.p. HiFi Cas9 Nuclease V3) [76], crRNA (Alt-R® CRISPR-Cas9 crRNA), and tracrRNA (Alt-R® CRISPR-Cas9 tracrRNA), the components necessary for CRISPR/Cas9, from Integrated DNA Technologies (IDT). We form gRNA by annealing the equimolar of custom-synthesized crRNA with universal tracrRNA using Nuclease Free Duplex Buffer (IDT), and mix the gRNA with the Cas9 protein to form ribonucleoprotein (RNP) complex. We inject the RNP complex into one-cell stage zebrafish eggs and raise them to adults and uses as founders (F₀).

It is important to minimize the possibility of off-target effects when we select target sequence for knockout. We use CRISPRdirect (<http://crispr.dbcls.jp/>) [77]. We typically select sequences having minimal similarity to other locations in the reference sequence by choosing “Zebrafish (*Danio rerio*) genome, GRCz11/danRer11 (May, 2017)”. After deciding target sequence, it is important to read actual DNA sequence surrounding the target region in our own zebrafish cohort because there could be SNPs in the target region from the reference genome.

To create knock-in zebrafish is attractive. For target size with smaller than 200 nt, there are reports [78]. However, accurate visualization of the localization of proteins tagged with fluorescent proteins such as EGFP and mCherry may still be particularly challenging, as the typical size of fluorescent proteins is ~750 nt.

We also have to be aware that there are some differences between humans and zebrafish. For example, in humans, rods are dominantly located in peripheral regions while rods are uniformly distributed in zebrafish. In addition, in zebrafish, there are four types of cones expressed and red and green cones form unique shape called double cone. Retinomotor movement reflecting circadian rhythm and/light is also very unique to fish, which is not necessarily disadvantage but needs to be note [79, 80]. One more important aspect is that photoreceptors have capability to regenerate throughout life in zebrafish [81–83]. This is advantageous for the study of regenerative medicine; this may make analysis a bit more difficult when we study retinal diseases because of continuous supply of new retinal cells.

7. Lrp5 protein and the visual cycle

EYS does not exist in rodents but does exist in zebrafish, which makes zebrafish valuable for EYS study. In humans, a case study was reported in 2017 describing

digenic form of the heterozygous mutations in *EYS* and *LRP5* genes might cause RP [53]. We took this advantage and generated mutant zebrafish, *eyes*^{+/-}; *lrp5*^{+/-} zebrafish, having the same form of *eyes* and *lrp5* mutations to test this possibility in zebrafish. In humans, the *EYS* gene is a cause of autosomal recessive RP and the *LRP5* gene has not been reported to be a cause of RP. From these facts, some possibilities are inferred that RP occurred by one changed gene of *EYS* or genetic interaction between *EYS* and *LRP5*.

We chose mutations in the C-terminal region of the *Eys* gene. This is because its C-terminal region is frequently mutated in the *EYS* patients (e.g., JV2) and other groups had tested if *eyes* knockout is pathogenic or not, but no groups had tested if mutations in the C-terminus are pathogenic in zebrafish at that time. Likewise, we checked disease mutations in *Lrp5* gene. Substitution mutation in the *Lrp5* seems to cause gain of function that leads primary osteoporosis or high bone density syndrome in humans, which are particularly located in the first β -propeller domain. Based on the information, we chose nonsense mutation after the first β -propeller domain to reduce potential phenotypes in other organs. After confirming mutant zebrafish are available from ZIRC, we obtained the *eyes*^{sa31957} and *lrp5*^{sa11097} from ZIRC. We crossed the zebrafish line and established the *eyes*^{+/-}; *lrp5*^{+/-} zebrafish.

In the digenic *eyes*^{+/-}; *lrp5*^{+/-} zebrafish, photoreceptor layer is mildly thinner than wild-type zebrafish at 14 months post-fertilization (mpf). This suggests that this form of mutation can cause RP in humans.

Then we subjected the whole eyes to an unbiased global gene expression analysis, microarray analysis, at 3.5 mpf. At this stage, zebrafish are sexually mature young adults and we expected that this stage would allow us to identify gene(s) that are primarily affected by the heterozygous digenic mutations. Indeed, retinol-binding protein 1 a (*rbp1a* (*rbp1.1*); <https://www.ncbi.nlm.nih.gov/gene/791484>) is remarkably downregulated in the digenic mutant eyes (less than 10% of wild-type eyes) [75]. This is remarkable in two aspects: (1) this kind of dramatic mRNA downregulation is unique to *rbp1a* and is not observed for other genes. (2) RBP1 protein is well-known as one of important proteins that is a transporter of atROL in the RPE as described in the above section.

This discovery highly motivated us to further investigate the reason(s) this gene is specifically downregulated in the *eyes*^{+/-}; *lrp5*^{+/-} eye. We first confirmed that Rbp1a protein is downregulated in the *eyes*^{+/-}; *lrp5*^{+/-} eye. We then analyzed the detailed localization of Rbp1a protein in the eye. The staining pattern is very unique and Rbp1a protein is localized to the string-like (or accessory-like) structure called microvilli of the RPE confirmed with a marker of microvilli of the RPE, Moesin (*Msn*). This is in good agreement with the literature where RBP1 is a transporter of atROL in the visual cycle. Of note, Rbp1a protein is concentrated in the tip of the microvilli and these most concentrated regions are *Msn* negative.

We then investigated localization of *Lrp5* protein in the eye. *Lrp5* protein colocalizes with Rbp1a protein at the most concentrated regions in the microvilli of the RPE, which is very close to the inner segment (IS) of photoreceptors in the outer retina. This is strong supporting evidence that *Lrp5* protein plays a role in the visual cycle as a receptor of atROL at the tip of the microvilli in the RPE. Using *in vitro* purified C-terminal intracellular region of *Lrp5* protein and full-length RBP1a protein involving a binding assay similar to Agrin and *Lrp4* [84, 85], the direct binding of Rbp1a and *Lrp5* proteins is further confirmed.

The expression level of the *rbp1a* gene was remarkably decreased (~10%) compared with the wild-type siblings in the *lrp5*^{-/-} adult eyes. We generated

rbp1a^{-/-} zebrafish using CRISPR/Cas9 system and examined the expression level of the *lrp5* gene in the *rbp1a*^{-/-} eyes. Contrary to *rbp1a* expression, the expression level of *lrp5* is not changed in the *rbp1a*^{-/-} adult eyes (~142%). These genetic studies clarified that *rbp1a* gene plays a role downstream of *lrp5* gene in the eye.

8. Perspective: potential involvement of EYS protein in the visual cycle and/or ciliary transport

Eys was originally found in *Drosophila* as a secreted protein present in extracellular matrix. Now two possibilities are raised: Eys is involved in (1) visual cycle and (2) ciliary transport/OS maintenance.

(1) Visual cycle: there are studies that support the idea that Eys protein is involved in the visual cycle. First, we have shown that Lrp5 is a strong candidate for the receptor of the atROL based on the possible genetic interaction between Eys and Lrp5. In this study, genetic studies suggest that the expression level of *rbp1a* gene is 10.9% in the *lrp5*^{-/-} eye and 6.8% in the *ey*^{+/-}; *lrp5*^{+/-} eye compared with wild-type eye. These observations suggest that the remarkable decrease of *rbp1a* expression in the *ey*^{+/-}; *lrp5*^{+/-} eye is not solely attributed to *lrp5* gene but also from the contribution of the *ey* gene.

Indeed, Eys and Lrp5 proteins are structurally related to Agrin (AGRN) and LDL receptor-related protein 4 (LRP4) proteins, respectively, and AGRN interacts with LRP4 in neuromuscular junction [84, 85]. Applying this to photoreceptors and the RPE, Eys and Lrp5 could interact with each other in the interphotoreceptor matrix between photoreceptors and the RPE. Based on the above evidence and suggestions from the previous study, we proposed in 2020 that Eys could be involved in the visual cycle (**Figure 1**).

Studies in good line with our proposal are now emerging in both zebrafish and humans. First, human clinical trial published recently shows that administration of vitamin A worsened the photoreceptor function in the *EYS* patients [86]. This study is strong evidence for the possible involvement of EYS proteins in the visual cycle, particularly in the step where atROL accumulates and its dysregulation could kill photoreceptors [75]. Second, human fundus image employing artificial intelligence-guided diagnosis (machine diagnosis) shows that fundus images of *EYS* patients is closer to those of *ABCA4* patients than those of RP1 like 1 (*RP1L1*) patients, a protein thought to be involved in transport of OS protein in the connecting cilia [87]. This would suggest that EYS causes a phenotype resemble to the disease caused by accumulation of retinal species (i.e., vitamin A derivatives) in photoreceptors and/or the RPE [88, 89], and therefore again supports the idea that EYS is involved in the visual cycle, particularly the conversion of atRAL to atROL and/or the clearance of atROL from the OS. Third, in zebrafish, Eys is found to be localized to the cone periciliary membrane, cone accessory outer segment (AOS), and photoreceptor ribbon synapse using immunoelectron microscope [90]. AOSs are structures described in teleost fish that extend from the ISs of cones, run along the cone OSs towards the RPE. AOSs are thought to be structural support to the cone OSs towards the RPE and are thought to be involved in the exchange of metabolites between cones and the RPE [91–93]. Indeed, numerous vesicles accumulate in AOSs in the *ey* knockout medaka [94]. This is in good agreement with the idea that Eys protein is involved in interaction between photoreceptors and the RPE. Fourth, early functional abnormality in cones is observed in *EYS* patients using 30 Hz Flicker electroretinogram (ERG) and macular

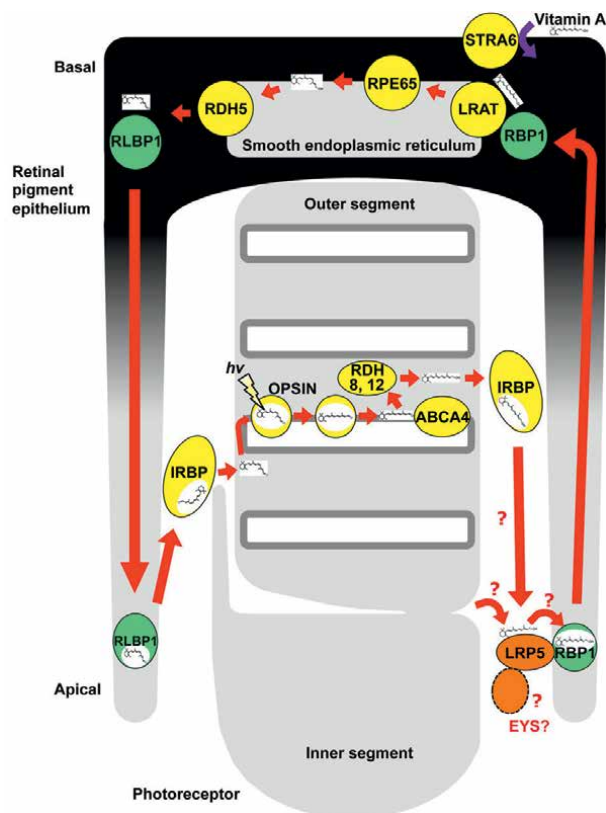


Figure 1.

A proposed model of LRP5 function in the visual cycle. LRP5 protein (orange in right bottom) is a strong candidate for the receptor of atROL in the visual cycle that forms a complex with RBP1 protein (the transporter of atROL; light green in right bottom) is involved in the uptake of atROL in the microvilli of the RPE (adopted from reference [75]). The flow of retinoid is shown by red arrows and the unknown possible interactions are indicated with “?” as well as possible involvement of EYS protein in the visual cycle. atROL, all-trans retinol. ABCA4, ATP binding cassette subfamily A member 4; IRBP, inter-photoreceptor retinol-binding protein (also known as RBP3, retinol-binding protein 3); LRAT, lecithin retinol acyltransferase; LRP5, LDL receptor related protein 5; RBP1, retinol binding protein 1 (also known as CRBP, cellular retinol binding protein); RDH, retinol dehydrogenase; RLBP1, retinaldehyde binding protein 1 (also known as CRALBP, cellular retinal binding protein); RPE65, retinoid isomerohydrolase RPE65; STRA6, stimulated by retinoic acid gene 6.

cone-mediated focal ERG (fERG) [95]. This suggests that EYS is involved not only in rod function but also in cone function in humans even though it does not cause cone degeneration and therefore, the patients may be unaware of it. With respect to retinal vasculature, it is reported that EYS does not seem to cause alteration of retinal vasculature using OCT [96].

(2) Ciliary transport/OS maintenance: the speculation that Eys protein is involved in ciliary transport may have arisen from two observations in the early studies. One is that, in Eys knockout zebrafish, ciliary pocket disappears in the cones [67], and opsins are mislocalized to IS to some extent in cones in terms of cell number [94, 97]. With respect to mislocalization of OS proteins, careful examination would be necessary because it is widely known that mislocalization of OS proteins to the IS and even non-OS proteins to the OS are rather a common phenomenon frequently observed in degenerative photoreceptors [98–102]. With this in mind, in the *eys* knockout medaka, ciliary pocket is not always affected and remains observable [94]. This would

raise a possibility that loss of ciliary pocket is a secondary effect rather than a primary cause of the cone degeneration in zebrafish. In addition, in both zebrafish and medaka, the affected cells appear to be sparsely distributed in both eyes knockdown and knockdown larvae [94, 97]. Theoretically, if defects in ciliary transport were the direct cause of degeneration, mislocalization of OS proteins would be expected to be uniformly distributed throughout the photoreceptor cell layer as observed in defects in intraflagellar transport (IFT) proteins [103, 104]. Careful reexamination would be necessary in the future study.

9. Future direction: to investigate possible involvement of EYS protein in the visual cycle in terms of AOS and/or other aspects

Based on the current knowledge, the most clinically important aspect of the Eys protein is whether Eys is indeed involved in the visual cycle. If Eys is involved, the search for the existence of other proteins that might be involved in the visual cycle would be a meaningful topic from both clinical and biological perspectives.

In this regard, a possible interaction between Eys and the Prom1 protein has long been proposed based on studies in *Drosophila* [66]. Based on the assumed EYS function, Prom1 protein appears to be an attractive candidate that functions in AOS. Since there is no genetic interaction observed between *ey*s and two zebrafish orthologs of prominin 1 (*Prom1*), *prom1a* (<https://www.ncbi.nlm.nih.gov/gene/322857>) or *prom1b* (<https://www.ncbi.nlm.nih.gov/gene/378834>), in the zebrafish eye [75], it would be necessary to test the possibility of interaction between them at protein level. It should be noted, however, that Prom1 is thought to be localized to the open rim in cones, which is opposite to the AOS.

Other possibly intriguing candidate proteins are Usher proteins. It is interesting that in humans, similar cone abnormality is observed in *USH2A* (Usherin) patients [105, 106], and *EYS*-RP and *USH2A*-RP patients showed better-preserved rod function compared with *PDE6A*-RP and *RPGR*-RP patients in chromatic pupil campimetry [106]. These studies indicate that phenotypes are overlapped at least to some extent between *EYS*-RP and *USH2A*-RP patients. In mice, *USH2a* knockout mice shows slow photoreceptor degeneration and *Eys* does not exist in mice. This aspect could be key to elucidate functional roles of Eys in zebrafish in the context of the visual cycle, and this might be true for the ciliary transport as well. It seems reasonable to assume that atROL is transported from the OS to the RPE directly through the AOS or through the boundary between the OS and IS (connecting cilium region), calyceal processes (the structure Usher proteins are localized to and is nearly missing in mice), and AOS (see Figure 1).

In either case, AOS seems to be the crucial structural component for understanding EYS function and its possible involvement in the visual cycle. This is an interesting but unknown structure that has not been reported outside of fish, and even in fish it has been little studied. This may be an excellent opportunity to open up a new field of research. On the other hand, assuming that EYS functions in the visual cycle in humans based on previous studies, the presence of AOS seems natural, while it would be surprising if AOS is present in humans and other primates but has not been found before. Future studies on AOS are awaited.

It is a little bit surprising that photoreceptor degeneration is observed in zebrafish while this is not observed in medaka. It might be interesting that this is also observed when mutations are introduced in other loci in the medaka *ey*s gene.

In addition, there are studies showing mislocalization of OS proteins in the IS in *eys* knockout zebrafish or knockdown medaka in larval stages [94, 97, 107]. This might suggest that Eys protein could also be involved in proper localization of OS proteins in the larval stage. Whether this observation is the actual primary cause of photoreceptor degeneration awaits definitive corroboration and/or further supporting evidence in the future.

In humans, cystoid macular edema (CME) is a well-known symptom frequently observed in RP patients [108, 109]. Involvement of WNT signaling is suggested in CME [110, 111], and LRP5 is one of the important WNT signaling molecules. If edema is observed in the outer retina rather than the inner retina, especially at early stages in the progression, it may suggest that both EYS and LRP5 play some roles in the retina.

There are a non-negligible number of studies claiming sector RP as one of possible phenotypes in *EYS*-RP patients [112, 113]. Sector RP is common in some forms of rhodopsin RP where inferior region is typically more severely affected than other regions. This could be due to higher exposure to light that can activate larger number of rhodopsin molecules [114]. In *EYS* patients, superotemporal region could be more likely to be affected [112, 115], or inferior retina may be more affected than superior retina [96]. Careful follow-up studies would be needed in the possible effect of regional differences on photoreceptor degeneration. In any case, it would be noteworthy to mention that vitamin A supplementation has different effect on *RHO*-RP patients and *EYS*-RP patients [86].

10. Conclusions

In this review, we summarized utilization of zebrafish in vision science and IRDs focusing on our recent findings in the digenic *eys*^{+/-}; *lrp5*^{+/-} zebrafish. Our group shows that *Lrp5* is a potent candidate for the receptor of atROL in the RPE, and have discussed for the first time the possible involvement of Eys protein in the visual cycle. We think that the highlights of recent studies obtained in medaka and humans that seek to understand the functional role of EYS are all in good agreement with our proposal in 2020 [75]. In this review, we have taken the discussion a bit further with the intention of contributing to the understanding of the functional role of the EYS. Because EYS is a major cause of arRP in Europe and Asia, we hope to make a significant contribution to the cure of *EYS* patients through basic research that seeks to understand the function of EYS, and we hope this review will help in this regard.

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Conflict of interest

The authors declare there is no conflict of interest or no competing interest in this study.

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Application of Zebrafish in Mitochondrial Dysfunction

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Abstract

This chapter provides an overview of the zebrafish (*Danio rerio*) as a model organism for studies of mitochondrial dysfunction. Zebrafish possess a genetic similarity with humans and have conserved mitochondrial genomes, rendering them a valuable research tool for examining the intricate mechanisms that govern mitochondrial processes at diverse developmental stages. The chapter explores several methods for evaluating mitochondrial health and function. Examples include *in vitro* cell culture and *in vivo* analysis in embryos, larvae, and adults. The chapter discusses the use of zebrafish models in toxicological research to investigate mitochondrial reactions to environmental stressors and xenobiotics. The importance of implementing standardized protocols, validating marker, integrating different omics data, and using *in vivo* and *in vitro* approaches to advance mitochondrial research will be highlighted. In summary, zebrafish are suitable for analyzing both mitochondrial function and dysfunction, as well as their impact on human health.

Keywords: zebrafish, mitochondrial dysfunction, model organism, mitochondrial research, mitotoxicology

1. Introduction

Mitochondria are indispensable organelles for eukaryotic organisms, enabling aerobic metabolism, thus consuming oxygen and oxidizing molecules, such as sugars, to produce adenosine triphosphate (ATP), the energy currency used by cells [1, 2]. In addition, mitochondria actively participate in the control of cell homeostasis and in signaling processes, including cell injury [3], the intrinsic apoptosis pathway, and mitophagy [2, 4].

Given their crucial importance, exposure to toxic agents that can affect mitochondria is a factor that has been explored in toxicological analyses of chemical substances and compounds [5, 6]. Damage to mitochondria, often of a structural nature [3], triggered by exposure to toxic agents, can lead to inhibition of oxidative phosphorylation, production of reactive oxygen species, alterations in the mitochondrial permeability transition, mitochondrial DNA damage, and inhibition of beta-oxidation of fatty acids [7–9]. The adverse effects derived from this damage and dysfunction are wide-ranging, including hepatotoxicity [8], cellular aging,

senescence, genomic instability, inflammation [10], as well as metabolic, neuro-degenerative, and cardiac disorders [9, 11] and hereditary diseases that affect the mitochondrial network and cell metabolism [11].

For regulatory purposes and to understand the toxicity of compounds, the definition of mitotoxicity is relevant and can help in the weight of evidence and in defining the safe use of substances, for which various experimental models and organisms are used. Various organisms are currently used to assess mitotoxicity. Among these model organisms, the Zebrafish (*Danio rerio*) has stood out worldwide [12, 13].

The advantages of using this model are related to its genetic, mitochondrial, and xenobiotic metabolism similarities when compared to humans [12–15], as well as aspects related to scientific research. In addition, zebrafish are a model applied to One Health, which is based on linking human, animal, and environmental health [16]. This makes it possible, by examining possible mitochondrial dysfunction in zebrafish, for researchers to obtain information about the potential implications for human health, for the fish themselves, and for their ecosystems [16].

In view of the growing application of zebrafish in mitochondrial dysfunction analysis, the object of this chapter is to present the principles of mitochondrial function and dysfunction, the application of the model organism, and the main techniques used in this context.

2. Mitochondria: function and dysfunction

Occupying up to 20% of the cytoplasmic volume, mitochondria are important ubiquitous organelles for the evolution of complex animals, since they carry out the complete metabolism of sugars, making the production of ATP 15 times greater than anaerobic glycolysis. Their shape is similar to that of small bacteria, and because they have their own genome (circular DNA), produce tRNAs (RNA transporters), and have ribosomes, it is the most widely accepted theory that their origin is from an endosymbiotic relationship between a primitive eukaryote and aerobic bacteria [1, 2, 8].

Mitochondria have two different membranes that surround specific compartments. The outer membrane contains pores that allow small molecules and ions to pass but prevent larger molecules like proteins. Located between the outer and inner membranes is the intermembrane space. The inner membrane defines the mitochondrial matrix and is highly impermeable to small molecules, including H⁺ ions. The mitochondrial cristae, invaginations in the inner membrane, greatly increase its surface area and house the machinery responsible for energy conversion. Here we find the complex of electron transport proteins of the respiratory chain (CTE) (complex I–V), ADP-ATP translocase, ATP synthase, and other membrane transporters. The mitochondrial matrix contains important processes, including the Krebs cycle, which converts pyruvate and fatty acids into NADH. In this space, we find the pyruvate dehydrogenase complex and various enzymes related to these processes. In addition, the matrix houses mitochondrial DNA and ribosomes, which are essential for mitochondrial function and replication [1, 2].

Mitochondrial DNA has undergone substantial changes during evolution, so most of the genes underwent migration to the nucleus through gene transfer. However, current mitochondria still contain smaller genes that encode hydrophobic proteins forming the electron transport chain (ETC) in addition to genes related to the mitochondrial genetic system like ribosomal proteins. Some important mitochondrial genes include *rns* and *rnl*, which encode ribosomal RNA, *cob* (cytochrome b), and *cox1* and *cox3*, which encode two subunits of cytochrome oxidase [1, 2, 17].

As dynamic and plastic organelles, mitochondria move within cells *via* microtubules of the cytoskeleton, change shape, and are able to divide and fuse. They possess the ability to change their shape and undergo division and fusion, collectively known as mitochondrial dynamics. These processes involve mitochondrial fission and fusion. These processes enable the regulation of mitochondrial number and shape according to cell requirements while preserving the integrity of mitochondrial compartments [1, 18].

The essential function of mitochondria is related to the energy conversion machinery located in the inner membrane. This process is known as chemiosmotic coupling, in which ATP synthesis is directly linked to transport processes across the membrane. In addition, in the complex context of cellular homeostasis, mitochondria interact with each other, including the endoplasmic reticulum, during fusion and lipid trafficking, and with lysosomes during the process of mitophagy [1, 2, 19].

The presence of mitochondrial permeability transition pores (mPTP) is a characteristic feature of mitochondrial permeability to compounds, especially with respect to passage through the outer membrane. The mPTPs maintain mitochondrial homeostasis and are composed of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase (ANT), and cyclophilin D (Cyp-D) [20].

The mitochondrial matrix houses crucial enzymes that facilitate conversion of pyruvate and fatty acids into acetyl-CoA. Subsequently, acetyl-CoA is oxidized in the citric acid cycle to produce NADH, which provides high-energy electrons for the respiratory chain. In the inner membrane, the respiratory chain utilizes these electrons to transport protons out of the matrix, thereby creating an electrochemical gradient. In this process, NAD-linked dehydrogenases contribute electrons by oxidizing substrates like α -ketoglutarate and malate. These electrons are passed to the respiratory chain that reduces oxygen to create water [1, 2].

The respiratory chain is composed of three primary enzyme complexes that transfer electrons through protein carriers. In each complex, energy is released for the pumping of protons. Technical term abbreviations will be explained on first use. The sequence of electron flow is as follows: NADH $\rightarrow$ Complex I (NADH dehydrogenase) $\rightarrow$ Ubiquinone $\rightarrow$ Complex III (Cytochrome c reductase) $\rightarrow$ Cytochrome c $\rightarrow$ Complex IV (Cytochrome c oxidase) $\rightarrow$ Molecular oxygen (O_2). NADH transfers its reducing equivalents to ubiquinone, which then guides the electrons through complexes III and IV. In this phase, oxygen undergoes a reduction and becomes water. Observe that the language is objective, clear, and avoids complexity [1, 2].

The electrochemical gradient promotes the proton-motive force, propelling hydrogen ions back into the mitochondrial matrix. ATP synthase utilizes this force to produce ATP while serving as “rotational catalysis” coupled with the proton flow. Along with ATP production, the electrochemical gradient enables the active transport of metabolites, efficiently converting ADP into ATP. This sustains elevated cellular levels of ATP, furnishing energy for a diverse array of cellular processes [1, 2]. **Figure 1** illustrates the processes of CTE and OXPHOS and the mitochondrial structures.

The oxygen reduction pathway processes naturally generate ROS, including superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$). These compounds necessitate speedy inactivation to avoid reactivity and potential destruction of various cellular structures. Mitochondria contain antioxidant enzyme complexes such as superoxide dismutase and glutathione peroxidase to neutralize ROS and prevent damage. On the other hand, low levels of ROS play a role in signaling and coordinating mitochondrial oxidative phosphorylation with other metabolic pathways [2, 8, 21].

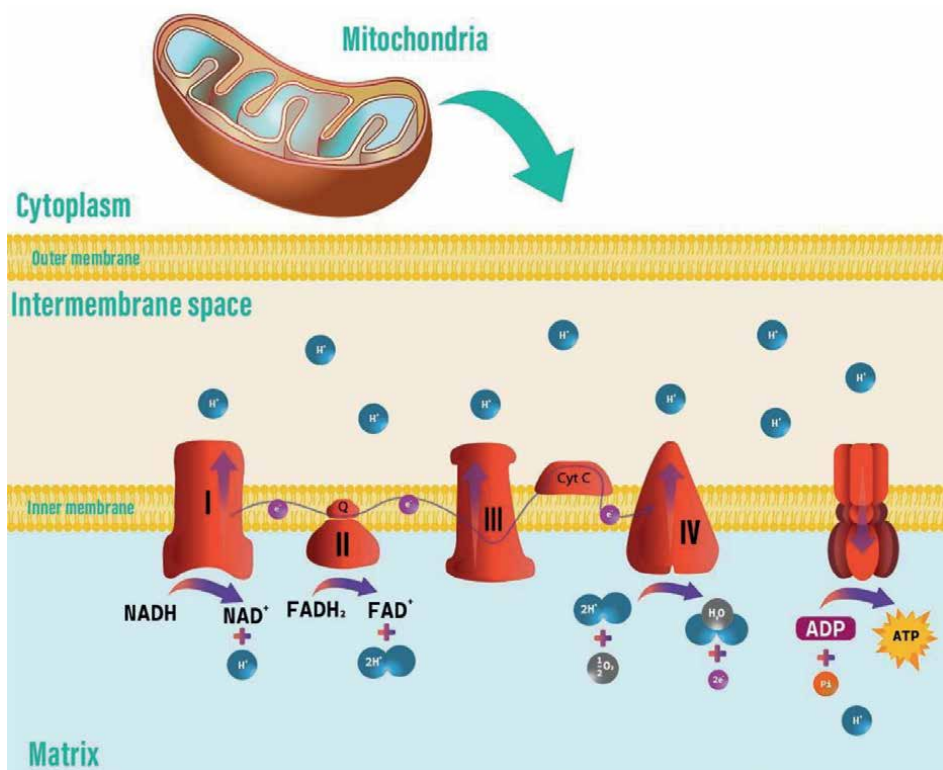


Figure 1.
Mitochondria: Structure and oxidative phosphorylation.

Furthermore, mitochondria perform various metabolic functions, such as redox buffering in the cytosol and calcium buffering, as well as play a critical role in biosynthesizing heme groups and membranes, the urea cycle, and metabolic adaptation [1, 19]. The intricate nature of mitochondria underscores their diverse significance in cellular metabolism, and consequently, any injury to these organelles by toxic agents can disrupt cell homeostasis in numerous ways.

One of the important mechanisms of action of toxic agents in mitochondria is mediated by oxidative stress caused by excessive production of ROS that exceeds the capacity of the antioxidant system. ROS can impact different cellular and mitochondrial structures, and they are genotoxic while interfering with protein structures. Excess ROS production can result from mtDNA damage and protein synthesis alteration, activating apoptotic pathways [8, 22, 23].

Another mechanism involves the mPTP induction, which leads to the release of pro-apoptotic proteins into the cytosol, initiating cell death. The cascade may or may not be mediated by the caspase. The primary proteins involved in this context are cytochrome C (Cyt C), apoptosis induction factor (AIF), and endonuclease G (EndoG), which are prevalent in the intermembrane space. Various mechanisms facilitate the permeabilization of the outer mitochondrial membrane, including BAX/BAK, which produce pores, the activation of BH3-only proteins, and others that contribute to mitochondria-mediated intrinsic cell death. Disruption of calcium homeostasis or increased production of mtROS results in the disruption of both the inner and outer mitochondrial membranes. This causes a decrease in membrane potential,

a reduction in ATP production, and ultimately a cellular demise through apoptosis or necrosis [8, 24, 25].

The induction of mutations in mtDNA by toxic agents may represent a molecular initiating event (MIE) of the mitotoxicity of these agents, as this induction can result in the abnormal synthesis of proteins involved in oxidative phosphorylation. mtDNA is especially susceptible to damage due to its proximity to CTE, which generates mtROS, and its limited repair mechanism. Consequently, mtDNA is prone to various types of damage, including strand breaks and base deamination, among others. These modifications can impact the function of CTE complexes, heighten the generation of reactive oxygen species (ROS), and ultimately initiate phenomena such as mitophagy, apoptosis mediated by mitochondria, or necrosis [8, 26–28].

The primary mechanism behind the mitotoxic effects of toxic agents is the inhibition of oxidative phosphorylation. These chemicals can impact mitochondrial respiration in multiple ways, such as by uncoupling it, inhibiting CTE complexes, or blocking ATP synthesis directly. Uncouplers transport H⁺ ions, changing the membrane potential, lowering ATP production, increasing oxygen consumption, and releasing energy as heat. Inhibition of ATP synthase and CTE results in ATP depletion, which disrupts calcium homeostasis. This leads to cytoplasmic calcium overload, activates proteases, and causes cell dysfunction or death. The decrease in ATP generation linked to this process is related to various pathologies, aging, and adverse medication effects. Moreover, blocking CTE raises the production of superoxide radicals and mtROS, which leads to cellular impairment [8, 29].

Ultimately, toxic substances could target fatty acid oxidation (FAO) in mitochondria. Inhibiting this process can occur directly through interaction with beta-oxidation enzymes or indirectly by depleting the coenzymes necessary for the electron transport chain (ETC). This inhibition of FAO oxidation can result in the uncoupling of oxidative phosphorylation, leading to potential consequences and apoptosis through the intrinsic mitochondrial pathway. Extended and severe reduction of mitochondrial beta-oxidation is associated with steatosis causing liver failure, coma, and mortality [8, 30].

3. Zebrafish as a model for mitochondrial toxicology

The fish species *Danio rerio*, popularly called zebrafish, is a small, blue, and gray striped fish native to the south of the Asian continent, first described by the Scottish physician Francis Hamilton in India [31]. Its typical habitat consists of shallow, slow-moving streams and, in particular, still pools that form along streams during the monsoon, a very rainy season when the waters become turbid. Having co-occurred with humans for thousands of years, zebrafish are also found in rice paddies, drainage ditches, storage tanks, and the like, although they certainly also suffer from the effects of pollution and habitat loss [32]. In terms of their natural diet, these fish are omnivorous, consuming larvae and adult insects, as well as small crustaceans and other zooplankton, while also consuming algae, plant materials, and various detritus [33]. Many studies have been carried out since their description, in relation to their diet, habitat, and cultivation, involving fish farming, and they have also been much investigated in relation to their genetics, mainly by George Streisinger (University of Oregon, USA), who made a significant contribution in the area of developmental biology and the use of zebrafish (*D. rerio*) as a model organism [34–36].

Since then, zebrafish have also been recognized as a useful organism for research into human diseases. Among the model organisms most commonly used in research, fish are already in second place in many countries, just behind rodents [37]. The zebrafish is widely used worldwide for a number of reasons. It is a low-cost model organism to keep in the laboratory; it is easy to cultivate and maintain because of its small size; it has a high reproduction rate, which allows several experiments to be carried out at the same time; fertilization is external, making it possible to collect eggs for studies such as toxicological tests, and its embryos are transparent, which allows the entire embryonic development to be visualized, observing its organs, heartbeat, and movement of the bloodstream; it is within the 3Rs (Replacement, Reduction, and Refinement), mainly because of the tests carried out with embryos up to 120 hpf, which is considered an *in vitro* test, and, above all, because it shares common organs and 71.4% of the same genes as humans and 84% similarity with disease genes [14, 38, 39].

The genetic similarity leads to the use of zebrafish as a model to study genetic functions and disorders in mammals, such as metabolic syndromes [40]. In the last two decades, zebrafish have been shown to be useful for discovering and studying toxicities in a wide range of organs and systems. Teratogenicity, cardiotoxicity, hepatotoxicity, nephrotoxicity, neurotoxicity, gastrointestinal toxicity, myotoxicity, and carcinogenicity have all been successfully explored in zebrafish [14]. We can see that there is a great demand for research in various areas of toxicology using this model organism, and in addition to these, mitotoxicity is also gaining ground today.

We can say that mitotoxicity is a new term, used in scientific articles since 2011, when we searched scientific platforms such as PubMed, with 147 scientific publications in total and only 21 review articles. Parallels between human and zebrafish mitochondria extend to their composition and genetic organization, encompassing genes for rRNAs, tRNAs, and proteins integral to oxidative phosphorylation [12, 13, 41, 42]. Consequently, zebrafish serve as an apt model for scrutinizing mitochondrial function, enriching the arena of comparative genomics and providing insights into genetic pathways and key molecular processes among vertebrates [43, 44]. Zebrafish can be used to study mitotoxicity at different stages of development and also in cultured cells from embryos, larvae, or adult tissues (**Figure 2**).

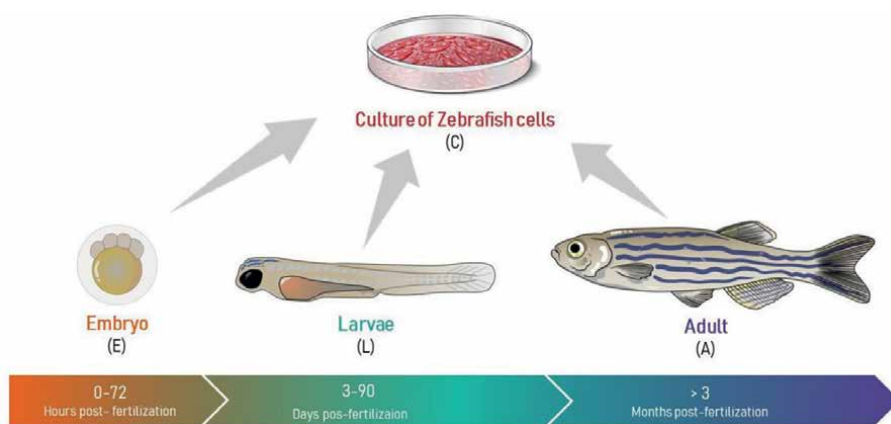


Figure 2.
The zebrafish model organism, stages of development, and the possibility of cell culture.

Xenobiotics, such as pollutants and drugs, target multiple macromolecules (DNA, proteins, lipids), including mitochondrial components, suggesting that their toxicity mechanisms may also be related to mitochondrial dysfunction [45–48]. Scientific publications evaluating mitochondrial dysfunction in zebrafish have grown exponentially since 1994, according to data from the PubMed platform. Most of these studies are for the evaluation of xenobiotics, which are often substances that we come into contact with on a daily basis [49–54]. The use of zebrafish as a model organism, in addition to all the advantages already described in this topic, is also of great importance for assessing mitotoxicity, and according to Azevedo et al. [12], it is because of the contribution of this small teleost that our knowledge of the intricate patterns linking mitochondrial function to the balance between health and disease is now more easily acquired. In the next topic, we will look more precisely and in detail at existing studies involving mitotoxicity and zebrafish [12, 42].

4. Approaches for mitochondrial analysis in zebrafish

Zebrafish enable researchers to investigate mitochondrial function and dysfunction using a range of methodological approaches. Established methodologies can be adapted, and new experimental procedures can be developed using various experimental models with the zebrafish organism. The structure is clear, with a logical progression and causal connections between statements. Analyses can be performed on primary and secondary cell cultures, embryos up to 48 hours after fertilization, larvae from 72 hours after fertilization, as well as juveniles and adults. Tissue extracts from adult zebrafish are utilized for the isolation of mitochondria, which allows for various analyses to investigate mitochondrial functions and potential dysfunctions.

This method is widely employed in mitochondrial toxicology research. Previous studies have focused on comprehending the similarity of isolated zebrafish mitochondria. In this context, certain studies have achieved the isolation of zebrafish mitochondria *via* methodologies, including differential centrifugation. This technique employs a series of centrifugation steps to divide cellular components by size and density and has successfully resulted in the isolation of mitochondria from zebrafish embryos. Another method for organelle isolation is density gradient centrifugation. This approach involves layering samples onto a density gradient medium, then separating organelles through centrifugation based on density fluctuations. Mitochondria can also be isolated utilizing specific commercial kits [55–57].

Microscopy and flow cytometry are important tools in mitochondrial toxicology studies, permitting visualization and analysis of mitochondrial morphology and dynamics and quantification of mitochondrial function and integrity at the single cell level [58]. The most commonly used microscopy techniques in this field include epifluorescence, confocal, and electron microscopy.

Fluorescence microscopy serves as an auxiliary technique for various experimental procedures, like immunofluorescence. A particular application of this method involves analyzing real-time and *in vivo* mitochondrial transport and dynamics in zebrafish larvae. The two-photon laser axotomy technique was utilized to axotomize M axons while concurrently recording the regeneration process and mitochondrial transport. In this context, it was observed that axons that have a greater ability to regenerate maintain higher levels of mitochondrial mobility [59].

Confocal microscopy can analyze the effects of toxic agents on mitochondrial fusion and fission processes, providing data on the regulation of mitochondrial

segregation [58]. In addition, electron microscopy can be used to explore mitochondrial ultrastructure, with a focus on zebrafish cells. This approach allows for precise imaging of mitochondrial cristae morphology, size, shape, and architecture, providing essential insights into mitochondrial function and identifying any abnormalities associated with dysfunction [60].

Additionally, flow cytometry has been utilized to quantitatively measure mitochondrial function and integrity in individual cells, as well as to assess mitochondrial content and mass in zebrafish cells. This method enables the examination of mitochondrial biogenesis and regeneration, making it a useful tool for assessing the impact of harmful substances on mitochondrial metabolism and energy generation [61].

Combined with microscopy and flow cytometry, and in addition to blotting techniques, the use of fluorescent dyes constitutes a particularly sensitive method for detecting various structures and homeostasis conditions. In this context, immunofluorescence and immunoblotting techniques are noteworthy, as they allow for the evaluation of specific proteins within mitochondrial complexes. Immunoblotting analyses concentrate on the expression and localization of proteins that relate to mitochondrial function. For instance, the levels of mitochondrial apoptotic proteins in zebrafish testes following exposure to carbon ion radiation were evaluated using immunoblotting [62].

In immunofluorescence of cell cultures, MitoTracker is a commonly employed fluorescent dye. This dye enables the visualization and quantification of mitochondria, as it infiltrates the mitochondria depending on the mitochondrial membrane potential and continues binding even after this potential dissipates [63]. In another study, various fluorescent dyes or fluorescent reporters were employed to analyze neuronal mitochondria *in vivo*, in Zebrafish embryos and larvae, following the procedure in the study. To this end, Mandal et al. refined techniques for visualizing mitochondria and analyzing their life, health, and function using parameters including movement, location, size, and function [64].

The evaluation of co-localization of mitochondria in relation to other cellular components using fluorescent dyes and immunostaining is pertinent for understanding mitochondrial dynamics. Ito et al. employed immunostaining with an anti-raldh2 antibody to investigate the localization of raldh2 in zebrafish and medaka subsequent to cardiac injury. Periostin-b from zebrafish and periostin-b from medaka, which are markers of fibrillogenesis, were detected at the wound site where collagen deposition occurred, implying their co-localization with mitochondria [65].

To evaluate mitochondrial health and the potential mitotoxic effect of a compound, measuring mitochondrial membrane potential ($\Delta\Psi$ M) is a fundamental analysis. Fluorescent dyes are frequently employed for this purpose. For instance, researchers developed a novel cyanine dye, ZMJ214, for live zebrafish larvae to detect mitochondrial membrane potential [66]. In addition, zebrafish embryos were subjected to another fluorescent dye; JC-10 is a lipophilic cationic dye that indicates mitochondrial depolarization [67].

Assessing ROS production is another parameter used to determine mitochondrial toxicity, and zebrafish can be used as a model organism to identify changes in ROS levels. Fluorescent probes, such as 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) and diamino fluorophore 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM), are commonly used because they can be internalized by cells and oxidized by ROS to generate fluorescent signals [68]. Finally, another frequent assessment is the analysis by biochemical assays of the activity of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), which are responsible for scavenging ROS [68, 69].

In studies that concentrate on mitochondrial genetics, the main techniques used are mtDNA knockout and knockdown. Zhou et al. developed a tissue-specific knock-out approach, which is an exemplar of this method. The main objective of the study was to explore essential developmental genes related to mitochondrial function in zebrafish [70]. Furthermore, the manipulation of genes responsible for generating or scavenging ROS can be employed to examine the impact of altered ROS concentrations on zebrafish physiology and development [71]. Moreover, transcriptome and proteome analyses can provide information on the molecular mechanisms underlying ROS production and its effects on zebrafish [72].

Oxygen Consumption rate (OCR) measurement provides valuable information on the mitochondrial function and bioenergetics of zebrafish. It enables the quantification of oxygen consumption rate (OCR) measurement, a direct measure of mitochondrial respiration and ATP synthesis [73]. The measurement of OCR using respirometry instruments permits the quantification of mitochondrial respiration in zebrafish. OCR measures the metabolic activity of organisms and provides important insights into mitochondrial function and bioenergetics. It has been utilized to explore the impact of temperature on the bioenergetics of zebrafish embryos and to evaluate how environmental stressors affect mitochondrial function [74]. OCR measurements can be utilized to evaluate the metabolic activity of zebrafish in response to different interventions or treatments [75, 76].

One method of determining OCR in zebrafish is by employing plate-based respirometry with the use of an extracellular flux analyzer like the Seahorse XF96 [74, 77]. This approach enables the evaluation of OCR in multi-well plates, providing a high-throughput method for measuring mitochondrial respiration in zebrafish embryos and larvae. The Seahorse analyzer employs Islet Capture microplates where a single embryo is situated in each well, permitting the measurement of bioenergetic parameters such as oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) [74].

Another innovative method involves utilizing the high-resolution Oroboros O2k Oxygraph instrument and microfluidic devices, which are combined with light modulation systems for precise measurement. This technology enables the calculation of OCR through the measurement of oxygen decrease over time. This method is supported by various studies. Using mitochondrial modulators that act at different stages of respiratory chain and oxidative phosphorylation, technology allows for the assessment of various mitochondrial components and complexes [78, 79]. This method enables precise and sensitive evaluation of mitochondrial oxygen consumption, enabling measurements of mitochondrial respiratory capacity, integrity, and energy metabolism [80].

4.1 Evaluation and enhancement of existing methodologies

Numerous techniques are employed to investigate mitochondria using zebrafish, as previously mentioned. However, in order to provide further insights into their applications, the developmental stages utilized, as well as the advantages and disadvantages associated with each, are elaborated. Below, **Table 1** is presented.

An overview of techniques employed for investigating mitochondrial function within diverse experimental models of zebrafish is presented. The experimental models considered include zebrafish cell culture (C), embryos up to 48 hours post-fertilization (E), larvae from hatching to 1 month (L), and adults utilizing tissue

Technique	Model	Application	Advantage	Limitation
Immunoblotting and immunofluorescence techniques using fluorescent dyes	C [63, 81] E [64] L [82] A [83]	Visualization and quantification of mitochondrial proteins and respiratory chain components [84]	Quantitative results and small sample research [85]	Requires specific antibodies Not so fast and sensitive [86, 87]
Genetic approaches	C, E and A [88]	Investigate effects of mitochondrial gene absence or reduction [88]	Efficient and accurate tool for targeted genetic manipulation [89]	Potential for nonspecific side effects [89]
Assessment of Mitochondrial Membrane Potential ($\Delta\Psi$ M)	C [90]	Measure the electrical potential across the mitochondrial membrane. Evaluate mitochondrial functional integrity [91]	Direct indicator of mitochondrial health [91]	Does not provide insights into other parameters. Limits in terms of high throughput performance and the need for multiple washing steps [92]
Assessment of Reactive Oxygen Species (ROS)	C [93] E [94] L [95]	Monitor mitochondrial oxidative stress [96]	Detection of oxidative damage [96]	Analytical and coordination limitations as well as the interference of concomitant ozone [97]
Assessment of mitochondrial co-localization	C [98] E [99] L [100]	Investigate mitochondrial interactions with other cellular structures [101]	Efficiency and prediction when analyzing and quantifying mitochondrial movements [102]	Challenges in image enhancement, trajectory tracking, and quantitative analysis [103]
Oxygen consumption rate (OCR) measurement techniques	C [104] E [105] L [106]	Comprehensive understanding of mitochondrial metabolism [79, 107]	Direct and quantitative measurement Live analysis and real-time monitoring Possibility of using mitochondrial modulators [79, 107, 108]	Requires specialized equipment. The cost of equipment and in some cases analysis well plates. Robustness of statistical inference [109].

Table 1.

Methods for assessing mitochondrial function in different experimental models of zebrafish (Danio rerio).

grafts and isolated mitochondria (A). For each technique, a description of its specific application, associated advantages, and limitations is provided.

Within this context, it becomes evident that there is substantial room for advancement in the realm of mitochondrial studies using the zebrafish model organism. Below, we delve into several potential areas that warrant exploration and development.

- **Standardization and reproducibility:** many of the mentioned studies have employed a wide array of techniques, reagents, and equipment to assess mitochondrial function. The standardization of experimental protocols and reagents

is paramount to ensuring result reproducibility across various laboratories and studies. This is particularly crucial in the realm of toxicological analyses, where assay reliability and reproducibility hold pivotal importance. In this context, the goal is for the collected data to constitute a substantial body of evidence, informing regulatory agencies' decision-making processes [33].

- Validation of markers: despite the utilization of numerous fluorescent markers and probes to visualize mitochondrial function, validating these markers in terms of specificity and sensitivity is imperative. Moreover, employing multiple markers to address distinct facets of mitochondrial function can provide a more comprehensive understanding. Another avenue in this regard involves research and development focused on creating novel markers for analyzing new mitochondrial characteristics and functionalities while simultaneously reducing costs and enhancing analysis robustness [34].
- Real-time quantification of mitochondrial function: the adaptation of assay protocols that leverage extracellular flux analysis and oxygen concentration detection is pivotal for advancing the field of these analyses using the *in vivo* and *in vitro* models facilitated by zebrafish. Considering the potential to employ methodologies with enhanced cost-effectiveness, in contrast to established evaluations with substantial costs, holds promise. This approach aligns with the broader goal of accessibility and affordability in mitochondrial function assessments, in addition to leveraging the advantages offered by Zebrafish models.
- Integration of multi-omics data: mitochondrial function is influenced by a myriad of genetic and metabolic factors. The integration of data derived from multi-omic approaches, including genomics, transcriptomics, proteomics, and metabolomics, has the potential to offer a more comprehensive understanding of the mechanisms underpinning mitochondrial function and the mitochondrial action mechanisms of xenobiotics throughout the various developmental stages of zebrafish. This holistic approach facilitates unraveling the intricate interplay of molecular events driving mitochondrial responses and contributes to a more nuanced comprehension of the impact of external agents on mitochondrial dynamics within the zebrafish model system [35].
- Integration of *in vivo* and *in vitro* studies: while numerous studies are centered on *in vivo* systems, particularly focusing on embryos and larvae, supplementation with *in vitro* studies employing zebrafish cell cultures can yield invaluable insights into mitochondrial function under more controlled conditions. This complementary approach enhances our understanding of mitochondrial dynamics, allowing us to discern intricacies that might be obscured by the complex milieu of whole organisms. It also facilitates the dissection of specific cellular responses and contributes to a more comprehensive appreciation of the molecular underpinnings of mitochondrial processes in the context of zebrafish models [36].

5. Application of zebrafish in mitochondrial toxicology analysis

Most of the studies that began to study mitochondrial dysfunction in zebrafish were aimed at understanding human diseases, as in the work of Van Raamsdonk et al.

[110], Guo et al. [111], and Bretaud et al. [112], who used this model mainly to analyze neurological diseases. According to the dates of these publications, we can say that the evaluation of mitochondrial alterations using *D. rerio* has been recent. One of the first studies to be published on mitotoxicity using zebrafish as a model organism was by Oulmi and Braunback [113], who at the time were microinjecting *D. rerio* embryos to evaluate an organochlorine compound (4-chloroaniline) used to make dyes, insecticides, and various industrial products. In this work, for the toxicological analysis, the researchers evaluated the size, shape, and function of the mitochondria in relation to the quantity of other organelles in the liver and kidney, concluding that, as well as the zebrafish being a suitable model for microinjection tests, it is also suitable in terms of histological and cytological methods for diagnosing the potential dangers of environmental chemical products [113].

We can observe that over the years, mitochondrial assessment has evolved, and new methodologies and techniques have been improved, as in the work of Mendelsohn et al., who investigated the role of energy detection in the response of the zebrafish embryo to anoxia, testing the arrest of development in mitochondrial inhibitors, through oxygen consumption measured by respirometer equipment [114]. Since then, several studies have evaluated mitochondrial dysfunction, mainly by analyzing mitochondrial oxygen consumption for mitochondrial toxicity, such as the work of Bourdineaud et al. [115], evaluating environmental pollutants; the work of Geffroy et al. [116] and Ladhar et al. [117], evaluating nanoparticles, already entering the area of nanotoxicology; the work of Bestman et al. [118], also evaluating an organic compound for the manufacture of pesticides; and Cowie et al. stating mitochondrial dysfunction of zebrafish caused by the pesticide Dieldrin [119]. Other studies have also evaluated pesticides using mitochondrial dysfunction as a method of toxicological analysis [49, 53, 54, 120–133].

In the literature, there are still few studies related to the toxicity of xenobiotics and mitochondrial dysfunction in zebrafish, especially in relation to mitochondrial oxygen consumption. Most of the existing publications have evaluated human diseases and drugs. Therefore, more studies are needed in the area of mitochondrial dysfunction and xenobiotic toxicity in zebrafish because of the importance of this analysis complementing conventional analyses and because of the help this analysis provides in understanding the mechanisms of action of substances.

6. Concluding remarks

This chapter discusses the function and dysfunction of mitochondria and how the zebrafish model organism can be utilized in various stages of development as well as in cell cultures. Zebrafish have proved to be a crucial tool for examining the intricate nature of mitochondrial function in the presence of xenobiotics.

Moreover, the model's distinctive features render it a valuable tool for studies on mitotoxicity. Due to its close genetic similarities to humans and shared mitochondrial structure, combined with the zebrafish's capacity for genetic manipulation, researchers are able to observe firsthand mitochondrial dynamics in action. Additionally, the zebrafish's capability to monitor mitochondrial respiration directly *in vivo* and in real-time serves as a valuable tool for thoroughly examining the effects of xenobiotic exposure on mitochondrial function. This approach offers valuable insights into the underlying mechanisms of mitochondrial dysfunction.

In summary, the research focused on analyzing mitochondria in zebrafish shows great potential. By illuminating the potential effects of xenobiotics targeting mitochondria, this research reveals complex molecular responses.

Conflict of interest

The authors declare that they have no conflict of interest.

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Section 4

Zebrafish Applications for the One Health

Zebrafish as Model Organism in Aquatic Ecotoxicology: Current Trends and Future Perspectives

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Abstract

Assessing the welfare of aquatic ecosystems has gained immense importance within the scientific community, elevating ecotoxicology to a fundamental discipline. This field utilizes bioindicators, often referred to as model organisms, to investigate the harmful effects of environmental pollution. Amid this array of biological markers, the zebrafish stands out as an exceptional contender. The primary objective of this present investigation is to highlight the complete information available in the literature about the utilization of zebrafish in ecotoxicology. Zebrafish are commonly utilized in research to study reproductive and embryonic toxicity due to their intricate sex-determination mechanism, which is significantly impacted by environmental factors. Similarly, this particular fish species is also employed in studying neuronal signaling, behavior, and DNA damage from pollutants due to their susceptibility and swimming abilities around day five post-fertilization. In addition to the established methodologies, recent advancements in environmental toxicology have incorporated zebrafish into emerging genome editing techniques. These innovative approaches provide fresh and compelling avenues for exploring detoxification mechanisms and organisms' intricate adaptive responses to environmental stressors. In conclusion, zebrafish are important as model organisms, providing profound insights into diverse facets of ecotoxicological investigations. Furthermore, their relevance is further underscored by their adaptability to evolving genome editing technologies.

Keywords: zebrafish, ecotoxicology, aquatic toxicology, model organism, bioindicator

1. Introduction

The proliferation of anthropogenic activities, such as industrialization, urbanization, and agriculture, has led to the release of diverse pollutants into aquatic ecosystems, including heavy metals, pesticides, pharmaceuticals, and organic compounds [1]. These pollutants disrupt the natural equilibrium of ecosystems, leading to impaired water quality, loss of biodiversity, and potential bioaccumulation of toxic substances throughout food chains [2]. Consequently, the need to assess the health of aquatic environments has become a pressing issue. Ecotoxicology serves as a crucial

discipline in addressing the adverse effects of environmental pollution on aquatic organisms [3]. By systematically evaluating the toxicity, distribution, and persistence of contaminants in ecosystems, ecotoxicological studies provide essential insights into the potential risks posed by pollutants. These insights inform policy decisions, guide regulatory frameworks, and aid in developing effective pollution mitigation strategies.

Bioindicators, alternatively called ecological indicators or biological markers, are living organisms that exhibit heightened sensitivity to environmental shifts primarily induced by human activities [4]. This diverse spectrum of organisms, encompassing avian and amphibian species, as well as insects and fish, is pivotal as indicators of the overall well-being of ecosystems. Among this varied assemblage of bioindicators, the zebrafish emerges as a particularly noteworthy candidate, owing to its distinctive characteristics and genetic resemblance to humans [5]. As an illustration, the transparency of zebrafish embryos permits direct observation of developmental processes, facilitating the detection of abnormalities resulting from environmental stressors [6]. Moreover, their swift reproductive cycles enable the examination of multi-generational impacts, unveiling chronic exposure effects [7]. This chapter aims to highlight the importance of zebrafish as a model organism in ecotoxicology.

2. Zebrafish

2.1 Zebrafish as model organism

2.1.1 Phylogeny

Zebrafish is classified within the kingdom *Animalia*, the phylum *Chordata*, and the subphylum *Vertebrata*. This taxonomic classification pertains to multicellular eukaryotic organisms possessing a vertebral column and a central nervous system [8]. Furthermore, it is classified under Actinopterygii, a group of ray-finned fishes that includes diverse species from *Cypriniformes*, such as goldfish and barbs [9]. At a more specific taxonomic level, the zebrafish belongs to the family *Cyprinidae*, which comprises a group of freshwater fishes that commonly lack mandibular teeth, such as carp [10]. Ultimately, *Danio*'s genus epitomizes a more sophisticated taxonomic categorization, denoting a closely affiliated species assemblage. The appellation “*Danio*” emanates from the Bengali expression “*Dhani*,” which precisely designates a piscine organism exhibiting zebra-like pigmentation.

2.1.2 Morphology

Danio rerio, commonly referred to as the zebrafish, is a small freshwater fish characterized by its elongated and slender body shape. Adult zebrafish typically exhibit a length of approximately 4 to 5 cm in captivity, although specific wild specimens have been documented to attain sizes of 6 to 7 cm [11]. As previously mentioned, the term “zebrafish” is derived from its prominent characteristic of possessing five identical, horizontally arranged stripes on its flanks, reminiscent of the lines observable on zebras. These conspicuous stripes, which manifest in either blue or black hues, span from the operculum (located behind the gills) to the base of the caudal fin (tail) [12]. The zebrafish demonstrates a fusiform morphology characterized by a streamlined, tapering shape in both anterior and posterior regions. Furthermore, it exhibits lateral compression, resulting in an upward-oriented mouth [13]. It possesses multiple fins, such as dorsal,

anal, caudal, pectoral, and pelvic fins [14]. Sexual dimorphism is apparent in this fish species, enabling easy differentiation between males and females [11]. Male zebrafish possess a slenderer, torpedo-like shape and yellow stripes along the ventral side. Whereas females can be identified by their distended white abdomen and silver lines [15].

2.1.3 Life cycle

In the presence of a male, adult female zebrafish exhibit a reproductive pattern wherein they can spawn every 2 to 3 days, resulting in the deposition of up to 200 eggs per week. This mating occurs in the morning, and light is the primary stimulus that triggers this behavior. Within 30 minutes after dawn, the female zebrafish can lay the eggs, which are fertilized by the male's released sperm. After fertilization, immediate embryonic development commences. If the eggs remain unfertilized, their growth halts following the initial cell divisions, ultimately leading to death. The zebrafish life cycle encompasses four principal stages: embryo, larva, juvenile, and adult (**Figure 1**) [6].

- Embryo:

Fertilized eggs (<0.7 mm) become transparent and develop rapidly [16]. Embryonic cells divide synchronously every 20 minutes [15]. Within the first 4 hours postfertilization (hpf), the fertilized egg transforms from a single cell into 1000 cells. Organogenesis (cell division and differentiation) occurs between 15 and 24 hpf. At 15.5 hpf, the tail bud becomes more prominent. Pigment formation starts in the

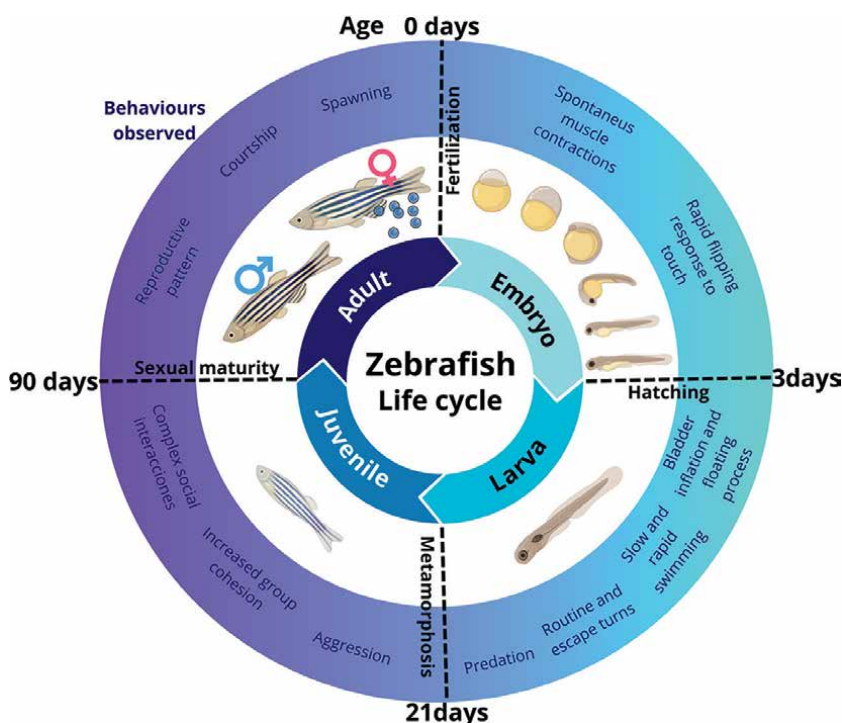


Figure 1.
 Zebrafish life cycle: Embryo, larvae, juvenile, and adult.

retinal pigmented epithelial cells and dorsolateral melanophores in the skin by 28 hpf [17]. The heart begins to beat at approximately 30 hpf. Shortly after (36 hpf), all major organs and tissues formed within the transparent embryos [18]. By 37 hpf, the brain has nearly fully developed. The pancreas, liver, and bladder are recognizable at 49 hpf [19]. Eventually, the embryos sporadically hatch between 48 and 72 hpf.

- Larva:

This stage extends from hatching to 21 days postfertilization (dpf) [20]. After hatching, the continuous anterodorsal protrusion of the mouth begins. Several days later, the swim bladder inflates with air, initiating the “floating” process [17]. The early larva starts swimming and moves its fins, jaw, and eyes. These actions promote respiration, the development of both rapid and slow movements, quick escape responses, food seeking, and predation on small prey [21].

- Juvenile:

This stage is characterized by the zebrafish larvae undergoing metamorphosis (22 dpf), such as the complete loss of the larval fin fold. Juvenile characteristics are acquired, such as scales on the body and the formation of two rows of melanophores along the edge of each scale [6]. Furthermore, the pigment pattern on their skin becomes more pronounced during this process. The juvenile zebrafish possesses all the physical characteristics of an adult of its species except for sexual maturity. Social interactions among young fish become more complex, involving aggressive encounters and increased group cohesion [22].

- Adults:

Approximately 90 days after fertilization, fish achieve sexual maturity and progress into reproductive adulthood [23]. Furthermore, within this temporal frame, discernible sexual dimorphism becomes apparent as males and females demonstrate the capacity to generate functional gametes and manifest secondary sexual attributes [24].

2.1.4 Advantages and constraints

The utilization of zebrafish as a biological model constitutes a profoundly invaluable asset within the realm of scientific investigation. This is attributed to its unique capacity to furnish insights across immediate and protracted *in vivo* research endeavors. **Table 1** expounds upon the merits and demerits inherent in employing the zebrafish (*Danio rerio*) as a biological indicator in juxtaposition to other animal models.

2.2 Zebrafish in aquatic ecotoxicology

2.2.1 Reproductive and embryonic toxicity

Mammals exhibit dimorphic sex chromosomes and follow a male heterogametic system for sex determination [34]. In this system, a particular gene known as SRY (sex-determining region Y) plays a pivotal role by acting as a genetic switch to initiate

Aspect	Justification	Reference
<i>Advantages</i>		
Rapid Development	The zebrafish undergoes a brief three-day embryonic development and attains reproductive maturity within 3 months. This species demonstrates a remarkable capacity for prolific embryo production over a concise timeframe.	[6]
Embryonic Transparency	The inherent transparency of zebrafish embryos enables the direct and noninvasive monitoring of intricate biological phenomena, such as the development of organs or the advancement of tumors. However, the opacity of tissues in other organisms constrains the extent of feasible observation.	[25]
Genetic Manipulation	The comprehensive characterization of the zebrafish genome facilitates the manipulation of genetic elements, thereby providing a robust platform to investigate the precise roles of individual genes across diverse biological processes.	[26]
Homology with Humans	Roughly 70% of the human gene repertoire possesses at least one discernible homolog in the zebrafish genome. Although precise gene-to-gene correspondence may not be uniform across the two species, many pivotal genetic pathways and fundamental processes exhibit conservation. This genetic analogy facilitates the elucidation of human biological mechanisms and the exploration of genetic afflictions and anomalies.	[27]
Cost and Space Efficiency	The zebrafish presents itself as a miniature and easily manageable organism within laboratory settings, affording the advantage of diminished expenses and spatial prerequisites for its upkeep and experimental utilization, in stark contrast to mammalian models. This financial economy can manifest substantially, ranging from ratios of 1:100 to 1:1000 when compared against rodent counterparts.	[28]
Fluorescent Labeling	Fluorescent markers possess the capability to designate proteins within zebrafish, facilitating noninvasive imaging and enabling the examination of intricate cellular and molecular mechanisms.	[29]
<i>Disadvantages</i>		
Limited Biological Complexity	The zebrafish exhibits a comparatively reduced level of biological intricacy compared with organisms such as mice or rabbits. These latter animals possess biological systems more akin to human physiology, rendering them more suitable for conducting intricate investigations into complex diseases and physiological processes.	[30]
Complex Behavior Studies Limitation	Zebrafish manifests comparatively less intricate behavioral patterns than highly evolved organisms such as mice or primates. This disparity in behavioral complexity imposes constraints on the comprehensive investigation of specific facets of animal behavior.	[31]
Immune System Dissimilarity	The zebrafish's immune system exhibits notable distinctions compared to that of high vertebrates. This dissimilarity could pose limitations for research into immune responses and exploring precise immune mechanisms, as the zebrafish possesses a less complex immune system.	[32]
Tissue Size and Manipulation Restrictions	The small size of zebrafish and its tissues and organs could limit the execution of experiments requiring larger tissue quantities or more complex surgical procedures.	[33]

Table 1.
Pros and cons of zebrafish as a model system compared with other species.

the male developmental pathway within mammals' bipotential gonads [35]. This means that the presence or absence of the SRY gene determines whether an individual develops as male or female during embryonic development. Unlike mammals, zebrafish do not possess a straightforward sex determination cascade. Instead, their sex-determination system is intricate and influenced by genetic and environmental factors. Research has shown that various elements, such as surrounding temperature [36], exposure to sex hormones such as estrogen and androgen [37], and oxygen availability [38] play significant roles in gonad ontogenetic differentiation in zebrafish. Endocrine-disrupting chemicals (EDCs) are external agents that profoundly impact the reproduction and development of organisms by interfering with endogenous hormones' natural synthesis, release, metabolism, and functioning [39]. These chemical compounds have been identified in various environmental compartments, such as air, soil, water, wildlife, and human tissues [40]. Thus, EDCs' effects on living organisms are of significant concern. Over the past few decades, investigations have concentrated on the adverse implications of EDCs on zebrafish after brief or prolonged exposure [41–45]. Most of these studies have targeted specific developmental stages in the zebrafish life cycle: adult, larvae, or embryo. In the broader context, it has been observed that adult zebrafish exhibit comparatively lower sensitivity to endocrine disruption when compared with developing embryos [46]. Nonetheless, this does not exempt them from experiencing detrimental effects on their gonads because of exposures to EDCs. Recent investigations have shed light on various consequences of such endocrine disruption in adult zebrafish. These effects include a reduced proportion of mature spermatozoa and spermatids [47]. Moreover, there is a decrease in the proportion of vitellogenin oocytes, coupled with an increased occurrence of atretic follicles, indicating a disruption in the normal ovarian follicle development process [48].

A clear difference between adult zebrafish and their embryonic and larval counterparts is evident in reproductive testing. Notably, during the early life stages, all zebrafish, regardless of gender, possess ovary-like gonads [46]. It is during the later stages of development that the definitive establishment of bisexual differentiation takes place [49]. In light of this information, recent research has suggested that the exposure of zebrafish to EDCs during sexual differentiation can lead to a skewed sex ratio, often resulting in an excess of female individuals [50, 51]. Although an initially inconspicuous skewed sex ratio might not manifest immediate detriment to the environment, it can potentially perturb the reproductive cycle of organisms and engender transgenerational effects. Transgenerational effects pertain to the potential negative consequences of exposures during early development that extend beyond the initial generation, leading to enduring repercussions on reproductive and developmental processes in subsequent generations. Several authors, for instance, have described that subjecting zebrafish to EDCs can lead to diverse effects, including elevated vitellogenin levels [51], feminization [52], and delayed production and development of their offspring [7, 53]. From the latter one, it is noteworthy to indicate that not only the transgenerational exposure nor the EDCs can delay or affect the embryonic development of zebrafish. Over the past decade, several authors have extensively documented the impact of various pharmaceutical and personal care products on zebrafish growth and development [54]. These studies have consistently revealed that exposure to these substances can significantly alter zebrafish's normal developmental processes, ultimately leading to the prompt occurrence of malformations in these organisms [55]. For instance, a study carried out in 2022 demonstrated that zebrafish embryos exhibited scoliosis, pericardial edema, and yolk deformation following a 96-hour exposure to microplastics [56]. Herein, it is pertinent to highlight that the

authors emphasize these deformations, and these deformations could arise from a reduction in the availability of oxygen and nutrients because of the accumulation of plastic particles on the surface of the embryos' chorion. Nevertheless, several other authors have also suggested that alternative mechanisms, including DNA damage, hormonal disruption, oxidative stress, epigenetic modifications, and changes in cell adhesion and migration, can also lead to the development of malformations in zebrafish embryos [57–59]. Consequently, establishing and formalizing a protocol to investigate the potential adverse effects of pollutants on fish has been regarded as a foremost priority in our times. The Fish Embryo Acute Toxicity (FET) test, as outlined in the Organization for Economic Co-operation and Development (OECD) TG 236, stands out for its standardized and efficient approach to assessing the acute toxicity of chemicals in early-stage fish embryos [60]. This test plays a pivotal role in efficiently identifying potential hazards while adhering to ethical considerations by reducing the need for adult fish testing. Within the framework of this test, zebrafish offer a unique opportunity to capture several teratogenic toxicity indicators, encompassing crucial embryonic development events, such as cell movement during the gastrula stage, formation of brain regions, heartbeat, and blood circulation [61]. These indicators effectively serve as pertinent endpoints for toxicological evaluation.

2.2.2 Behavioral changes

The incidence of neuroactive substances in aquatic environments is increasing, giving rise to apprehensions regarding potential hazards to hydrobionts [62]. According to reports, approximately 70,000 identified neurotoxic chemicals exist, but only a tiny proportion, roughly 10%, have thoroughly recognized toxicological mechanisms [63]. Therefore, precise behavioral assays become imperative to comprehend neuronal signaling, identify emerging neuroactive compounds, and forecast their modes of operation. Neurotoxicity testing utilizes various model organisms, encompassing invertebrates such as fruit flies and nematodes and vertebrates such as chickens, mice, and other mammals. Nevertheless, invertebrates lack intricate neural structures, including myelin sheaths, which imposes limitations on *in vivo* neural activity research [64]. Furthermore, the substantial expenses and spatial demands associated with testing on mice and chickens considerably hinder the efficient screening of neurotoxic compounds in large quantities. Therefore, carefully selecting suitable model organisms is particularly important in neurotoxicity studies. In the realm of neurotoxicity research, the zebrafish has emerged as a highly potent model for investigating alterations in the nervous system from diverse perspectives [65]. It provides a comprehensive examination of chemical mechanisms of action. This efficacy is primarily attributed to its analogous central nervous system (CNS) arrangement, neurogenesis process, and specific responsiveness to pollutants, which closely mirror those observed in other vertebrates [66]. Moreover, the zebrafish possesses a notable advantage in its capacity to engage in unrestricted swimming approximately 5 days postfertilization, during which its juvenile brain tissue undergoes differentiation into distinct segments, such as telencephalon, diencephalon, mesencephalon, hindbrain, and rhombencephalon [67]. This particular characteristic greatly facilitates the assessment of various aspects, including locomotion, learning, memory behavior, and responsiveness to photoelectric stimulation.

In the last 10 years, there has been a notable emphasis on using sophisticated video-tracking tools and data-analyzing software to enable efficient and rapid screenings of adult and larval zebrafish locomotion. This emphasis stems from the necessity to explore toxicological impacts on behavior, encompassing the investigation of

fundamental motor responses, sensorimotor reactions, and learning and memory processes [68]. Various diverse tests have been devised to fulfill these objectives, such as the novel tank diving test, the social interaction task, the shoaling test, the aggression test, and the predator avoidance test. The novel tank diving test is a behavioral experiment that utilizes the concept of thigmotaxis, a commonly observed behavior in aquatic species, including zebrafish [69]. Thigmotaxis refers to the instinctual tendency of fish to seek comfort and safety by staying close to the walls or bottom of an unfamiliar tank or environment [70]. In this experiment, thigmotaxis is employed as a measure of anxiety or stress levels in zebrafish. Zebrafish are highly social animals, and their complex social behaviors are investigated through the social interaction task. This task provides valuable insights into various aspects of zebrafish sociability, including their preference for social interaction, the establishment of dominance hierarchies, and territorial behavior [71]. Researchers can gain significant information about their social dynamics and behavior by observing their interactions in a controlled setting. Like many other social animals, zebrafish exhibit aggressive behaviors when defending their territories or striving for dominance within their social group [72]. The aggression test, thus, is employed to study the aggressive tendencies of zebrafish, particularly during territorial disputes or competition for resources. Despite their small size, zebrafish demonstrate sophisticated predator avoidance strategies, which are evaluated through the predator avoidance test. This experiment aims to assess their ability to detect and respond to cues from predators, such as visual or chemical signals [73]. Understanding the neural pathways and sensory mechanisms involved in evading predation is crucial for uncovering evolutionarily conserved survival traits in prey animals.

2.2.3 DNA damage

Per Guideline Numbers 487 and 489 issued by the OECD, genotoxic effects refer to a broad spectrum of damage inflicted upon DNA or chromosomes [74, 75]. This damage encompasses various types, including, but not limited to, DNA breaks, deletions, adducts, nucleotide modifications, linkages, rearrangements, gene mutations, chromosome aberrations, and aneuploidy [76]. Nonetheless, genotoxic effects have also been associated with other health conditions, such as hematology disorders, liver disease, endocrine disorders, and autoimmune diseases [77]. Thus, identifying pollutants capable of inducing genetic damage in somatic and/or germ cells is essential, and for this purpose, genotoxicity tests play a crucial role. Zebrafish have become a highly esteemed model system for conducting genotoxicity studies. Notably, they possess orthologous genes with eukaryotic organisms, indicating a shared genetic similarity with other organisms containing cell nuclei [78]. Moreover, zebrafish encompass nearly all the genes relevant to various DNA repair pathways, rendering them exceptionally well-suited for comprehending the mechanisms of DNA repair and damage response [79]. The micronucleus and comet assays are two of the most frequently employed tests to evaluate mutagenic and genotoxic effects induced by chemical agents in zebrafish. The micronucleus test in fish is a cytogenetic assay that examines abnormal little nuclei frequencies in nucleated erythrocytes, a characteristic unique to fish compared to mammals [80]. The primary objective of this test is to detect the potential cytogenetic damage induced by pollutants. Specifically, it aims to identify substances that lead to micronuclei forming acentric chromosome fragments or entire chromosomes [81]. Research data indicate that various substances, such as nanomaterials, metals, effluents, pesticides, pharmaceuticals, alkylating

agents, steroids, hormones, minerals, and polycyclic aromatic hydrocarbons, exhibit mutagenic properties by inducing micronuclei formation in fish erythrocytes [82–85]. The comet assay, also known as single cell gel electrophoresis (SCGE) protocol, represents a widely used and well-established technique employed for the evaluation of DNA damage and DNA repair mechanisms. The fundamental principle underlying the comet assay lies in the fact that when DNA undergoes damage induced by genotoxic agents such as radiation or chemicals, it experiences fragmentation [86]. Consequently, during electrophoresis, these damaged DNA fragments move away from the nucleus, giving rise to a distinct “tail” formation, whereas undamaged DNA remains closer to the nucleus, forming the “head” of the comet structure. DNA damage is quantified by measuring various parameters, including DNA tail rate, tail moment, total area, tail length, and tail intensity [87]. However, the comet assay also enables the analysis of DNA repair mechanisms. Researchers can investigate the efficiency of DNA repair processes in different cell types or under various conditions by observing the rate at which the comet tail diminishes after DNA damage [88]. Based on the findings presented by Canedo and Rocha in 2021 [78], the comet assay has emerged as the most widely utilized method for identifying genetic material damage in zebrafish. This technique was more prevalent than other approaches, such as the 8-OHdG ELISA kit, RAPD-PCR, and qRAPDPCR [78]. Thus, despite the continuous development of novel techniques, data indicate that the comet assay remains the primary choice for assessing genetic material damage in zebrafish studies.

2.2.4 Histopathological damage

Histopathological studies are crucial in environmental toxicology, assessing pollutants' impact on living organisms. These investigations involve a thorough microscopic examination of tissues, enabling researchers to identify and quantify cellular alterations and damage caused by toxic exposure. One significant advantage of histopathology is its capability to detect subtle tissue changes at early stages of toxicity, even before visible signs or clinical symptoms become apparent [89]. Moreover, by analyzing cellular and tissue changes, histopathology offers valuable insights into the health of populations in the wild and assesses the effectiveness of pollution control measures [90]. Zebrafish have garnered significant interest as a valuable model organism for conducting toxicological assays, encompassing the evaluation of administered compounds and environmental contaminants. This heightened attention stems from the remarkable physiological responses and histological features of zebrafish, which closely resemble those observed in mammals [91]. Furthermore, a significant advantage of utilizing zebrafish in histopathology is the ability to observe multiple organs on a single slide, facilitated by their small size [92]. Two distinct methodologies are employed to quantify tissue modifications in the organs of zebrafish. These methodologies involve the estimation of the histological alteration index and the calculation of tissue alteration prevalence. The first approach, known as the histological alteration index, assesses the average tissue alterations by employing a predefined list of potential changes, further categorized into three distinct levels of severity [93]. The second approach involves calculating the occurrence of tissue alterations in zebrafish organs. In other words, this method analyzes more tissue samples to determine the frequency of specific changes or abnormalities [85]. In general, these quantitative methodologies significantly contribute to enhancing comprehension of organ pathology. Nonetheless, their efficacy is contingent upon being accompanied by molecular techniques, such as immunohistochemistry, TUNEL assay, in situ hybridization, among other relevant methodologies.

2.2.5 Hematotoxicity

The evaluation of hematologic effects in environmental toxicology assumes paramount importance because of its potential to elucidate the adverse impacts of environmental contaminants on the blood and blood-forming tissues in living organisms. The hematologic system, comprising various blood cells and components, is critical in maintaining essential physiological functions within the body [94]. Any disruption in this intricate system can lead to severe health implications, including the onset of anemia, impairment of immune function, and heightened susceptibility to infections [95]. Consequently, a comprehensive examination of hematologic impacts is imperative in enhancing our comprehension of the potential risks posed by environmental contaminants. Zebrafish have become an exceptional model organism for exploring pollutants' hematologic toxic effects. One of the key reasons for its prominence is its developmental hematopoiesis, which remarkably mirrors the hematopoietic system's development in mammals [96]. In addition, this process is regulated by molecular pathways that are conserved and shared with higher vertebrates, further enhancing its utility for research in this field [97]. Nevertheless, akin to any model system, zebrafish are not exempt from limitations. A prominent challenge lies in obtaining sufficient blood volume from these organisms. Fortunately, some authors have endeavored to overcome this obstacle by developing innovative methodologies for obtaining larger volumes of blood [98, 99].

2.2.6 New trends

Transgenic zebrafish have emerged as an advantageous model within ecotoxicology. These genetically modified organisms originate by introducing exogenous genes, which may arise from diverse sources, including other animals or humans [100]. The integration of the transgene into the host genome can be accomplished through various methodologies, wherein the most prevalent approaches encompass the utilization of transposons or nucleases, such as Zinc Finger Nucleases (ZFNs), Transcription Activator-like Effector Nucleases (TALENs), and RNA-guided endonucleases such as CRISPR/Cas9 (Clustered Regularly Interspersed Short Palindromic Repeats Associated Protein 9) [101]. The utilization of transgenic organisms in the realm of environmental monitoring and toxicological studies presents a multitude of advantages. This methodology facilitates the swift acquisition of real-time outcomes, primarily achieved through detecting color and fluorescence signals [101, 102]. Moreover, by employing these organisms, researchers can also delve into the functions of genes that play critical roles in detoxification processes and responses to environmental stressors [102]. Specifically, this approach allows for the controlled disruption or alteration of genes in model organisms, leading to the observation of phenotypic changes that provide crucial insights into the roles of these genes in mitigating the effects of environmental toxins [102]. As outlined in the research conducted by Brito et al. in 2022 [101], existing literature describes 97 distinct transgenic zebrafish lines. Among these variants, the Tg(fli1: EGFP) strain commands the highest degree of utilization (comprising 16.02% with $n = 33$), succeeded by Tg(kdrl: EGFP; 5.34% with $n = 11$), Tg(cyp19a1b: GFP; 3.88% with $n = 8$), as well as both Tg(myl7: GFP) and Tg(hsp70: GFP; each accounting for 3.4% with $n = 7$). These transgenic zebrafish strains have demonstrated notable efficacy in the exploration of diverse toxicities encompassing vasotoxicity, neurotoxicity, systemic toxicity, hepatotoxicity, endocrine disruption, cardiotoxicity, immunotoxicity, hematotoxicity, ototoxicity, and pancreo-toxicity [103]. As an illustration, the exposition of Tg(kdrl: EGFP) embryos to malachite green (MG) and leucomalachite green (LMG) dyes spanning 14 hours generated the

aberration of intersegmental vessels (ISVs) within the upper trunk region dorsal longitudinal anastomotic vessels (DLAVs) and posterior cardinal veins (PAVs). This exposure also elicited perturbations in the spatial differentiation of the dorsal aorta (DA) and posterior cardinal vein (PCV) [104]. In addition, MeHg affected dopaminergic neurons and mitochondrial activity of dopaminergic neurons in the caudal hypothalamus region in exposed Tg(slc6a3: EGFP) and Tg(slc6a3: MITO-mCherry-NPT) zebrafish larvae of 120 hpf [105]. Hence, the amended data substantiate the significance of the Tg(kdrl: EGFP), Tg(slc6a3: EGFP), and Tg(slc6a3: MITO-mCherry-NPT) strains in the scrutiny of diverse biomarkers, facilitating the elucidation of mechanisms underlying vasotoxicity and neurotoxicity induced by various chemical agents or pollutants.

3. Conclusions

Zebrafish exhibit a sophisticated sex-determination system influenced by their environment, making them a highly relevant model for investigating reproductive and embryonic toxicity from exposure to endocrine-disrupting chemicals (EDCs). In addition, their well-established behavioral assays enable efficient screening of neurotoxic compounds and examination of neuronal signaling and behavior. Zebrafish also possess orthologous genes and proficient DNA repair pathways, which gives them a significant advantage in genotoxicity testing compared with other organisms. Consequently, their heightened suitability for detecting and evaluating DNA damage induced by diverse pollutants is noteworthy. In conjunction with the above-established techniques, emerging trends in environmental toxicology, such as genome editing methods (TALENs, ZFNs, and CRISPR-Cas9), introduce exciting new possibilities. These cutting-edge techniques facilitate studying detoxification processes and organismal responses to environmental stressors. One particularly promising avenue is the use of genetically engineered zebrafish as living biosensors. This approach holds the potential for real-time detection and reporting of environmental pollutants, revolutionizing our ability to monitor and respond to ecological hazards effectively.

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The *Danio rerio* (zebrafish) is an alternative vertebrate animal model for in vitro and in vivo research methodologies. In the last decades, notably due to increased laboratory investigation, novel discoveries, and complete genome sequencing, the zebrafish has attracted great interest from the scientific community. Its exceptional genetic homology to humans and the large number of mutations and diseases that can be reproduced in the make zebrafish an effective research model. This book examines the advantages of using zebrafish in scientific applications

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