DOI: 10.1590/1809-6891v24e-74134E

Ciência Animal Brasileira
Brazilian Animal Science

e-ISSN 1809-6891

Section: Veterinary medicine
Review

Good practices in the rearing and maintenance of zebrafish (*Danio rerio*) in Brazilian laboratories

Boas práticas na criação e manutenção de zebrafish (*Danio rerio*) em laboratório no Brasil

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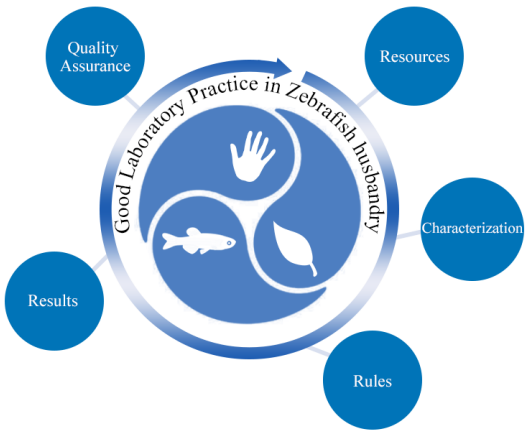
Abstract
Good Laboratory Practice (GLP) is a management quality control system that encompasses the organizational process and conditions under which non-clinical health and environmental studies are carried out. According to the World Health Organization, GLP must contain five topics: resources, characterization, rules, results, and quality control. The aim of this work is to address a review according to WHO standard to the implementation of Good Laboratory Practices in *zebrafish* (*Danio rerio*) facility. Considering that the promotion of one health (animal, human, and environmental) associated with an education plan, protocols, and records are fundamental to guarantee the safety and integrity of employees, animals and the environment as well as reliability in the results generated. In a way, Brazil still needs improvements related to the wellfare of aquatic organisms (national laws, international agreements, corporate programs, and others); especially in relation to its use in research and technological development. In this way, the implementation of GLPs provide valuable guidance for improving animal welfare and worker safety, facilitating the standardization of research.
Keywords: *Danio rerio*; standardization; welfare; regulatory guidelines; legislation.

Resumo
As Boas Práticas de Laboratório (BPL) são um sistema de controle de qualidade gerencial que abrange o processo organizacional e as condições sob as quais os estudos não clínicos de saúde e meio ambiente são desenvolvidos. Conforme a Organização Mundial da Saúde (OMS) as BPL devem conter cinco tópicos: recursos, caracterização, regras, resultados e controle de qualidade. O objetivo deste trabalho foi apresentar uma revisão conforme o padrão da OMS para a implementação das BPL em biotério de *zebrafish*. Considerando que a promoção da saúde única (animal, humana e ambiental) associada a um plano de educação, protocolos e registros são fundamentais para garantir a segurança e a integridade dos trabalhadores/pesquisadores, animais e meio ambiente assim como confiabilidade nos resultados gerados. De certa forma o Brasil ainda necessita de melhorias relacionadas ao bem-estar de organismos aquáticos (leis nacionais, acordos internacionais, programas corporativos e outros); especialmente em relação à utilização destes na pesquisa e desenvolvimento tecnológico. Desta forma, a implementação de BPL fornece uma orientação valiosa para a melhoria do bem-estar animal, e segurança do trabalhador vindo a facilitar a padronização da pesquisa.
Palavras-chave: *Danio rerio*; padronização; bem-estar; diretrizes reguladoras; legislação.



Graphical abstract: Good practices in the rearing and maintenance of zebrafish (*Danio rerio*) in Brazilian laboratories

Received: September 23, 2022. Accepted: November 8, 2022. Published: January 9, 2023.

1. Introduction

The use of fish as models for biological studies was disseminated during the 19th century. One of the precursor species was the goldfish, *Carassius auratus*, on which toxicology and physiology studies were carried out [1-4]. In addition to *C. auratus*, other species have stood out as models for scientific studies, such as *Oryzias latipes*, *Rutilus rutilus*, *Gasterosteus aculeatus*, *Takifugu rubripes*, *Xiphophorus hellerii,* and *Danio rerio*. The latter is the most studied [5].

Although *D. rerio* (zebrafish) was proposed as a species for use in science for the first time in 1934 [6], the model began to become popular after the publication of the work by George Streisinger's team in 1981, where they described methods for generating mutations through gymnogenesis [7]. However, the significant consolidation of zebrafish as a "world biomedical model" occurred from 1996 onwards, when a volume was published in the Journal of Development (December 1996, Vol. 123) containing 37 articles on the genetic screening of more than 4,000 mutations. In Brazil, the first work published with zebrafish as a model was in 1999 [8]. Furthermore, Brazil's first transgenic fish was also a zebrafish strain [9]. Currently, in the country, several research institutions use this species as a biomodel, with the following main areas of study: (1) Neuroscience and Behavior, (2) Pharmacology and Toxicology, and (3) Environment and Ecology [10].

The use of vertebrates in scientific research in Brazil is governed by the National Council for the Control of Animal Experimentation (CONCEA), created by Law nº 11,794/2008 and regulated by Decree nº 6,899/2009 [11-12]. CONCEA regularly edits Normative Resolutions (*Resoluções Normativas*, RNs, in portuguese), which have the force of law in Brazil that deal with guidelines and procedures for the scientific use of animals in educational institutions or scientific research. CONCEA RN nº 34/2017 [13], for example, presents regulations directly related to the creation and maintenance of zebrafish in teaching activities or scientific research. In addition, several reviews and books have addressed methods for rearing zebrafish in the laboratory [14-16]. Recently Canedo et al. [17] presented a paper describing the principle of the 10 Rs and the importance of its implementation in zebrafish research. However, the single health approach (animal, human, and environmental), together with a standardization of creation, management, and experimental procedures in zebrafish in Brazil, considering ethical and legal principles, is still limited.

Good Laboratory Practice (GLP) has been regulated by various institutions and agencies around the world, such as the United States Food and Drug Administration (USFDA), the United States Environmental Protection Agency (USEPA), and the Organization for Economic Cooperation and Development (OECD), for example. This regulation promoted a monitoring system for studies carried out with animals to guarantee the safety of the products developed. However, although GLPs are widely used in studies involving terrestrial mammals, they have been comparatively underutilized in research with aquatic organisms [18]. For example, in Brazil, the Integrity and Good Practices guidelines for the Production, Maintenance, or Use of Animals in Teaching or Scientific Research Activities are described in RN nº 32/2016 of the CONCEA [19]. However, the document only presents the values and principles of conducting scientific research without a more detailed explanation of the particular recommendations for each group of organisms used.

This work aims to present a standard in accordance with the World Health Organization (WHO) for implementing Good Laboratory Practices in zebrafish facilities.

2. Good Laboratory Practices (GLP)

The activities carried out in the laboratory require a series of precautions from the professional, justified by the risk to the health of the worker, as well as to animal welfare and the environment. The prevention of these risks requires the application of modern technological advances in designing facilities and in work routines. Among these advances, we highlight the animal health standard, genetic characterization, and less invasive techniques. Unfortunately, few establishments in the country have human resources with appropriate training and basic research infrastructure that includes laboratory animal breeding centers equivalent to those in the United States and Europe [20].

GLP is a management quality control system that encompasses the organizational process and conditions under which non-clinical health and environmental studies are planned, carried out, monitored, recorded, reported, and retained (or archived) [21]. Despite considerable advances in quality control, errors arising from laboratory investigations continue to occur in large numbers annually. In this way, a strategy adopted is the continuous search for quality improvement through regulatory guidelines such as GLP, which can serve as a key to reducing errors.

Tropical diseases are a significant public health problem in developing countries. For many of these diseases, there are no new, effective and affordable medicines, while older therapies are beginning to lose ground due to the emergence of resistance against traditional medication. Multinational pharmaceutical companies have traditionally not invested in new formulations in their development programs, which is why the WHO has created research and development programs in several priority areas, such as malaria [21]. In this way, the WHO published documents on good manufacturing practices (GMP) and good clinical practices (GCP). However, these documents do not define any quality standard that governs the non-clinical phases of drug development.

In animal experimentation, GLP encompasses adherence to ethical aspects of methods used in experiments

with laboratory animals, including experimental design, adherence to guidelines issued by animal ethics bodies, dosage, number of animals used in each study group, analysis of statistically significant data obtained, data measurement and quality assurance. For didactic purposes, the GLP was divided into five major topics as recommended by the WHO [22] described below.

3. Resources

3.1. Personnel Management

In general, all regulations referring to personnel management describe the team and the requirements that are integral to all GLP studies, including the provision of test facility management, a study director, a quality assurance unit and access to professional assistance [21,22]. In this context, the requirements for developing studies with zebrafish are the same for experiments with mammals, for example, rodents. That is, they must follow the prerogative that all personnel involved in the care and use of animals must have qualifications and training based on the principles of laboratory animal care to subsidize animal welfare and, consequently, the quality of research [17-23].

Furthermore, the need for veterinary care is an essential part of using animals for experimentation. A veterinarian's primary focus is overseeing animal welfare and clinical care used in research, testing, teaching, and production. This responsibility extends to monitoring and promoting animal welfare during animal use and all animal life stages [24]. According to Kuzel et al. [25], the guidance a veterinarian gives to technicians and users reduces the risks to which animals and professionals are exposed during experimentation.

3.2. Facilities and equipment

The installations of a fish vivarium must present critical care with the layout to maximize the use of the available space, facilitate access and traffic, guarantee the safety of the workers, maintain the environmental conditions and be suitable for future expansions. These general principles are independent of the size of the facility and must be consistent with the research objective and available space. In this sense, the facility for keeping zebrafish can vary from a room with a few aquariums to a center composed of several rooms containing rack systems with dozens of tanks.

Breeding and keeping zebrafish is more demanding than keeping invertebrates and less than keeping mammalian stocks [14]. For efficient operation, failure prevention is one of the primary concerns. The greater the control over external influences such as water supply, air supply, food, and new fish, the better the stability and security. This must be balanced against considerations of space, cost of equipment, labor, cost of maintenance, technical skills of staff, the usefulness of research, as well as efforts invested in a specific strain of fish [26]. In this context, the most significant demand occurs to keep the water in good condition. Thus, we can have two types of systems: (1) without filtration and (2) with water filtration [14]. Filters have no installation and maintenance costs in the former, making them relatively inexpensive to set up. On the other hand, they are very demanding in terms of space because the fish must be kept at low densities. In addition, they require high maintenance and, in practice, only work in areas where clean water can be produced or collected at a low cost. The system with filters, on the other hand, has filtering elements and means for the aggregation of bacteria that degrade toxic compounds in the water, allowing the reuse of the breeding medium through the recirculation of the water, the continuous passage of the water through the various filters and the constant availability of treated water for the animals.

One of the main advantages of recirculation systems is that they provide high-quality water without needing a water change. Furthermore, this system allows the rearing and maintenance of a larger number of fish in a comparatively smaller space. However, regardless of the type of system adopted, the type of tank must allow for the animals' normal physiological and behavioral needs, including excretory function, body temperature control and maintenance, typical movement and postural adjustments, and, where indicated, reproduction [24]. Zebrafish breeding systems have evolved considerably in recent decades due to the popularization and diversification of research using it as a model. Given these increasing levels of complexity, both in housing systems and zebrafish's experimental uses, choosing, designing, and planning a new system or upgrading an existing one is vital to successful research. According to Lawrence and Mason [27], above all, selected fish housing systems must function to (1) provide a stable, supportive environment that produces and maintains healthy, productive fish and (2) support the specific objectives of the research team.

4. Characterization

Considering that water is the “environment” in which the fish are and that it is the means of support for their life, maintaining adequate conditions is essential for the quality of the research and the well-being of the fish [28]. For this reason, the main parameters to be regularly evaluated and monitored in an animal house intended to house zebrafish will be presented below.

4.1. Abiotic Parameters

4.1.1. Temperature

In fish, temperature affects virtually every aspect of behavior and physiology [29]. The zebrafish is classified as a eurythermic fish that supports a wide temperature gradient. In the natural environment, it inhabits places with temperatures from ~6 °C in winter to ~38 °C in summer [30].

In the laboratory, room or water temperature is usually maintained between 26-28.5 °C [41]. However, the optimum temperature recommended for reproduction and embryonic development in the laboratory is 28.5 °C [15, 31]. Studies with zebrafish embryos have shown that oxygen consumption, heart rate, and toxicity of compounds are modified by temperature [32,33]. Furthermore, the temperature has a strong influence on sexual differentiation during embryonic development [29]. Scott and Johnston [34] demonstrated that the embryo's incubation temperature could have dramatic and persistent effects on the capacity for thermal acclimatization at various levels of biological organization, from the molecular to the morphological level. According to Zhang et al. [35], larvae incubated at lower temperatures (24 °C) during initial development have a worse survival rate and innate immunity.

4.1.2. Photoperiod

Although zebrafish has been described as a diurnal species [36], later studies have revealed that the species is capable of exhibiting diurnal or nocturnal behavioral rhythms depending on rearing conditions (such as feeding and temperature cycles) [37,38]. However, the photoperiod has a strong influence on the reproductive behavior of zebrafish [40]. In the laboratory, zebrafish usually spawn in the first few hours of daylight. According to Francis [39], one of the quickest ways to ensure that fish do not lay eggs is to keep the lights on full-time. Thus, the ideal circadian light cycle in the laboratory for zebrafish is generally defined as 14 hours of light and 10 hours of complete darkness [15]. This light-dark cycle mimics the natural environment and is ideal for maintaining the zebrafish's circadian clock.

4.1.3. Water quality

The water quality parameters directly affect the organisms and keeping them within the appropriate levels for the crop species is necessary. The maintenance of animals in inadequate water conditions causes the organisms to change their physiological state, which may lead to the emergence of diseases, epizootic outbreaks, low growth, reproductive failures, and mortality [28].

Regardless of the type of rearing system adopted, the water quality parameters must follow strict control as described below.

4.1.4. pH

The pH of water in aquatic systems profoundly affects the physiological processes of fish and the functioning of the microbial community that supports them. Furthermore, the pH of the water strongly influences the toxicity of some compounds, such as ammonia, nitrite, metals, and drugs [42-44]. Most laboratories maintain a pH between 7.0 and 8.0 [40]. However, Brand et al. [18] suggest a more restricted level between 6.8 and 7.5 (never below 6.0 or greater than 8.0).

4.1.5. Alkalinity

Alkalinity represents the measure of all titratable bases present in water. It describes water's ability to neutralize strong acids, which helps maintain pH stability. It is recommended to keep alkalinity values within a range of 50-150 mg of CaCO3/L [41].

4.1.6. Hardness

Water hardness is a measure of the amount of divalent ions, primarily calcium and magnesium, and to a lesser extent, iron and selenium [45]. Fish require these ions for physiological functions, and they must be provided in the water and diet of captive fish. Zebrafish are considered “hard water” fish, preferring water with concentrations above 100 mg CaCO3/L. According to Lawrence [40], it is recommended to keep hardness values within a range of 75-200 mg of CaCO3/L, the same range generally recommended for a variety of freshwater fish.

4.1.7. Nitrogenous compounds

Total ammoniacal nitrogen consists of two compounds, ionized ammonia (NH_4^+), called the ammonium ion, and non-ionized (NH_3), widely known as ammonia. The ratio relative to the proportion of each form of ammonia depends on the pH, temperature, and salinity [46]. The main metabolic product excreted by fish is ammonia, which is eliminated through the gill epithelium by diffusion and, to a lesser extent, in the feces. Ammonia production also occurs during the decomposition of organic matter (mainly feces, uneaten food, and dead fish) by bacteria [27]. Non-ionized ammonia is highly toxic to fish and must be removed from the system. Ammonia levels above 0.02 ppm should be avoided [41]. In recirculating systems, these levels are reached through the oxidation of ammonia to nitrite and then to nitrate due to the action of nitrifying bacteria in a process known as nitrification. The intermediate product of this conversion, nitrite, is also toxic to fish and can be problematic at concentrations greater than 0.1 ppm [41]. Nitrate toxicity occurs only at very high concentrations. Thus, partial water changes are recommended as a maintenance routine to keep the nitrate level below 50 mg/L [41].

4.1.8. Oxygen

Low dissolved oxygen levels are more responsible for fish mortality in culture than any other parameter [46]. The solubility of oxygen varies with atmospheric pressure, salts, and temperature. Elevated temperatures reduce the solubility of oxygen in the water. In zebrafish farming, the relatively high temperature of maintenance, the density of animals in the aquariums, and the high feeding frequency typical of high-activity facilities create the need for dissolved oxygen levels close to saturation (~7,8 mg/L in 28 ºC) [40].

4.1.9. Salinity and conductivity

Salinity is measured in parts per thousand or the

capacity of water to conduct electricity (conductivity) expressed in microSiemens per centimeter (µS/cm). Salinity levels should be stable and maintained at < 5 g/L [27]. Most zebrafish systems use dechlorinated city water; however, some systems use deionized water. Conductivity should be 150-1,700 µS/cm [47], although a narrower range of 300-1,500 µS/cm is recommended, as recommended by Avdesh et al. [41].

4.1.10. Source of water

Special care must be taken with the sources of water supply to zebrafish breeding and maintenance vivariums. Although filtration systems are an option for treating collected water, these systems do not remove many contaminants. In addition, there are many reports of impacts on zebrafish behavior, physiology, reproduction, and development for drug residues [48,49] and agrochemicals [50].

4.2. Handling and containment

Almost all laboratory-reared fish must be physically handled at some point, and it must be considered a stressful event for the fish. Activities such as exposure to air, transferring tanks, social isolation, reproductive management, anesthesia, blood, and semen collection, among others, are episodes that cause stress. Each handling activity is characterized by intensity and frequency, so its influence on experimental results must be considered when planning an experiment. Even in episodes of acute stress, fish can take a few hours to return to their initial state. Indeed, many of the routine activities of a fish house, such as chasing and netting, transferring aquariums, and exposure to air, are stressful for zebrafish and are even used in standard stress protocols [51-55].

Zebrafish subjected to different chronic stressors show physiological and behavioral changes [54]. Furthermore, Kirsten et al. [55] demonstrated that chronic stress activates some genes related to the pro-inflammatory response in zebrafish. This way, exposure to chronic stress can be minimized through personnel training, standardization in the management routine, and acclimatization. According to the recommendation of the Canadian Council on Animal Care (CCAC) [28], "fish should be handled only when necessary, and the number of episodes involving handling should be minimized."

4.3. Population density

Fish stocking density is influenced by factors such as water quality, food, feeding rate, size, and age of the fish. The optimization of these factors varies from one vivarium to another. Adults can be maintained at a density of 5-8 fish/L. Juveniles (< 45 days) can be housed at a higher density. Table 1 presents density recommendations according to some studies. However, at the moment, there is not enough information about the proper density for young developing fish. If the growth or health of the fish is not as expected, the density must be modified.

Table 1. Stoking density of *zebrafish* (*Danio rerio*). Adapted from Canadian Council on Animal Care [28]

Stoking density	Reference
Adults	
In large-scale recirculating systems, families of sibling adult fish are kept in serial tanks at densities of 5 fish/L	14
Maintenance: 5 fish/L Reproduction, a couple overnight in 1.5 L, or 6 fish in 2.3 L	15
Recirculation system with biofilter, with regular water exchange, good diet and water quality: 5 fish/L For reproduction purposes, it is better to have less fish per tank: 2-3 fish/L Static tank: 1-2 fish/L	56
25 fish in 45 L tank	57
Reproduction in static tank: 2-8 fish in 2 L tank	58
6-7 fish/L	41
5 fish/L in 10 L tank	59
4-10 fish/L	47
Juveniles	
Growing: 5 fish/L	15
Larvae	
250 larvae 5-10 dpf/L	47
20 larvae in 400 ml until the juvenile stage	15
Eggs and embryos	
20 eggs/embryos in 100 ml of water	15
100 embryos in 35 ml using Petri dish of 9 cm diameter	47

4.4. Feeding

Of all aspects of zebrafish husbandry and management, nutrition has shown minor development despite the great popularization of zebrafish as a biomedical model. “Standard” nutrition protocols for zebrafish, cited in various publications, generally describe the administration of a diet consisting of *Artemia* spp. nauplii and processed feed, usually flocculated, developed for tropical fish [27,57]. However, due to the lack of specific information about the nutritional requirements of zebrafish, it may be unwise to feed an artificial diet exclusively. In nature, zebrafish feed on small crustaceans and insect larvae. In this way, the supply of live food allows the fish to express the species' natural behavior and meets the need for active search and capture of food, fitting as a measure of increment to environmental enrichment. The laboratory's most commonly used live foods are paramecium, rotifers, and nauplii of *Artemia* spp.

The amount of food offered at each feeding and the frequency are essential components of feeding protocols and are often specific according to the objective to be achieved, such as, for example, growth, reproduction, or maintenance. In terms of feeding rate, there are two general approaches used in zebrafish care: (1) feeding to satiation (*ad libitum*) and (2) feeding proportionate to body weight. In zebrafish care guides, the recommended feeding rate ranges from 3-8% of body/feed weight/day [59]. According to Lawrence [26], feeding frequency should be continuous from 5 to 14 dpf (days post-fertilization), moving to 3-5 times a day from 15 to 60 dpf, and finally 1-3 times a day after 60 dpf. Another critical factor in zebrafish farming is the size of the food. Feeding the larvae should start from 5 dpf. In this development period, the digestive system is already open at both ends, and the yolk is almost or entirely consumed [16]. The transition from endogenous to exogenous feeding and from live food to feed are critical periods for larviculture. It is crucial to offer invertebrates such as *Paramecium* spp., *Brachionus plicatilis*, and *Artemia* spp. nauplii during this period. Young larvae can be fed on dry foods ~100 µm in size (e.g., ZM-100, ZM systems, UK) and live foods such as paramecia and rotifers (which stimulate growth). The feed size can be slowly increased to 200 µm (e.g., ZM-200, ZM systems, UK) or 300/400 µm (e.g., ZM-300, ZM systems, UK) [40].

In addition to the nutritional aspects related to the composition of diets used for zebrafish, feeding management of fish stocks is equally or more relevant, as it is known that both the feeding frequency used and the time interval between the behavioral tests and the last feeding, can strongly interfere with zebrafish behavior [60]. Thus, it is imperative to standardize and faithfully report the protocol used to avoid biases, especially in experiments that evaluate the behavioral responses of fish to stimuli of different natures.

4.5. Reproduction

Reproduction is a complex process influenced by many behavioral and abiotic factors. Behavioral factors, such as mate choice, are determined by cohort behavior and are related to the interaction and perception of various elements, including visual, tactile/auditory (through the lateral line), and olfactory perception. Abiotic environmental factors include water quality, temperature, photoperiod, feeding, environmental enrichment, and pond size [61]. The first step in reproduction is the sexing of individuals. Under ideal conditions, zebrafish reach sexual maturity at around 3 to 4 months [62]. Adult zebrafish do not have a clearly visible sexual dimorphism. In general, dimorphism can only be observed in individuals in the already well-developed adult stage. At this stage, the following characteristics can be distinguished: males have a thinner and shorter body; anal fin with a more vibrant color (orange-yellow); pectoral-fin rays yellowish in color. Females, on the other hand, have a plumper and longer body with a lighter and more distended belly; the presence of a small genital papilla and pectoral fins with more tenuous rays; absence of vibrant coloration on the anal fin; pectoral-fin rays pale in color [63]. According to McMillan et al. [64], a practical method of sexual differentiation is the presence of tubercles on the pectoral-fin rays of males. According to these authors, these tubercles develop after sexual maturation.

According to Hutter et al. [65], in large tanks, fish usually form pairs rather than groups, demonstrating sexual selection, while in conventional aquariums with high densities, group spawning is more common. An important factor to be considered in the reproductive management of zebrafish colonies is inbreeding and genetic drift, always striving to increase genetic diversity.

For mating, fish are usually housed in the afternoon or early evening in the spawning tank and kept until the next morning. If collected embryos need to be precisely synchronized at the same developmental stage, a pair can be separated by a plastic divider in the tank. This divider is removed in the first hour of light for spawning and fertilization of the eggs. Group or pair utilization for mating should consider tank design, such as size and shape. Another important consideration is the fish density used as it influences water quality. Zebrafish maintenance and breeding recommendations are summarized below (Box 1).

Box 1. Main recommendations for breeding *zebrafish* (*Danio rerio*)

About environment
Water parameters must be in accordance with fish tolerance
Photoperiod should be adjusted once fish breed in first hour of light
Environmental disturbance, such as the traffic of people and noise, must be minimized in the room during reproduction period
Breeders must receive a balanced diet
During the period that fish are in breeding tank they must not be fed
About biology/behavior
Fish are separated the day before breeding
Healthy fish should be chosen, preferentially those with secondary sexual characteristics: ex. brightly colored males, females with distended abdomens
Sexual maturity occurs from 3 months
Males reach sexual apices at 10 months
Ideal spawning frequency is once a week
Spawning interval for females must be at least one week
Group mating (> 1 couple) has higher fertilized eggs and greater genetic variability
Tanks > 10 L: density of 2-3 fish/L
Small group: 2-8 fish in 2 L tank
Couple in 1 L

4.6. Hygiene and prophylaxis

A clean environment is essential to maintain a high animal health and welfare standard. To achieve this goal, special care must be taken to avoid cross-infection during husbandry and routine procedures. Many diseases can be transmitted through physical contact between individual fish, tanks, and water systems. Therefore, any fomite or equipment in physical contact with the fish (such as nets, mating boxes, heaters, etc.) should be used in only one system and cleaned/disinfected periodically [46].

Waste accumulation can encourage the growth of algae, cyanobacteria, and other organisms that can be pathogenic to fish. Although some designs are more efficient than others at removing waste, in any case, some solid waste will build up and require tank cleaning or replacement [26]. Cleaning aquaria and filters are one of the essential features to keep fish healthy and in breeding condition. In addition, to prevent the spread of disease, all containers, and tools that fish may come into contact with should be kept clean [14]. Any recommendations for cleaning practices will be influenced not only by the type of tank or system but also by the feeding regime and quality of the water entering the system.

Specifically, regarding the hygiene of utensils, all fomites used in zebrafish rearing must remain free of detergents, as these products are complex, contain many different compounds, and are highly toxic to zebrafish [66,67]. Instead of detergents, chlorine solutions can be used for washing items made of plastic and glass [66]. It is important to note that sodium hypochlorite is the most widely used chlorine-based compound for disinfection worldwide. Still, this compound can release chlorine gas from exposure to some degree to organic matter, ultraviolet (UV) light, or contact with some metallic surfaces. Chlorine gas is highly irritating to the eyes and the respiratory tract, becoming a risk for those who frequent the facility [68]. In addition, sodium hypochlorite is known to be corrosive to metals [69], which can be harmful to certain utensils and equipment sanitized through prolonged use of this compound. Recently, peracetic acid has been considered an efficient and safer substitute for sodium hypochlorite, even applied to aquaculture, the dose being dependent on the alkalinity and hardness of the water used [70]. Also, disinfection with 70% ethanol, autoclaving, and UV light irradiation remain good options for use in materials that support these processes.

Several methods of cleaning and disinfecting fomites have been proposed for zebrafish-keeping facilities. Garcia and Sanders [71] offer two protocols for cleaning and disinfecting fomites used in zebrafish rearing. The first is directed towards materials that do not absorb, do not deteriorate with chlorine, and are easy to dry, such as tanks and their lids, apparatus used for feeding animals, and other diverse materials. This protocol establishes an initial wash with reverse osmosis water under pressure, immersion for 30 minutes in a 1.98% chlorine disinfectant solution (Clorox® 5.25% diluted in reverse osmosis water), a new rinse with reverse osmosis water, and natural drying. The other protocol is directed towards nets routinely used for handling fish, which are difficult to dry (due to high demand and turnover) and absorb chlorine, which is toxic to fish even in low amounts. In addition, these utensils are made of thin nylon filaments, which deteriorate when in contact with chlorine. This protocol establishes an initial wash with reverse osmosis water under pressure, immersion for 1 hour in a commercial disinfectant solution for aquariums (Net Soak, Jungle Labs, USA, 4.93 mL/3.8 L of reverse osmosis water, active ingredients: benzalkonium chloride and methylene blue) and a new rinse with reverse osmosis water. The bath of disinfectant solution for nets is kept in opaque buckets and covered when not in use to avoid light and evaporation. The disinfectant bath is replaced once a week.

Chang et al. [72] tested the effectiveness of chlorine solutions (Clorox®); hydrogen peroxide, and povidone-iodine (PVPI) against *Mycobacterium* spp., a group of bacteria very prevalent in zebrafish colonies (see below). Of the treatments tested, disinfection with 25 ppm PVPI for 5 minutes was the most effective (< 1% survival) against *M. abscessus*. Disinfection with a chlorine solution at 100 ppm for 10 minutes was the least effective. Against *M. chelonae*, the most effective disinfection (< 1% survival) used chlorine solution at 150 ppm and PVPI at 100 ppm, both for 5 minutes. Still, for *M. chelonae*, the least effective protocol used PVPI at 12.5 ppm for 5 minutes. Finally, the authors evaluated the effectiveness of these three disinfectant agents for *M. gordonae*, the most effective disinfectant being chlorine at 150 ppm for 10 minutes and PVPI at 25 ppm for 5 minutes. The least effective protocol used 1.5% hydrogen peroxide for 5 minutes. Another prevalent infectious agent, the microsporidian *Pseudoloma neurophilia*, has > 95% of its spores inactivated after treatment with chlorine at 100 ppm for 10 minutes. When the pH of the solution is adjusted to 7 with glacial acetic acid, the effectiveness of the disinfection increases, and > 99% of the spores are inactivated [73].

Based on the above, it can be seen that several active principles can be used to prevent the proliferation of undesirable microorganisms in a zebrafish facility. However, the effectiveness of the method used will depend on factors such as the parameters of the solution, such as the concentration used and the pH, the microorganism for which the treatment is intended, the time of exposure of the fomites to the disinfectant agent, the material composition of the fomite and adequate storage and replacement of the disinfectant solution. Furthermore, when more than one microorganism is the specific treatment target, an association of protocols can be adopted. For example, as already demonstrated, the disinfection of utensils with 100 ppm of chlorine for 10 minutes is highly effective against *P. neurophilia*. Still, it leaves much to be desired when dealing with *M. abscessus*. In this sense, each laboratory must develop its cleaning and maintenance protocol containing the standard operating procedures for these routines.

4.7. Origin of animals

Every supplier of zebrafish in the Brazilian territory, whether for scientific purposes or just for aquarium hobby, must have a veterinarian as the technician responsible for the animals [66]. Furthermore, the supplier must be consulted about the practice of bleaching before sending eggs of a particular strain [76]. Originally bleaching was proposed as a method of disinfecting eggs using chlorine as the active ingredient. Westerfield et al. [57] recommend a bleaching solution containing approximately 0.003% chlorine. In these authors' protocol, four containers are prepared, two with the bleaching solution and two with water from the facility housing system. The embryos are collected and placed in a sieve, which will pass through the sequence of baths to submerge and emerge all the embryos quickly and simultaneously. Embryos are submerged in the first bath, which contains the bleaching solution, for 5 minutes. Soon after, the embryos are placed in a bath with water from the system. The process is repeated once more to use all four prepared containers. The protocol proposed by Brand et al. [18] presents minor variations in the described procedure. This protocol uses a bleaching solution containing between 0.0038 and 0.0049% sodium hypochlorite. Instead of 4 baths, this protocol uses five baths in the following sequence: bleaching bath; tap water bath; new bleaching bath; new tap water bath, and, finally, another tap water bath. Each bath is applied for 5 minutes. After the baths, the embryos must be washed with an E3 medium (which will be used as the embryos' incubation medium). Embryos must undergo the bleaching procedure aged between 10 and 28 hpf. After 28 hpf, the chorion begins to be degraded by enzymes that prepare the embryo for hatching, so this protective membrane is already permeable to chlorine. The penetration of chlorine by the chorion kills the embryo. It is important to note that when performed between 10 and 28 hpf, the bleaching process hinders hatching, making the chorion more rigid. Therefore, 30 mg/ml of pronase should facilitate hatching. After the bleaching process, the embryos can be incubated at 28.5 °C. If the embryos have not hatched the next day after the bleaching process, hatching must be performed manually by removing the chorion using tweezers.

An alternative to using chlorine-containing solutions is bleaching with PVPI as the active ingredient [74]. This protocol prepares four baths in the following sequence: a bath of buffered PVPI at a concentration of 12.5 or 25 ppm and three baths of sterile Milli-Q water. As previously explained, the embryos are transferred through the sieve baths and remain in the PVPI bath for 2 minutes. The passages in water are quick baths of about 5 seconds. Before and after the described baths, the embryos are maintained in an embryo culture medium, and this protocol does not require using pronase. Bleaching is an important sanitary barrier in zebrafish breeding. It can be used as a requirement for sending eggs from external suppliers and for carrying out external disinfection of any spawning obtained in an aquatic facility. In addition to the technical responsibility and the practice of bleaching, animals purchased from external suppliers must have a health certificate about their state or health conditions [22]. In Brazil, the issuance of this type of certificate is a private activity of the veterinary medical professional [77].

Given this, healthy colony also depends on reputable suppliers committed to the health and well-being of their animals. Knowledge about the fish's origin

and life is highly relevant when they arrive at the laboratory. Some factors during rearing can influence the fish for life and justify the search for reputable suppliers with a well-controlled prior history of the fish. Keeping zebrafish in facilities where the fish can see possible predators substantially impacts their behavior and reactivity for life [78,79]. In the same way, the type of installations, the practice of keeping the fish (mixed or separate sexes), and possible environmental enrichments can also impact the animals' behavior and reactivity to stimuli [80-82]. Also, even if the fish are raised in high-quality, contaminant-free water, if their parents (parental generation) have suffered any contamination with drug or pesticide residues, the effects may be passed on to the next generation [48,83].

4.8. Transport

According to Aleström et al. [47], exchanging fish between laboratories presents three main challenges: (1) organizing the safe shipment of fish, (2) ensuring compliance with fish welfare as well as national and international legislation, and finally, (3) prevent the spread of pathogens between laboratories.

During transport, the challenge is maintaining temperature and water quality parameters within the zebrafish requirements, particularly regarding dissolved oxygen, carbon dioxide, and nitrogen. Shipping times should be as short as possible, and it is generally easier to send and receive embryos than adult fish. Embryos prepared for shipment must be disinfected by bleaching prior to shipment. Transporting adult fish requires a relatively low fish density (two adult fish/0.5 L) and a 1:3 or greater volume ratio of air or oxygen to water in each container [84]. Juvenile and adult fish should be fasted for 24 hours before packaging to reduce excretion and prevent the degradation of container water [47]. For transport times longer than one day, adding ammonia binders to the transport water is recommended to reduce the fish's health risks. Upon arrival at the destination, animals must be inspected for number, sex, general health, and stress.[76].

4.9. Quarantine

After safely receiving embryos or fish, the next challenge is to prevent the spread of pathogens in the laboratory herd. Disinfection of eggs by bleaching, although highly recommended for the safe insertion of new strains in a facility, is inefficient for eliminating intracellular pathogens, such as *P. neurophilia*, for example [47]. Newly arrived fish may bring pathogenic organisms, even if the hosts initially show subclinical conditions. In this case, the stress of handling and crowding during transport can cause a depression in the fish's immunity, leading to a disease outbreak. A combined approach to acclimation and quarantine should be used, as far as possible, so that both are carried out simultaneously. New stocks should undergo general health screening, and the health assessment protocols used may be influenced by research objectives [33]. Keeping newly imported animals in quarantine is strongly recommended to limit the risk of spreading pathogens in the main facility [47], and according to Matthews et al. [15], once introduced into their new tank, quarantined fish should remain there for three to four weeks. For laboratories with only a few tanks where lower biosafety levels are employed, the animals are kept under observation in quarantine for some time, awaiting the finding of clinical signs or mortality. In this case, it is appropriate to dedicate a tank with its own water circulation as a quarantine unit to reduce the risk of spreading pathogens during the introduction of new individuals. The quarantine unit should be kept as separate from other units as possible, preferably in a different room from the main colony. All equipment used must be for the exclusive use of the quarantine unit. It is recommended that they are clearly identified and never come into contact with fomites used in the main facility.

Facilities that are more demanding in terms of biosecurity apply quarantines between 3 and 4 weeks [85,86] and, in addition, perform some tests by sampling on the animals received, such as molecular, histological, bacteriological, and fecal analyses [87]. However, perhaps the most important recommendation for introducing new animals to the facility is not to integrate new exogenous animals into experiments or main colony. A safer alternative is to create and reproduce these new animals in quarantine/isolation and incorporate only the offspring of these animals [76,84,88, 89] or even the offspring of the offspring (F2 generation) [84] after verifying the health status of the fish.

4.10. Animal health monitoring

Another recommendation for evaluating the health of the colony created in an aquatic facility is the practice, now traditional in rodent facilities, of creating and maintaining sentinels [90]. Sentinel animals are individuals that indicate the health status of the entire colony. In zebrafish facilities, sentinels are maintained so that the primary sources of colony health risks are presented to them in a more intensified manner so that they are evaluated frequently without the need to dispose of animals from the main colony. Sentinel zebrafish are usually exposed in isolated aquaria to the effluent of the entire housing system with partial water changes that allow for optimal welfare, but always using the effluent as the rearing medium [85,86]. Ideally, individuals should be selected as sentinels from hatching onwards. This reduces the risk that they are indicating problems external to the facility, in the case of exogenous animals and those introduced at later stages. The minimum maintenance period for these animals is three months [86,91]. However, older animals can be kept so that they also indicate

conditions that may be age-related or problems arising from chronic exposures. Sentinels have their destiny mapped out since hatching, and that destiny must not be diverted. Therefore, it is recommended that sentinels are not used for purposes other than detecting health and well-being problems in the facility environment, such as reproduction or experimentation.

On the other hand, some authors argue that, for some diseases, the best method for detecting an etiological agent causing a subclinical disease is testing by representative sampling instead of using sentinel animals or animals rescued from the sump of recirculation systems. Another promising source of material to be used for colony health surveillance is moribund or recently dead individuals [92]. Periodic sanitary monitoring does not necessarily require that clinical signs to be observed. However, the personnel must monitor the general characteristics of the animals in the colony to perceive any abnormality.

In addition to the sanitary aspect discussed above, establishing a routine for monitoring and collecting dying and dead fish is justified because odors released from dead fish can cause behavioral responses in live fish in this aquarium. Indeed, odors from dead zebrafish induce defensive behavior in living ones. These responses coincide with the destruction of epidermal cells, indicating that defensive and stress reactions can occur as an effect of substances emanating from decaying flesh and alarm substances released due to the rupture of epidermal cells. Thus, if the monitoring and collection of dead fish are not standardized, we may have behavioral changes that may bias experiments with these animals [93].

4.11. Normal biological characteristics of laboratory zebrafish

To identify signs of stress, clinical signs, and anatomical and behavioral abnormalities, the bioterist must know the animals kept in the facility. Adult zebrafish prefer to swim in schools, are curious, and explore the entire aquarium [94]. The species is highly social and exhibits "shoaling" behavior, which is the simple aggregation of several animals in the same area. The species still shows "schooling" behavior, which is synchronized swimming with coordinated movements; however, it is unclear whether this behavior is normal or due to excitement [95]. When transferred to a new aquarium, the animals soon occupy the bottom and gradually begin exploring the water column's upper levels.

Well-defined hierarchies are formed in the group and space (tank). In addition, other resources can be disputed and defended, generating aggression and injuries [95-97]. Groups of males and females prefer environments with floating plants to sterile environments, although females also show a preference for submerged plants [95]. Zebrafish have scototaxis; they prefer dark environments to very bright ones. In addition, they have thigmotaxis; they prefer to swim close to walls, such as the walls of an aquarium, for example. In nature, these behaviors are essential to avoid predators. However, when the animals are already acclimatized to the rearing environment, the shoal explores the entire water column.

In addition, zebrafish have cells called melanophores, containing granules of the pigment melanin, melanosomes. Depending on the environmental condition, zebrafish can change color. This phenomenon also aids in the escape of predators. When in a stressful environment, the melanosomes aggregate, and the color of the fish becomes lighter and duller. When in a quiet environment, the melanosomes disperse, and the animals have a brighter color [96]. Therefore, color can be a real-time indicator of stress, fear, or anxiety. Although generally, the coloring of males is brighter and more golden and that of females is less colorful and more silver, this is not a rule for all animals, and the differences between males and females are more significant during the morning. That is the color of the animals changes throughout the day. Furthermore, in blind tests, more colorful males show more intense reproductive behavior [98]. Therefore, knowledge of the color pattern can also be used to infer the reproductive health of some animals.

Many facilities worldwide opt for aquaria without environmental enrichment in favor of the hygiene of housing system. Gravel at the bottom and "plastic plants", even if artificial, can facilitate the accumulation of organic matter in the water, which makes it difficult to clean the aquaria. This preference for sterile environments interferes with the welfare of the animals, as it is well-documented that zebrafish prefer enriched environments [95,99,100]. An alternative to improve the animals' welfare without compromising the aquarium's controlled hygiene is to use printed images of gravel at the bottom of the aquarium and plants on the walls of the aquarium [100]. Another option for environmental enrichment, which has the advantage of not impacting the cleaning routines of the facilities, is auditory or musical enrichment, which has proven to be effective in making the fish calmer and less anxious, including reducing the stress caused by isolation, common in tests, behavioral tests such as the Tanque Novo test, for example [101,102].

The characteristics presented are a generalization for the species. However, it is essential to point out that there are variations and individual particularities of each specimen of a colony. Given the above, it is recommended that the bioterist knows the animals under their supervision and knows how to identify the primary anatomical and behavioral variations at the group and individual levels. This will enable the bioterist to diagnose pathological scenarios or not and decision-making towards the solution of intercurrences imposed by

the breeding routine in the facility.

4.12. Diseases and clinical signs of laboratory zebrafish

The susceptibility of zebrafish to various etiological agents is well documented (Table 2). These agents can trigger morphological, physiological, and behavioral changes, causing the normal patterns described above to be slightly or intensely destabilized. Among the main clinical signs associated with diseases in zebrafish is abnormal swimming; emaciation; protrusion of dorsal scales; distention of the coelomic cavity; skin congestion and hemorrhages; dyspnoea; fins close to the body; spinal curvature; rectal prolapse; surface swim; bottom swim; isolation; lack of appetite; weight loss/body score; external ulcers; group mortality [76,86,89]. Of the infectious diseases, the most prevalent in zebrafish facilities seem to be caused by *P. neurophilia* and *Mycobacterium* spp. [76]. For the bioterist to learn more about zebrafish diseases, we recommend the studies by Esmail et al. [103], Kent and Sanders [88], and Kent et al. [104].

Table 2. Main pathogens identified in zebrafish (*Danio rerio*) and information about affected organs, clinical signs, and potential risks to human health

Group	Disease agent	Infection/infestation	Clinical signs	Zoonotic risk	Reference
Bacteria	*Aeromonas salmonicida masoucida*	Coelomic cavity	Erratic swimming; superficial swimming; tachypnea; high mortality rate	-	76;107;175
	Aeromonas hydrophila	Coelomic cavity	Distension and hemorrhages in the coelomic cavity	+	76;92;134;151
	Aeromonas sobria	Coelomic cavity;			
	Aeromonas veronii	skin ulcers	Petechial hemorrhages in the skin; muscle and fins; distension of coelomic cavity	+	76;133;152
	Staphilococcus aureus	Multiple organs	Hemorrhages and severe injuries on the surface of the body; distension of the coelomic cavity	+	92;134
	Plesiomonas shigelloides	Multiple organs	Distension of the coelomic cavity; perianal edema; growth of the initial lesion that served as a gateway for infection; superficial swimming; tachypnea; high mortality rates	+	107
	Pseudomonas spp.	Multiple organs	Septicemia com distensão da cavidade celômica; úlceras cutâneas; dispneia; morte	+	92;153
	Pseudomonas fluorescens	Skin ulcers	Septicemia with distension of the coelomic cavity; skin ulcers; dyspnoea; death	+ (rare)	76;153;154
	Shewanella putrefaciens	Coelomic cavity; skin ulcers	Skin ulcers	+	76
	Vibrio alginolyticus	Skin	Skin ulcers	+	76
	Vibrio metschnikovii	Coelomic cavity; skin	Skin ulcers	+	76
	Vibrio parahaemolyticus	Coelomic cavity; swim bladder	Spinal curvature; internal hemorrhages with distension of the coelomic cavity	+	76;145
	Vibrio cholerae	Intestine	Pale feces; diarrhea; lethargy; weight loss; aquarium water becomes cloudy over the course of the infection as a result of mucus increase in the feces	+	109;110
	Vibrio vulnificus	Coelomic cavity	Distension of coelomic cavity with hemorrhage septicemia	+	76;176
	Edwardsiella tarda	Multiple organs	Mortality of embryos and larvae up to 8 dpf; embryos and larvae show whitish color and lethargy; high mortality rates in adults; petechial hemorrhages; discoloration of the initial injury that served as a gateway for infection; lethargy; superficial swimming; distention of the coelomic cavity; perianal edema	+	106
	Flavobacterium columnare	Skin; gills	Loss of pigmentation and scale near dorsal fin (saddleback lesion); high mortality	-	155
	Mycobacterium abscessus	Multiple organs	Subclinical signs; low mortality; illness/death when in a stressful environment with poor water quality	+	86;92;103;156
	Mycobacterium chelonae	Multiple organs	Subclinical signs; low mortality; illness/death when in a stressful environment with poor water quality	+	92;103;104;156;157;158
	Mycobacterium fortuitum	Multiple organs	Subclinical signs; low mortality; illness/death when in a stressful environment with poor water quality	+	86;92;103;156;158
	Mycobacterium peregrinum	Multiple organs	Subclinical signs; low mortality; illness/death when in a stressful environment with poor water quality	-	86;103;158
	Mycobacterium haemophilum	Multiple organs	Lethargy; severe wasting; outbreaks with high mortality; muscular and skeletal deformities	+	86;92;103;104;157
	Mycobacterium marinum	Multiple organs	Distension of the coelomic cavity; outbreaks with high mortality; muscle and skeletal deformities	+	86;92;103;104
	Mycobacterium saopaulense	Multiple organs	Signs not reported in the bibliography	+	86;156

To be continued...

...continuation

Table 2. Main pathogens identified in zebrafish (*Danio rerio*) and information about affected organs, clinical signs, and potential risks to human health

Group	Disease agent	Infection/infestation	Clinical signs	Zoonotic risk	Reference
Ciliate	*Coleps* sp.	Whole body of embryos and larvae	Embryos and larvae up to ~7 dpf are preyed; low hatching rates and high mortality rates in larviculture	-	159;160
	Ichthyophthirius multifiliis	Body surface; gills	Whitish nodules on the surface of the skin and gills; increased mucus production; dyspnoea; loss of balance; anorexia; lethargy; death	-	161;162;163
	Tetrahymena sp.	Whole body of larvae	~30 days old larvae are preyed on by the ciliate; mass mortality of larvae	-	164
	Trichodina spp.	Body surface; gills	Dermatitis and dyspnoea with gill inflammation; usually outbreaks related to increased water temperature	-	86;161
Dinoflagellate	*Piscinoodinium pillulare*	Body surface; gills	Skin with a "velvety" appearance; skin discomfort/ itching, represented by animals rubbing against objects or the walls of the aquarium; excess skin mucus; lethargy; dyspnoea; superficial swimming; skin darkening; petechial hemorrhages; outbreaks with high lethality	-	88
Oomycete	*Saprolegnia brachydanis*	Body surface; gills	Growth of thin white filamentous hyphae that accumulate on the surface of the body and on the gills forming a cotton-like mass	-	86;103
	Saprolegnia ferax	Body surface; gills	Idem *S. brachydanis*	-	86;103
Myxozoa	*Myxidium streisingeri*	Mesonephric ducts; kidney lumen	Subclinical condition	-	88;104;165
Microsporidia	*Pleistophora hyphessobryconis*	Primarily in skeletal muscle but also in kidney; spleen; intestine; and ovary	Muscle necrosis and deformation, emaciation, lethargy; predominant subclinical conditions	-	86;88;103
	Pseudoloma neurophilia	Generally nerves; spinal cord; and hindbrain, but also in skeletal muscle; esophagus; kidney; and ovary	Emaciation; lordosis; scoliosis; muscle necrosis; reduced growth; reproductive deficit; predominant subclinical conditions; wasting unrelated to anorexia; stress-related illness	-	86;92;103;104; 158;166;167
Monogenea	*Gyrodactylus* spp.	Body surface; gills	Dermatitis and dyspnoea with gill inflammation; usually outbreaks related to increased water temperature	-	161
Digenea	*Clinostomum* sp.	Muscles	Twisted swimming; lateral curvature of the spine; yellowish lumps under the skin	+	89
	Transversotrema patialense	Skin	Subclinical condition; local lesions at the parasite's adhesion points were found only in experimental infections	-	88
	Centrocestus formosanus	Gills	Respiratory abnormalities; subclinical condition	+	125;168;169;170
Nematode	*Pseudocapillaria tomentosa*	Intestine	Emaciation; lumps in the belly	-	92;104;158;171; 172
Virus	Red-spotted grouper nervous necrosis virus (RGNNV)	Central nervous system	Erratic swimming; light opaque spot with a creamy appearance on the back of the head; high lethality of adults	-	104;173
	Infectious spleen and kidney necrosis virus (ISKNV)	Spleen and kidney	Lethargy; loss of appetite; abnormal swimming; distention of the coelomic cavity; tachypnea; pale gills; petechial hemorrhages at the base of the fins; death	-	104;174

When analyzing Table 2, it is possible to notice that many of these conditions present possible clinical signs in common, even those caused by very different etiological agents (such as, for example, infections caused by *Mycobacterium marinum* and *Pseudocapillaria tomentosa*) and several have a latency of several months or present subclinically (such as *P. neurophilia*; *Mycobacterium* spp.; *Myxidium* spp. and *P. tomentosa*). Therefore, it is important that, in addition to simple observation and clinical examination, the responsible for the animals also use as many complementary methods as are available that help in the definitive diagnosis [92].

Many of these disease-causing agents in zebrafish are part of the normal microbiota of the species [103,105] and are characterized as opportunistic agents. Furthermore, several pathogens, including some zoonotic ones, have been used for experimental modeling of infections in zebrafish, for example, *Edwardsiella tarda*; *Vibrio cholerae*; *Staphylococcus aureus*; *Aeromonas salmonicida*; Spring viremia of carp virus (SVCV) and several others [106-111]. Therefore, the maintenance of good practices in the facility related to factors such as water

quality, sanitary barriers, and welfare is ratified so that outbreaks of acute diseases for the colony or handlers do not occur as a result of the immunological weakness of the animals caused by the imbalance of these factors.

In addition to these infectious diseases, several diseases can develop in zebrafish from the maintenance of creation under unfavorable environmental parameters; they are called environmental diseases. Among them are branchial lesions resulting from exposure to ammonia, nitrites, nitrates, and chlorine; nephrocalcinosis, resulting from an excess of environmental/nutritional calcium and an excess of carbon dioxide (CO_2) in the water; gas bubble disease due to supersaturation of oxygen in the water; ultimobranchial tumors, probably resulting from electrolyte imbalance in the environment; thyroid tumors caused by iodine deficiency; and finally, hepatic megalocytosis and seminomas, possibly caused by exposure to xenobiotics and carcinogens, respectively [104]. Based on this series of diseases, the importance of maintaining a healthy culture medium and controlling and correcting water quality parameters as practices favorable to the complete health of colony is highlighted.

Still, there are idiopathic diseases, that is, with an unknown cause, such as, for example, spinal deformations; egg-associated inflammation; opercular malformations; proliferative bile and pancreatic duct lesions; chordoma; peripheral nerve sheath tumors and lymphosarcoma [104]. Due to the lack of knowledge of the origin of these conditions, it is recommended to avoid sudden changes in water quality or general handling so that the animals do not get into a state of stress or low immunity that could trigger such diseases.

Several of the diseases presented in Table 2 are related to the age of the animals. Up to 24 months of age, the prevalence of pathogens and associated lesions generally increases with the age of the animals [75]. Therefore, it is not recommended to keep elderly animals in the main squad. When experimental procedures require senescent animals, they must be isolated from others to reduce the risk of transmission of age-related diseases.

Thus, the simple perception of an individual or collective fish abnormalities can indicate to the bioterist that the colony is being affected by something and can also guide decision-making toward the solution of the problem. In cases where the researcher notices any clinical sign and behavioral deviations, the most appropriate recommendation is to separate the affected fish from the others as soon as possible and then plan the best approach to the problem. In this way, the transmission of the agent to healthy animals is interrupted in cases of infectious diseases whose cycle is still at the beginning. In cases of aggression between companions in the same aquarium, the aggressor animal must be isolated from the others so that the hierarchy is broken, and this animal loses its dominance over the others in a possible return to the same aquarium. An alert substance is released into the water when a fish is injured, signaling to all fish sharing the environment that there is an imminent threat [96]. Therefore, if the attacked fish is significantly injured, it can also be isolated in a hospital tank for treatment or rest before being returned to the aquarium to reduce the stress on the animal and the aquarium companions. It is worth mentioning that zebrafish are a social species and that isolation can generate stress in the animals [46].

4.13. Microbiota Control

To avoid health problems with several of the pathogens mentioned earlier, zebrafish facilities can adopt the creation of specific pathogen-free (SPF) or even “germ-free” colonies (free of germs, totally free of microbiota). Facilities with colonies of this type undoubtedly pay attention to the general and specific state of health of their animals and, therefore, require greater investment in installations, materials, feed, sanitary barriers, personnel, procedures, and, mainly, testing and definitive diagnosis [87,92]. For example, SPF colonies regularly engage in careful periodic monitoring of health status. In addition, they choose not to use live food since the maintenance of invertebrate species with a controlled microbiota becomes a more significant challenge than zebrafish breeding itself [85].

However, as much as the complete or partial elimination of associated microorganisms seems to be an advantage for raising any animal, adverse effects also exist. The health status of animals is cited as a variable capable of affecting the immune system and the response to experimental treatment in laboratory animals [112,113]. Just as the presence of pathogenic organisms can affect animals' general physiology and development, so too may their total absence [114]. Germ-free zebrafish have no natural activation of essential pathways for the maturation of the immune system and homeostasis of different tissues [114]; lower immune resistance to infectious agents [115]; reduced proliferation of intestinal epithelial cells [116]; lower lipid absorption capacity [117] and have drastically altered locomotor behavior [118,119] for example. For this reason, some authors state that models with a more natural microbiota should be advocated in translational studies. SPF models should be directed to mechanistic studies on the relationship between microbiota and different pathogenesis [120]. In this way, researchers and institutions must adapt the form of their creations and their sanitary barriers and monitoring programs to the objective of the studies developed. Likewise, it is essential that each study be planned to bear in mind the capacity, structure, and sanitary standard of the facility that will subsidize the research. For this, joint and collaborative planning between researchers and personnel is essential.

4.14. One health advice related to zebrafish facilities

Recommendations on zebrafish health should not be limited to animal health but to the one health. The concept of one health was created in 2004 and is directly derived from the concept of one medicine, which advocates the union of human medicine and veterinary medicine in response to zoonoses [121,122]. In 2008, the WHO, the World Organization for Animal Health (OIE), and the Food and Agriculture Organization of the United Nations (FAO) created the "One World, One Health" initiative, in which the term "One Health" expresses the inseparability of human health, animal health, and ecosystem health (or environmental health) [123,124]. Parasitic diseases of zebrafish have relevance to one health [89,125]. From this point of view, the health of a zebrafish colony can impact the health of researchers and bioterists, as well as the people they live with and the environment; therefore, some recommendations are essential.

4.14.1. Human health

Several of the infectious agents already identified naturally or experimentally in zebrafish have the potential to be transmissible both from humans to zebrafish and from zebrafish to humans (that is, they are both zooanthroponotic and anthropozoonotic, respectively) such as, for example, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia cepacia* and fungi of the genera *Aspergillus, Candida*, and *Cryptococcus* [126,127]. It is not by chance that zebrafish are being used as study models on human infectology [128]. Therefore, the bioterist must bear in mind that the animals in the colony may be naturally or experimentally harboring a human pathogen. The aquaria must be duly identified in the case of experimental infections by these pathogens. Only trained professionals involved in the project must come into contact with the used aquaria and fomites. The zoonotic potential of the primary zebrafish diseases is shown in Table 2.

Bioterists who develop their activities with zebrafish are constantly exposed to potentially significant occupational hazards. The concern is further intensified by the fact that these infectious agents are present in water, which, is a major source of disease spread to humans [129,130]. Given this, it is recommended that handlers always wash their hands before and after contact with animals, breeding water, or any fomite that has come into contact with water used by the animals and use gloves to carry out these activities. The use of a protective mask is also necessary since the environment of an aquatic facility can often have the formation of droplets that can be inadvertently inhaled or ingested. Another important recommendation is that, as soon as dead animals are noticed in aquaria, they are removed and used in surveillance programs (as already discussed) and disposed of correctly (see below). Hygiene and disinfection of fomites that have entered or will come into contact with the rearing water, as well as using a footbath at entry and exit, are important measures for biosafety and biosecurity. That is, avoiding the infection of animals with possible pathogens from outside the facility or avoiding the infection of handlers and other people with potential pathogens coming out of the aquaria/facility, respectively. Hygiene and disinfection of benches that routinely receive dripping water from aquaria are also effective measures that prevent contamination of the environment and infection of researchers.

Keeping the vaccination schedule up to date is also recommended. It has already been reported that the BCG (Bacillus Calmette-Guérin) vaccine has been shown to reduce the lethality of *M. marinum* in zebrafish [131]. Furthermore, the BCG vaccine can provide cross-protection against several *Mycobacterium* species in zebrafish colonies [132]. This shows that the BCG vaccine has a broad protective spectrum with respect to bacterial species and potential host species present in a zebrafish facility. In more specific cases of outbreaks located in certain facility or in experiments that use zebrafish as a study model for human infectology, vaccination against cholera may be indicated.

In addition to the risk of transmitting the zoonosis itself, there is still the problem of the handler becoming infected with agents resistant to the treatments available in most health services, as is the case with infection by bacteria resistant to antibiotics [133,134]. In addition, even if they do not become infected, handlers can carry infectious agents (resistant or not to available treatments) to environments frequented by immunocompromised individuals, newborns and their mothers, the elderly, or those hospitalized in general. In fact, it is recommended that the bioterist never leave the laboratory/facility to visit a hospitalized patient on the same day or without first having rigorously been bathed [135].

4.14.2. Environmental health

As already mentioned, an aquatic facility that does not prioritize good practices and the biosafety of its facilities can become a risk to the health of animals and experimenters and environmental health. It is already widely advocated that experimental evaluations of transgenic fish, for example, be carried out in facilities so that physical restraint is adequate to ensure the safety of the application of these animals [136]. However, it has recently been reported that transgenic zebrafish have reached the Brazilian Atlantic Forest's natural environment due to inadequate containment barriers by ornamental fish breeders, causing concern regarding the health of the ecosystem [137,138].

Furthermore, transgenic or wild zebrafish are potential reservoirs of pathogens that can contaminate the

natural environment, consequently infecting wild fish and other animals in general, including humans (Table 2). Indeed, the widely distributed occurrence of zebrafish worldwide is already causing concern about introducing diseases that were not reported in the past in specific geographic regions [89,125]. To avoid contamination of the environment and the risk of infection, it is recommended that zebrafish aquatic facilities coordinators be aware of the provisions of current legislation. In Brazil, the National Health Surveillance Agency (ANVISA) and the National Council for the Environment (CONAMA) made public the Resolution of the Collegiate Board nº 222 [139] and Resolution nº 358 [140], respectively, which regulate good practices in the management of waste from health services and have various guidelines on the disposal and destination of waste from activities of maintenance and experimentation with laboratory animals. Furthermore, the National Technical Commission on Biosafety published the RN nº 18/2014, which provides for the classification of risks of genetically modified organisms (GMOs) and the levels of biosafety to be applied in activities with GMOs, aiming to contain these organisms and their derivatives [141]. In addition, the National Council for the Control of Animal Experimentation has two particularly important regulations that help guide the containment of biological material in facilities that keep zebrafish for scientific purposes; they are RN nº 55/2022, which presents the Brazilian Guideline for the Care and Use of Animals in Teaching or Research Activities (*Diretriz Brasileira para o Cuidado e a Utilização de Animais em Atividades de Ensino ou Pesquisa*, DBCA, in Portuguese) [142] and RN nº 34/2017 [13], which presents relevant information on the breeding and maintenance of zebrafish in teaching and scientific research facilities. [13]

However, although zebrafish facilities pose risks, they also favor environmental health and one health. Furthermore, zebrafish from different lineages have been used as promising models in studies of animal [110,143], human [144,145], and ecotoxicology [146,147] diseases. Therefore, if well managed and paying attention to good practices and biosecurity, an aquatic zebrafish facility can bring more benefits to society than risks.

5. Rules

5.1. Study plans, protocols, and procedures

The study plan details the purpose of the research, how the work will be organized, what data will be collected during the experiment, and who is responsible for various aspects of the study. This is the central document through which the researcher communicates the objectives and conduct of the study to the work team and third parties. In addition, this document must contain a general description of the experiment with the activity schedule, material and methods, and responsibility of each team member involved. Therefore, approval of the study plan is vital before research can begin. This document is routinely prepared in Brazil as a monograph, thesis, and dissertation project.

Standard Operating Procedures (SOP) are developed for conducting studies, recording, and reporting. These protocols are part of routine laboratory tasks and describe how procedures should be performed. According to Andrade et al. [148], working with laboratory animals requires using and contacting chemical substances and allergens that are potentially dangerous for the health of the personnel involved, the facilities, and the animals themselves. These hazards can be minimized or eliminated by strictly following standard operating procedures to ensure safety.

An SOP is prepared for the people directly linked to the task to efficiently and safely meet the quality requirements. In SOP, it is essential to observe the critical activities that must be summarized and contain only those basic steps that cannot be ignored. Necessary activities should be detailed in the training manual, where figures, photos, and diagrams can be used. The training manual can also be placed in video or audio-visual form to facilitate training [149].

In the case of zebrafish, an online platform was developed by the Zebrafish Information Network (ZFIN). This site provides information on the biological data of zebrafish and some protocols for their maintenance in the laboratory.

One of the most important study protocol differences between mammalian and fish projects involves the increased attention that must be given to the physical environment (water) in fish studies. The aquatic environment is equivalent to a life support system, so housing and testing systems can be quite complex. The need for verification protocols for the previously described water quality parameters, as well as their registration, becomes necessary for greater reliability of the data generated. In this regard, the type of protocol will be adjusted to the type of maintenance system adopted. Thus, the main items that must be included in the SOP of a zebrafish vivarium are listed below.

- Emergency contacts.
- Equipment creation and checking systems.
- Cleaning of utensils and tanks.
- General care and feeding of fish.
- Verification of abiotic parameters and water quality.
- Receiving and transporting fish.
- Assessment of fish health.
- Euthanasia.
- Approval, identification, and registration of experiments.

The SOP must be revised periodically and present the last revision's number and date as a form of registration. By considering the data provided by scientific research, protocols with practices and standards for animal welfare and safety at work can be developed. Thus, each zebrafish experimentation/breeding laboratory must work in accordance with current legislation in pursuit of animal welfare to qualify research results.

6. Results

Given the potential importance of knowledge derived from a study, the data must be complete, have integrity, and be kept secure [29]. Although less common in early articles published with zebrafish, there is a growing trend towards including detailed information about rearing and the parameters used in the studies in the "Material and Methods" section of publications [47]. With the notion that parameters such as power supply, temperature, photoperiod, etc., may affect experimental research results [150], it is always recommended to include a detailed description of the zebrafish-rearing parameters used. In the case of facilities, the registration of activities such as feeding and abiotic data is of paramount importance for resource management and activity planning.

The report and the generated data are the final results of the experiment. This information often becomes part of the scientific knowledge base as soon as the results reach the public domain, often through journal publication. Given the potential importance of the knowledge derived from the study, the data must be complete, have integrity, and be maintained securely [28]. The files resulting from a study must be stored so that when accessing the information, it is possible to repeat the experiment.

7. Quality control

GLPs define the minimum quality assurance requirements necessary to guarantee the validity of experimental results. To adhere to GLP principles, quality control must review all preclinical research phases – from planning to reporting and archiving documentation. In summary, the fundamental mission of quality control is that of an independent witness to the entire preclinical research process and its organizational structure. In this regard, the animal research ethics committees of higher education institutions, the Federal Council of Veterinary Medicine, and the peer review of scientific journals play an important role in fulfilling this requirement. It should be noted that it can rarely be done by just one person and usually requires input from scientific experts in the study area.

8. Conclusion

Generally speaking, GLP implementation should begin with an assessment of system-wide risks and opportunities by searching for improvements that will be practical in given situations. This assessment should include a scientific basis for the needs and welfare of the animals and a risk assessment to identify the causes of poor animal welfare. In many cases, the most effective approach is likely to be an ongoing process of improvement based on achievable goals. In a way, Brazil still needs improvements related to the well-being of aquatic organisms (national laws, international agreements, corporate programs, and others), especially concerning its use in research and technological development. In this way, GLP implementations provide valuable guidance for improving animal welfare and worker safety by facilitating the standardization of research. As part of the risk and opportunity assessment, GLPs should consider the possible role and benefits of such protocols, as well as any training required to facilitate the workers involved to comply with the standards.

Conflict of interests

The authors declare that there is no conflict of interest.

Author contributions

Conceptualization: M.T. Kutter and T. Silveira. *Data curation:* M.T. Kutter. *Formal analysis:* T. Silveira. *Methodology:* M.T. Kutter. *Writing (original draft):* M.T. Kutter, T. Silveira and L.J.G. Barcellos. *Writing (proofreading and editing):* M.T. Kutter, T. Silveira, L.F. Marins and R.T. Boyle.

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