



Canadian Council on Animal Care
Conseil canadien de protection des animaux



**CCAC guidelines: Zebrafish and
other small, warm-water laboratory fish**

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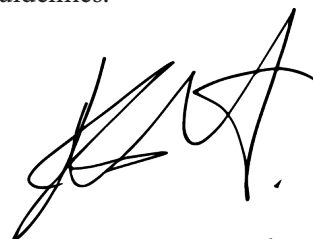
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Zebrafish and other small, warm-water laboratory fish

PREFACE

The Canadian Council on Animal Care (CCAC) is the national peer-review organization responsible for setting, maintaining, and overseeing the implementation of high standards for animal ethics and care in science throughout Canada.

The *CCAC guidelines: Zebrafish and other small, warm-water laboratory fish* provides information for investigators, study directors, animal care committees, facility managers, veterinarians, and animal care staff to help facilitate improvement in both the care given to laboratory fish and the manner in which experimental procedures are performed. These guidelines address conditions normally present in laboratories housing zebrafish and other small, warm-water fish; where experimental conditions required by studies differ from the guidelines, they must be justified to, and approved by, the animal care committee.

CCAC guidelines are intended to provide assistance in the implementation of Russell and Burch's Three Rs (Replacement, Reduction and Refinement) principles for animals in science (Russell and Burch, 1959). The guidelines are based on expert interpretation of current scientific evidence, and have been subject to peer review. They are intended to provide a framework for the implementation of evidence-based practices, which are constantly evolving. Implementation of evidence-based practices should result in continual improvement in animal welfare.

For studies outside of Canada, Canadian investigators are subject to these guidelines as well as to the relevant legislation and regulations pertaining to animal ethics and care in the country where the study is conducted.

SUMMARY OF THE GUIDELINES LISTED IN THIS DOCUMENT

The following list of guideline statements serves as an executive summary covering the most important aspects of the care and use of zebrafish and other small, warm-water laboratory fish. These guideline statements are included throughout this document alongside details and references that provide support and context for their implementation.

1. INTRODUCTION

Guideline 1

Institutions must strive to sustain an institutional culture of respect for animal life.

p.6

2. AQUATIC FACILITIES

Guideline 2

Facilities must ensure an adequate amount of water of suitable quality is provided at all times for the species and life stage of the fish being housed.

Section 2.1 Water, p.12

3. FACILITY MANAGEMENT AND PERSONNEL

Guideline 3

Water quality variables must be routinely monitored to permit predictive management of water quality. Contingency plans must be in place to deal with deviations from acceptable limits for the species being held.

Section 3.1 Water Quality Monitoring and Management, p.18

Guideline 4

Sufficient numbers of competent personnel must be available for daily care and observation of the fish and the safe operation and maintenance of the water and life support systems, 365 days a year.

Section 3.6 Personnel, p.25

4. PROCUREMENT

Guideline 5

Facilities and investigators acquiring or transporting fish, or conducting research on fish, must be familiar with, and comply with, relevant international, federal and provincial/territorial legislation and policies.

Section 4.2 Regulations, p.26

Guideline 6

The health and welfare of the fish must be checked upon arrival by competent animal care personnel and the fish must undergo quarantine and acclimation for a period appropriate to assure the health of the fish.

Section 4.5 Receiving Fish, p.29

5. BROODSTOCK AND BREEDING

Guideline 7

Breeding must be managed to minimize inbreeding and genetic drift and to increase genetic diversity.

p.32

Guideline 8

For female zebrafish, there should be an interval of at least one week between breeding attempts.

Section 5.1 Breeding, p.33

6. HUSBANDRY

Guideline 9

Zebrafish must not be individually housed without scientific or veterinary justification and should be housed at densities appropriate to their life stage.

Section 6.3 Housing Management, p.38

Guideline 10

Consideration must be given to providing zebrafish with environmental enrichment suited to the housing conditions.

Section 6.5 Environmental Enrichment, p.43

Guideline 11

Detailed Standard Operating Procedures must be developed for the care of all fish and for maintenance and cleaning of tanks, rooms and equipment.

Section 6.8 Record Keeping; 6.8.1 Standard Operating Procedures, p.46

7. HANDLING AND RESTRAINT

Guideline 12

Fish must be handled only when necessary, and the number of handling episodes should be minimized.

p.48

Guideline 13

Personnel involved in handling fish must be competent in methods that expose fish to air for the shortest time possible, and that minimize injury, including damage to the mucus-skin barrier.

p.48

8. WELFARE ASSESSMENT

Guideline 14

Basic physical and behavioural indicators of fish welfare must be monitored daily and written records must be maintained. Any changes must be investigated and the causes identified and corrected.

p.50

9. HEALTH AND DISEASE CONTROL

Guideline 15

All facilities must have a fish health program that includes a system of regular monitoring and reporting for health assessment purposes, aimed at early detection of disease conditions and the causal agents, stressors and mechanisms of disease, so that correct control measures can be initiated.

p.53

Guideline 16

A management plan must be in place to deal with unanticipated disease outbreaks.

Section 9.3 Disease Management in the Event of an Infectious Outbreak, p.59

10. EXPERIMENTAL PROCEDURES

Guideline 17

Endpoints must be defined for studies that involve potential pain and/or distress to fish or where morbidity and mortality are expected, and a list of parameters for objective assessment of the health and well-being of the fish must be established.

p.61

Guideline 18

Anesthetics must be used in procedures where there is expected to be noxious stimuli, and in experiments entailing extensive handling or manipulation with a reasonable expectation of trauma and physiological insult to the fish.

Section 10.4.1 Anesthesia, p.67

Guideline 19

Following the precautionary principle, fish should be provided with analgesia for procedures that are likely to be painful, based on the best available scientific evidence.

Section 10.4.2 Analgesia, p.70

11. EUTHANASIA

Guideline 20

Euthanasia of zebrafish must be carried out by competent personnel only, using the method that minimizes pain and distress for the particular fish and is suited to the study data.

p.73

1 INTRODUCTION

Throughout this document, the term ‘should’ is used to indicate an obligation, for which any exceptions must be justified to, and approved by, an animal care committee. The term ‘must’ is used for mandatory requirements.

The zebrafish (*Danio rerio*) is a species of freshwater fish of the family *Cyprinidae*. Although zebrafish are the most common small, warm-water fish held in institutions in Canada for scientific purposes, other small, warm-water fish, such as guppies, killifish, medaka and cichlids, are also present in Canadian laboratories in significant numbers. These guidelines primarily focus on zebrafish, and provide additional guidance specific to other small, warm-water fish where there is evidence that their requirements differ from those of zebrafish. Since this document does not provide inclusive guidelines for species other than zebrafish, investigators must perform a literature search and consult those working with the species of interest to determine appropriate husbandry requirements prior to initiating rearing and studies in those species. Where data on optimal conditions for a species is lacking, guidelines for zebrafish may provide an appropriate starting place, with careful monitoring and adjustment.

As stated in the [CCAC guidelines on: the care and use of fish in research, teaching and testing](#) (CCAC, 2005), “Fish eggs, embryos or larvae that have not developed beyond exclusive reliance on their own yolk nutrients are not covered by these guidelines”. For zebrafish, larvae are capable of independent feeding at five days post-fertilization when kept at a standard laboratory temperature of 28°C (development may be slower at a lower temperature); at this point, the yolk sac has greatly decreased (Vargesson, 2007; Lindsay and Vogt, 2004; Jones et al., 2008). The timing of this stage differs among species; for example, it takes seven days for medaka to emerge from a tough chorion, where they are protected from the environment and fully supported by the yolk (Furutani-Seiki and Wittbrodt, 2004).

Understanding the behaviour and biology of experimental animals is crucial to improving both animal welfare and the quality of scientific research (Olsson et al., 2003). Relatively little is known about the natural behaviour or biology of zebrafish and few studies have been conducted on wild populations (Graham et al., 2018a). However, some of the work that has been undertaken suggests the conditions under which zebrafish are often kept in laboratories differ significantly from their natural preferences (Delaney et al., 2002). Additionally, the scope of studies involving zebrafish has recently expanded from the field of vertebrate development, which has focused on embryos due to their transparency and rapid development, to include studies on adult zebrafish in areas such as pharmacology, disease and drug discovery (Kinth et al., 2013).

Guideline 1

Institutions must strive to sustain an institutional culture of respect for animal life.

This statement can be found in the [CCAC guidelines on: training of personnel working with animals in science](#) (CCAC, 2015), and is fundamental to all CCAC guidelines.

The present guidelines represent current understanding of best practices for zebrafish. Investigators and animal care personnel should keep abreast of advancements relevant to the ethics and care of zebrafish, which is a rapidly evolving field. Cost and convenience must not take precedence over the animal's physical and mental well-being (CCAC, 1989). The following sections provide a brief overview of behavioural biology, where it has importance for the welfare of zebrafish and other small, warm-water fish (Section 1.1, “Behavioural Biology”), as well as the particular anatomical and physiological characteristics (Section 1.2, “Anatomy and Physiology”) and sensory abilities (Section 1.3, “Senses”) of these fish. In addition, some potential inter-animal variations are described (Section 1.4, “Sources of Variation”). The information in these sections forms the basis for the more detailed guidance provided throughout this document.

1.1 BEHAVIOURAL BIOLOGY

In their natural habitat, zebrafish are typically found in standing or slow-moving bodies of water, such as pools, ponds, lakes, ditches or rice paddies (Vargesson, 2007; Delaney et al., 2002; Spence et al., 2006a). Field studies have found zebrafish at sites that are silt-bottomed and well-vegetated, with shallow and relatively clear water where they appear to occupy the whole of the vertical water column (Engeszer et al., 2007; Spence et al., 2007). However, they also appear in streams and rivers (Suriyampola et al., 2016). The habitat in which they are found appears to have an impact on both the size and social behaviour of the fish (Suriyampola et al., 2016).

Zebrafish are omnivorous. Their natural diet consists primarily of zooplankton and insects, although gut content analyses have also revealed phytoplankton, filamentous algae, vascular plant material, spores, invertebrate eggs, fish scales, arachnids, detritus, sand and mud (Spence et al., 2008). While it is thought that these fish feed within the water column, it has also been suggested that they feed at the surface and from the substrate (Spence et al., 2008).

When zebrafish perceive a threat, behaviours displayed may include shoal cohesion, either agitated swimming or freezing on the substrate, decrease in feeding rate, and increase in aggression (Spence et al., 2008).

In the wild, zebrafish have been observed in small shoals of six to seven individuals, as well as large shoals of up to 300 individuals, depending on the habitat (Suriyampola et al., 2016). It has been proposed that stripes are a shoaling cue (Rosenthal and Ryan, 2005) and pattern preference is learned rather than innate, with individuals preferring to associate with individuals having the colour pattern with which they have been raised (Spence and Smith, 2007). In a laboratory setting, zebrafish have shown a preference for being with groups of four fish versus being with one fish; however, this may be influenced by water temperature (Pritchard et al., 2001). Isolated zebrafish have been observed to have a higher stress response and more variable behaviour than fish that were able to shoal (Pagnussat et al., 2013). Mansur et al. (2014) noted that zebrafish in groups of eight demonstrated less avoidance of light areas than single zebrafish or groups of two or four, possibly indicating that shoaling plays a role in reducing anxiety and stress.

Zebrafish exhibit a robust circadian pattern of daytime activity and night-time rest, a state which is said to have important similarities with sleep in mammals (Zhdanova, 2006). Activity patterns are also dependent on feeding time (Blanco-Vives and Sánchez-Vázquez, 2009).

Observations in the laboratory suggest that the mating behaviour of zebrafish is influenced by the exposure of mating partners to one another during the 24 hours before spawning begins (at sunrise), with males stimulated to perform courtship behaviour by the detection of female pheromones in the water (Delaney et al., 2002). Courtship in zebrafish involves the male swimming quickly in close proximity to the female, often touching her flanks with his snout, and circling tightly in front of her while attempting to lead her to a spawning site. Once over the spawning site, the male swims closely alongside and slightly behind the female, and may oscillate his body at high and low amplitude. Both territorial and non-territorial males show the same courtship behaviour, but whereas non-territorial males have been observed to pursue females all around the aquarium, territorial males confine their activities to within a few body lengths of the spawning site and chase other males away when they try to approach (Spence et al., 2006b).

In laboratory settings, some male zebrafish are territorial during mating, and a single male may aggressively attempt to control rivals' access to a spawning site and to females (Spence and Smith, 2005). While territorial defence by males confers a fitness advantage at low densities, it may not always do so at high densities (Spence et al., 2006a). However, most mating behaviour research has been conducted under artificial laboratory conditions, and it is possible that zebrafish engage in different mating behaviour in different environments, including the possibility that they may nest and show little aggression in semi-natural environments (Graham et al., 2018a).

Female zebrafish use chemosensory cues (Hutter et al., 2010; Bloom and Perlmutter 1977) and body shape to distinguish between the sexes, and appear to show a preference for males with a larger body (Turnell et al., 2003). However, male body size does not appear to be correlated to either dominance rank or clutch size. Female preference may be overridden by dominant males who do not allow females to access other males (Spence and Smith, 2006).

For zebrafish, there is no parental care of the offspring post-laying. However, parental care has been demonstrated in both male and female cichlids (Budaev et al., 1999). Fish from the family *Poeciliidae* (e.g., guppies and swordtails) differ from many other small, warm-water species in that fertilized eggs are retained in the follicle throughout gestation (Liu and Lee, 2014).

For a review of studies on social motivation and behaviour of zebrafish, see Graham et al. (2018a). Basnet et al. (2019) provide a review of the behavioural repertoire of larval zebrafish, which is considerably similar to that of adults.

1.2 ANATOMY AND PHYSIOLOGY

The rate of development of individual fish is affected by genetic and environmental factors. Therefore, indicators other than age, such as size and various anatomical changes, have been used to identify specific milestones in the stages of zebrafish development (Parichy et al., 2009).

After fertilization, the basic body plan of the animal develops within 24 hours. Newly hatched 'early' larvae (two to three days post-fertilization) are largely inactive, negatively buoyant and lay immobile on the bottom, although they can respond to touch. On, or just before, day five (depending on the water temperature), the larvae inflate their gas bladders by swimming up and gulping air at the surface (Lawrence, 2007). After this point, larvae are neutrally buoyant and are capable of continuous swimming and maintaining their position within the water column. As mentioned, this is also the stage where the yolk sac has decreased and larvae are capable of independent feeding. This is the point at which these guidelines apply, and when the juvenile fish should be counted for submission on animal use data forms.

Swimming involves regular but discontinuous beating of the tail, which is characteristic of the method of swimming observed in these fish as they continue to grow and age (Lindsay and Vogt, 2004). The period of metamorphosis from larvae, through juvenile, to adult includes the complete loss of the larval fin fold, remodelling of features such as the gut and nervous system, the acquisition of scales, and the production of viable gametes and appearance of secondary sexual characteristics in fish that are in breeding condition (Parichy et al., 2009).

Adult zebrafish are usually less than 5 cm in length (De Tolla et al., 1995). Though similar in size and coloration, the sexes can be distinguished fairly reliably by appearance. Reproductively mature females have a fuller abdomen due to the developing eggs in the ovaries. Males are generally more slender and darker in colour than females, and have more yellow coloration in the anal fin (Ruhl et al., 2009; Schilling, 2002). Mature males can also be identified by the presence of tubercle clusters on the pectoral fins, as depicted in McMillan et al. (2015). The most reliable way to distinguish females from males is by the presence of a small genital papilla, but this can only be definitively determined after death (Laale, 1977) or under anesthesia.

1.3 SENSES

Zebrafish possess all of the classes of senses: touch, balance, hearing, smell, vision and taste (see Moorman [2001] for a description of the development of these senses). Like many other fish, zebrafish possess a lateral line, which is a series of mechanosensory receptors located on or just beneath the skin. The neuromasts of the lateral line are first recognizable two days after fertilization. Each is a mechanosensory end-organ that is sensitive to low-frequency (1-200 Hz) vibrations. Information reaches the brain via the rostral and caudal lateral line nerves on each side and is used to detect water movement and vibrations, which helps to guide behaviours such as shoaling, prey capture, and predator and obstacle avoidance (Whitfield, 2002; Moorman, 2001).

Zebrafish can respond to external chemical cues within 24 hours of hatching (Lindsay and Vogt, 2004). Studies on other fish species, such as the fathead minnow and Arctic charr, have indicated that these species perceive chemical cues from the environment prior to hatching, and therefore this may be a consideration for zebrafish.

Zebrafish use olfactory cues to distinguish between kin and non-kin (Mann et al., 2003). They show a preference for associating with kin during the larval and early juvenile stage, but this changes to avoidance of kin and preference for non-kin once they reach sexual maturity (Gerlach and Lysiak, 2006). Olfactory cues are also important for feeding and reproduction (Whitlock, 2006). It is thought that when selecting mates, males may rely more on olfactory cues than visual cues (Turnell et al., 2003).

The zebrafish visual system appears to be similar to other vertebrates (Bilotta and Saszik, 2001). Visual behaviour is displayed approximately 68 hours after fertilization and visual acuity appears to improve with age (Fleisch and Neuhauss, 2006; Easter and Nicola, 1996 in Bilotta and Saszik 2001; Bilotta 2000). The retina consists of both rod cells that support vision in low light levels, and cone cells that support vision in bright light and colour perception. Zebrafish possess at least four different cone photoreceptors, including an ultraviolet photoreceptor (Bilotta, 2000). Studies have indicated that zebrafish are able to distinguish colours, and exhibit colour preferences (Oliveira et al., 2015; Peeters et al., 2016). They have also been observed to make eye movements that track the stripes on a rotating drum, thus providing evidence for pattern vision (Moorman, 2001).

Zebrafish do not possess outer or middle ears, but have a fairly typical vertebrate inner ear. Together with visual cues, this is used to maintain balance (Whitfield, 2002). Four small bones (the Weberian ossicles) link the swim bladder to the inner ear, and enhance hearing (Moorman, 2001).

1.4 SOURCES OF VARIATION

Consideration should be given to sources of variation in zebrafish when designing experiments and analyzing data. In addition to the sources noted below, researchers should take into account the potential for genetic drift and/or genetic selection in isolated colonies over time, which can result in the genetic background of standard lines being different among different institutions. For example, Lange et al. (2013) report variation in locomotor activity between zebrafish of the same strain from two different laboratories.

1.4.1 Strain

Among wild-type zebrafish, different strains have shown differences in their response to various behavioural tests (Vignet et al., 2013). Additionally, manipulation of zebrafish breeding has produced various strains, including long fin, golden, casper and albino. Some of these strains lack pigmentation (e.g., the casper strain) or are hypopigmented (the golden and albino strains), which allows better visibility of stains (e.g., vital dyes, fluorescent tracers, antibodies and riboprobes) that are applied to the fish in some studies (Whitfield, 2002). Wilson et al. (2014) determined that two domesticated strains of zebrafish (TU and AB) do not possess the same WZ/ZZ sex determinant as strains found in the wild.

It has been suggested that growth rates of domesticated strains in the laboratory are higher than that of wild fish (Spence et al., 2007, 2008). Standard laboratory strains of zebrafish have been found to have a faster growth rate, greater sexual dimorphism, reduced predator avoidance behaviour, and a greater degree of surface orientation compared with a population obtained directly from their natural habitat in India (the Nadia strain).

In the laboratory, zebrafish have an average lifespan of three and a half years, with a maximum recorded lifespan of five and a half years (Gerhard et al., 2002). In contrast, zebrafish may survive for one to two years in the wild; their shorter live span is possibly due to predation and/or parasites (Spence, 2007, cited in Reed and Jennings, 2011). Zebrafish are routinely only kept in laboratories for 18 to 24 months, after which they are considered to be of lower reproductive value and may carry higher pathogen loads.

Spinal curvature has been observed in both domesticated zebrafish and wild-type zebrafish after their second year of captivity in the laboratory. This has not been reported in the literature for wild populations, possibly due to their shorter lifespan.

1.4.2 Effects of the Environment

There are numerous environmental conditions that can impact physical and behavioural variation among individual zebrafish. Examples include the following:

- tank size and fish density can influence reproductive behaviour (zebrafish have been observed to spawn in pairs when in large tanks (1,100 litres) of 8 fish, whereas ‘group spawning’ was found to be prevalent in smaller tanks (17 litres) with higher fish densities (20 fish/tank) (Hutter et al., 2010);

- environmental temperature (Sfakianakis et al., 2012) and density (Ribas et al., 2017) can influence sex ratios;
- structural enrichment, in the form of a gravel substrate and plants, can have an impact on survivorship and behaviours associated with anxiety (Lee et al., 2018); and
- pigmentation can change to blend in with the background as a camouflage response (zebrafish with visual defects appear to be much darker than wild-type fish, presumably because the absence of visual input is interpreted as being in a dark environment [Goldsmith and Solari, 2003]).

AQUATIC FACILITIES

2

Facilities required for zebrafish and other small, warm-water fish are detailed throughout this section. Where the information is applicable to all facilities holding fish and is sufficiently covered in the [CCAC guidelines on: the care and use of fish in research, teaching and testing](#) (2005), cross references are provided.

Aquatic facilities are complex systems that must be well designed to minimize stress to the fish and maintain their health, promote efficient operation, and ensure a safe working environment for personnel. Providing an environment that minimizes stress for the fish and promotes a healthy fish population requires knowledge of the natural history of the species of interest, fish welfare and behaviour, and the type of studies being conducted.

2.1 WATER SUPPLY

Guideline 2

Facilities must ensure an adequate amount of water of suitable quality is provided at all times for the species and life stage of the fish being housed.

Requirements for the analysis of the quality of the source water when setting up an aquatic facility is described in Section C.2, “Water Quality”, in the [CCAC guidelines on: the care and use of fish in research, teaching and testing](#) (2005). It is important to have full knowledge of the origin and properties of the water used for maintaining zebrafish. The chemical and physical properties of the source water will vary widely, depending on whether water is obtained from municipal or natural sources and whether it is distilled or desalinated.

When using water from a municipal source, the municipality should be contacted to determine whether chlorine and/or chloramines are present in the water. If chlorine or chloramines are present, they must be removed by a confirmed effective method, such as passing the water through a carbon filter before use. Catalytic or activated charcoal is commonly used for this purpose. Other products are available that bind chlorine and chloramines, as well as ammonia. For a large laboratory where carbon filters are used to supply dechlorinated water to the tanks, destructive UV dechlorination can also be used, in case some of the source water penetrates the activated carbon filters.

Unlike chlorine, chloramines cannot be removed through off-gassing. Where municipal water is treated with ozone to remove taste and odor-causing compounds, periodic supersaturation of the water with oxygen can occur and should be addressed (see Section 3.1.2, “Supersaturation”).

The purification of water through reverse osmosis, alone or in combination with deionization for increased removal of ions, is described by Lawrence and Mason (2012). Water treatment procedures may result in water that is osmotically imbalanced and unsafe for fish. They may also remove trace elements required for fish

metabolism and growth, which will need to be added back into the water (Lawrence and Mason, 2012). The use of reverse osmosis is common in recirculation systems designed for zebrafish, with ions re-introduced into the water via an automated dosing system.

Monitoring requirements depend on the system (e.g., a static system using municipal water will require more extensive monitoring than a recirculating or flow-through system with well-established water quality). For flow-through systems, it is important to monitor seasonal changes in the source water. Frequent monitoring is required when a system is set up, but the monitoring intervals may become longer once the system is stable. Typically, biological activity in filtration systems takes six weeks to become effective. For acceptable ranges of water quality variables and monitoring requirements, see Section 3.1, “Water Quality Monitoring and Management”, and Appendix 1.

2.2 ANIMAL ROOMS AND PROCEDURE ROOMS

2.2.1 Engineering and Design

The design and construction of aquatic facilities requires specific expertise. Input from both investigators and operational personnel is critical to ensure it meets the unique requirements of the fish being held and of the studies being conducted (Sanders, 2013). General requirements for rooms housing fish are provided in Section C.3, “Engineering and Design”, in the [CCAC guidelines on: the care and use of fish in research, teaching and testing](#) (2005). Lawrence and Mason (2012) provide an overview of basic considerations specific to the design of zebrafish housing systems.

Work requiring the containment of aquatic animal pathogens requires special design considerations, as detailed in *Containment Standards for Facilities Handling Aquatic Animal Pathogens* (CFIA, 2013).

2.2.2 Structural Materials

General criteria for materials to be used in construction that are resistant to corrosion and water damage and are safe for fish are outlined in Section C.3.1, “Structural Materials”, in the [CCAC guidelines on: the care and use of fish in research, teaching and testing](#) (CCAC, 2005).

While polycarbonate tanks are standard in laboratory facilities, investigators and facility managers should be aware of their potential for leaching bisphenol A (BPA), a known endocrine disruptor that can influence sex ratios, fish development, physiology and reproduction. Particular caution should be taken during the initial use of plastic material and when it is exposed to autoclaving or washing at high temperatures.

2.2.3 Room Ventilation and Airflow in Aquatic Areas

Requirements for air handling systems are provided in Section C.3.2, “Room Ventilation and Airflow in Aquatic Areas”, in the [CCAC guidelines on: the care and use of fish in research, teaching and testing](#) (2005). For additional information on using airflow direction to minimize the spread of potential aerosols and to allow for safe handling of dangerous substances, see Section 12.3.6, “Differential Pressure”, in the [CCAC guidelines on: laboratory animal facilities – characteristics, design and development](#) (2003).

2.2.4 Mechanical and Electrical Requirements

Requirements for mechanical and electrical systems in aquatic environments are detailed in Section C.3.3, “Mechanical and Electrical Requirements”, in the [CCAC guidelines on: the care and use of fish in research, teaching and testing](#) (2005).

2.2.5 Redundancy in Aquatic Life Support Systems

It is critical that aquatic facilities have the capacity to maintain water quality, and other elements important to the health of the fish, during a power or equipment failure. The specific requirements to address these situations are outlined in Section C.3.5, “Redundancy in Aquatic Life Support Systems”, in the [CCAC guidelines on: the care and use of fish in research, teaching and testing](#) (2005).

In small facilities that do not have redundancy in the electrical feed to life support systems, uninterrupted power supply (UPS) ports should be used for essential equipment such as air pumps. These ports can hold electrical charge and supply electricity in the event of an outage.

2.3 PRIMARY ENCLOSURE

Prior to adding fish to a tank that currently does not hold fish, the tank must be commissioned through the full battery of water quality tests over a 24 hour period (see Section 3.1, “Water Quality Monitoring and Management”). When the results show that the water quality is suitable for the fish, only a small number of fish should be added, with the numbers increased gradually if the water quality remains stable. The same level of care must be given to the initial fish as to all others who will occupy the tank.

2.3.1 Types of Systems

The three types of housing for fish, recirculation systems, static tanks and flow-through systems, are described in Section C.4, “Types of Systems”, in the [CCAC guidelines on: the care and use of fish in research, teaching and testing](#) (2005). The choice of system should take into consideration the types of studies that are intended to be carried out and the species involved, as each system has different challenges, requirements and applications.

In the wild, zebrafish are found in slow-flowing waters (Spence et al., 2008), and in the laboratory they have shown a preference for moving water (DePasquale et al., 2019). Therefore, the position of in-flow and out-flow taps in the tanks and the rate of water flow should be set to produce a gradient of flow to allow the fish to regulate their own exposure to water turbulence and flow. The set-up of the tank to produce this effect will depend on the tank design.

2.3.1.1 Recirculation Systems

Recirculating systems use a series of processing stages, as described by Lawrence and Mason (2012):

- 1) mechanical separation and removal of large suspended solids (uneaten food and feces), followed by removal of suspended solids through filtration;
- 2) biological filtration, involving nitrifying bacteria that oxidize toxic nitrogenous waste in the water to nitrite, and then to nitrate;

- 3) chemical processes to bind and reduce dissolved organic materials or harmful chemicals;
- 4) aeration to ensure adequate levels of dissolved gases in the water; and
- 5) disinfection through UV sterilization.

To help reduce the spread of disease between interconnected tanks in recirculation systems, water should be sterilized by UV radiation before redistribution (Brand et al., 2002). UV equipment must be properly maintained and back-up bulbs should be available. Records of UV sterilization (e.g., when the bulb is changed) must be kept to confirm that the system is operating.

Consultation with experts in recirculation systems designed for zebrafish is encouraged to gain an understanding of the life support systems available and their application.

2.3.1.2 Static Tanks

In static tanks (e.g., a stand-alone rectangular glass aquarium) there is no new water added automatically from outside or recirculation sources. These tanks are commonly used for small-scale research, for quarantine purposes, or for drug trials where the fish need to be held in water containing a dissolved drug without impacting the rest of the system. Many warm-water species, such as guppies, medaka, killifish, etc., can be held in static tanks.

Tank heaters or environmental chambers may be used to keep the water temperature constant. Static systems require frequent cleaning and/or lower stocking densities than flow-through or recirculation systems.

Tanks maintained by manual water changes can be equipped with filtration units to continually remove undesirable material from the water (Matthews et al., 2002), including biofiltration for ammonia and nitrite detoxification. A separate tank filtering system should not be necessary if a portion of the water is replaced each day by siphoning up debris from the bottom of the tank. The amount of water that needs to be replaced depends on factors such as stocking density; however, one third of the total water volume can be used as a starting point and adjusted based on daily measurements of pH, ammonia and temperature. If a filter is used in the tank, approximately half the water should be changed at least once a week (Westerfield, 2007).

In addition to the measures to assure appropriate water quality that are noted in Section C.4.3, "Static Systems", in the [*CCAC guidelines on: the care and use of fish in research, teaching and testing*](#) (2005), water quality variables (see Section 3.1, "Water Quality Monitoring and Management") and animal condition (see Section 8, "Welfare Assessment") must be monitored daily, although water quality can be monitored less frequently if the water quality parameters stabilize with establishment of a biofilter. Regular partial volume water changes with water of the same temperature as the tank water should be used to improve water quality. Devices should also be incorporated to create water movement within the tank, such as corner filters, under-gravel filters, hanging filters, air bubblers, internal impeller or jet pumps. In addition, establishment of beneficial bacterial communities should be encouraged by providing physical structures for the bacteria to grow on, and by seeding the system with ammonia prior to introducing the fish.

2.3.1.3 Flow-Through Systems

Flow-through systems are less common for zebrafish and other small, warm-water laboratory fish; however, they are used for specific applications, such as experiments involving exposure to substances, genotyping, and isolation or quarantine. In flow-through systems, levels of toxic waste are kept low and solid waste (in suspension) can be drained continuously or on a regular basis.

Flow-through systems require a continuous supply of water of a consistently high quality and stable temperature. Therefore, these systems use a lot of water and require constant monitoring of the quality of the input water. As well, the supply water usually needs to be heated to meet the requirements of the species. Therefore, these systems have a high energy demand and may be impractical for some situations.

2.3.2 Tank Design and Spatial Requirements

The basic requirements for fish housing are detailed in Section C.5, “Fish Housing”, in the [*CCAC guidelines on: the care and use of fish in research, teaching and testing*](#) (2005). As noted, aquatic environments should be designed to meet the physical and behavioural requirements of the particular species and life stage in terms of such factors as the shape, depth, volume and colour of the tank, the provision of shelter, social grouping, overhead cover and lighting.

Zebrafish and other small species are generally housed in small plastic tanks as part of a recirculation system; however, they are also housed in rectangular glass aquariums for some studies or situations (e.g., quarantine). Care should be taken to ensure none of the materials used in setting up the system, such as tanks, pipes, plastic connections, tubing, siphons and pumps, leak toxic compounds into the water (Brand et al., 2002). Copper and other toxic metals, such as brass, should not be used in systems housing fish. Newly manufactured tanks should be rinsed before use, in accordance with the manufacturer’s recommendations.

Tanks must be of sufficient size to accommodate the physical and behavioural needs of zebrafish and to allow appropriate social interactions. The necessary dimensions depend on the size and age of the fish, but are also affected by variables such as water quality and the food and feeding regime (Matthews et al., 2002).

Tanks should have smooth, inert, sealed interior surfaces to facilitate tank cleaning; however, this should be balanced with the preferences of the fish for a more complex environment. Polycarbonate tanks can become scratched during cleaning, which can reduce visibility of the fish during monitoring and facilitate mold and algal growth within tanks.

Zebrafish can jump (Brand et al., 2002), and all tanks must be provided with a cover that prevents them from jumping from the tank. A translucent lid, which allows light in while reducing the risk of alarm to the fish from movements of personnel working nearby, is the most suitable (The Berlin Workshop, 1994, cited in Reed and Jennings, 2011). If tank lids have a small hole, then feeding can be carried out using a squirt bottle or a spatula without having to open the lid, thus reducing disturbance (Brand et al., 2002).

Tank drains or baffles must be designed and maintained in a way to prevent the fish escaping the tank. Build-up of material on the baffles can cause overflow of water.

2.3.2.1 Volume and Population Density

Zebrafish should not be kept in crowded conditions as it is detrimental to their welfare. Adults kept in large tanks (76 litres) at high densities (40 fish/L, as compared to 0.25 fish/L) were observed to have an increase in whole body cortisol levels; however, the results may have been influenced by tank size and the density at which the fish were previously held (Ramsay et al., 2006). Stocking density has been shown to influence the male:female ratio of offspring (Vargesson, 2007; Ribas et al., 2017). Development is also affected by density, with zebrafish maintained at higher densities growing slower than those maintained at lower densities (Vargesson, 2007).

Appropriate fish density depends on the age of the fish and the type of system being used. Adult zebrafish can be kept at a density of 5-8 fish/L. Juvenile zebrafish (<45 days) may be housed at a higher density; however, at present there is insufficient information on the appropriate density for young, developing fish. If the growth or health of the fish is not as expected, the density should be modified accordingly. For breeding, the density mentioned above should be reduced (Vargesson, 2007; Matthews et al., 2002). See Appendix 3 for a summary of the recommendations on density in the literature.

2.3.2.2 Depth

Zebrafish are often described as surface-living fish, yet field studies show that they occupy the whole of the water column, with no significant difference in their distribution according to depth (Spence et al., 2006a). Therefore, water depth as well as surface area should be considered in designing tanks for long-term housing.

2.3.2.3 Colour and Transparency

Tank design and material should ensure that the impact of movements and disturbance outside the tank are minimized. Zebrafish have shown a preference for black walls and demonstrate behavioural signs of anxiety in the presence of white walls (Blaser and Rosemberg, 2012). However, if all the walls are opaque or very dark colours, hygiene problems may not be obvious (The Berlin Workshop, 1994, cited in Reed and Jennings, 2011) and the fish will have reduced visual stimulation.

Transparent containers have the advantage of allowing easy observation and monitoring of the fish, but zebrafish find transparent tank bottoms to be aversive (Schroeder et al., 2014). In addition, individual zebrafish have been shown to exhibit fear behaviours in response to the sight of a distressed conspecific in an adjacent tank (fear contagion; Fernandes Silva et al., 2019). This suggests that when transparent tanks are used, and the welfare of one tank is compromised, other tanks may be affected, which has implications for studies where adjacent transparent tanks of fish are considered to be independent.

One option is to cover the bottom and three of the four walls of the tank with a dark opaque material, to facilitate observation of the fish and monitoring of tank hygiene, while accommodating the preference of the fish. In the case of stacked systems, a dark surface on the bottom of a tank will decrease the light intensity to lower tanks, and therefore additional above-tank lights are required to maintain consistent lighting for all tanks.

The type of study should also be a factor in tank design; for example transparent tanks (or tanks with one transparent wall) are particularly useful for studies where good experimental visibility is essential (e.g., studies with a behavioural component or toxicology studies).

2.3.2.4 Substrate

Zebrafish have been shown to prefer environments with substrate see Section 6.5, “Environmental Enrichment”. Gravel and sand have been added to the bottom of tanks to replicate the natural environment; however, the use of such substrates can impede cleaning and require procedures to ensure water quality is maintained. As a compromise, some facilities have positioned photographs of natural surroundings under the tanks. Pictures of gravel on the bottom of the tanks have been found to be almost as attractive to zebrafish as actual gravel (Schroeder et al., 2014). However, images of sand did not elicit a similar response, suggesting that the variety of textures and patterns in the picture is important (Schroeder et al., 2014).

3 FACILITY MANAGEMENT AND PERSONNEL

Requirements for the management of facilities housing zebrafish and other small warm-water fish are detailed throughout this section. Where the information is applicable to all facilities holding fish and is sufficiently covered in the *CCAC guidelines on: the care and use of fish in research, teaching and testing* (2005), cross references are provided.

Aquatic facilities must have standard operating procedures (SOPs) that detail routine maintenance and a preventive maintenance program, developed specifically for the systems and supporting equipment. All operating manuals for special equipment, such as pumps, chillers and computer control systems, must be available to those in charge of running the facility. Architectural and engineering specifications and drawings of the facility should also be available.

Facilities must be kept in a clean and orderly manner. Movement through the facility must follow SOPs to minimize the potential for cross contamination. Facility-specific clothing and footwear or foot and hand sanitizing stations should be located in the entry area. Personnel should wash their hands as soon as they enter the facility. Tanks must be cleaned and potentially disinfected, depending on the disease transfer risk, before and after every exposure to animals (see Section 6.7, “Cleaning and Sanitation”).

3.1 WATER QUALITY MONITORING AND MANAGEMENT

Water quality is one of the most important factors for the health and wellbeing of fish. Poor water quality can lead to stress and disease, and may affect breeding (Kreiberg, 2000; Bilotta et al., 1999). Levels of contaminants need to be minimized, and this can be facilitated by good water exchange, removal of excess food from tanks, keeping tanks and systems clean, and ensuring the biofilter is healthy (Vargesson, 2007). The use of self-cleaning tanks is encouraged to minimize disturbance of the fish; however, regular cleaning of the tanks is still necessary and the frequency will depend on the water quality. Biofilters used in recirculation systems are biological entities containing microorganisms; their care requires expertise to ensure they operate effectively.

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Water quality variables must be routinely monitored to permit predictive management of water quality. Contingency plans must be in place to deal with deviations from acceptable limits for the species being held.

The most common water quality factors known to affect fish are temperature, dissolved oxygen, pH, suspended solids/sediment, carbon dioxide, nitrogen supersaturation, ammonia, nitrite, nitrate (Wedemeyer, 1996; Kreiberg, 2000) and chlorine. Once the system is set up, these factors must continue to be monitored on a regular basis, and the frequency of monitoring should be based on the stability of the system (i.e. the frequency may be decreased if the system is confirmed to be stable). Instruments used in measuring water quality should be calibrated regularly, in accordance with manufacturers’ instructions.

Facilities should have alarm systems with remote notification when water quality parameters fall outside of set ranges, or other monitoring systems and procedures should be in place to prevent fish mortality incidents caused by system failures. When municipal water sources are used directly, chlorine monitoring should occur constantly in flow-through systems (with alarms in place) and at least daily in other systems, to ensure the level does not exceed 0.01 mg/L. If the water is treated by reverse osmosis, constant monitoring of chlorine and chloramines is unnecessary, but regular monitoring should be conducted. The removal of chloramines significantly reduces the lifespan of the membranes on reverse osmosis/deionization units, and as a result, membranes may rupture. This effect can be reduced by placing a small carbon filter before the unit.

Appropriate water conditions for individual species can vary depending on life stage (i.e. larvae, juveniles and adults) or according to physiological status (e.g., spawning, feeding or recent exposure to high levels of a particular water quality variable such as ammonia). These situations should be taken into consideration when assessing water quality.

The variables and frequency of measurement used to assess water quality depend on the type of system used. For example, while there may be no pressing need to measure nitrite and nitrate in a high volume flow-through system (depending on the source of the water), such measurements are critical with recirculation systems, which are more commonly used for zebrafish research. For recirculation systems, variables such as conductivity, pH, ammonia, nitrite and nitrate must be monitored (Avdesh et al., 2012). Other important variables that contribute to water quality and should be considered in water quality assessment include total dissolved gases, dissolved oxygen, alkalinity, hardness, and salinity (Lawrence and Mason, 2012), although some of these are closely related. Under certain applications, such as disease studies, total dissolved solids should also be measured. Other variables to be measured should be relevant to the health of the species housed in the system, and must be taken at a frequency that allows adjustments to be made well in advance of any morbidity and mortality (Wedemeyer, 1996). In addition, the ability to conduct rapid tests should be in place in case a change in water quality is suspected.

Water quality analysis should be done at a time that reflects the greatest demand on the system (usually after feeding) to identify potential problems.

Records of water quality monitoring must be maintained and kept for a period suited to the type of research and institutional requirements; a minimum of one year is required, or as long as necessary to meet the requirements of government agencies, relevant professional associations and the research (CCAC, 2017).

For further information, see Section D. 3, “Environmental Monitoring and Control”, in the [CCAC guidelines on: the care and use of fish in research, teaching and testing](#) (2005).

3.1.1 Oxygen

Zebrafish should be kept in water with a minimum dissolved oxygen content of 6.0 ppm (mg/L) (Matthews et al., 2002; Cartner et al., 2019). Oxygen concentration will vary according to temperature, atmospheric pressure and salinity. As temperature increases, water’s capacity to carry oxygen decreases; in addition, the fish’s demand for oxygen increases, due to an increase in metabolic rate. A variety of other factors can dictate the amount of oxygen required by fish; for example, the age, health and activity rate of the fish, and handling procedures.

The congregation of fish at the tanks’ water inlet or gasping behaviour at the surface is an indication of critically insufficient oxygen (Abdallah et al., 2015). In some instances, low oxygen levels can be remedied

by aeration, reducing the stocking density, and decreasing feeding. Balancing these responses is essential to prevent inadvertent harm to the fish. Airstones can be used to improve aeration of the water; however, placement and type of stone should be chosen to avoid disrupting the fish and the self-cleaning action of the tank. It is best not to put airstones in tanks, but rather to place them in a part of the system that does not house fish (e.g., sumps).

3.1.2 Supersaturation

Conditions that may cause supersaturation, as well as the effect of supersaturated water on fish and measures to address the problem, are described in Section D.3.4, “Supersaturation”, in the [CCAC guidelines on: the care and use of fish in research, teaching and testing](#) (2005).

Monitoring nitrogen gas, or preferably total dissolved gases, is valuable. Supersaturation of nitrogen or oxygen may occur seasonally or in response to system issues, and can cause chronic health problems or even acute death in zebrafish. Total gas pressure should be no greater than 100% (Lawrence and Mason, 2012); higher levels can cause gas bubble disease.

Temporary measures that can be taken in emergency situations while the source of the problem is being identified include adding airstones to degas the water (see Section 3.1.1, “Oxygen”, for application of airstones), or turning off the system to stop the inflow of supersaturated water.

3.1.3 pH

Water pH should be maintained at a stable and optimal level, as changes in pH may influence other water quality variables, such as ammonia. Water pH within a tank fluctuates in response to fish respiration and the addition of nitrogenous compounds, which can vary with feeding practices and the health and age of the fish. It is important to monitor the pH of the water in the tanks regularly, using a colorimetric test kit or preferably, a precise electronic pH meter.

Systematic studies detailing growth and reproductive performance of zebrafish at different levels of pH have not been conducted; however, most laboratory facilities aim to maintain pH between 7.0-8.0 (Lawrence, 2007; Cartner et al., 2019). Others suggest aiming for 6.8-7.5 and not allowing pH to be lower than 6 or higher than 8 (Brand et al., 2002). If the pH falls outside of the target range, mitigation measures should be applied to ensure a slow change in pH as it returns to previous levels.

3.1.4 Salinity, Conductivity, Water Hardness and Alkalinity

Salinity, conductivity or water hardness should be measured in the supply water at least weekly, unless the water source has consistent conductivity/hardness (e.g., some deep wells). Salinity levels should be stable and maintained at <5 g/L (Harper and Lawrence, 2010, cited in Lawrence and Mason, 2012). Conductivity should be 200-3,000 $\mu\text{S}/\text{cm}$ (Cartner et al., 2019), although a narrower range of 300-1,500 $\mu\text{S}/\text{cm}$ is recommended by Avdesh et al. (2012). Total hardness (CaCO_3) should be stable at 75-200 mg/L (Lawrence and Mason, 2012; Avdesh et al., 2012; Cartner et al., 2019).

Alkalinity (CaCO_3) should be monitored to ensure the system has sufficient buffer capability to maintain good water quality when parameter changes occur (e.g., ammonia) or if there is a need to medicate the system. Total alkalinity should be stable at around 50-75 mg/L (Cartner et al., 2019); however, 50-200 mg/L may be acceptable (Lawrence and Mason, 2012; Avdesh et al., 2012).

3.1.5 Nitrogen Compounds

Nitrogenous compounds include ammonia, nitrite and nitrate. The sources of these compounds and the use of biological filters to support nitrifying bacteria in the conversion of ammonia to nitrite and then to nitrate are discussed in Section D.3.6, “Nitrogen Compounds”, in the *CCAC guidelines on: the care and use of fish in research, teaching and testing* (2005). The conversion of ammonia to nitrite (NO₂) and then nitrate (NO₃) is a process requiring two types of aerobic bacteria, *Nitrosomonas* and *Nitrobacter* (Fisher, 2000). Free ammonia and nitrite are toxic to fish and their accumulation must be minimized, while nitrate is much less toxic to fish (Learmonth and Carvalho, 2015).

When the system is being set up, ammonia, nitrite and nitrate levels in the source water should be relatively low. Target levels for on-going monitoring are: unionized ammonia < 0.05mg/L (or ppm), nitrite <0.1 mg/L and as close to 0 as possible, and nitrate < 50 mg/L (Cartner et al., 2019; Avdesh et al., 2012). Monitoring should be in line with the capacity of the life support system and performed at least twice a week for a recirculation system, or monthly for a flow-through system. Monitoring frequency for all systems can be monthly once the system is stable, unless there is a change in the system, a large increase in fish density, or a problem is detected. Monthly monitoring of ammonia and nitrite/nitrate can ensure the biofilter continues to be effective. While adding live plants to a tank may reduce the concentration of nitrogenous wastes, they pose other risks, such as the introduction of pathogens.

If a large increase in ammonia or nitrite is detected, a large water exchange must be carried out. Standard practice for this situation is to change 30-50% of the water per day while monitoring ammonia and nitrite, and continue daily water changes until the levels are suitable. Additional measures to reduce the accumulation of ammonia include increasing the flushing rate, reducing fish density or the temperature, or using ammonia-absorbent compounds in fresh water. As an emergency back-up in the event of biofiltration failure, resins and ion exchange apparatus can be used to remove ammonia.

3.1.6 Carbon Dioxide

Carbon dioxide (CO₂) is produced by fish during respiration and dissolves in water to form carbonic acid, thus lowering the pH. If allowed to accumulate above a certain level, it can increase the potential for hypercapnia, a condition of abnormally elevated CO₂ levels in the blood (Miller et al., 2014; Qin et al., 2010). Although high CO₂ concentration can be fatal to fish, in general this is not likely to be a problem in aquatic facilities with adequate ventilation. However, when recirculation technology is applied to high density fish culture systems, CO₂ may become the major limiting factor. Cartner et al. (2019) state that CO₂ should be <15-20 ppm, but kept as low as possible.

3.1.7 Toxic Agents

Provisions should be made to ensure toxic agents that are potentially harmful to fish do not enter the fish's environment. Section D.3.9, “Toxic Agents”, in the *CCAC guidelines on: the care and use of fish in research, teaching and testing* (2005) reviews common sources of toxic agents, and actions to be taken if there is reason to believe a hazardous material or infectious agent has accidentally entered the water system.

3.2 TEMPERATURE AND RELATIVE HUMIDITY

3.2.1 Temperature

Fish should be maintained stably within their preferred temperature ranges, and should be acclimated to any changes that occur. Zebrafish are classified as eurythermal, which means that they can tolerate a wide temperature range; however, the temperature range at which an animal can survive is different from its preferred temperature range. Maintenance at suboptimal temperatures will have a metabolic cost that may affect breeding, development and welfare.

In their natural habitat, zebrafish have been observed to survive at temperatures as low as 6°C in winter and over 38°C in summer (Spence et al., 2008). Studies in the laboratory have shown that wild-type zebrafish have a maximal acclimated thermal tolerance range¹ of 5.3-39.3°C (Cortemeglia and Beiting, 2006). Additionally, zebrafish raised in a variable thermal environment have shown a greater tolerance for temperature changes than those raised in a stable environment, but they also have smaller body size, suggesting an associated energetic cost (Schaefer and Ryan, 2006).

Zebrafish should be maintained at a stable temperature in the range of 26-29°C (Cartner et al., 2019; Lawrence, 2007). A water temperature of 28.5°C is typical and widely cited as the optimum temperature for breeding zebrafish (see Appendix 2 for a summary of recommendations in the literature). Rearing zebrafish at temperatures as low as 22°C or as high as 31°C from spawn to metamorphosis can skew sex ratios (Sfakianakis et al., 2012). Whatever the system of water exchange used, incoming replacement water should be the same temperature as the water it is replacing.

Changes in ambient temperature for fish are much more significant to many vital functions than for terrestrial animals. Susceptibility to diseases, parasites and toxicants is greatly affected by temperature, and the further the temperature shifts in either direction from the optimal range, the greater the potential for stress and disease.

When zebrafish do experience a viral infection, they have been found to move into warmer water if given the opportunity (a response known as behavioural fever), and this upregulates anti-viral genes (Boltaña et al., 2013). This response has also been found in larvae at 18-20 days post-fertilization (Rey et al., 2017). However, another study found zebrafish showed a reduced preference for warmer areas immediately following a stressful event (Jones et al., 2019).

3.2.2 Humidity

Humidity in the room should be controlled to prevent condensation and the growth of mold. In addition, external life support systems and other related system components must be appropriately protected from humidity.

From a fish welfare perspective, there is little need to control humidity levels in the room; however, room humidity can have an effect on temperature maintenance in the fish tanks. Dry rooms will have cooler tanks, or a larger temperature variation in tanks due to evaporative cooling at the surface.

¹ The specific figure slightly varies depending on the temperature at which the groups of fish had previously been acclimated. Also note that this study involved aquarium-sourced fish, rather than research lines.

3.3 LIGHT

Appropriate lighting facilitates good breeding success and minimizes stress for zebrafish (Villamizar et al., 2014). Light influences almost all physiological and behavioural processes in fish, including growth, development and reproduction. The response of fish to light is complex, and light often acts synergistically with other environmental factors, such as temperature, making it difficult to predict the effect that inappropriate lighting regimes can have on the fish or the experimental results (Stickney, 1994).

Fish are easily startled when light switches are activated at night. Where artificial lighting is used, a gradual brightening and dimming period of 20-30 minutes in the morning and evening can be incorporated to avoid startling the fish, rather than switching lights abruptly on and off (The Berlin Workshop, 1994, cited in Reed and Jennings, 2011). Alternatively, brightening and dimming can be created by turning on a small lamp (manually or automatically) before the room lights go on and leaving it on for a period after the room lights go off.

3.3.1 Photoperiod

A standard lighting regime of either 14 hours light:10 hours dark or 12:12 cycle should be used. Continuous 24-hour light or dark regimes must not be used. Having a night cycle that is completely dark has been shown to be important to reproductive success, and care should be taken to ensure that fish are not disturbed by night-time lighting entering through windows in the fish holding facilities. Even the presence of a red exit light can have a negative effect (Adatto et al., 2016). When possible, work should be conducted during the light phase. Where task lighting is needed for people working in the room, it should be restricted in its dispersion throughout the room.

Initiation of light triggers zebrafish to breed, so periods of darkness are important for allowing animals to rest (Vargesson, 2007; Brand et al., 2002). If the lights are left on continually, zebrafish will not lay eggs (Francis, 2008). Screening that occludes light from entering the tank (e.g., a curtain or red film) can be used to protect the fish's natural photoperiod.

The importance of circadian rhythm in zebrafish is discussed by Frøland Steindal and Whitmore (2019). Light-dark preference in zebrafish is influenced by their circadian clock (Wang et al., 2014), and this can be affected by ambient light levels as well as olfactory stimulation, such as the presence of food (Stephenson et al., 2011). Fish facilities should have timed lights to switch on and off at consistent times each day to mimic natural circadian cycles found in the wild.

Zebrafish larvae alter their movement in response to changes in light levels, and this has been suggested to be linked to optimizing opportunities to feed (Burgess and Granato, 2007). However, zebrafish larvae reared in constant light have been observed to show severe deficits in visual acuity and behaviour, though no anatomical abnormalities (Bilotta, 2000). They appear to be able to recover from the effects of early rearing in abnormal lighting if they are subsequently housed under a normal light cycle (Bilotta, 2000). Constant darkness has been shown to delay general development of embryos, with hatching not observed at 7 days post-fertilization (Bilotta, 2000). Larvae kept under constant darkness died within 18 days of hatching, but the survival rates improved if they were transferred to a light-dark cycle within 5-10 days of hatching (Villamizar et al., 2014).

3.3.2 Spectrum

Light spectrum appears to affect the development of zebrafish following hatching. Hatching rate has been shown to be highest under blue and violet light, while the total length of zebrafish at 30 days after hatching appears to be greatest under blue, white, and violet light (Villamizar et al., 2014). Red light has been shown to result in reduced feeding and poor survival, while constant white light or a light-dark cycle with violet light resulted in a higher proportion of malformations (Villamizar et al., 2014).

Adult zebrafish have the necessary mechanisms for colour vision (Saszik et al., 1999), but no specific requirements with regard to the light spectrum of their environment have been determined. One study has indicated that “cool white” fluorescent light (colour temperature 4,100 K) can lead to an increase in immune function and inflammation (Gonzalez et al., 2018); however, more research is needed. Until further research is available to guide light selection, standard laboratory lighting is acceptable (Matthews et al., 2002).

3.3.3 Intensity

Little, if any, research has been carried out to determine the effect of light intensity on zebrafish health and welfare. Matthews et al. (2002) have cited a broad range of 54-324 lux as being appropriate at the surface of the water. Some facilities maintain a low intensity of lighting with the aim of minimizing algal growth in tanks. Further evidence is required before any particular regime can confidently be considered most beneficial.

3.4 SOUND AND VIBRATION

Zebrafish appear to grow accustomed to their surroundings and can habituate to certain vibrations, but this does not mean they are unaffected by them (e.g., Vandenberg et al., 2012). They can also react strongly to loud noises or novel vibrations, and steps should be taken to avoid such disturbances. Spawning in zebrafish may be affected by noise or other types of disturbance (Vargesson, 2007). The sensitivity of zebrafish to sounds such as talking or music is uncertain (Matthews et al., 2002; Barcellos et al., 2018).

3.5 SECURITY AND ACCESS

Facilities must have SOPs for access that addresses biosecurity risks. Access to fish facilities should be designed to minimize traffic through the area, and should be restricted to authorized personnel required for facility maintenance, the care of the fish, and the use of fish in approved studies. A security system for physical access to the facilities should be in place that is appropriate for an aquatic system. Environments with high humidity can cause problems for card access and keypad security systems.

3.6 PERSONNEL

Guideline 4

Sufficient numbers of competent¹ personnel must be available for daily care and observation of the fish and the safe operation and maintenance of the water and life support systems, 365 days a year.

The inherent nature of aquatic facilities requires that there be constant maintenance, repair and upgrading. Competent personnel need to be on call and accessible 24 hours a day, 7 days a week, and facilities must have a method for assuring a rapid emergency response outside of normal operating times.

¹ Competency is defined in the [glossary](#).

4 PROCUREMENT

4.1 SOURCE

Fish should be obtained from a captive colony bred for scientific purposes, with a defined health status, and preferably known genetic history. For environmental reasons, animal health and welfare, and the quality of science, it is better that fish are obtained from captive colonies bred specifically for research purposes. Pet store sourced fish are generally carriers of numerous disease agents and have unknown genetic background and rearing history. As noted in the [CCAC guidelines on: procurement of animals used in science](#) (2007), “animals used for scientific purposes should not be obtained from pet stores or their suppliers due to the potential health risks associated with disease transmission and the potential health problems for these animals themselves”. Exceptions to this guideline include if experimental protocols require aquarium suppliers or collection sourced fish, or if there are no alternatives for a particular species of fish (e.g., some tropical non-zebrafish species).

Institutions using zebrafish are encouraged to establish liaisons with others to facilitate the sharing of strains. As zebrafish can be bred and reared under laboratory conditions and there are limited commercial sources for research-grade fish, many institutions routinely breed their own (the potential for genetic drift and/or genetic selection in isolated colonies is discussed in Section 1.4, “Sources of Variation”). In addition, a large range of specific zebrafish mutants and wild-type strains can be obtained from other laboratories or stock centres (Matthews et al., 2002). Even a modest facility can generate millions of embryos per year (Goldsmith and Solari, 2003), and many researchers and facilities will provide fertilized eggs of strains they hold. Institutions receiving zebrafish must determine any applicable requirements of the Material Transfer Agreement for the original license holder of the line.

To improve animal welfare and reduce health risks, exchange of embryos that have been surface sanitized using a process that has been documented as effective is a safer option than exchanging adult fish between laboratories. Matthews et al. (2002) suggest surface sanitizing embryos with a mild bleach solution (35 mg/L sodium hypochlorite) for five minutes; however, spores of some fish microsporidia are highly resistant to chlorine (Ferguson et al., 2007). Iodine can also be used (Chang et al., 2016). It should be noted that surface sanitization is ineffective at removing all pathogens, and additional screening, breeding and biosecurity measures should be taken.

4.2 REGULATIONS

Guideline 5

Facilities and investigators acquiring or transporting fish, or conducting research on fish, must be familiar with, and comply with, relevant international, federal and provincial/territorial legislation and policies.

It is important to verify current legal requirements with the regulatory agencies to ensure compliance. An aquatic animal health import permit must be obtained for all shipments of zebrafish imported into Canada.

The Canadian Food Inspection Agency (CFIA)'s *Health of Animals Act* and Regulations and the *National Code on Introductions and Transfers of Aquatic Organisms* provides the basis for federal and provincial/territorial regulations and policies related to the movement of live aquatic organisms. The Code is implemented through Introduction and Transfer Committees, which may issue transfer licenses with conditions listed (e.g., containment requirements). The *Health of Animals Regulations* is administered by the CFIA and Aquatic Animal Health Import Permits are issued by the CFIA's Centre of Administration.

The CFIA has developed the *Containment Standards for Facilities Handling Aquatic Animal Pathogens* (CFIA, 2013), and quarantine standards for import of aquatic animals originating from countries with an unknown animal health status.

Institutions planning to import zebrafish from outside Canada must meet CFIA import requirements. These requirements were implemented in 2011 as a result of this species being listed in the OIE *Aquatic Animal Health Code*, as a susceptible species for the spring viremia of carp virus (SVCV) (World Organisation for Animal Health – OIE, 2019). The specific import requirements depend on the country of origin and can be found in CFIA's Automated Import Reference System. Information on obtaining an aquatic animal health import permit, and on becoming a CFIA-approved quarantine unit is available on the CFIA website. Information on becoming a CFIA-approved Aquatic Containment Level (AQC) ACQ level 2 *in vivo* or AQC small scale (AQCss) *in vivo* containment laboratory is available by contacting the CFIA's Office of Biohazard Containment and Safety.

Genetically modified organisms are regulated under the *Canadian Environmental Protection Act* (CEPA) *New Substances Notification Regulations (Organisms)* [NSNR(O)]. Use of genetically modified fish must be notified under NSNR(O) prior to import or manufacture. However, genetically modified fish used for research and development are exempt from this notification under Section 2(4) of NSNR(O), provided there is no release of the organism or its genetic material from the facility to the environment. Further information can be found on the NSNR(O) fact sheet (Government of Canada, 2017).

For shipping fish by air, the International Air Transport Association (IATA) produces the *IATA Live Animal Regulations* annually, which includes information concerning documentation, containers and other requirements for humane transportation of live animals. While the IATA specifically provides information for air transportation, this information is also useful for land transportation.

4.3 PRE-SHIPMENT PROCEDURES

Prior to importing zebrafish from outside Canada, the importer must obtain an aquatic animal health import permit from the CFIA's Centre of Administration and at the time of application indicate if the shipment will be:

- accompanied by a zoosanitary certificate from the country of origin;
- imported into a CFIA pre-approved quarantine unit; or
- imported into a CFIA-approved AQC level 2 *in vivo* containment laboratory or AQCss (small scale) *in vivo* containment laboratory.

Information relating to the transport, welfare and care of the fish must be communicated between supplier and receiver before shipment of fish takes place. Sufficient preparation and communication between the supplier and receiver regarding obtaining a zoosanitary certificate to meet the CFIA's import requirements (if applicable), the transport itinerary and conditions, and the breeding history, dietary background and health status (for pathogens other than SVCV) of the fish should take place before shipment. There must also be assurance that suitably trained and informed animal care personnel will be on hand to receive the shipment and check the health and welfare of the fish upon arrival.

Ideally, zebrafish should be transported during temperate months, as transport in hot summer temperatures is associated with an increased risk of mortality. An insulated container should be used for transport, and during winter months an appropriate heat pack should be included.

4.4 TRANSPORTATION

To avoid the potential risks to health and welfare associated with transport, zebrafish should be shipped as embryos.

Fish bred off-site and transported to the place of use may have to endure a long journey, sometimes from another country or continent. Extended travel times have the potential to cause health and welfare problems for fish, for example, the stress of transportation can adversely affect the immune system (De Tolla et al., 1995). Where in-house breeding is not possible, the transport of fertilized eggs or early larvae (which will arrive at the receiving institution before 5 days post-hatch) is recommended in preference to juvenile or adult fish.

4.4.1 Packing and Insulation

When transporting zebrafish, the potential to cause injury or stress must be minimized. The following guidance should help achieve this (adapted from Matthews et al., 2002):

Adult Fish

- Fish should be double-bagged in a good quality, plastic, fish bag at a density of about 10 fish/ 1.9 L (a half gallon) of water.
- The bag should be about two-thirds full of air or pure oxygen (pure oxygen is a better choice if available, as it will not deplete as rapidly if there are delays in transit).
- Food should be withheld for a day before shipping, so that fish will produce less ammonia while confined.
- An ammonia sequestrator can be added to detoxify any ammonia that is produced during transport.

Eggs and Larvae

- Eggs that have been surface disinfected (using bleach or iodine) should be packed in a 250-500 ml tissue culture flask at a density of no more than 1-2 eggs/ml of sterile water (or preferably a sterile embryo media).
- The container should be filled with 50% system water or embryo media (which allows air to form the remaining 50% of the space).

- Methylene blue (0.5 mg/L or 0.5 ppm) can be added to the solution to reduce fungal growth.
- The packing box should be insulated and any extra space filled with packing chips or similar packing material.

4.5 RECEIVING FISH

As noted in Section 4.4, “Transport”, zebrafish should be shipped as embryos. For those situations where shipping adults or juveniles is approved by the animal care committee, the following guidelines apply.

Guideline 6

The health and welfare of the fish must be checked upon arrival by competent¹ animal care personnel and the fish must undergo quarantine and acclimation for a period appropriate to assure the health of the fish.

If animals are to be imported into a CFIA-approved quarantine unit, all approvals must be obtained prior to receipt of fish. An appointment must be made with the responsible CFIA Veterinary Inspector for an inspection at the time of arrival. Visit the CFIA website for information regarding the [location of CFIA Animal Health offices](#).

If animals are to be imported into a CFIA-approved containment laboratory, no veterinary inspection is required. However, if the disease status of the fish is not known, liaising with zebrafish-specific diagnostic or veterinary facilities is important to determine the presence of zebrafish-relevant pathogens.

It is important to know the water temperature and quality upon arrival to facilitate acclimation of the fish to the conditions in the receiving facility. If water quality allows, a gradual change is preferred. However, the stress of abrupt changes must be balanced with risks to fish health when maintained in poor quality water.

On arrival in a facility, new fish should be handled as little as possible, and care should be taken to prevent thermal shock (Wedemeyer, 1996). For practical purposes, thermal shock may be defined as an abrupt change in temperature of more than 2-3°C. The pH of the water should also be determined, along with the ammonia level, prior to releasing the fish to make sure the change will not be too great.

The unopened transport bag should be floated in water of the same temperature as the receiving water. The temperature of the water inside the bag can be determined by a temperature gun or by folding the bag around a probe. Once there is no risk of thermal shock, the bag can be opened, the bag water poured through a net and disposed of in a biosecure manner, and the fish transferred to the receiving environment. For animal health purposes and to prevent transmission of disease, it is preferable not to introduce the transport water into the receiving environment, particularly if the receiving environment contains other animals, unless the fish have been certified as free of disease.

While some have suggested opening the transport bag and gradually adding small amounts of the receiving water while it is floating in the receiving tank to prevent sudden change, this is generally not necessary and may potentially compromise the welfare of the fish. Gradual transfer of the fish can increase the risk

¹ Competency is defined in the [glossary](#).

of asphyxiation or hypoxia stress. If ammonia levels in the transport bag are high, chemical changes in the water that result in ammonia becoming more toxic can occur immediately when the bag is opened. The addition of an ammonia sequestrator (mentioned above with regard to packaging) and airstones (see Section 3.1.1, “Oxygen”) may be useful.

4.5.1 Quarantine and Acclimation

Once the health and welfare of zebrafish have been checked upon arrival, the fish must be closely monitored and isolated from existing resident colonies to avoid introducing disease. A combined approach for acclimation and quarantine should be used, as far as possible, so that both are accomplished simultaneously. Acclimation involves ensuring a gradual adjustment of the living conditions for the fish, before any experimental manipulations are performed. In general, fish brought into a facility should be allowed to adjust to their new environment (including water quality, temperature, illumination and diet). Human activity and handling should be minimized during acclimation. This period should also ensure that any problems related to the stress of transport (e.g., anorexia, unanticipated morbidity and mortality) have been resolved.

If the fish are to be imported into a CFIA-approved quarantine unit, the CFIA import quarantine standards must be met and verified by a CFIA veterinary inspector prior to obtaining an aquatic animal health import permit and importing the fish. These standards include mitigation measures that address the main risk pathways for disease introduction (animals, water, food, fomites, and vectors), and include release from the quarantine once negative results for SVCV are obtained. Visit the CFIA's website for [additional information on their quarantine process](#).

If the animals are to be imported into a CFIA-approved AQC level 2 or AQCss containment laboratory, the applicable *Containment Standards for Facilities Handling Aquatic Animal Pathogens* containment level requirements must be met (CFIA, 2013), resulting in CFIA certification. In the case of AQCss containment, CFIA-issued compliance must be met. Transfer of susceptible species is only permitted with CFIA written approval between a CFIA-approved containment laboratory at the same or higher initial containment level.

If the zebrafish are approved for importation into Canada without requiring CFIA quarantine (animals come with disease certification), or if fish are being moved domestically, then the following guidelines on general quarantine are to be followed. General quarantine is defined as a period or place of isolation for the fish. The following are also important guidelines to adhere to when fish are imported under CFIA requirements.

The purpose of general quarantine after receipt of shipments of fish is to isolate those fish from the main populations in the facility, to permit observation and testing if necessary, until such time as the newly arrived fish are determined to be healthy and free from communicable disease. Newly arrived fish may bring pathogenic organisms with them, either in an active or subclinical state, to which the resident populations have not been exposed. As a result of the stress of handling and crowding during transport, the fish's immune system may be depressed and a disease outbreak may occur.

New stock should undergo general health screening, some of which may be influenced by the aims of the research. For example, testing for *Pseudoloma* spp., which is transferred vertically between generations (i.e. passed from maternal parent to offspring), is important for studies where its presence may impact results (e.g., behavioural and neurological research) (Spagnoli et al., 2017), but may not impact other types of research.

The importance of quarantining newly-arrived fish away from those already present in an institution, in order to reduce the opportunity for transmission of infection or disease, is widely agreed upon. However, the period of general quarantine is debated, with suggestions ranging from 2 weeks to at least 30 days for all fish (Vargesson 2007, De Tolla et al 1995). When determining an appropriate period for each incoming batch of fish, consideration should be given to the source of the supply, their life history, and the housing system in use at the receiving institution. Minimum quarantine time should be established based on the holding temperature, source of fish, and the anticipated timeframe for expression of the pathogens of concern. If there is a communicable disease present, it is more likely to be observed following transport stress; however, quarantine without testing does not guarantee freedom from any communicable fish disease. When fish are received from a health-certified source, the duration of the quarantine period may be decreased depending on the health status, but quarantine is highly advisable for any fish introduction regardless of the source.

Fish under quarantine should be monitored with increased frequency, and records should be kept of any health problems detected, with documentation of the response.

Quarantine is primarily a measure to ensure that fish are isolated and sanitary measures are put in place to ensure there is no escape of viable pathogens or their hosts from the facility and into the surrounding waters, or transfer of pathogens to other animals in the facility. Information specifically for containment of organisms used in disease studies or incidentally infected with transmissible diseases is given in *Containment Standards for Facilities Handling Aquatic Animal Pathogens* (CFIA, 2013).

Quarantine must involve the isolation of fish, ideally in separate areas. The water supply must be separate so that water from quarantined tanks is not circulated to other tanks. Effluent should also be separate when there is a need to treat the water prior to recirculation or release.

Quarantine areas should be managed according to rigorous biosecurity practices. Rigorous SOPs for disinfection should be in place for general cleanliness and to avoid the potential transfer of pathogens to the main areas of the facility. Particular vigilance should be paid to practices such as dedicated accessories (e.g., nets and feeding bottles) and hand implements, effluent disinfection, footbaths, hand washing stations, and clean to dirty traffic flow in the quarantine area. Quarantine areas should be serviced after other rooms containing fish.

As a precaution, fish from external sources should be introduced into a facility as surface sanitized embryos. Matthews et al. (2002) offer the following protocol for introducing the eggs of new fish to the general population: “Once introduced into their new tank, the quarantined fish remain there for three to four weeks. The new fish are mated and the embryos are surface-sanitised with a mild bleach solution (35 mg/L sodium hypochlorite for five minutes). Only these sanitised embryos will be introduced into the main aquarium facility.” While this is a commonly used disinfection dose, it may be insufficient for the disinfection and prevention of the transmission of SVCV and other zebrafish relevant pathogens (e.g., *Pseudoloma*).

5 BROODSTOCK AND BREEDING

Holding systems and environmental conditions for broodstock must be appropriate for the species. Particular attention should be paid to the importance of environmental cues for the maintenance (or manipulation) of endogenous reproductive rhythms. Environmental factors, such as temperature, light cycle, light intensity, habitat/tank design, nutrition (i.e. a high-quality diet for egg production), holding density, and sex ratio, are all critical to reproductive success.

For broodstock, a strict disease and health control program must be implemented with veterinary oversight to ensure the production of healthy progeny and prevention of disease transfer through water sources, fish or eggs.

Guideline 7

Breeding must be managed to minimize inbreeding and genetic drift and to increase genetic diversity.

Considerations for the propagation of a desired line of zebrafish, including pair mating versus group crosses and inbreeding versus outbreeding strategies, are discussed by Nasiadka and Clark (2012) and Monson and Sadler (2010). Additional information is also provided by Lawrence (2011). Important aspects of the propagation of wild type lines are discussed by Nasiadka and Clark (2012).

As noted in Section 1.4, “Sources of Variation”, there is the potential for genetic drift and genetic selection in isolated colonies over time, which can result in the genetic background of standard lines being different among different institutions (e.g., Lange et al., 2013).

5.1 BREEDING

Mating behaviour and breeding of zebrafish in their natural habitat is outlined in Section 1.1, “Behavioural Biology”. For additional information, see Engeszer et al. (2007).

Ovulation in zebrafish is thought to be induced via the presence of male gonadal pheromones in the surrounding water (van den Hurk and Resink, 1992). The dark part of the diurnal cycle allows zebrafish to rest and the return of light will trigger fish to breed (Vargesson, 2007), allowing for gathering of age-matched embryos.

Zebrafish are broadcast spawners that release eggs and sperm in a cloud over the substrate (Ruhl et al., 2009). Female zebrafish will release eggs directly onto a bare substrate, but when provided with an artificial spawning site, such as a plastic box filled with gravel or marbles, they preferentially use it (Spence et al., 2006a).

Marbles have been used to prevent the fish from eating their eggs (Matthews et al., 2002); however, it is now more common to use spawning tanks or mass spawning chambers with inserts that have perforated bottoms for this purpose (Nasiadka and Clark, 2012), particularly as spawning tanks designed for zebrafish

are available. In spawning tanks greater success has been reported with groups of up to five fish, compared to single mating pairs (Lawrence, 2007).

When zebrafish are bred in a spawning tank, the water variables can change significantly over night and precautions should be taken when returning the fish to their original tank.

A generalized description of the techniques and equipment used in relation to the spawning process can be found in Lawrence (2007):

- a small (<1 L) plastic cage or box with a mesh or grill bottom is placed inside a slightly larger container that is filled with water;
- breeding pairs or small groups of fish are added to the cage or box in the evening; and
- when the fish spawn (usually the following morning), the fertilized eggs fall through the ‘floor’ of the inner cage or box, preventing the fish from eating them.

Egg production per fish is typically highest in small shoals (Kurtzman et al., 2010). However, shoaling is not practical where 1:1 pairings are required (e.g., heterozygote screens, developmentally time-critical studies, or studies involving embryo microinjection). If timing of embryo production is critical (e.g., for microinjection of embryos), dividers can be used to keep males and females separate until a specific time for spawning. Wild-caught zebrafish held in tanks have been observed to reproduce only for a 1-2 hour period in the morning (Hutter et al., 2010).

Unlike many temperate fish species, zebrafish do not require a seasonal change in day length to bring them into a breeding state. When maintained under laboratory conditions, zebrafish can be encouraged to breed throughout the year, with females spawning every one to three days and releasing all mature ova within a single hour (Matthews et al., 2002; Spence et al., 2006a).

Eggs have a diameter of about 1.0-1.5 mm (Matthews et al., 2002). A female generally produces 70-300 transparent eggs in a single spawning, of which at least 80% are fertilized (Brand et al., 2002); although the number of eggs can range from a few to over 1,000 (Reed and Jennings, 2011). Females consistently spawn more frequently, and produce larger clutches of eggs with some males than others; however, this effect does not appear to be related to male dominance rank (Spence and Smith, 2006). Ruhl et al. (2009) observed that eggs were significantly more likely to be absent in tanks in which aggressive interactions had occurred between fish (either between competing males or between the male and the female). For a review of studies on mate choice, see Nasiadka and Clark (2012).

Guideline 8

For female zebrafish, there should be an interval of at least one week between breeding attempts.

Although zebrafish females are capable of spawning on a near daily basis (Lawrence, 2007), a female that lays eggs daily is unlikely to produce a good quantity of eggs, nor good-quality eggs. The full impact on the fish of maintaining such a rigorous egg production schedule for more than two to three weeks has yet to be evaluated (Kurtzman et al., 2010). Given the likelihood that such a regime places a significant metabolic cost on females, some suggest they should not be bred more than once every one to two weeks to maintain a healthy, fecund breeding population (Kurtzman et al., 2010). The breeding interval is less of a concern for males, as the metabolic energy cost is not as high as it is for females.

When kept in mixed-sex groups, fish mate naturally and therefore it is not necessary to maintain a breeding schedule when embryo production is not needed (for information on breeding strategies, see Westerfield, 2007). However, when sexes are kept separately this baseline breeding frequency must be maintained, even when demand for embryos is low, to avoid the visceral cavity of gravid females from becoming inflamed due to excessive egg retention (Kent et al., 2016).

There is a need to more fully evaluate the health and welfare effects of encouraging females to spawn over a range of time intervals. For laboratory breeding, it is likely that the frequency at which eggs of good quality can be collected from females, and the impact of this process on the animals, is heavily influenced by water quality and nutrition.

Sessa et al. (2009) found that simulating the natural environment of zebrafish by tilting the breeding tank to produce a depth gradient (simulating stream banks) had a positive influence on embryo production, and Wafer et al. (2016) found that environmental enrichment in the form of plastic grass positively affects reproduction under certain conditions.

The sex of zebrafish is seemingly determined by a combination of genetic and environmental factors. There is a tendency, at least in some strains, for male offspring to significantly out-number females unless inbreeding is eliminated by continually introducing a few individuals from other sources into the fish breeding stock (Overstreet et al., 2000). Additionally, water temperature during the period from spawning until after metamorphosis affects the sex ratio of zebrafish, with lower temperatures (22°C) favouring the production of males and higher temperatures (31°C) producing a larger proportion of females (Sfakianakis et al., 2012).

5.2 EMBRYO HARVESTING

Obtaining good quality embryos is important for meeting research objectives and minimizing the number of fish used. The number of eggs laid is less important than the quality of the embryos. Since the majority of zebrafish embryos produced at institutions are for research purposes rather than reproduction, ‘quality’ typically refers to the ability of the embryos to remain viable after experimental manipulation, such as pharmacological treatment or microinjection. For most research involving zebrafish embryos (e.g., developmental biology, anatomy, and genetics studies), it is particularly important that a high percentage (>80%) of the eggs are fertilized successfully, that the embryos undergo a clean and even first cleavage, and that they remain normally developed at gastrulation. Healthy eggs and embryos have a translucent, yellowish appearance (Pelegri, 2002).

Numerous husbandry variables can be manipulated to improve embryo quality, including diet, lighting, water salinity, water flow, frequency of tank cleaning, tank size, frequency of embryo collection, and age of females (Reed and Jennings, 2011). Embryos should be kept at the same temperature as adults (i.e. 28.5°C). Hygiene is especially important as zebrafish embryos are highly susceptible to fungal infections (Overstreet et al., 2000). Embryo quality can have a direct effect on the quality of scientific data, and researchers are encouraged to share and publish information regarding factors that affect embryo quality.

5.3 MANUAL EXPRESSION OF EGGS FROM FEMALES

It may be necessary to manually express eggs from females for certain procedures (e.g., when producing haploid or clonal diploid animals, when using reconstituted frozen sperm, where synchronous fertilization is a requirement of the study, or where females are not engaging in mating). If eggs are to be manually expressed, fish must first be anesthetized.

This process must only be carried out by people experienced and trained in the manual expression of eggs, or someone overseen by an experienced person. Individuals must be assessed as competent prior to manually expressing eggs from zebrafish, as injury to fish can be caused easily through improper handling or incorrect or too heavily applied pressure. To protect the health of the fish, unpowdered, non-textured gloves should be worn during handling.

Pelegri (2002) illustrates the typical egg expression process. Once under anesthetic, fish can be removed from the water using a plastic spoon and placed onto a lightly dampened paper towel to absorb excess water. The fish can then be transferred to a small plastic dish. Fingers must be slightly dampened. One finger is placed on the dorsal side of the fish and a finger of the other hand is used to express the eggs by gently pressing on the ventral side of the fish, starting just behind the pectoral fins and moving toward the tail. Only gentle pressure is needed. If the fish has eggs, they will come out easily.

5.4 OBTAINING SPERM FROM MALES

Sperm collection must only be performed by people competent in the procedures, which involve either dissecting the testes of euthanized males or collecting milt from live, anesthetized males. Pelegri (2002) provides protocols for both methods and suggests that the first method is more reliable, less laborious and requires fewer males (a larger volume of sperm can be obtained from dissected testis than from an anesthetized fish). Dissecting the testes of euthanized males also has less potential for individual animal pain or distress, as the fish do not have to be manipulated and single or repeated recovery from anesthesia is not required. To provide enough sperm to fertilize about 40 egg clutches, 40-60 males would be needed if expressing the sperm from anesthetized males, while only 10 males would be required if using the testes of dissected males (Pelegri, 2002).

5.5 AGE OF FISH FOR BREEDING

Both males and females typically reach sexual maturity within two to six months of hatching (Nasiadka and Clark, 2012); two months of age is common when rotifer feeding protocols are followed (Lawrence et al., 2015; Best et al., 2010; Lawrence et al., 2012). Although institutions may begin using fish for breeding at this age (Kurtzman et al., 2010), initial batches of eggs from such young females may not be of optimal quality. The highest number of embryos is reported to be obtained from females between 6-18 months of age (Vargesson, 2007; Nasiadka and Clark, 2012), with optimal production from age 6-12 months (Nasiadka and Clark, 2012).

Males can be maintained in breeding stocks until they are approximately one year old and then replaced with younger breeders (even though they are capable of producing sperm, albeit at reduced levels, beyond one year of age) (Kurtzman et al., 2010). Females can be maintained in breeding stocks until 18 months old.

5.6 CARE OF LARVAL ZEBRAFISH

Rearing zebrafish from the time the larvae first feed independently until they develop into juvenile fish is particularly challenging due to the high mortality rate during their transition from skin metabolism to gill and organ function (Lawrence et al., 2016; Best et al., 2010). Although there have been improvements in commercial food for zebrafish, live-food may reduce the risk of starvation among larval zebrafish, as they readily hunt live prey in the wild and may not ingest dry food. Rotifers and paramecium are live prey options

for zebrafish less than 10 days old. Lawrence et al. (2016; 2012) and Best et al. (2010) describe procedures for the production of rotifers within the same tank as larval zebrafish to provide a preferred, nutrient-rich food source that can result in greater survival and/or growth than rearing on stage-specific commercial food. Early larvae can also be fed newly-hatched brine shrimp once they are large enough to consume it (usually after 10-12 days old, depending of the size of the fish).

5.7 RECORD KEEPING

Record keeping is extremely important to ensure effective and efficient management of breeding and broodstock, including detection and spread of disease, and for reproducibility of research experiments. The age and strain of the fish and the name of the investigator should be recorded on tank labels. If this information is the same for all tanks in a room, it can be recorded on the door, rather than on each tank. Similarly, if all tanks in a rack or room belong to one investigator, the name of the investigator can be labeled at that level rather than on every tank.

Tank labels are important as a quick visual reference, but additional records are necessary for managing breeding programs. It is useful to know whether fish were successfully bred and when they were last bred. This is typically recorded at the tank level, indicating that possibly only some of the fish in that tank were successfully bred. Records kept at the room level for fish involved in a breeding program should include the parents' genotype and information about the date of fertilization.

A complete tracking system of all derivatives of the fish, which includes husbandry records and any records of experimental procedures, provides important information for use in addressing the Three Rs (particularly reduction and refinement). Fish involved in breeding must be documented within the institution, and reported at least annually to the animal care committee as part of protocol renewal. Annual reports should include information on the breeding strategy, reproductive performance (including any sudden change that could indicate altered genetic background of the line), morbidity and mortality, criteria used for culling fish, and other factors related to the way the breeding program is managed, with the aim of remaining within the number of fish designated in the animal use protocol. Where there are numerous inbred strains in a facility or room, record keeping can be complex and computerized systems may be useful.

Where investigators have approval to undertake breeding, they must demonstrate competence in managing breeding and demonstrate that they and their personnel keep complete records. Husbandry logs must be available to the veterinary team, animal care committee and animal care personnel to ensure appropriate procedures are followed. For general husbandry records, see Section 6.8.3, "Records".

HUSBANDRY

Good fish husbandry requires attention to detail and the rigorous and consistent performance of routine tasks. The importance of a high standard of husbandry cannot be over emphasized and should be regularly reinforced to personnel responsible for fish care.

6.1 IDENTIFICATION

An individual fish must only be tagged if it is required as part of the research protocol or husbandry management. The least invasive method of responding to the need for identification should be used.

Marking techniques can affect animal welfare through the act of marking itself, through the wearing of the mark and/or through the procedures required for observing the mark (Mellor et al., 2004). If marking individual fish is necessary, the method of identification must:

- cause minimal suffering or impact on the animal, both during the marking process and subsequently;
- last an appropriate time (dependent upon the duration of the study);
- be reasonably quick and simple to apply; and
- be easy and quick to read/identify.

While the literature concerning pain in fish is contentious (Chatigny, 2018), the precautionary principle should be applied: fish should be assumed to perceive pain in a way analogous to mammals, and therefore anesthetics and analgesics should be considered for methods that may cause pain (see Section 10.4, “Anesthesia and Analgesia”).

Table 1 lists possible identification methods. The optimal method will depend on the experimental conditions and outcomes. The available methods must be reviewed by the investigator and approved by the animal care committee.

Table 1 Methods of Identification in Order of Preference

METHOD	POINTS TO CONSIDER	REFERENCES
Subcutaneous dye injection	<p>Appropriate in clear or light coloured fish.</p> <p>Marks can be distinguished for approximately 30 days.</p> <p>Does not appear to affect social interactions, such as shoaling behaviour.</p> <p>Fish must be anesthetized during the procedure.</p>	Cheung et al., 2014

METHOD	POINTS TO CONSIDER	REFERENCES
Elastomer marking	<p>An elastomer material containing pigment is injected in liquid form beneath an area of translucent skin. Over a short period this becomes a pliable solid.</p> <p>Fish must be anesthetized during the procedure.</p>	Hohn and Petrie-Hanson, 2013
Fin clipping	<p>Small clip(s) from different fins or at different positions can be used to identify individuals within tanks.</p> <p>Fins can regrow quite rapidly.</p> <p>Fins are innervated, so clipping could be painful. Fish must be anesthetised during the procedure.</p>	<p>Delcourt et al., 2018</p> <p>Schroeder and Sneddon, 2016</p>
Radio frequency identification (RFID) microtags	<p>Where necessary, microtags may be used but less invasive methods are preferred. There is the potential for fish mortality, loss of microtags and the inability to read tags.</p> <p>Intracoelomic implantation of microtags (1 mm diameter, 6 mm length, ~10 mg mass) in juvenile zebrafish has been shown to result in 82% survival rate and microtag loss of 11% after 5 1/2 months, with no negative effects on growth or behaviour. Success rate for reading microtags was 73% for zebrafish weighing 350-450 mg (26 mm in length).</p> <p>Fish must be anesthetized during the procedure.</p>	<p>Cousin et al., 2012</p> <p>Delcourt et al., 2018</p>

Other more invasive methods are available (e.g., tattooing); however, their use must be justified to the animal care committee.

6.2 ANIMAL OBSERVATION

Fish must be observed daily for signs of illness and changes in behaviour. This is most easily accomplished during feeding. See Section 8, “Welfare Assessment”, for details.

6.3 HOUSING MANAGEMENT

Guideline 9

Zebrafish must not be individually housed without scientific or veterinary justification and should be housed at densities appropriate to their life stage.

Zebrafish are highly social animals and engage in shoaling behaviour in the wild (Graham et al., 2018a). In the laboratory, they have shown a preference for shoaling with other fish, regardless of shoal composition or even species, rather than being on their own (Ruhl et al., 2009). Important social interactions occur during

shoaling and spawning (Spence and Smith, 2007). Observation in the laboratory of “heightened-shoaling”, characterized by tight groups with synchrony of behaviours and low agonism, suggests that it may be an internally driven behaviour that may confer positive emotional benefits (Franks et al., 2018).

If fish are to be housed alone or in pairs, the addition of enrichment items such as floating plastic plants may help minimize stress once the fish have adapted to their presence, which could take several days (Keck et al., 2015; Collymore et al., 2015). The effects on water flow dynamics also need to be considered when enrichment items are added (see Section 6.5, “Environmental Enrichment”).

Female zebrafish have shown a preference to be with a group of three other fish, as opposed to one other fish, regardless of the sex of the fish (Ruhl et al., 2009). Observation of female zebrafish in large tanks has shown that they avoid staying alone and prefer to be with one or two males without other females present; they were found in female-only groups only 5% of the time (Delaney et al., 2002). Females can behave aggressively towards each other and develop a dominance hierarchy. There does not appear to be a significant difference in aggression among females between the morning and afternoon, suggesting dominance is not linked to the spawning period and may play a general role in maintaining social structure among females (Paull et al., 2010). This behaviour is density dependent and occurs at low densities (Lawrence, 2011).

Ruhl et al. (2009) observed that single males apparently preferred shoaling with single females rather than groups of three. In the laboratory, males appear to change female partners on a daily basis and social grouping can influence egg production (Delaney et al., 2002). While dominance hierarchies occur for both males and females, male aggressive behaviour is usually limited to the spawning period (approximately one hour after lights are turned on in the laboratory); they often shoal together at other times of day without incident (Spence and Smith, 2005; Paull et al., 2010). Aggressive territoriality as a normal feature of spawning behaviour does not usually cause physical harm; however, chasing and biting may result in the shedding of scales (Ruhl et al., 2009). Displays by territorial males are usually brief and serve only to deter others from approaching the spawning site (Spence and Smith, 2005). For other species of small warm-water fish, male aggression may be a greater concern.

In the laboratory, males appear to display different rates of aggression depending upon the number of other males in close proximity. At low densities, territorial males follow and actively court females, periodically returning to the spawning site. At high densities, territorial males confine their activities to within a few body lengths of the spawning site and vigorously defend the area from other males (Spence, 2007, cited in Reed and Jennings, 2011). However, genetic analysis of male reproductive success has shown that under high-density conditions in the laboratory, males with territories are no more successful than those without (Spence et al., 2006b).

Housing density for zebrafish is described in Section 2.3.2, “Tank Design and Spatial Requirements”. Fish density needs to be optimized to reduce aggressive behaviour. If zebrafish need to be housed at low densities, careful consideration must be given to structures that the fish can use as hiding places to help minimize aggressive encounters.

Zebrafish kept together for breeding should have some means of escape from more aggressive fish (Matthews et al., 2002). Providing extra space will help, as will plant-like materials or other structures that can be used as hiding places (see Section 6.5, “Environmental Enrichment”).

The implications of housing conditions on zebrafish are complex and need to be taken into account in studies (e.g., for comparative models of anxiety [Parker et al., 2012]).

6.4 NUTRITION AND FEEDING

Nutritionally balanced diets and appropriate feeding regimes are critical in ensuring that fish remain healthy. Essential nutrients include protein and amino acids, lipid and fatty acids, vitamins, and minerals. Deficiency of these nutrients can reduce growth rate and food consumption, and lead to development of disease (NRC, 1993; Conklin, 2000). As fish are ectothermic, feeding rates and quantities need to take temperature into consideration (Alanära et al., 2001; Kestemont and Baras, 2001).

6.4.1 Nutrition

Zebrafish must be fed a quality aquaculture diet developed for zebrafish (Francis, 2008); food from a commercial pet store is not appropriate and lacks consistency. Flake foods lack precision in nutrient content and are unstable in water, resulting in water soluble nutrients leaching into the water (Lawrence, 2011). Some commercial foodstuffs claim to offer a nutritionally complete food; however, the precise nutritional requirements of zebrafish have yet to be determined (Lawrence, 2007; Fernandez et al., 2016).

Research on the basic nutritional requirements of zebrafish is ongoing (Fernandez et al., 2016; Lawrence, 2007; Watts et al., 2016), so it is important to keep up-to-date with the scientific literature. The effects of different commercial and laboratory-prepared diets on the growth of zebrafish have been demonstrated by Siccardi et al. (2009), with suggested implications for both the welfare of the fish and consistency of research results. Additionally, Smith et al. (2013) found the source of protein in commercial diets to affect the growth and body composition (lean mass versus body fat) of zebrafish. Fernandes et al. (2016) showed that food containing 35%-40% protein is optimal for juvenile zebrafish, and levels in excess of this can result in increased excretion of ammonia.

In the wild, adult zebrafish usually feed on small crustaceans, insect larvae and, to some extent, algae (Engeszer et al., 2007; Spence et al., 2007; Arunachalam et al., 2013). In most cases, provision of live foods in the laboratory requires culture of the prey item, in addition to the fish. Other potential disadvantages to be considered include the possibility of variable nutritional planes and the potential for introduction of disease (Peterson et al., 2013) or contaminants (Tye et al., 2018). Frozen food can also introduce disease.

Findings regarding the best foods for zebrafish differ, and facilities are encouraged to use both dry and live food in a combination that best suits the needs of fish, taking into account such factors as the type of food and age of the fish. One comparison of diets and feeding regimes found that zebrafish fed only brine shrimp experienced weight loss throughout the study, while those on commercial diets gained weight, with differences noted between the commercial diets tested (Gonzales and Law, 2013). In another study comparing different commercial dry foodstuff alone and in combination with live food, one dry foodstuff was found to be superior for all welfare parameters evaluated (Farias and Certal, 2016). This study also indicated that the best outcomes resulted from the combination of dry food and live food; however, satisfactory results were also found for adult zebrafish that were only given dry food (Farias and Certal, 2016).

Fish require ions such as calcium, magnesium, iron and selenium to maintain health. As mentioned in Section 2.1.2, “Water Quality”, reverse osmosis and deionization remove trace elements required for fish metabolism and growth, and those ions need to be added back into the water. These can be provided through diet or environmental additions such as shells or commercially available composites.

6.4.2 Feeding

Fish must be fed at appropriate intervals with a nutritionally adequate, properly-sized food. Optimal feeding techniques are essential for good welfare and to prevent fouling of water with uneaten food. The practice of feeding involves determining the proper size and appropriate properties of the food for the species, the ration size, the feeding frequency, the preferred time for feeding, and the most efficient means of distributing the food.

All food should be consumed within five minutes. Feeding techniques for captive fish have the general aim of encouraging rapid consumption, thus increasing feed ingestion, preventing leaching of water-soluble nutrients, and reducing waste. The feeding response depends not only on a suitable diet, but also on the environment. Temperatures at the low and high ends of the tolerance range inhibit feeding, as do stressful conditions such as low oxygen levels and the development of social hierarchies within the population (Kestemont and Baras, 2001). When new food is introduced, it should be mixed with the accepted food until the transition is made between the two different types of food.

The frequency of feeding depends on the life stage of the fish, the research goals and the type of food. Adults are generally fed once to twice a day; juveniles and larva are fed more frequently. Zebrafish-specific commercial diets usually come with recommended feeding frequency per life stage, and the scientific literature should be consulted for optimal feeding strategies for other food types. In a study of feeding frequency, 30 day post-fertilization zebrafish (particularly females) showed significantly more growth (increased weight and length) when fed at least once a day, compared to those that were fed the same total amount every other day, or the controls that were fed three times a day to satiation (Lawrence et al., 2012). This study also showed that breeding success was not correlated to the same feeding practices as for growth. Adults can tolerate a few days without food but require daily feeding for optimal egg production (Matthews et al., 2002).

Fish must be observed regularly to determine whether they are feeding as expected, and whether the ration is appropriate. Feeding time is often used as an opportunity to observe the health and behaviour of the animals. Fish must not be overfed, and excess food should be removed soon after the feeding period. Overfeeding can cause fish to become obese and reduce breeding performance (Vargesson, 2007), and adversely affect water quality, which increases the risk of disease. Food or feces retention in the environment is a particular concern, both in static and recirculation systems. The influence of food quality and quantity on water quality must be addressed in the study design. It is good practice for housing system designs to incorporate an effective mechanism (e.g., self-cleaning tanks) for removing any solids after the last feeding (for approaches to removing solids, see Lawrence and Mason, 2012).

Feeding should be optimized to ensure all fish have the opportunity to feed. In the event of prolonged food refusal, alternative plans should be in place, including consultation with a fish nutritionist, a veterinarian or a food manufacturer. The primary modes of food detection by fish are through olfaction and sight, but the taste and texture of the food is the key factor that determines whether the food will be swallowed or rejected.

Studies involving food restriction must undergo careful consideration. The [*CCAC policy statement on: ethics of animal investigation*](#) (CCAC, 1989) states that for “experiments requiring withholding of food and water for periods incompatible with the species-specific physiological needs, such experiments should have no detrimental effect on the health of the animal”. Endpoints for food restriction studies should be based on visual assessments of the fish using a body condition scoring system (e.g., Clark et al., 2018), rather than on a percentage decrease in body weight, which would result in unnecessary handling of fish. As with any other studies, the animal care committee is responsible for approving the endpoints of the proposed study,

in consultation with the investigator and veterinarian or fish health specialist. Studies involving over-feeding (i.e. obesity research) should also be carefully considered, as they can have varied health effects depending on the type of diet provided (Landgraf et al., 2017).

6.4.3 Larval Rearing

Larvae are capable of independent feeding by 5 days post-fertilization when kept at a standard laboratory temperature of 28°C (development may be slower at a lower temperature); at this point, the yolk sac has greatly decreased (Vargesson, 2007; Lindsay and Vogt, 2004; Jones et al., 2008).

In the wild, zebrafish larvae chase and catch their prey (e.g., *Paramecium*) in a process that appears to be predominantly visually guided (McElligott and O'Malley, 2005). Keeping larvae in the dark greatly impairs their ability to feed.

The change from endogenous feeding to exogenous feeding and weaning from a live diet are critical transition periods for fish. While the death of large numbers of fish at these times is observed to occur naturally in the wild, in a laboratory setting where practices are aimed at meeting the needs of the fish, large mortalities should not occur and individuals judged unlikely to thrive must be euthanized. Endpoints should be set, such as low weight, lethargy, not actively feeding, sitting at the bottom of the tank, etc.

The timing of first feeding and availability of suitable prey is critical. The most crucial factor in the weaning process is the provision of live invertebrates, such as rotifers and *Artemia* of the appropriate size for the fish. Ideally, *Artemia* should be decapsulated for maximum digestibility. These food organisms are nutritious when enriched with limiting nutrients (e.g., essential fatty acids and amino acids) to promote larval growth and survival (Best et al., 2010). If young fish are deprived of exogenous food, the effects become manifest as irreversible starvation.

Zebrafish larvae reared on rotifers following methods described by Aoyama et al. (2015) and Best et al. (2010) have shown high survival and growth rates. Other studies have shown good results rearing zebrafish on formulated diets; however, the results differ according to the particular diet (Kaushik et al., 2011; Hensley and Leung, 2010).

6.4.4 Use of Medicated Foodstuff

Medicated food must only be used under veterinary prescription and supervision. Medicated food may be used to treat clinical conditions, such as bacterial infections, or be used in studies involving models of disease control, toxicity testing, disease model creation, etc. (e.g., Collymore et al., 2014a). Approaches to administering medication include “gut loading” live food (e.g., Samaee, 2015), gelatin-based feeding (e.g., Chang et al., 2017), and spray-coating pelleted food.

In recirculation systems, the use of chemotherapeutic agents should be carefully considered due to the possible detrimental effects of these compounds on nitrifying biofiltration flora and their potential impact on subsequent studies (Yanong, 2012; Schmidt et al., 2007).

6.4.5 Food Quality and Storage

Food stock must be labeled with the date of manufacture and guaranteed analysis information.

Processed food must be stored in dedicated areas that are dark, temperature and humidity controlled, and pest-free to ensure nutritional quality. Food for immediate use should be similarly protected. Food used for daily feeding should be kept in sealed containers to protect it from humidity and light.

All foodstuffs, whether moist, semi-moist or dry, are susceptible to degradation with time. Dry food should be stored at temperatures <20°C and humidity <75%. High humidity increases susceptibility to mold, and high temperatures destroy certain vitamins and enhance the degradation of lipids. Vitamins in foodstuff can also be destroyed by oxygen, ultraviolet light and lipid peroxidation (DSM, n.d.).

Only very small quantities of dry food should be held in the fish room (i.e. enough for 1-2 days) to prevent food from deteriorating and becoming moldy, which happens rapidly in warm, humid rooms. Under no circumstances should moldy food be used, as it is highly toxic (Wedemeyer, 1996).

Oxidative rancidity is one of the most serious changes that can occur in stored food (Wedemeyer, 1996; O'Keefe, 2000). In the absence of antioxidant protection, lipids rich in polyunsaturated fatty acids, including the essential fatty acids, are highly susceptible to auto-oxidation, which produces harmful breakdown products that include free radicals (Hardy and Roley, 2000). The pathological effects of feeding oxidized oils are summarized by Tacon (1992).

Some foods can be frozen to extend shelf life. This is an option when relatively low amounts of food are required for a specific research project. However, certain micronutrients such as B complex vitamins are degraded by freezing and thawing, and therefore supplements may be required. Very small portions of frozen food do not require thawing before being added to the tank; however, larger frozen food items, such as bloodworms, should be thawed in advance of feeding.

Live food must be held in clean tanks, with different species kept in separate tanks (e.g., brine shrimp kept separate from rotifers). Live food should also be checked daily to ensure proper hatch and health of the cultures.

Special care needs to be undertaken to ensure best practices when maintaining live food cultures, as poor management can impact fish welfare. Record keeping for production of live food should be in place and include information to date cultures, such as when cultures were started. Details for the production of live food are not within the scope of this document, and the current literature should be reviewed for information on best practices (e.g., Lawrence et al., 2016). It is important to be aware that live food is a potential source of pathogens (Peterson et al., 2013). Precautions should be taken to minimize contamination when preparing live food, such as using clean equipment dedicated to the food, and working in an area away from fish housing. In the event of a disease, food should be tested.

6.5 ENVIRONMENTAL ENRICHMENT

Guideline 10

Consideration must be given to providing zebrafish with environmental enrichment suited to the housing conditions.

Social housing and substrate are basic requirements for zebrafish and are addressed in Section 6.3, “Housing Management”, and Section 2.3.2.4, “Substrate”. When it is necessary to singly house zebrafish, social contact

should be simulated by housing them next to conspecifics or adding mirrored paper to the side of their enclosure (Krueger et al., 2020).

As noted in Section 2.3.2.4, “Substrate”, zebrafish have been shown to prefer environments with substrate (sand or gravel) and plants (Kistler et al., 2011; Schroeder et al., 2014). Compared to transparent bottoms, they even prefer pictures of gravel fixed to the exterior of the tank (Schroeder et al., 2014). While zebrafish were found to respond slightly differently to potential sources of environmental enrichment depending on whether they were housed in pairs or groups of eight, fish always showed a preference for substrate, particularly gravel.

The provision of environmental enrichment beyond these basic requirements of social housing and substrate is an emerging field; however, numerous studies have indicated potential benefits of various forms of enrichment, such as plants and water movement. The benefits of additional enrichment can be species-specific (Kistler et al., 2011), and it is important to understand the natural requirements of the species. It is recognized that there is insufficient published information on the effects of different forms of enrichment on the various types of systems currently in use. Facilities housing zebrafish should therefore keep up-to-date with research in this area, and where possible, test different forms of enrichment, with documentation of both positive and negative outcomes.

Field and laboratory-based studies have shown both wild and captive-bred zebrafish prefer habitats with vegetation. In the wild, the vast majority of sites where zebrafish were observed had submerged or overhanging vegetation (Engeszer et al., 2007) and zebrafish have shown a preference for spawning in sites associated with aquatic vegetation (Spence et al., 2008). In laboratory studies where zebrafish were given the choice between barren areas and areas containing artificial plants (Delaney et al., 2002) or plants and substrate (Kistler et al., 2011), they showed a clear preference for inhabiting the enriched environments. In addition, zebrafish provided with enrichment (in the form of gravel and plastic plants) have been shown to be less vulnerable to chronic stress than those held in a barren environment (Marcon et al., 2018). A further study has shown zebrafish have a preference for an environment with water flow (14 m/s), plants and substrate, over a similar environment with calm water (DePasquale et al., 2019). Furthermore, recent work has suggested that zebrafish may also experience positive welfare benefits from the opportunity to freely explore a new environment (Graham et al., 2018b).

There is evidence that environmental enrichment can enhance survival of larval zebrafish (Lee et al., 2018) and reduce inhibitory avoidance behaviour in young fish (Manuel et al., 2015), while less complex environments appear to stunt cognitive and social development (Graham et al., 2018a; Spence et al., 2011). A study that reared larvae in either enriched (plants and gravel) or barren tanks showed those in enriched tanks had higher survivorship and exhibited less anxiety behaviour when exposed to a new environment (Lee et al., 2018). However, this study also showed that fish reared in enriched tanks tended to monopolize resources to a greater extent. Similarly, a study of adult fish reared with different levels of enrichment showed those reared in the most complex environment displayed more aggression (Woodward et al., 2019).

Plants have been shown to be particularly beneficial when zebrafish are held in suboptimal social groups (Collymore et al., 2015). Plants can provide an important refuge, especially for females to avoid males, or for males to avoid aggressive encounters with other males. Additionally, fertility and fecundity have been found to be greater in zebrafish in a breeding tank containing plants than in a bare tank, under certain conditions (Wafer et al., 2016).

Before introducing enrichment objects to a tank, careful planning and consideration should be given to the relevance of the object to the fish, the potential effect on water quality (i.e. disruption of water flow, overgrowth of algae, or accumulation of food or feces), the method and frequency of cleaning the objects, the potential for chemicals to leach into the water, and the ability of animal care staff to observe and assess the welfare of the fish.

When using plants, plastic plants are generally preferred; however, there is the possibility of contaminants leaching into the water. Some contaminants can be removed through bleaching the plants, followed by soaking the plants in water before adding them to the tank. The risk of toxins from plastic plants in the water may present a problem for some types of research; however, in general the level of toxicity is not a concern. There are also pathogen-free plants being developed.

6.6 HUMAN CONTACT AND HANDLING

During the performance of husbandry tasks, the handling of fish should be kept to a minimum and precautions should be taken to avoid causing stress or injury. The majority of zebrafish in research facilities are the descendants of many generations of captive-bred fish. Although they appear to exhibit reduced 'nervousness' or predator avoidance behaviours, being handled represents a potential stress and damages their outer mucus layer. Repeated daily handling has been shown to negatively affect growth and reproductive performance, particularly in females (Abdollahpour et al., 2020).

For information on handling equipment and procedures, see Section 7, “Handling and Restraint”.

For hygiene reasons, each room or rack should have its own dedicated equipment for handling fish (e.g., nets) or the equipment must be sanitized, and if possible sterilized, between use with different groups of fish. The need for sterilization depends on the health status of the fish, as some disease-causing organisms might be resistant to some chemical disinfectants (e.g., *Pseudoloma*). A biosecurity protocol and SOPs should be in place.

6.7 CLEANING AND SANITATION

Cleaning strategies to address biosecurity requirements should be designed to minimize disturbance and distress to the fish. Procedures for cleaning and disinfection should be selected according to such factors as the type and frequency of equipment used, husbandry practices, number of zebrafish, tank system, water quality and the type of research (Garcia and Sanders, 2011). For larger tanks, increasing flow during cleaning can help flush infectious material. Common procedures for cleaning and disinfecting nets used to collect zebrafish have been evaluated by Collymore et al. (2014b) and Garcia and Sanders (2011).

Chemical disinfectants (e.g., bleach) and detergents must be used with extreme caution. Unless disease transfer is a concern, soaps and chemicals should be avoided as they can introduce toxins into the water. When soaps and chemicals are used, tanks must be thoroughly rinsed several times with clean, cold water that has undergone dechlorination or reverse osmosis, before being refilled. Work surfaces should be cleaned before and after use, and where fish will be in contact with the cleaned surfaces or odours from cleaning products, the same cautions apply. Cleaning and disinfectant products must be stored away from tanks holding fish.

Although the majority of tanks holding zebrafish are made of polycarbonate, most facilities do not autoclave them (Francis, 2008). If a cage washer is used to clean polycarbonate tanks, they should be thoroughly rinsed with water, as soap residues in the aquatic environment may be easily absorbed into the bodies of zebrafish, causing illness and possibly death.

6.8 RECORD KEEPING

6.8.1 Standard Operating Procedures

Guideline 11

Detailed Standard Operating Procedures must be developed for the care of all fish and for maintenance and cleaning of tanks, rooms and equipment.

Each facility must prepare a facility SOP manual for acceptable fish husbandry practices and standards, including contingency plans for disease outbreaks, power failures, proper disinfection and storage of tanks between experiments, etc. The manual should be reviewed and updated regularly. Personnel assigned to fish care should be trained in relation to its contents and the animal care committee and facility management should ensure that users follow SOPs.

6.8.2 General Checklists

Checklists should be used to facilitate record keeping for husbandry, cleaning and maintenance activities, and experimental procedures. They should cover each group of fish at the level that is relevant to the research (e.g., room, rack or tank).

At a minimum, checklists must be maintained for the following, as a record that tasks have been completed:

- daily checks of the welfare of fish – generally performed at the time of feeding; for details, see Section 8, “Welfare Assessment”, and information on endpoints in Section 10, “Experimental Procedures”;
- water quality – see Section 3, “Facility Management”, for water quality parameters to be monitored, frequency of monitoring and acceptable ranges; and
- tank and room cleaning – see Section 6.8, “Cleaning and Sanitation”.

6.8.3 Records

Tanks should always be clearly labelled and the genetic background of the fish should be available on the label or included in a record kept in close proximity to the tank. If the fish are currently being used in a project, reference to that research and the person responsible should be clearly identifiable. Personnel caring for the fish should know where to find relevant information relating to the project, such as the experimental procedures involved, the objectives of the work, the potential adverse effects the fish may experience and the agreed endpoints (where applicable).

The following information should be maintained at the room level and be readily accessible:

- source of fish and date of arrival if imported;

- species and sex (if identifiable);
- estimated date of fertilization;
- name of principal investigator and list of emergency contacts;
- protocol number, including expiration date;
- daily records of husbandry (including feeding schedule), maintenance, experimental procedures and water quality parameters as required;
- morbidity/mortality; and
- history of the fish, including disease and ongoing health status.

The approach used for record keeping will depend on the scale of the facility. For example, barcode systems have been developed to record husbandry activities in extensive zebrafish housing systems (Anderson et al., 2010).

As noted in the [CCAC guidelines: Husbandry of animals in science](#) (CCAC, 2017), “Retention of records is important for research accountability, and requires collaboration between investigators and facility personnel regarding the type of records and length of time they need to be retained. Health records and records for food, water and [housing] should be retained for a period of time suited to the type of research and institutional requirements; a minimum of one year is required, or as long as necessary to meet the requirements of the government, relevant professional associations and the research. Investigators should also be aware of any additional requirements by publishers or granting agencies funding the research regarding record retention, publication and availability.”

HANDLING AND RESTRAINT

7

Guideline 12

Fish must be handled only when necessary, and the number of handling episodes should be minimized.

Nearly all fish held in the laboratory need to be physically handled at some point; however, handling and disturbance appear to be stressful events for fish. A study by Ramsay et al. (2009a) showed an increase in cortisol in zebrafish following handling that persisted for one hour post-handling. Even following a brief stressful event, the physiological response may significantly affect blood chemistry for as much as 24 hours (Wedemeyer, 1972, in Kreiberg, 2000).

Long-term effects of stress can include loss of appetite, inhibition of growth, reduced reproductive success (Abdollahpour et al., 2020) and impaired immune response (Reddy and Leatherland, 1998). Depending on the species and the frequency and intensity of the stressor involved in handling, it may take a few hours to resume normal feeding and fish should be given sufficient time to recover before feeding.

If possible, fish should not be fed prior to handling to lessen the risk of water contamination from gut contents. The length of time that fish should be fasted prior to handling depends on the procedures involved: a couple of hours may be sufficient for simple breeding procedures, while more invasive procedures, such as those requiring anesthetic (e.g., respirometry, intramuscular or intraperitoneal injections, elastomer tagging or fin clipping), may require up to 24 hours. Fasting is not necessary when fish are only moved between tanks.

Guideline 13

Personnel involved in handling fish must be competent¹ in methods that expose fish to air for the shortest time possible, and that minimize injury, including damage to the mucus-skin barrier.

Manual restraint may be a practical means of performing rapid, minimally stressful procedures, but requires skilled and careful handlers. Even routine handling procedures may cause morbidity and mortality if carried out by personnel who are not competent.

Appropriate handling equipment, such as a soft net with a smooth inner surface or a container, should be used to minimize damage to fish during handling. Containers have been used to scoop fish out of holding tanks so that they do not experience the stress of air exposure. This may also reduce the potential for scales to be lost due to abrasions caused by the transfer net (Ruhl et al., 2009).

¹ Competency is defined in the [glossary](#).

When performing procedures, sanitizable tables should be used. Wet, soft sponges that are free of disinfectants can provide restraint (e.g., Collymore et al., 2013; Kinkel et al., 2010). Dedicated equipment should be used for groups of fish to minimize the potential for cross contamination, and should be cleaned and allowed to dry between uses. See Collymore et al. (2014b) and Garcia and Saunders (2011) for information on acceptable net cleaning practices. Personnel must wear powder-free gloves if there is the possibility of contact with fish or the water, to prevent contamination from lotions, soaps and/or hand sanitizers.

Measurements that involve hands-on manipulation, such as weight and length, must be conducted quickly and in a manner that is minimally stressful. Procedures that involve more than momentary restraint, or require that large numbers of fish be handled should be conducted under sedation (see Section 10.4.1, “Anesthesia”), unless the fish have been conditioned to the handling. Prolonged physical restraint without sedation can damage the skin and mucous membranes of adult zebrafish and larvae that are able to feed independently, and should be avoided. Fish are highly reliant on the integrity of their mucus and epidermal body covering as a barrier to osmotic stress and infectious agents. As the skin is relatively delicate, anesthetics and sedatives are frequently used to prevent external damage during procedures.

When fish are to be handled repeatedly, a period of recovery that allows normal feeding behaviour to be resumed should occur between handling procedures. Recovery from stress can be prolonged. Repeated handling may require an increased level of monitoring, and the stress may be alleviated by the use of sedation (Kreiberg, 2000).

The length of time fish are held out of water should be minimized, as it is a stressful event for fish. In general, fish should be exposed to air for the shortest time possible, and no longer than a few seconds. If fish are held out of water for more than a few seconds, efforts should be made to re-wet the fish and the gill lamellae should be kept moist.

Where feasible, fish should be protected from direct light and rapid changes in lighting while being handled and/or restrained. Many fish are sensitive to visual stimuli, especially light. Exclusion of light, wholly or in part, has been recommended as a practice to reduce stress in fish undergoing handling (Wedemeyer, 1985; Hubbs et al., 1988); however, this needs to be balanced with providing sufficient light to properly conduct the procedure.

Due to the chronic stress imposed by small static tanks without water changes, fish should only be held in these conditions for a maximum of 24 hours without verification of water quality or a water change. As a general rule, water should be changed at a rate of 30% per day.

WELFARE ASSESSMENT

8

Guideline 14

Basic physical and behavioural indicators of fish welfare must be monitored daily and written records must be maintained. Any changes must be investigated and the causes identified and corrected.

It is important to be aware of an animal's welfare before, during and following the conduct of any studies. The objective evaluation of key behavioural and physical variables should help in the detection of abnormalities, whether related to environmental/husbandry factors or to the effects of the experimental procedures. Changes in fish behaviour or physical indicators could be a sign of poor welfare.

A good understanding of zebrafish biology and behaviour, including diseases, clinical signs and treatments, is necessary to minimize suffering or death. Zebrafish should be observed at least daily for indicators of poor health (see Section 8.1, “Welfare Indicators”). Sick fish should be removed from the tank as quickly as possible and veterinary advice sought.

The assessment of fish welfare is challenging because responses of fish to adverse conditions are not always displayed, particularly if significant observational restrictions are imposed by the rearing environment. It is not uncommon for a fish to appear healthy one day, only to die on the next (ASPI, 2006, cited in Reed and Jennings, 2011). This suggests more work needs to be done to improve knowledge regarding definition and recognition of visual signs, and the assessment of welfare. Matthews et al. (2002) acknowledge that while it is accepted that fish have the capacity to experience pain, their responses can be difficult to interpret.

Standardizing the terms used to describe the welfare of zebrafish will help limit subjectivity among observers and improve the sharing of information within a facility and between facilities, which will lead to better welfare for the fish and support for scientific studies (Goodwin et al., 2016).

8.1 WELFARE INDICATORS

8.1.1 Health Indicators

Table 2 Some Key Signs of Ill Health in Zebrafish

CLINICAL SIGNS	POSSIBLE CAUSE								
	BACTERIAL INFECTION	VIRAL INFECTION	PARASITES	CHEMICAL OR ENVIRONMENTAL STRESS	GAS SUPERSATURATION	OXYGEN DEPLETION	HORMONAL INFLUENCES	MECHANICAL TRAUMA	STARVATION
Changes in body colour ¹ and skin appearance	*	*	*	*			*		*
Clamped fins			*	*					
Emaciation/altered body condition	*	*	*	*					*
Exophthalmos	*		*		*				
Improper buoyancy or abnormal swimming	*		*		*				
Opercular flaring	*		*	*	*	*			
Petechiation or haemorrhage	*	*	*	*	*			*	
Scale loss	*		*					*	
Sloughed mucus	*		*	*					
Sudden death	*	*	*	*	*				
Surface breathing			*	*	*	*			
Activity level (including lethargy)	*	*	*	*	*	*	*		*
Failure to feed	*	*	*	*	*	*	*	*	
Flashing or rubbing against the tank			*	*			*		
Spinal curvature ²	*		*				*		

1 Social hierarchy and the tank colour can also affect the colour of the fish.

2 Also related to age and genetics.

Adapted from Astrofsky et al., 2002.

8.1.2 Behavioural Indicators

When zebrafish become aware of an actual or perceived threat, behaviours displayed may include shoal cohesion, agitated swimming, freezing on the substrate, decrease in feeding rate, or increase in aggression (Spence et al., 2008). Regular occurrence of such behaviours may indicate a chronic welfare problem. However, zebrafish can habituate to a change in environment or a noxious substance, and therefore, a chronic welfare threat can continue to exist even though there is a decline in their response to the stressor.

Signs of pain or distress in zebrafish may include escape behaviour, frantic movements, significant reduction in activity, increased respiration (rapid movement of opercula), blanching of colour (Matthews et al., 2002; Reilly et al., 2008), and occupying lower locations in the water column (Egan et al., 2009).

The use of behavioural thermoregulation as a welfare indicator has been debated in the literature. Rey et al. (2015) observed that zebrafish exposed to a stressful handling event spent more time in an area with a higher temperature, which resulted in an increase in their own body temperature, compared to controls that were not handled. However, a further study in this area did not replicate this observation and found zebrafish showed a reduced preference for warmer areas immediately following a stressful event (Jones et al., 2019).

HEALTH AND DISEASE CONTROL

9

Guideline 15

All facilities must have a fish health program that includes a system of regular monitoring and reporting for health assessment purposes, aimed at early detection of disease conditions and the causal agents, stressors and mechanisms of disease, so that correct control measures can be initiated.

Healthy fish are prerequisites for reliable data (Jenkins, 2000). Fish used for research must be free of any notable disease agents that could lead to a diseased condition (unless it is part of the experimental protocol). Several infectious disease agents, such as *Pseudoloma neurophilia* and some species of *Mycobacterium*, have been found at the subclinical level in apparently healthy zebrafish (Kent et al., 2012; Whipps et al., 2012), confirming the importance of acquiring fish of known health status.

If a disease condition is part of the experimental design, the potential effects of the pathogen or parasite on the research results should be predictable or constitute a variable that is being tested through the research protocol.

Institutions housing fish for research, teaching and testing must have access to a veterinarian with fish training. This individual can assist in the development of SOPs to limit the introduction of disease into the facility, and should be available for consultation on matters relating to the health of the fish. Elements to be considered in developing a fish health monitoring program are described by Collymore et al. (2016a).

9.1 DISEASE PREVENTION

Stress, nutritional problems, water quality problems, disease outbreaks from infectious causes, and cannibalism can all cause major problems in captive fish (Wedemeyer, 1996). Methods for early detection of emerging health problems should be implemented to facilitate mitigation and restoration of fish health. Good health management is important for the welfare of the fish and for study results, which can be adversely affected or compromised by sub-optimal health or disease events.

Many diseases in fish holding systems can be prevented through good husbandry and biosecurity procedures, starting with stock that have been pre-screened for infectious disease agents, subjecting incoming animals to a period of quarantine, disinfection of embryos, and low-level handling to minimize stress. Chronic crowding and handling stress have been shown to increase clinical disease from mycobacterial infections in zebrafish (Ramsay et al., 2009b).

9.1.1 Containment of Animals

Fish entering a facility should be quarantined, as noted in Section 4.5.1, “Quarantine and Acclimation”. Quarantine is also important when there is the potential for pathogens that could affect other aquatic animals to escape in the effluent of the holding facility.

Contagious fish or those suspected to be contagious should be separated from healthy fish, and fish with different health statuses should be separated. For infectious disease research, containment standards are specified in the *Canadian Biosafety Standard* (CBS), 2nd edition (CFIA, 2015).

9.1.2 Immunizations

Immunizations are not standard for zebrafish at this time, but it is important to keep up-to-date with changes in this area.

9.1.3 Precautions for Personnel in Prevention of Disease Transmission to Fish

An SOP should be in place to prevent the transmission of disease from aquariums in the homes or offices of personnel working with zebrafish. The SOP should describe preventative measures suited to the particular facility, such as the use of dedicated outerwear (lab coats), dedicated footwear or a footbath (with the solution changed regularly and sufficient contact time between footwear and the solution) and forearm-length gloves for added protection. There should also be dedicated equipment for handling fish.

Zoonotic concerns for people working with fish (e.g. *M. marinum*) are addressed in Section 13, “Human Safety”.

9.2 HEALTH MONITORING AND DISEASE DETECTION

When confined in an artificial environment, fish are particularly sensitive to variations in water quality, nutrition, the presence of pathogens and management practices of the facility. The expression of disease, whether infectious or non-infectious, cannot be considered in isolation from any of these factors. Microorganisms are distributed throughout any aquatic environment; however, their presence may only be obvious under sub-optimal conditions.

Health monitoring of zebrafish populations within facilities may involve the use of sentinel fish or the testing of colony fish, aged fish, sick fish or moribund/dead fish (Collymore et al., 2016a; Kent et al., 2009; Borges et al., 2016). Water or substrate samples can also provide useful information on the presence of some disease agents, but should be used in conjunction with information gained from direct testing of fish (Collymore et al., 2016a; Crim et al., 2017). Certain pathogens can be detected in fish using polymerase chain reaction (PCR) tests, while others appear more prominently in environmental samples, and therefore a combination of sample types is useful (Miller et al., 2019).

9.2.1 Disease Diagnosis and Identification of Pathogens

Disease management protocols should include a reliable system for the detection and reporting of clinical signs and criteria to distinguish between acceptable and unusual levels of mortality. Isolation and rapid removal of sick or dead fish will help reduce the spread of disease.

If unexpected losses of fish occur, water, food and fish samples should be taken immediately, in case they are required for later analysis. Samples of affected fish should be retained for diagnostic purposes. It is advisable to develop an SOP with the assistance of an experienced fish health or veterinary clinician to standardize sample collection methods.

With active clinical problems, consultation with a clinical veterinarian is preferred. When submitting samples to diagnostic laboratories, there should be communication with the laboratory to determine the best type of sample to submit. Some diagnostic facilities have PCR tests for specific zebrafish pathogens, so live fish are not always necessary. Additionally, environmental samples may be appropriate for some pathogens (Crim et al., 2017; Miller et al., 2019). Where submission of fish is necessary, live untreated fish with and without symptoms may be required, rather than dead specimens.

Fish health management programs should strive to identify clinical and subclinical/adventitious pathogens that may occur as a result of experimental stressors. The presence of infectious diseases in an experimental population, even one not showing clinical disease, may lead to results that are difficult to interpret due to the potential confounding variables caused by subclinical disease. For example, *Pseudoloma neurophilia* has been shown to affect shoaling behaviour in zebrafish (Spagnoli et al., 2017) and *Mycobacterium chelonae* may alter fluorescence and lead to misinterpretation of imaging results (Whipps et al., 2014). The scientific validity and reproducibility of experiments made in a morbid or subclinically affected population is questionable. The application of treatments may also be an experimental variable.

Particular attention should be paid to monitoring fish following any potentially stressful event. All fish in the facility must be monitored on a daily basis; however, fish undergoing stressful procedures have an increased risk of developing opportunistic infections. Additional care should be taken in observing fish for up to five days following a potentially stressful event. Reductions in feeding, unusual behaviour, discoloration of the integument and lesions are signs of possible developing health problems (see Section 8, “Welfare Assessment”).

Physical trauma is one source of stress, but more common stressors are major and abrupt changes in environment, crowding, handling, transportation, and degradation of water quality (such as sub-lethal changes in temperature, reduced dissolved oxygen, and increased ammonia [Wedemeyer, 1996; Reddy and Leatherland, 1998; Speare, 1998]). Such stressors can suppress the immune response, allowing disease organisms to proliferate (Tort, 2011). However, stress can also lead to mortality in the absence of infectious disease agents.

9.2.1.1 Common Disease Agents

Clinical signs of some common pathogens in zebrafish and potential treatments are detailed in the Table 3. A veterinarian should be consulted if any of the clinical signs in Table 2 (see Section 8.1.1, “Health Indicators”) or Table 3 are observed.

Table 3 Common Disease Agents

Disease Agent	<i>Pseudoloma neurophilia</i> (microsporidiosis)
Background	<ul style="list-style-type: none"> • Common in laboratory colonies (Collymore et al., 2016a). • Likely transmitted from maternal parents to progeny, even when eggs are surface cleaned with chlorine because the parasite is abundant in ovaries; larvae are extremely susceptible to infection and chlorine levels used to treat eggs are not entirely effective for killing the spores (Kent, 2007, cited in Reed and Jennings, 2011). • Infects the central nervous system, cranial and spinal nerves, and skeletal muscle of zebrafish.
Clinical signs	<ul style="list-style-type: none"> • Clinical signs include chronic emaciation (or ‘skinny disease’), reduced growth, ataxia, spinal malformations, and decreased reproductive fitness (Collymore et al., 2016a). • Infected zebrafish may appear clinically normal but still able to transmit the disease (Murray et al., 2011). • Clinical signs are not specific and may be found to be common to other causes, such as nutritional deficiencies, environmental problems, genetic factors, physical trauma, toxins, and various infectious agents (Murray et al., 2011).
Detection	<ul style="list-style-type: none"> • Quarterly testing of fish that may have been exposed to <i>Pseudoloma</i> starting three months after the potential exposure is suggested, as it may take at least three months for identification of infection following exposure (Collymore et al., 2016a). • Exposing sentinels to the effluent with overlapping six-month periods of exposure is also useful (Collymore et al., 2016a). • If histopathology is available, spores can be seen (it may also be possible to see them on wet mounts).
Proposals for prevention/treatment	<ul style="list-style-type: none"> • There is no known effective treatment (Collymore et al., 2016a), but UV light sterilization of the water, with particulate filtration prior to UV exposure, has proven to be reasonably helpful in reducing the incidence of infection (Murray et al., 2011). • Ideally, these pathogens should be excluded through import screening and quarantine. PCR-based tests can be used to screen for carriers (such that <i>Pseudoloma</i>-free facilities may be established and maintained), but the process required is particularly laborious (Barton et al., 2016). • Surface sanitation of embryos (as noted in Section 4.1, “Source”) will help limit horizontal transmission of <i>Pseudoloma</i>; however, disinfection of embryos with chlorine will not destroy spores (Murray et al., 2016) or prevent transmission of <i>Pseudoloma</i> vertically (Collymore et al., 2016a).

Disease Agent	Mycobacteriosis (or fish tuberculosis)
Background	<ul style="list-style-type: none"> • Several mycobacterium species have been implicated, including <i>M. chelonae</i>, <i>M. peregrinum</i>, <i>M. marinum</i> and <i>M. haemophilum</i>, and observations from outbreaks and experimental transmission studies indicate that the latter two are of the most concern, while <i>M. chelonae</i> usually causes opportunistic infection (Whipps et al., 2012). • Frequently present in aquaria, with varying impact depending on the species of pathogen and the health of the fish (Whipps et al., 2012). • High risk of horizontal infection between fish (Vargesson, 2007). • Some evidence that mycobacteriosis is zoonotic (i.e. can be spread to humans [Mason et al., 2016; Whipps et al., 2012]); therefore, if dealing with infected fish, gloves must be worn to avoid cross-contamination (Vargesson, 2007); see Section 13, “Human Safety”. • Also infects medaka (Kent et al., 2009) as well as many other small warm-water fish, and different fish species may have varying susceptibilities to different mycobacterium species.
Clinical signs	<ul style="list-style-type: none"> • Fish may look unwell (e.g., open sores, lethargic, raised scales or emaciated appearance) (Vargesson, 2007; Whipps et al., 2012); however, they can also be asymptomatic (Whipps et al., 2012).
Detection	<ul style="list-style-type: none"> • Methods include sentinel monitoring programs, sampling biofilms (Whipps et al., 2012) and environmental sampling (water and detritus) (Crim et al., 2017). • PCR-based detection protocols have been developed (e.g., Meritet et al., 2017). • Diagnosis is suggested by granulomas on wet mounts, but confirmed by histology and culture (culture should be done in consultation with a clinical pathologist because cultures of aquatic organisms often require specific media, incubation temperatures, and/or long incubation periods).
Proposals for prevention	<ul style="list-style-type: none"> • Impact is reduced when the system is kept clean, with a good water supply, and the fish are healthy (Vargesson, 2007; Whipps et al., 2012). • Some level of disease control can be obtained by removing sick fish, following a biosecurity protocol and standardized cleaning and sterilization procedures, and reducing stress caused by moving fish between tanks or by changes in temperature, water flow, or feeding regimen. • UV lamps can be incorporated into the circulation system, which kills 99% of all <i>Mycobacterium tuberculosis</i> when delivered at a dose of at least 10,000 W/s/cm² (Brand et al., 2002). • Embryo surface disinfection with chlorine bleach is a common practice in controlling the spread of infection when fish are imported; however, pathogens can enter the system through means other than fish (Whipps et al., 2012). • An alternative to bleach, povidone-iodine, has been shown to effectively kill <i>Mycobacterium</i> spp. (Chang et al., 2016; 2015), but another study found it not to be effective for eliminating <i>Mycobacterium marinum</i> (Mason et al., 2016), suggesting that this in an area for further investigation.
Recommendations for treatment	<ul style="list-style-type: none"> • There is currently no known successful treatment (Vargesson, 2007).

Disease Agent	<i>Pseudocapillaria</i>
Background	<ul style="list-style-type: none"> • <i>Pseudocapillaria tomentosa</i> is a common nematode pathogen that affects a wide range of fish species, including zebrafish. • Transmitted to other fish through ingestion of eggs of infected fish that have been expelled into the environment (Collymore et al., 2016a).
Clinical signs	<ul style="list-style-type: none"> • Associated with emaciation and decreased reproductive efficiency (Collymore et al., 2016a), as well as an increased incidence of tumor formation in zebrafish that have been exposed to carcinogens (Kent et al., 2002); however, fish can also be asymptomatic.
Detection	<ul style="list-style-type: none"> • Sources include concentrated water samples, intestinal squash or wet mount preparations (Collymore et al., 2016a; Crim et al., 2017). • Microscopic examination of intestine (via necropsy) and feces for adult nematodes and eggs and PCR-based detection from fish or environmental samples are used (e.g., Mocho et al., 2017).
Proposals for prevention	<ul style="list-style-type: none"> • Fish imported into a facility should be quarantined and if infected with <i>P. tomentosa</i>, they should be treated before research use or spawning (Collymore et al., 2016a). • Within the facility, the use of sentinels in the effluent for at least three months is suggested to detect the presence of the parasite. Treatment can be costly and labour intensive (Collymore et al., 2016a).
Recommendations for treatment	<ul style="list-style-type: none"> • Treatments suggested in the literature include use of fenbendazole, ivermectin, emamectin benzoate, mebendazole and praziquantel (Collymore et al., 2016a).

Table 3 describes three of the most common diseases and infections found in zebrafish. More detailed information is provided by Cartner et al., 2019; Esmail et al., 2015; Kent et al., 2016; Lawrence et al., 2016; Noga, 2010; and Ostrander, 2000.

9.2.2 Injuries and Other Disorders

9.2.2.1 Handling Injuries

Efforts must be made to minimize morbidity and mortality caused by osmoregulatory compromise, systemic acidosis, and opportunistic infections of damaged skin that can result from handling and traumatic injuries. Handling techniques should minimize the potential for injury. Traumatic injuries can result from handling procedures or abrasions from contact with tanks and equipment or other fish (Speare, 1998). Malfunctioning equipment or inexperienced fish handlers can turn even routine procedures into events that cause disease outbreaks. Additional factors that can increase the risks to fish during handling include: dry or abrasive surfaces that fish contact during handling (e.g., measuring boards, balances, knotted nets, etc.); unplanned rapid change in water temperature; prolonged handling times; and repetition of procedures on the same individual.

9.2.2.2 Behavioural Interactions Causing Injury

Health management measures should be used to ensure that behavioural interactions with negative consequences such as aggression are avoided. Some fish exhibit territorial behaviour, which can lead to wounds

(Speare, 1998). Social interactions and density can lead to compromised behaviours, for instance the suppression of feeding (Speare, 1998). Various steps, such as size-sorting fish, adjusting density or providing visual sight barriers, can be employed to minimize aggressive encounters.

9.2.2.3 Feed-Related Disorders

Nutrition can influence the health of fish by causing nutrient deficiencies, imbalances or toxicoses (Tye, 2018), or by introducing infective agents (Peterson et al., 2013). For example, when brine shrimp containing high levels of cadmium were fed to adult zebrafish, there was a decline in health and survival of larva (Tye, 2018).

9.2.3 Treatment

Treatment of fish should be carried out in consultation with a veterinarian. An SOP must be established for any standard medical interventions, and include endpoints when fish are adversely affected. SOPs for standard treatments should be developed in consultation with the veterinarian or fish health professional.

Medication is usually delivered to groups of fish rather than to individual sick fish, which puts more animals at risk of unexpected effects of the treatment. As far as possible, and in consultation with the veterinarian, fish that need to be treated should be isolated until the treatment is completed. When bath treatments are administered, there should be close observation and maintenance of water quality, as this is a major source of problems. In cases where the anticipated effects are unknown, a veterinarian should be consulted and a small number of fish should be tested before application to the group as a whole.

9.3 DISEASE MANAGEMENT IN THE EVENT OF AN INFECTIOUS OUTBREAK

Guideline 16

A management plan must be in place to deal with unanticipated disease outbreaks.

A management plan must be developed to deal with serious disease outbreaks within the facility and from outside sources, and to prevent pathogen transmission and infection recurrence. Plans should include a communication strategy involving veterinarians, veterinary and animal care personnel, investigators, the facility manager and the animal care committee. Access to quarantine facilities or a means of isolating the animals must be available.

For infectious disease outbreaks, the veterinarian must be consulted to ensure that the techniques employed will eradicate the pathogens and all appropriate groups are treated. Typical procedures may include quarantining the rack or room in which the disease is discovered, and tracking the movement history of infected fish, the source of the water and the direction of water circulation to determine the source and/or other stocks affected. Follow-up actions, such as treatment, depopulation, re-derivation, etc., will depend on the nature and extent of the outbreak, the health status of the animals, and the type of research. If infected animals are to be euthanized, proper containment measures must be in place for handling and disposal of the animals and the filter material, as well as decontamination of tanks, recirculating systems and rooms to prevent the spread of disease.

10

EXPERIMENTAL PROCEDURES

The quality of housing and husbandry during experimental procedures should be maintained at the level described in these guidelines. If different conditions are required for a particular study, scientific or veterinary justification must be provided to and approved by the ACC. As shown by Goodwin et al. (2016), water quality deteriorates quickly when fish are held in static containers for studies, as opposed to the recirculating system used for their normal housing.

Researchers and animal care personnel must be well informed regarding adverse effects that zebrafish may experience as a result of experimental procedures, including the responses zebrafish are likely to show and probable effects of scientific interventions. They also need to be aware of the requirements for appropriate monitoring of the fish, the humane endpoints of the project, and actions to be taken when the endpoints are reached.

A review of the literature provides evidence that similar to other vertebrates, fish experience pain, rather than just nociception (Braithwaite, 2010). Fish have been shown to possess nociceptors that are physiologically identical to those found in mammals, brain structures and opioid compound receptors necessary to feel pain, and a capacity to associate specific events with noxious stimuli (Sneddon et al., 2003; Sneddon, 2009). Although fish lack some of the structures associated with pain perception in mammals (e.g., a well-developed cortex and neospinothalamic tract), there exists evidence that fish respond in a similar manner to noxious stimuli, learn to avoid "unpleasant" experiences and respond with an amelioration of pain response after treatment with morphine and other analgesic drugs (Jansen and Green, 1970; Lopez-Luna et al., 2017). Fish also react to aversive stimuli with a full scale of endocrine and metabolic responses. Changes in corticosteroid and catecholamine levels, as well as increases in plasma glucose and lactic acid, as demonstrated in some fish species, are generally recognized to be indicators of acute stress. Studies on pain in zebrafish are further discussed in Section 10.4.2, "Analgesia".

Manipulations that provoke stress or avoidance/escape behaviour in fish may be causes of distress. Fish respond to noxious stimuli with altered behavioural, physiological and hormonal parameters, in accordance with the intensity of stimuli.

Invasive, potentially painful procedures should be subject to appropriate ethical review and accompanied, where appropriate, by anesthesia and peri-operative care, including analgesia (see Section 10.4, "Anesthesia and Analgesia").

Recognition and evaluation of pain and/or distress in fish can sometimes be difficult, though obvious signs of distress (thrashing, rapid breathing/opercular beat rate, extended mouth and/or gills) should not be ignored. Zebrafish species are prey animals and may be genetically predisposed not to exhibit signs of injury or pain, thus if such signs do appear, immediate action should be taken to alleviate their distress and identify the source. See Section 8, "Welfare Assessment", for potential indicators.

Guideline 17

Endpoints must be defined for studies that involve potential pain and/or distress to fish or where morbidity and mortality are expected, and a list of parameters for objective assessment of the health and well-being of the fish must be established.

For situations that have not been previously demonstrated in similar studies, pilot studies should be carried out to identify clinical signs to be used as the humane intervention points and to establish how best to monitor the fish (e.g., 3Rs-Centre Utrecht Life Sciences, 2016).

Whenever live animals are used for research, teaching and testing, investigators have an ethical obligation to minimize any pain or distress experienced. The importance of identifying the scientific objectives for a study, and ensuring that these reflect a clear understanding of the mechanisms being studied and the consequences to the animals used, is underlined in the *CCAC guidelines on: choosing an appropriate endpoint in experiments using animals for research, teaching and testing* (CCAC, 1998). Selection of appropriate endpoints that meet the scientific goals but minimize the adverse effects for the fish requires the ability to identify signs of pain and/or distress for fish. As discussed earlier, this can be a challenge in many fish species.

Establishing a checklist or scoring sheet may involve a literature review and compilation of documented clinical and behavioural signs (also see Section 8, “Welfare Assessment”). Information accompanying the checklist should include instructions concerning actions that should be taken when abnormalities are observed (including who to contact) and instructions about additional procedures to be taken if a fish is to be euthanized.

It is the responsibility of the investigator, working with the animal care committee and veterinarian, to decide when a clinical sign is a reliable predictor of an event, such as death (i.e. to prevent death as an endpoint) and what margin of error is acceptable.

The use of fish in toxicological research and toxicity testing is well established. Lethal endpoint tests may be required by regulatory agencies, for example in the assay of environmental toxicant mixtures and in fish vaccine efficacy tests. Such tests have the potential to cause pain and/or distress in fish; therefore, the development of pre-lethal scientific endpoints is expected and the fish should be observed more frequently than once per day. If the morbidity and clinical signs are known, then the point at which an intervention (likely euthanasia) becomes essential can be defined; for instance, anorexia of “x” days duration alone or in combination with certain other clinical signs (e.g., more than 10 parasites per fish in a parasite infection study) or a certain number of days post-infection (e.g., parasite load number times number of days infected).

Regulatory agencies should be contacted during the development of the study protocol to agree on the scientific endpoints. In general where lethal endpoints are required, studies should make provision for euthanasia of animals expected to die before the next scheduled observation.

Fish must be monitored at least daily. The frequency of monitoring must be increased where mortality is expected to be high and must allow for the timely removal of fish before severe morbidity occurs. If the timeframe for morbidity is unknown, a pilot study should be conducted under veterinary and animal care committee oversight to determine the most critical period for observation of the fish during the study.

10.1 ADMINISTRATION AND REMOVAL OF SUBSTANCES

Morton et al. (2001) should be consulted for guidance on best practices for the administration of substances. Although principally focused on mammals, there are recommendations for fish and a useful checklist to consult when planning procedures. As with any procedure, administration of substances must be carried out by competent individuals under expert supervision.

10.1.1 Administration of Compounds and Devices

10.1.1.1 Branchial Diffusion ("Inhalation")

The most prevalent route of exposure of fish for chemical agents is via the gills. Fish gills have a large surface area due to a series of lamellae protruding from the surface. The epithelium of the lamellae is extremely thin and designed to facilitate the diffusion of respiratory gases. In addition to gas transfer, fish gills also permit uptake of other molecules. Diffusion or uptake efficiency of chemicals by the gills depends primarily on their hydrophobicity and molecular size (Black, 2000).

10.1.1.2 Oral

If a treatment compound is to be administered orally, the volume dose rate should not exceed 1% body weight (100 μ l/100 mg). Fish may be force-fed liquids and semi-solid solutions; however, many methods produce inaccurate results in zebrafish (Collymore et al., 2013). A gavage method to deliver precise amounts of a substance to anesthetized zebrafish with 88% effectiveness is described by Collymore et al. (2013).

10.1.1.3 Injection

There is little information available on the refinement of procedures for the injection of substances specifically relating to zebrafish, other than limited information on injection techniques in Morton et al. (2001). The most common route for injection in fish is intracoelomic (intraperitoneal). For details of intracoelomic injection techniques, see Kinkel et al. (2010). Intracoelomic injections should avoid penetrating abdominal viscera, as substances that cause inflammation may lead to adhesion formation.

Chemicals to be injected should be dissolved directly in sterile physiological saline appropriate for the species (Westerfield, 2007). However, hydrophobic chemicals should be dissolved in very small quantities of co-solvent (e.g., ethanol, methanol, or dimethyl sulfoxide [DMSO]) prior to dilution in saline. For chemicals that are not soluble or stable at neutral pH, the pH of the injection solution must be adjusted with an acid or base (Perry and Reid, 1994).

Final injection volumes should be as small as possible to minimize physiological disturbances to the fish. In addition, control fish (vehicle and/or sham injected) should be part of the experimental protocol to correct for any effects of the injection procedure or the vehicle. For more information, see Turner et al. (2011).

10.1.1.4 Implants

Implanted materials should be biocompatible and aseptic, and should be implanted using sterile techniques. Bioabsorbable pellet implants of bioactive compounds in absorbable and nonabsorbable matrix vehicles can be surgically implanted in the peritoneal cavity or implanted with a trocar introducer into muscle masses of

fish. However, most implants are suitable for fish weighing over 50 grams, and therefore, are not appropriate for small tropical fish such as zebrafish. For zebrafish, microbead implants can be used to administer compounds as early as embryo stage (Gerlach et al., 2015).

10.1.2 Collection of Body Fluids

Blood collection should be carried out only under anesthesia and should be followed by euthanasia. It is not practical to obtain blood from live zebrafish as a survival procedure since it generally involves causing irrevocable harm. Where the animal care committee approves the collection of blood as a survival procedure, based on scientific justification, the procedure must be developed with the veterinarian and approved by the ACC. Zang et al. (2013) describe a technique for this procedure; however, the method still involves a large incision relative to the small size of zebrafish.

Blood collection must only be undertaken by trained personnel using sterile equipment.

Both restraint and anesthesia may alter physiological parameters such as serum glucose and various hormone levels. In a comparison between tricaine methanesulphonate (TMS; also known as MS 222) and clove oil, Davis et al. (2015) showed that serum cortisol levels and the volume of blood collected vary with the type of anesthetic used.

For maximum blood collection from euthanized zebrafish, retro-orbital bleeding has been shown to be reliable in providing a significantly higher blood yield than collection from a lateral incision (Vliegenthart et al., 2014). Severing the caudal fin of euthanized fish is a common method; however, collection is slower and there is a greater possibility of the sample becoming contaminated with other fluids.

10.2 OTHER EXPERIMENTAL PROCEDURES

10.2.1 Restricted Environments

When it is necessary to keep zebrafish in physically restricted environments such as swim tunnels (e.g., for cardiovascular research), every effort should be made to ensure the environment is as non-stressful as possible. These fish should be acclimated to the restricted environment before the study, and should be kept in such environments for the shortest duration possible. Fish that fail to thrive in these environments (see Section 8, “Welfare Assessment”) should be removed. A protocol for assessing swimming performance is described by Tierney (2011).

10.2.2 Use of Infectious Disease Agents, Tumorigenic or Mutagenic Agents, and Toxic and Noxious Compounds

For studies involving infectious disease agents, tumorigenic or mutagenic agents, or toxic and noxious compounds, it is of particular importance that the protocols include the earliest scientific endpoints that meet the goals of the study; see [CCAC guidelines on: choosing an appropriate endpoint in experiments using animals for research, teaching and testing](#) (CCAC, 1998).

10.2.3 Behavioural Experiments

If fish are to be moved to a new tank for a behavioural study, consideration must be given to the impact of any transportation and/or environmental changes on the behaviour of the fish.

Certain studies may entail the application of a noxious substance, an alarm substance added to the tank water, or electrical shock to induce swimming or fear/avoidance responses in fish. For behavioural studies using negative stimulation, literature searches and pilot studies should be used to establish the least invasive method of obtaining a consistent response. The guiding principle of ethical research in these instances is to avoid excessive or unavoidable stress where possible, and to use procedures that minimize distress. For example, when designing an experiment, a preference test with an avoidable negative stimulus should be substituted for an unavoidable negative stimulation schedule.

10.2.4 Exercise to Exhaustion

Studies involving forced swimming of fish to the point of exhaustion should be conducted with strict adherence to guiding principles of minimizing distress. Fish used in exercise to exhaustion studies should be monitored continuously.

Recovery of fish after exercise to exhaustion may entail special holding and handling arrangements, such as segregation from normal conspecifics and provision of low current environments.

10.2.5 Environmental Extremes

Studies involving the exposure of fish to environmental extremes must select the earliest scientific endpoint possible. In some instances, studies may require that fish are subjected to environmental conditions that are outside of the recommendations in these guidelines. For example, extreme heat or cold in the holding environment may be part of an experimental design. As noted in Section 3.3.1, “Temperature”, zebrafish have been observed to survive in a range of temperatures in their natural habitat and in the laboratory; however, the temperature range at which an animal can survive is different from its preferred temperature range, and suboptimal temperatures will have a metabolic cost that may affect breeding, development and welfare. The rate at which the environment changes also affects the welfare of the fish.

This type of study would be regarded as highly invasive in most vertebrate species. When proposing studies involving exposure to extreme environments, endpoints are important and should be carefully determined to reduce the use of animals in future studies and the level of distress for fish involved in ongoing studies.

If scientific endpoints based on behaviour are proposed, fish behaviour must be observed and recorded in a repeatable fashion using rigorous methodology.

Animals undergoing environmental extremes are likely to experience a range of after-effects, including immunological and physiological changes, which may preclude their use as suitable animal models in subsequent studies.

10.3 GENETICALLY MODIFIED FISH

Newly generated genetically modified fish (i.e. first and second generation) that have changes in physiology and anatomy as the result of their genetic modification, with potential to adversely impact welfare, should be closely monitored.

Genetically modified fish may have different metabolic and environmental requirements compared to non-genetically modified fish. The normative tables generated for fish cannot be automatically applied to genetically modified fish (Stevens et al., 1998). As well, transgenic fish may be created such that they require chemical or other environmental treatment to manipulate transgene expression or function (for example, heat shock protein promoter use requires exposure to high temperature to turn on transgene expression (Shoji and Sato-Maeda, 2008)). Animal welfare concerns regarding these treatments must be considered and addressed.

10.3.1 Creation of Genetically Modified Fish

Genetically modified zebrafish can be created by a variety of methods. The most common is to inject DNA directly into the fertilized egg (Whitfield, 2002). However, injection of DNA at a high concentration can be lethal to embryos or cause abnormalities in their development. When abnormal fry are expected, endpoints must be in place. For more information on manipulating gene expression in the zebrafish, see Sassen and Köster (2015).

Advancements in the field of genetic modification have produced tools such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat - associated protein 9 (CRISPR/Cas9) technology. These tools have allowed increased precision in genetic modifications, as they enable manipulation of DNA at a specific location (Gaj et al., 2013). In particular, CRISPR/Cas9 has evolved to be highly site specific, efficient and easy to use, with application in a growing diversity of research areas (Liu et al., 2017). Liu et al. (2017) provide an overview of the development of CRISPR/Cas9 technology as a gene editing tool for zebrafish, and compare it to other commonly used techniques.

Mutagenesis is becoming less commonly used as new technologies evolve. It involves the induction of random or specific mutations which produce stable and heritable changes in animals. There are a variety of ways that this is done in zebrafish, including the exposure of sperm, embryos or adults to particular chemicals or radiation. Careful consideration must always be given to the type of mutagen chosen for a particular genetic screen¹, since this determines the efficiency of mutation induction. Efficient induction will minimize the number of fish involved.

The type of mutagen influences the type of mutation and potential harms induced. The use of some mutagens can significantly compromise the welfare of adult zebrafish, for example, the chemical mutagen N-ethyl-N-nitrosourea (ENU) is highly toxic (Pelegri, 2002). Fish can also become very agitated during ENU treatment and it is essential to ensure that refinements, such as reduction in external stimuli (e.g., noise and light), which may disturb the animals, are implemented to help reduce potential suffering and improve survival rates (Pelegri, 2002).

¹ Details on specific practical methodologies used to perform mutagenesis and genetic screens in zebrafish are available in Pelegri (2002).

For regulations related to genetically modified fish, see Section 4.2, “Regulations”.

10.3.2 Genotyping

The genetic composition of zebrafish is determined using breeding records, phenotypic classifications of the fish and their siblings, and genetic and molecular tests to determine whether fish carry particular recessive traits (Matthews et al., 2002). The latter require biopsies to obtain tissue for DNA isolation and PCR analysis, which may cause suffering and distress due to the need for capture, handling and surgical procedures.

The most common biopsy technique used for zebrafish is to cut off a small part of the caudal fin using a sterile razor blade, scalpel or surgical scissors. Fish should be anesthetized during this procedure. Only the minimum amount of tissue necessary (2-3 mm) should be taken, as the caudal fin is innervated and clearly important for locomotion. Personnel with practical experience in the fin clip procedure should be able to perform it very rapidly, without causing bleeding (Matthews et al., 2002). No pre-surgical cleansing of the caudal fin should be necessary; however, gloves should be worn and the surgical area should be clean. Before surgery, small (500 ml), individual tanks containing clean water from the holding tank should be set up for anesthesia and recovery of fish. It may be necessary to either singly house fish until the PCR analysis is completed, or ensure that individuals can be identified before regrouping (see Section 6.1, “Identification”). If single housing is required, this should be for the minimum period possible.

Skin swabs do not require anesthetic and may be a less invasive alternative to fin clipping for some studies (Breacker et al., 2017). However, care should be taken to minimize contamination and cross-contamination of the DNA sample when using a swab.

10.3.3 Cryopreservation

Cryopreservation of sperm, eggs and embryos is a useful tool for archiving genetically modified animal lines until they are required, and for providing a strategic reserve in case of genetic contamination or drift, pathogenic infection and natural disasters. Cryopreservation can also avoid potential logistical and animal health and welfare problems associated with the live transport of animals and can substantially reduce the number of animals used to re-establish a genetically modified line (RSPCA, 2008).

Several common pathogens have been shown to survive cryopreservation (e.g., *Mycobacteria*, *Edwardsiella* and *Pseudoloma*). It is therefore recommended that sperm samples or the donor fish be tested prior to cryopreservation (Norris et al., 2018).

10.3.3.1 Sperm

A number of cryopreservation and thawing protocols have been used and result in varying degrees of success in the preservation of viable sperm. Carmichael et al. (2009) review some of the common methods and provide a description of the protocol used at the Zebrafish International Resource Center.

A simple method uses 10% N,N-dimethylacetamide (DMA) diluted in buffered sperm motility-inhibiting solution (BSMIS) as a cryoprotective medium. A 14% fertilization rate has been observed following the use of this method (Morris et al., 2003).

An older method (Harvey et al., 1982) uses methanol and milk powder as the cryoprotective agent, but this protocol is more complicated and has been found to be difficult to reproduce. However, several adaptations

have been made to this protocol that show good potential (e.g. Draper and Moens, 2009; Yang et al., 2007; Matthews et al., 2018; Matthews et al., 2017).

Given the ongoing work in this area, the success rates of reconstitution using preserved and thawed sperm are likely to increase and investigators should keep up-to-date with the latest developments.

10.3.3.2 Eggs

Isayeva et al. (2004) demonstrated that zebrafish eggs are highly sensitive to chilling, and survival after chilling depends on the exposure temperature, exposure time period, developmental stage, and the individual female. It was concluded that sensitivity of zebrafish eggs to chilling may be one of the limiting factors in the development of a successful protocol for their cryopreservation.

10.3.3.3 Embryos

Cryopreservation of embryos has not been possible previously, due to problems associated with high egg yolk content and low membrane permeability. This stops water removal from the cell and penetration of the cryoprotective agent, which results in chilling injury. However, there has been success with freezing both zebrafish blastomeres and yolk-removed embryos, and research continues in this field (Lin et al., 2009; Higaki et al., 2010). In addition, a process involving the use of cryoprotectants, gold nanoparticles, and rapid warming post-cryopreservation has resulted in 10% viable embryos at 24 hours post warming (Khosla et al., 2017).

10.4 ANESTHESIA AND ANALGESIA

10.4.1 Anesthesia

Guideline 18

Anesthetics must be used in procedures where there is expected to be noxious stimuli, and in experiments entailing extensive handling or manipulation with a reasonable expectation of trauma and physiological insult to the fish.

Anesthesia is generally defined as a state caused by an applied external agent, resulting in depression of the nervous system, leading to loss of sensation and motor function. As with other species, zebrafish require appropriate anesthesia when undergoing or when exposed to potentially painful procedures. Anesthesia is also needed for some procedures involving handling in order to reduce stress and minimize the risk of injury due to escape behaviours. For procedures such as grading, sedation is also used during manipulation of the fish.

The use of anesthetics facilitates work with fish and is required for invasive procedures such as surgical preparations for physiological studies, where the fish must be held immobile for extended periods. In addition to holding fish immobile while being handled, anesthetics are also used to lower the level of stress associated with such procedures and may alleviate pain (Iwama et al., 1988; Davis, 1992; Iwama, 1992). Given that anesthetic overdoses are used routinely as an effective and humane means of euthanizing fish, care must be taken in calculating the appropriate concentration of the anesthetic dose to avoid accidental mortality.

During induction, spontaneous ventilation (e.g., gill movement) should be monitored closely and can be used as an indicator of the depth of anesthesia (Matthews et al., 2002).

For surgery, fish are usually kept on a moist cloth. A number of positioning aids are also available (Brattelid and Smith, 2000). Other than for brief anesthesia, care should be taken to continuously irrigate the gills with aerated water containing the anesthetic. Recovery should occur within 10 minutes of a return to clean, well-aerated water.

Water quality conditions during anesthesia must be maintained stable within ranges appropriate for the species, with minimal bacterial and organic burden. Water for anesthesia should be from the same source as the tank water to minimize shock caused by differences in temperature, pH, electrolytes, etc.

The most common anesthetic agent currently used is TMS in an aqueous solution. The level of anesthesia required will depend on the particular procedure being performed. See Table 4 for information on dosages of common anesthetics to achieve different levels of anesthesia in zebrafish.

Volumes for anesthesia should be carefully calculated and solutions should be made up fresh on each occasion. Where several fish are anesthetized serially in the same bath(s), there should be assurance that there is adequate oxygenation of the water and the level and speed of anesthesia are maintained.

Table 4 Anesthetic Agents Used for Zebrafish

DRUG	DOSAGE	ANESTHETIC STAGE	OBSERVATIONS & COMMENTS
Tricaine methanesulfonate (Chen et al., 2014; Collymore et al., 2014c; Matthews and Varga, 2012)	50 mg/L	Sedation	Must be buffered with sodium bicarbonate.
	50-100 mg/L	Light anesthesia	
	150-200 mg/L	Surgical anesthesia	Also used to anesthetize larval zebrafish; provides general anesthesia for larvae and does not paralyze muscle at standard dosage (Attili and Hughes, 2014). Has been found to induce developmental and behavioural alterations in zebrafish embryos (Félix et al., 2018).
Benzocaine (Readman et al., 2017)	25-100 mg/L	Light anesthesia	After induction with TMS, intermittent dosing with benzocaine, according to procedures used by Readman et al. (2017), maintains anesthesia for median time of 7.5h.
2-Phenoxyethanol (Ackerman et al., 2005)	200-300 µL/L	Light anesthesia	
Clove oil/eugenol/ isoeugenol (Grush et al., 2004)	2-5 mg/L	Sedation	
	60-100 mg/L	Surgical anesthesia	

DRUG	DOSAGE	ANESTHETIC STAGE	OBSERVATIONS & COMMENTS
Metomidate hydrochloride (Collymore et al., 2014c)	2-4 mg/L	Sedation	
	6-10 mg/L	Light anesthesia	
Lidocaine hydrochloride (Collymore et al., 2014c)	300 mg/L	Light anesthesia	A propofol/lidocaine combination has been shown to induce loss of equilibrium and anesthesia faster than TMS (Martins et al., 2018; Valentim et al., 2016); however, Martins et al. (2018) found no significant difference in recovery between propofol/lidocaine and TMS, while Valentim et al. (2016) found fish treated with TMS recovered quicker.
	325 mg/L	Surgical anesthesia	
Tricaine methanesulfonate and isoflurane (Huang et al., 2010)	65 ppm + 65 ppm	Light anesthesia	Maintains opercular movement for 20-60 min in adults.
	175 ppm + 175 ppm	Deep anesthesia	For imaging for approximately 10 min duration.

Gradual cooling slows movements and metabolic activity and has been used for some minor procedures; however, hypothermia is not an effective anesthetic as it does not completely block nerve impulses, and therefore should not be used for invasive procedures (Matthews and Varga, 2012).

Since fish are ectotherms, the environmental temperature during anesthesia will affect their metabolism. This, in turn, influences the rate of absorption and excretion of the anesthetic agent and its subsequent effectiveness.

Concerns have recently been raised that several anesthetics may be aversive to zebrafish (Wong et al., 2014; Readman et al., 2013), and the literature should be reviewed in selecting the most appropriate anesthetic. However, this should not influence the use of an anesthetic when necessary.

Researchers should ensure they stay informed of latest scientific literature on appropriate anesthetic regimes for fish. This should include the effectiveness of current dosing recommendations in terms of their ability to induce anesthesia or relieve pain, without causing distress.

Anesthetics should be chosen on the basis of their documented ability to provide predictable results, including immobilization, analgesia and rapid induction and recovery, while allowing for a wide margin of safety for the animals and the operators, without causing interference with the research project. The investigator should ensure that the anesthetic selected has no toxic side-effects for the fish or the handler; is biodegradable and can be cleared from the fish; and has no persisting physiological, immunological or behavioural effects.

Where anesthesia will be of long duration, a recirculation technique that ensures continuous delivery of oxygenated water and anesthetic to the gills should be employed (Iwama and Ishimatsu, 1994) and levels of dissolved oxygen should be monitored. Wynd et al. (2016) describe continuous and intermittent administration of benzocaine to immobilize adult zebrafish for long-term imaging. Additionally, the duration

of anesthesia using TMS has been shown to be extended when combined with isoflurane, which has also resulted in faster recovery time with minimal side effects (Huang et al., 2010; Lockwood et al., 2017).

Any application of anesthetics that is not covered by a facility SOP should be tested on a small sample of fish under veterinary supervision, as the effect of an anesthetic can vary with local water conditions, species, life stage, and size of the fish. The reaction of any fish to the proposed anesthetic should be well understood. Trials with healthy fish are recommended to ensure proper dosage, and to accurately calculate the time to reach the necessary anesthetic plane (Johnson, 2000).

Many of the anesthetics in use have the potential to cause harm to humans if they are misused; therefore, personnel working with anesthetic agents in fish must be competent and protected with personal protective equipment.

10.4.2 Analgesia

Guideline 19

Following the precautionary principle, fish should be provided with analgesia for procedures that are likely to be painful, based on the best available scientific evidence.

While limited studies have been carried out to determine the safety and efficacy of potential analgesics, routes of administration and dose rates for zebrafish, analgesics should be used as a precaution in situations that are likely to cause pain. Correia et al. (2011) found that the behavioural responses of zebrafish to a noxious substance such as acetic acid was reduced by injection of morphine, and Schroeder and Sneddon (2016) showed the potential of low-dose lidocaine immersion as an analgesic for zebrafish subjected to invasive procedures such as fin clipping. Lopez-Luna et al. (2017) showed that larval zebrafish had a behavioural response to noxious stimuli which could be ameliorated by analgesics. A few analgesics, such as morphine and ketoprofen, have been evaluated in fish species other than zebrafish (NRC, 2009).

Although little is known about the effect of analgesic drugs on fish, investigators are encouraged to use post-operative analgesia as suitable analgesic agents become available (see Chatigny et al. [2018] for a review of fish analgesia). Fish produce opioid substances in response to pain and fear, similar to terrestrial animals, i.e. Substance P, enkephalins and β -endorphins (Vecino et al., 1992; Rodriguezmoldes et al., 1993; Zaccone et al., 1994; Balm and Pottinger, 1995), and the response of goldfish to analgesia has been shown to be similar to that of rats (Jansen and Green, 1970). The response of carp (*Cyprinus carpio*) to electric shock, to the presence of alarm substance chemicals in water, and to hook and line fishing indicates that reactions to repeated shocks is graded, non-reflexive, and similar in nature to that in mammals (Verheijen and Buwalda, 1988).

10.5 SURGERY AND POST-OPERATIVE CARE

While surgery is not typically performed on zebrafish, surgery-type situations include regenerative studies involving tissues from such organs as the heart and brain.

10.5.1 Surgery

Surgery must only be performed by competent individuals. Surgery in fish can be complex and intricate, particularly in very small fish such as zebrafish. Anyone attempting any invasive surgery must be properly trained and competent in surgical aseptic technique, or must obtain the services of a veterinarian. Fish surgery should normally be covered under the institutional veterinary care program.

Water used to irrigate fish gills during prolonged anesthetic procedures should be circulated and treated to maintain proper anesthetic levels, oxygen, temperature, pH, and salinity, and to remove particulates. As water temperature can be affected by room air temperature and use of surgical lights, it should be carefully monitored and controlled. Water quality can be affected by the production of mucus, urine or feces during surgery, and should be changed or renewed accordingly.

In some instances, the addition of conditioning solutions to the water may be justified, particularly for the replacement of electrolytes following trauma or stress.

The site on the fish where the surgical incision will be made should be prepared to minimize tissue damage and contamination of the wound area. The removal of mucus and disruption of scales that occurs during surgical preparation may devitalize tissue and render the area more subject to attack by saprophytic agents, particularly fungal and bacterial invasion.

Although creating surgically clean skin is problematic in fish, the provision of sterile occlusive drapes will help to maintain a surgically clean operating field. Aseptic plastic food wrap is preferable to fabric drapes, as the latter is prone to absorption of water and introduction of bacteria in the water.

Attention must be paid to the use of asepsis, disinfection and sterile instruments to minimize wound contamination and maximize the healing response. Instruments may be autoclaved or gas sterilized, or where this is not possible, hot bead sterilized. Cold sterilization for 10 minutes using benzalkonium chloride or cold disinfectants, such as alcohol, iodine and hydrogen peroxide, could be used, although this technique is unlikely to be sporicidal. These latter agents are toxic to tissues and the instruments must be rinsed thoroughly in sterile water before being used. In multiple surgeries, two sets of instruments should be rotated through a cold sterilizing solution. A hot bead sterilizer may also be used and is the most practical method for many laboratory situations.

The mucus layer of fish is integral in healing, and it is important that it is maintained. Care must be taken to avoid surgical disinfectants (e.g., alcohol and iodine) from coming in contact with the surface of the fish, as they can cause devitalization or irritation and excessive mucus production.

Any incisions should avoid the lateral line and follow the longitudinal axis of the fish. When fish scales need to be removed, they should be removed individually by pulling in a posterior direction to minimize damage. Only the scales necessary to create the incision should be removed, as the scales provide protection and stability to the wound area.

Although sutures are not used in zebrafish surgery, tissue glue may have application in certain types of surgery. Cyanoacrylate surgical adhesives should be used sparingly and care must be taken to ensure they only come in contact with the intended site of application. It is important that the tissue is dry at the time of application to seal the wound.

Opercular beat rate should be monitored during surgery and if the opercular beating stops, the fish should be removed from the anesthetic.

10.5.2 Post-Operative Care and Monitoring

Factors that affect wound healing include:

- water quality (hardness, levels of salt and other osmotically active compounds, and water temperature), which regulates immune response and tissue metabolism rate;
- presence of tank mates, cannibalism of surgical wound area by conspecifics, and exclusion of fish with postoperative morbidity during competition associated with feeding;
- nutritional plane before surgery, good nitrogen balance, anorexia after surgery (stress response), and speed of return to normal feeding and other behaviours;
- hormonal status;
- changes in electrolyte balance due to open wounds and passive loss of electrolytes to the surrounding water;
- integrity of mucus layers and biofilms on fish; and
- presence/absence of opportunistic bacterial and fungal pathogens in water during and after surgery.

Following surgery, fish must be carefully monitored until they recover, and held in conditions that are appropriate for the species and their condition. Negative social interactions must be eliminated or minimized. Although recovering fish may appear to be normal, there may be prolonged metabolic effects following the stress of anesthesia and surgery. In situations where monitoring is not possible, pilot-scale evaluations of procedures should be considered. Where possible, fish should be allowed to recover from anesthesia until able to resume normal behaviour.

Fish require extra attention in the postoperative recovery period. A number of common complications may occur, including wound dehiscence and infection, osmotic imbalances related to surgical incisions, and anorexia. Transient postsurgical shock is a common problem in fish and includes problems with oxygen debt, catabolic processes, fluid and electrolyte loss, and hormonal imbalance. It is important to keep recovery water clean.

Recovery tanks should be designed to promote recovery with reduced risk of long-term effects from anesthesia. As anesthesia itself causes prolonged stress, careful procedures for recovery are vital, for example, a quiet, well aerated, and possibly darkened tank will facilitate recovery. Additional considerations for suitable recovery tanks include opportunity to observe the fish, good quality water (with removal of excreted anesthetic), avoidance of stressful stimulation, consistent temperature, and decreased exposure to other compromised fish that may be a source of infectious disease agents. The use of water conditioning agents to improve the buffering ability of the water and to supply lost electrolytes may help speed recovery.

The return of fish to the pre-surgery tank, including the presence of tank mates, should consider both the benefits of companion fish (e.g., social interaction and feeding activity stimulation) and any disadvantages (e.g., competition for feed and aggressive behaviour).

11 EUTHANASIA

Guideline 20

Euthanasia of zebrafish must be carried out by competent¹ personnel only, using the method that minimizes pain and distress for the particular fish and is suited to the study data.

Considerations in the selection of an appropriate method of euthanasia must include the size and developmental stage of the zebrafish, as these are both major variables in the effectiveness and humaneness of the method (Strykowski and Schech, 2015; Collymore et al., 2016b), and the effects of the method on research results (Köhler et al., 2017).

The preferred methods of euthanizing zebrafish are 1) use of lethal levels of a central nervous system depressant, followed by a physical method such as decapitation or maceration, or excessive exposure to a chemical agent to ensure death (see Table 5); and 2) rapid cooling in a water bath at 4°C or less, followed by prolonged exposure of at least 40 minutes (depending on the age of the fish) to cold temperature to ensure death (Strykowski and Schech, 2015). If the fish cannot be held for this length of time, a secondary method should be used.

Physical methods may be appropriate in exceptional cases when fish are in extreme distress and the time needed to prepare the anesthesia or water bath would result in prolonged distress (e.g., for immediate euthanasia of a fish that escapes from a tank and cannot be returned for biosecurity reasons).

Information on possible methods of euthanasia for adult zebrafish is provided in Table 5. Also, see Köhler et al. (2017) for the advantages and disadvantages of various methods in terms of effectiveness and impacts on research.

¹ Competency is defined in the [glossary](#).

Table 5 Possible Methods of Euthanasia for Adult Zebrafish

Method	Details of the Procedure	Comments
Chemical Agents	<ul style="list-style-type: none"> Overdose of an anesthetic using a route and an anesthetic agent appropriate for the size and species of fish, followed by a secondary method to confirm death. Agents listed as acceptable by AVMA (2020): immersion in buffered benzocaine or benzocaine hydrochloride, isoflurane, sevoflurane, quinaldine sulfate, buffered TMS, 2-phenoxyethanol, injected pentobarbital, lidocaine hydrochloride. Agents listed as acceptable with conditions by the AVMA (2020): eugenol, isoeugenol, clove oil. 	<ul style="list-style-type: none"> Concern has arisen that TMS may be aversive, it could be acting as a neuromuscular blocking agent, and it may not always be reliable (as noted in Section 10.4.1, “Anesthesia”). One study has shown metomidate hydrochloride and clove oil to be less aversive to zebrafish than TMS (Wong et al., 2014), while another study showed both TMS and isoeugenol (an active ingredient in clove oil) were aversive to zebrafish (Readman et al., 2013). This second study also found benzocaine to be aversive, while etomidate and 2,2,2 tribromoethanol were not aversive. Based on these studies, euthanasia using metomidate/etomidate appears to be more humane than TMS, and further evidence is needed on the humaneness of clove oil and its components (Hawkins et al., 2016). Matthews and Varga (2012) provide descriptions of various chemical agents used to kill fish.
Hypothermal Shock	<ul style="list-style-type: none"> Rapid chilling followed by an adjunctive method is listed as acceptable with conditions by AVMA (2020). The procedure involves immersing the fish in ice water at a temperature of 4°C or less, followed by prolonged exposure of at least 40 minutes (depending on the age of the fish) to cold temperature to ensure death (Strykowski and Schech, 2015). Direct contact between the fish and ice should be avoided through the use of an ice slush bath or by holding the fish in a mating cage with the ice at the bottom of the tank. 	<ul style="list-style-type: none"> Studies suggest that inducing hypothermal shock in adult zebrafish by placing them in a water bath at 4°C or less may be more humane than using TMS (Wilson et al., 2009). Also see Matthews and Varga (2012) for details of the method.
Physical Methods	<ul style="list-style-type: none"> Maceration and decapitation are listed as acceptable with conditions by AVMA (2020). 	<ul style="list-style-type: none"> Decapitation does not destroy the brain and should be followed by pithing. The small size of zebrafish makes a blow to the skull unsuitable (Köhler et al., 2017).

Euthanasia methods that use chemical agents (e.g., TMS) are generally less effective on fry than adults (Strykowski and Schech, 2015). For embryos (<3 days post-fertilization), hypothermal shock and immersion in TMS do not reliably result in euthanasia and should be followed by a secondary method, such as diluted sodium or calcium hypochlorite solution (500mg/ml) (AVMA, 2020). A study by Wallace et al. (2018) provides further evidence that younger fish require longer exposure to rapid cooling for euthanasia than adults. They also note that length of time required depends on the strain, genetic modifications and environmental factors (e.g., density, temperature and water quality), which all influence the rate of growth and maturity of young fish. Wallace et al. (2018) provide a conservative recommendation of 12 hours of exposure to euthanize zebrafish <14 days post-fertilization, while Matthews and Varga (2012) report that 20 minutes of hypothermic shock alone is sufficient for fish at 5-6 days post fertilization.

If a physical technique is used when euthanizing zebrafish, it should entail the physical destruction of brain tissue by maceration. Because many species of fish continue to have brain activity in the face of advanced cerebral and systemic hypoxia, physical euthanasia techniques such as decapitation alone should be avoided (Flight and Verheijen, 1993). It is therefore desirable to physically destroy or freeze the brain in fish that have been euthanized using a physical technique. Exsanguination under anesthesia is also an acceptable method of euthanasia (CCAC, 2010), but is not a preferred method because of the small size of these fish.

Use of carbon dioxide is not an acceptable method of euthanasia, nor is suffocation by draining the tank or removing the fish from water.

If other methods of euthanasia are being considered, they must be thoroughly researched and justified to the animal care committee.

12

END OF STUDY

12.1 FISH AS PETS

Where institutions allow the release of healthy research fish (not genetically modified fish) as companion species to individuals with the knowledge and ability to provide adequate care, they should develop an appropriate policy describing the conditions that need to be fulfilled and the required documentation, prior to release. No genetically modified fish may be removed from research facilities to private premises unless the specific strain is approved for commercial ornamental aquarium use under CEPA NSNR(O) (see Section 4.2, “Regulations”). Where institutions have policies against personnel having fish as pets at home to prevent potential transmission of disease, these must be followed.

12.2 TRANSFER OF FISH BETWEEN FACILITIES

Appropriate regulatory approval and permits must be obtained and fish should undergo a health assessment prior to movement from facilities. Further information on transport and reception of fish is detailed in Section 4, “Procurement”.

The transfer of unhealthy fish between facilities should be avoided, other than when requested by a veterinarian for the purposes of clinical investigation and diagnosis. To minimize the potential for disease transfer, transfer of disinfected embryos is preferable to transfer of adults or juvenile fish.

12.3 DISPOSAL OF DEAD FISH

Fish must be disposed of according to acceptable federal, provincial/territorial and municipal regulations for the disposal of biological materials.

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HUMAN HEALTH AND SAFETY

Institutions have occupational health and safety programs that are specifically tasked with addressing this topic through risk assessments. The responsibility of the animal care committee extends to ensuring there is an institutional occupational health and safety program in place so that any risks to human health and safety are properly assessed.

Those working with fish must follow institutional policies and standard operating procedures outlining appropriate measures of prevention and protection. They should seek professional knowledge on zoonotic diseases, as well as other risks or hazards that may be associated with a particular study, such as exposure to radiation, anesthetic gas, chemical hazards, and human cell lines. The use of TMS, clove oil, and other chemicals require adherence to chemical safety requirements, PPE and proper disposal, as they can be carcinogenic.

13.1 ZOOONOTIC DISEASES

Zoonoses, also called zoonotic diseases, are animal diseases that may be transmitted to humans. In general, transmission of fish-borne zoonoses is relatively rare, and the vast majority of these organisms produce localized wound infections due to contamination of cuts or abrasions while handling either live fish, fish tissues, or water fish reside in. However, a few of the more virulent organisms have the ability to produce systemic infections in humans and, in very rare instances, death (Nemetz and Shotts, 1993). Although zoonotic diseases are rare in healthy individuals, the risk is markedly increased in people with depressed immunity (e.g., people with AIDS or organ transplant recipients receiving immunosuppressive drugs).

Diseases of fish can be induced by a variety of bacteria, rickettsiae, roundworms (nematodes), cestodes (tapeworms), flukes (trematodes), protozoa, viruses, and fungi (Nemetz and Shotts, 1993; Fryer and Bartholomew, 1996). The type of infestation is dependent upon a myriad of factors, including the species, the supplier, the geographic origin, and the diet of the fish; other important issues are water quality and salinity.

Zoonotic organisms of concern when working with zebrafish and other small warm-water laboratory fish include:

- gram negative bacteria: *Aeromonas* spp., *Plesiomonas shigelloides*, *Pseudomonas fluorescens*, *Escherichia coli*, *Salmonella* spp., *Klebsiella* spp. and *Edwardsiella* spp.;
- gram positive bacteria: *Mycobacterium* spp., *Streptococcus* spp., *Erysipelothrix* spp., *Clostridium* spp., *Staphylococcus* spp. and *Nocardia* spp.; and
- protozoa: *Cryptosporidium* spp. (Byers and Matthews, 2002).

Erysipelothrix and *Mycobacterium* usually cause a localized skin infection but, under some circumstances, can cause a disseminated infection (i.e. septicemia). In contrast, skin contact with *Clostridium* or *Staphylococcus* does not usually cause an infection in humans. Mason et al. (2016) describe a case study of *Mycobacterium marinum* infection of zebrafish and personnel at a research facility and the effectiveness of the mitigation strategies that were implemented. Personnel should wear gloves and all personnel should wash their hands upon entering and exiting an aquatic facility.

Prompt and accurate diagnosis of fish-borne zoonoses expedites appropriate treatment. Because they are rare, most physicians, even those specializing in the treatment of infectious diseases, have little or no experience diagnosing them. Therefore, it is important for people working with fish to be aware of the existence of fish-borne zoonoses and, if being evaluated by a physician, to mention that they have occupational exposure to fish.

With the advent of fish as a source of implantable biomaterials, in xenotransplantation, or as bioreactors for large-scale production of human proteins, the possibility of emerging xenozoonoses transmitted from these sources should be considered. At the present time, disease agents recognized as a hazard in other xenotransplantation models, such as mammalian endogenous retroviruses, are not a recognized threat from fish tissues; however, the knowledge of this area is in its infancy.

The Centers for Disease Control and Prevention (CDC) provides further information on diseases of aquarium fish and their prevention.

REFERENCES

- 3Rs-Centre Utrecht Life Sciences (2016) [Humane Endpoints – Score sheets for zebrafish](#) (accessed 2020-10-02).
- Abdallah S.J., Thomas B.S. and Jonz M.G. (2015) Aquatic surface respiration and swimming behaviour in adult and developing zebrafish exposed to hypoxia. *Journal of Experimental Biology* 218:1777-1786.
- Abdollahpour H., Falahatkar B., Jafari N. and Lawrence C. (2020) Effect of stress severity on zebrafish (*Danio rerio*) growth, gonadal development and reproductive performance: do females and males respond differently? *Aquaculture* 522:735099.
- Ackerman P., Morgan J. and Iwama. G. (2005) [Anesthetics](#). Published as a resource for the CCAC guidelines on: the care and use of fish in research, teaching and testing. Ottawa ON: CCAC (accessed 2020-10-02).
- Adatto I., Krug L. and Zon L.I. (2016) The red light district and its effects on zebrafish reproduction. *Zebrafish* 13(3):226-229.
- Alanärä A., Kadri S. and Paspatis M. (2001) Feeding management. In: *Food Intake* (Houlihan D., Boujard T. and Joblings M., eds.). pp. 332-353. Oxford UK: Blackwell Science.
- American Veterinary Medical Association – AVMA (2020) *AVMA Guidelines for the Euthanasia of Animals*, 2020 edn. Schaumburg IL: AVMA.
- Anderson J.L., Macurak M.L., Halpern M.E. and Farber S.A. (2010) A versatile aquatics facility inventory system with real-time barcode scan entry. *Zebrafish* 7(3):281-287.
- Andrews C. (1999) Freshwater fish. In: *The UFAW Handbook on the Care and Management of Laboratory Animals*, 7th ed., volume 2 Amphibious and Aquatic Vertebrates and Advanced Invertebrates (Poole T., ed.). Oxford UK: Blackwell Science.
- Animals (Scientific Procedures) Inspectorate – ASPI (2006) *Animals (Scientific Procedures) Inspectorate - Annual Report 2005*.
- Aoyama Y., Moriya N., Tanaka S., Taniguchi T., Hosokawa H. and Maegawa S. (2015) A novel method for rearing zebrafish by using freshwater rotifers (*Brachionus calyciflorus*). *Zebrafish* 12(4):288-295.
- Arunachalam M., Raja M., Vijayakumar C., Malaiammal P. and Mayden R.L. (2013) Natural history of zebrafish (*Danio rerio*) in India. *Zebrafish* 10:1-14.
- Astrosky K.M., Harper C.M., Rogers A.B. and Fox J.G. (2002) Diagnostic techniques for clinical investigation of laboratory zebrafish. *Lab Animal* 31(3):41-45.
- Attili S. and Hughes S.M. (2014) Anaesthetic tricaine acts preferentially on neural voltage-gated sodium channels and fails to block directly evoked muscle contraction. *PLoS ONE* 9(8):e103751.
- Avdesh A., Chen M., Martin-Iverson M.T., Mondal A., Ong D., Rainey-Smith S., Taddei K., Lardelli M., Groth D.M., Verdile G. and Martins R.N. (2012) Regular care and maintenance of a zebrafish (*Danio rerio*) laboratory: an introduction. *Journal of Visualized Experiments* 69:e4196.

- Balm P.H. and Pottinger T.G. (1995) Corticotrope and melanotrope POMC-derived peptides in relation to interrenal function during stress in rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology* 98(3):279-288.
- Barcellos H.H.A., Koakoski G., Chaulet F., Kirsten K.S., Kreutz L.C., Kalueff A.V. and Barcellos L.J.G. (2018) The effects of auditory enrichment on zebrafish behavior and physiology. *PeerJ* 6:e5162.
- Barton C.L., Johnson E.W. and Tanguay R.L. (2016) Facility design and health management program at the Sinnhuber Aquatic Research Laboratory. *Zebrafish* 13(Suppl. 1):S39–S43.
- Basnet R.M., Zizioli D., Taweedet S., Finazzi D. and Memo M. (2019) Zebrafish larvae as a behavioral model in neuropharmacology. *Biomedicine* 7(1):23.
- Best J., Adatto I., Cockington J., James A. and Lawrence C. (2010) A novel method for rearing first-feeding larval zebrafish: polyculture with Type L saltwater rotifers (*Brachionus plicatilis*). *Zebrafish* 7(3):289-295.
- Bilotta J. (2000) Effects of abnormal lighting on the development of zebrafish visual behavior. *Behavioral Brain Research* 116:81-87.
- Bilotta J. and Saszik S. (2001) The zebrafish as a model visual system. *International Journal of Developmental Neuroscience* 19(7):621-629.
- Bilotta J., Saszi S., DeLorenz A.S. and Hardesty H.R. (1999) Establishing and maintaining a low-cost zebrafish breeding and behavioural research facility. *Behaviour Research Methods, Instruments and Computers* 31(1):178-184.
- Black M.C. (2000) Routes of administration for chemical agents. In: *The Laboratory Fish*. (Ostrand G.K., ed.). Chapter 31, pp. 529-542. San Diego CA: Academic Press.
- Blanco-Vives B. and Sánchez-Vázquez F.J. (2009) Synchronisation to light and feeding time of circadian rhythms of spawning and locomotor activity in zebrafish. *Physiology and Behavior* 98(3):268-275.
- Blaser R.E. and Rosemberg D.B. (2012) Measures of anxiety in zebrafish (*Danio rerio*): Dissociation of black/white preference and novel tank test. *PLoS ONE* 7:e36931.
- Bloom H.D. and Perlmutter A. (1977) A sexual aggregating pheromone system in the zebrafish, *Brachydanio rerio* (hamilton-buchanan). *Journal of Experimental Zoology* 199(2):215-226.
- Boltaña S., Rey S., Roher N., Vargas R., Huerta M., Huntingford F.A., Goetz F.W., Moore J., Garcia-Valtanen P., Estepa A. and Mackenzie S. (2013) Behavioural fever is a synergic signal amplifying the innate immune response. *Proceedings: Biological Sciences* 280(1766):1-11.
- Borges A.C., Pereira N., Franco M., Vale L., Pereira M., Cunha M.V., Amaro A., Albuquerque T. and Rebelo M. (2016) Implementation of a zebrafish health program in a research facility: a 4-year retrospective study. *Zebrafish* 13(Suppl. 1):S115–S126.
- Braithwaite V. (2010) *Do fish feel pain?* Oxford UK: Oxford University Press.
- Brand M., Granato M. and Nüsslein-Volhard C. (2002) Keeping and raising zebrafish. In: *Zebrafish – A Practical Approach*. (Nüsslein-Volhard C. and Dahm R., eds.). Chapter 1. Oxford UK: Oxford University Press.
- Brattelid T. and Smith A.J. (2000) Methods of positioning fish for surgery or other procedures out of water. *Laboratory Animals* 34:430-433.

- Breacker C., Barber I., Norton W.H.J., McDearmid J.R. and Tilley C.A. (2017) A low-cost method of skin swabbing for the collection of DNA samples from small laboratory fish. *Zebrafish* 14(1):35-41.
- Budaev S.V., Zworykin D.D. and Mochek A.D. (1999) Individual differences in parental care and behaviour profile in the convict cichlid: A correlation study. *Animal Behaviour* 58(1):195-202.
- Burgess H.A. and Granato M. (2007) Modulation of locomotor activity in larval zebrafish during light adaptation. *Journal of Experimental Biology* 210(14):2526-2539.
- Byers K.B. and Matthews J.L. (2002) Use of zebrafish and zoonoses. *Applied Biosafety* 7(3):117-119.
- Canadian Council on Animal Care – CCAC (1989) [CCAC policy statement on: ethics of animal investigation](#). Ottawa ON: CCAC (accessed 2020-10-02).
- Canadian Council on Animal Care – CCAC (1998) [CCAC guidelines on: choosing an appropriate endpoint in experiments using animals for research, teaching and testing](#). Ottawa ON: CCAC (accessed 2020-10-02).
- Canadian Council on Animal Care – CCAC (2003) [CCAC guidelines on: laboratory animal facilities – characteristics, design and development](#). Ottawa ON: CCAC (accessed 2020-10-02).
- Canadian Council on Animal Care – CCAC (2005) [CCAC guidelines on: the care and use of fish in research, teaching and testing](#). Ottawa ON: CCAC (accessed 2020-10-02).
- Canadian Council on Animal Care – CCAC (2007) [CCAC guidelines on: procurement of animals used in science](#). Ottawa ON: CCAC (accessed 2020-10-02).
- Canadian Council on Animal Care – CCAC (2010) [CCAC guidelines on: euthanasia of animals used in science](#). Ottawa ON: CCAC (accessed 2020-10-02).
- Canadian Council on Animal Care – CCAC (2015) [CCAC guidelines on: training of personnel working with animals in science](#). Ottawa ON: CCAC (accessed 2020-10-02).
- Canadian Council on Animal Care – CCAC (2017) [CCAC guidelines on: Husbandry of animals in science](#). Ottawa ON: CCAC (accessed 2020-10-02).
- Canadian Food Inspection Agency – CFIA (2013) [Containment Standards for Facilities Handling Aquatic Animal Pathogens](#), 1st ed. Ottawa ON: Government of Canada (accessed 2020-10-02).
- Canadian Food Inspection Agency – CFIA (2015) [Canadian Biosafety Standards](#), 2nd ed. Ottawa ON: Government of Canada (accessed 2020-10-02).
- Carmichael C., Westerfield M. and Varga Z.M. (2009) Cryopreservation and in vitro fertilization at the Zebrafish International Resource Center. *Methods in Molecular Biology* 546:45-65.
- Cartner S., Eisen J., Farmer S., Guillemain K., Kent M. and Sanders G. (eds.) (2019) *The Zebrafish in Biomedical Research: Biology, Husbandry, Diseases, and Research Applications*, 1st ed. American College of Laboratory Animal Medicine Series. San Diego CA: Academic Press.
- Chang C.T., Amack J.D. and Whipps C.M. (2016) Zebrafish embryo disinfection with povidone-iodine: evaluating an alternative to chlorine bleach. *Zebrafish* 13(Suppl. 1):S96-S101.
- Chang C.T., Colicino E.G., DiPaola E.J., Al-Hasnawi H.J. and Whipps C.M. (2015) Evaluating the effectiveness of common disinfectants at preventing the propagation of *Mycobacterium* spp. isolated from zebrafish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 178:45-50.

- Chang C.T., Doerr K.M. and Whipps C.M. (2017) Antibiotic treatment of zebrafish mycobacteriosis: tolerance and efficacy of treatments with tigecycline and clarithromycin. *Journal of Fish Diseases* 40(10):1473-1485.
- Chatigny F. (2018) The controversy on fish pain: a veterinarian's perspective. *Journal of Applied Animal Welfare Science* 22(4):400-410.
- Chatigny F., Creighton C.M. and Stevens E.D. (2018) Updated review of fish analgesia. *Journal of the American Association for Laboratory Animal Science* 57(1):5-12.
- Chen K., Wang C.-Q., Fan Y.-Q., Xie Y.-S., Yin Z.-F., Xu Z.-J., Zhang H.-L., Cao J.-T., Han Z.-H., Wang Y. and Song D.-Q. (2014) The evaluation of rapid cooling as an anesthetic method for the zebrafish. *Zebrafish* 11(1):71-75.
- Cheung E., Chatterjee D. and Gerlai R. (2014) Subcutaneous dye injection for marking and identification of individual adult zebrafish (*Danio rerio*) in behavioral studies. *Behavior Research Methods* 46(3):619-624.
- Clark T.S., Pandolfo L.M., Marshall C.M., Mitra A.K. and Schech J.M. (2018) Body condition scoring for adult zebrafish (*Danio rerio*). *Journal of the American Association for Laboratory Animal Science* 57(6):698-702.
- Collymore C., Banks E.K. and Turner P.V. (2016b) Lidocaine hydrochloride compared with MS222 for the euthanasia of zebrafish (*Danio rerio*). *Journal of the American Association for Laboratory Animal Science* 55(6):816-820.
- Collymore C., Crim M.J. and Lieggi C. (2016a) Recommendations for health monitoring and reporting for zebrafish research facilities. *Zebrafish* 13(Suppl. 1):S138-S148.
- Collymore C., Porelli G., Lieggi C. and Lipman N.S. (2014b) Evaluation of 5 cleaning and disinfection methods for nets used to collect zebrafish (*Danio rerio*). *Journal of the American Association for Laboratory Animal Science* 53(6):657-660.
- Collymore C., Watral V., White J.R., Colvin M.E., Rasmussen S., Tolwani R.J. and Kent M.L. (2014a) Tolerance and efficacy of Emamectin Benzoate and Ivermectin for the treatment of *Pseudocapillaria tomentosa* in laboratory zebrafish (*Danio rerio*). *Zebrafish* 11(5):490-497.
- Collymore C., Rasmussen S. and Tolwani R.J. (2013) Gavaging adult zebrafish. *Journal of Visualized Experiments* 78:e50691.
- Collymore C., Tolwani A., Lieggi C. and Rasmussen S. (2014c) Efficacy and safety of 5 anesthetics in adult zebrafish (*Danio rerio*). *Journal of the American Association for Laboratory Animal Science* 53(2):198-203.
- Collymore C., Tolwani R.J. and Rasmussen S. (2015) The behavioral effects of single housing and environmental enrichment on adult zebrafish (*Danio rerio*). *Journal of the American Association for Laboratory Animal Science* 54(3):280-285.
- Conklin D. (2000) Diet. In: *The Laboratory Fish*. (Ostrand G.K., ed.), pp. 65-78. San Diego CA: Academic Press.
- Correia A.D., Cunha S.R., Scholze M. and Stevens E.D. (2011) A Novel behavioral fish model of nociception for testing analgesics. *Pharmaceuticals* 4:665-680.

- Cortemeglia C. and Beitinger T.L. (2006) Projected US distributions of transgenic and wildtype zebra danios, *Danio rerio*, based on temperature tolerance data. *Journal of Thermal Biology* 31:422-428.
- Cousin X., Daouk T., Péan S., Lyphout L., Schwartz M.-E. and Bégout M.-L. (2012) Electronic individual identification of zebrafish using radio frequency identification (RFID) microtags. *Journal of Experimental Biology* 215: 2729-2734.
- Crim M.J., Lawrence C., Livingston R.S., Rakitin A., Hurley S.J. and Riley L.K. (2017) Comparison of antemortem and environmental samples for zebrafish health monitoring and quarantine. *Journal of the American Association for Laboratory Animal Science* 56(4):412-424.
- Davis K.B. (1992) Stress management in aquaculture. In: *The Care and Use of Amphibians, Reptiles and Fish in Research*. (Schaeffer D.O., Kleinow K.M. and Krulisch L., eds.). Bethesda MD: Scientists Centre for Animal Welfare.
- Davis D.J., Klug J., Hankins M., Doerr H.M., Monticelli S.R., Song A., Gillespie C.H. and Bryda E.C. (2015) Effects of clove oil as a euthanasia agent on blood collection efficiency and serum cortisol levels in *Danio rerio*. *Journal of the American Association for Laboratory Animal Science* 54(5):564-567.
- Delaney M., Follet C., Ryan N., Hanney N., Lusk-Yablick J. and Gerlach G. (2002) Social interaction and distribution of female zebrafish (*Danio rerio*) in a large aquarium. *The Biological Bulletin* 203:240-241.
- Delcourt J., Ovidio M., Denoël M., Muller M., Pendeville H., Deneubourg J.-L. and Poncin P. (2018) Individual identification and marking techniques for zebrafish. *Reviews in Fish Biology and Fisheries* 28(4):839-864.
- DePasquale C., Fettrow S., Sturgill J. and Braithwaite V.A. (2019) The impact of flow and physical enrichment on preferences in zebrafish. *Applied Animal Behaviour Science* 215:77-81.
- De Tolla L.J., Srinivas S., Whitaker B.R., Andrews C., Hecker B., Kane A.S. and Reimschuessel R. (1995) Guidelines for the care and use of fish in research. *ILAR Journal* 37(4):159-173.
- Draper B.W. and Moens C.B. (2009) A high-throughput method for zebrafish sperm cryopreservation and *in vitro* fertilization. *Journal of Visualized Experiments* 29:e1395.
- DSM (n.d.) [*Factor resulting in inadequate vitamin dietary intake*](#) (accessed on 2020-10-02).
- Easter S.S.Jr. and Nicola G.N. (1996) The development of vision in the zebrafish (*Danio rerio*). *Developmental Biology* 180(2): 646-663.
- Egan R.J., Bergner C.L., Hart P.C., Cachat J.M., Canavello P.R., Elegante M.F., Elkhayat S.I., Bartels B.K., Tien A.K., Tien D.H., Mohnot S., Beeson E., Glasgow E., Amri H., Zukowska Z. and Kalueff A.V. (2009) Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural Brain Research* 205(1):38-44.
- Engeszer R.E., Patterson L.B., Rao A.A. and Parichy D.M. (2007) Zebrafish in the wild: a review of natural history and new notes from the field. *Zebrafish* 4(10):21-38.
- Esmail M.Y., Astrofsky K.M. and Serluca F.C. (2015) The Biology and Management of the Zebrafish. In: *Laboratory Animal Medicine*, 3rd ed. (Fox J.G., Anderson L.C., Otto G.M., Pritchett-Corning K.R. and Whary M.T., eds.). Chapter 20, pp. 1015-1062. Amsterdam NL: Elsevier.

- Farias M. and Certal A.C. (2016) Different feeds and feeding regimens have an impact on zebrafish larval rearing and breeding performance. *International Journal of Marine Biology and Research* 1(1):1-8.
- Félix L.M., Luzio A., Themudo M., Antunes L., Matos M., Coimbra A.M. and Valentim A.M. (2018) MS-222 short exposure induces developmental and behavioural alterations in zebrafish embryos. *Reproductive Toxicology* 81:122-131.
- Ferguson J.A., Watral V., Schwindt A.R. and Kent, M.L. (2007) Spores of two fish microsporidia (*Pseudoloma neurophilia* and *Glugea anomala*) are highly resistant to chlorine. *Diseases of Aquatic Organisms* 76(3):205-214.
- Fernandes H., Peres H. and Carvalho A.P. (2016) Dietary protein requirement during juvenile growth of zebrafish (*Danio rerio*). *Zebrafish* 13(6):548-555.
- Fernandes Silva P., Garcia de Leaniz C. and Luchiari A.C. (2019) Fear contagion in zebrafish: a behaviour affected by familiarity. *Animal Behaviour* 153:95-103.
- Fisher J.P. (2000) Facilities and husbandry (large fish models). In: *The Laboratory Fish* (Ostrand G.K., ed.). pp. 13-39. San Diego CA: Academic Press.
- Fleisch V.C. and Neuhaus S.C.F. (2006) Visual behavior in zebrafish. *Zebrafish* 3(2):191-201.
- Flight W.G.F. and Verheijen F.J. (1993) The neck-cut (spinal transection): not a humane way to slaughter eel, *Anguilla anguilla*. *Aquaculture and Fisheries Management* 24:523-528.
- Francis M. (2008) Aquatics labs: five questions you don't want to have to ask. *CALAS/ACSAL Membership Magazine* 42(3):25-27.
- Franks B., Graham C. and von Keyserlingk M.A.G. (2018) Is heightened-shoaling a good candidate for positive emotional behavior in zebrafish? *Animals* 8(9):152.
- Frøland Steindal I.A. and Whitmore D. (2019) Circadian clocks in fish – what have we learned so far? *Biology (Basel)* 8(1):17.
- Fryer J.L. and Bartholomew J.L. (1996) Established and emerging infectious diseases of fish. *ASM News* 62: 592-594.
- Furutani-Seiki M. and Wittbrodt J. (2004) Medaka and zebrafish, an evolutionary twin study. *Mechanisms of Development* 121(7-8):629-637.
- Gaj T., Gersbach C.A. and Barbas C.F.III (2013) ZFN, TALEN and CRISPR/Cas-based methods for genome engineering. *Trends in Biotechnology* 31(7):397-405.
- Garcia R.L. and Sanders G.E. (2011) Efficacy of cleaning and disinfection procedures in a zebrafish (*Danio rerio*) facility. *Journal of the American Association for Laboratory Animal Science* 50(6):895-900.
- Gerhard G.S., Kauffman E.J., Wang X., Stewart R., Moore J.L., Kasales C.J., Demidenko E. and Cheng K.C. (2002) Life spans and senescent phenotypes of zebrafish (*Danio rerio*). *Experimental Gerontology* 37:1055-1068.
- Gerlach G. and Lysiak N. (2006) Kin recognition in zebrafish based on phenotype matching. *Animal Behaviour* 71:1371-1377.
- Gerlach, G. F., Morales, E. E., Wingert, R. A. (2015) Microbead Implantation in the Zebrafish Embryo. *Journal of Visualized Experiments* 101:e52943.

- Goldsmith P. and Solari R. (2003) The role of zebrafish in drug discovery. *Drug Discovery World*. Spring 2003.
- Gonzales J.M.Jr. and Law S.H.W. (2013) Feed and feeding regime affect growth rate and gonadosomatic index of adult zebrafish (*Danio rerio*). *Zebrafish* 10(4):532-540.
- Gonzalez T.J., Lu Y., Boswell M., Boswell W., Medrano G., Walter S., Ellis S., Savage M., Varga Z.M., Lawrence C., Sanders G. and Walter R.B. (2018) Fluorescent light exposure incites acute and prolonged immune responses in zebrafish (*Danio rerio*) skin. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 208:87-95.
- Goodwin N., Karp N.A., Blackledge S., Clark B., Keeble R., Kovacs C., Murray K.N., Price M., Thompson P. and Bussell J. (2016) Standardised welfare terms for the zebrafish community. *Zebrafish* 13(Suppl. 1):S164-S168.
- Government of Canada (2017) [*Canadian Environmental Protection Act: assessment of new substances fact sheet*](#) (accessed on 2020-10-02).
- Graham C., von Keyserlingk M.A.G. and Franks B. (2018a) Zebrafish welfare: natural history, social motivation and behaviour. *Applied Animal Behaviour Science* 200:13-22.
- Graham C., von Keyserlingk M.A.G. and Franks B. (2018b) Free-choice exploration increases affiliative behaviour in zebrafish. *Applied Animal Behaviour Science* 203:103-110.
- Grush J., Noakes D.L.G. and Moccia R.D. (2004) The efficacy of clove oil as an anesthetic for the zebrafish, *Danio rerio* (Hamilton). *Zebrafish* 1(1): 46-53.
- Hardy R.W. and Roley D.D. (2000) Lipid oxidation and antioxidants. In: *Encyclopedia of Aquaculture*. (Stockney R.W., ed.), pp. 470-476. New York NY: John Wiley & Sons Inc.
- Harper C. and Lawrence C. (2010) *The Laboratory Zebrafish*. Boca Raton (FL): CRC Press.
- Harvey B., Kelley R.N. and Ashwood-Smith M.J. (1982) Cryopreservation of zebrafish spermatozoa using methanol. *The Canadian Journal of Zoology* 60:1867-1870.
- Hawkins P., Prescott M.J., Carbone L., Dennison N., Johnson C., Makowska I.J., Marquardt N., Readman G., Weary D.M. and Golledge H.D.R. (2016) A good death? Report of the Second Newcastle Meeting on Laboratory Animal Euthanasia. *Animals* 6(9):50.
- Hensley M.R. and Leung Y.F. (2010) A convenient dry feed for raising zebrafish larvae. *Zebrafish* 7(2): 219-231.
- Higaki S., Eto Y., Kawakami Y., Yamaha E., Kagawa N., Kuwayama M., Nagano M., Katagiri S. and Takahashi Y. (2010) Production of fertile zebrafish (*Danio rerio*) possessing germ cells (gametes) originated from primordial germ cells recovered from vitrified embryos. *Reproduction* 139:733-740.
- Hohn C. and Petrie-Hanson L. (2013) Evaluation of visible implant elastomer tags in zebrafish (*Danio rerio*). *Biology Open* 2(12):1397-1401.
- Huang W.-C., Hsieh Y.-S., Chen I.-H., Wang C.-H., Chang H.-W., Yang C.-C., Ku T.-H., Yeh S.-R. and Chuang Y.-J. (2010) Combined use of MS-222 (Tricaine) and isoflurane extends anesthesia time and minimizes cardiac rhythm side effects in adult zebrafish. *Zebrafish* 7(3): 297-304.

- Hubbs C., Nickum J.G. and Hunter J.R. (1988) Guidelines for the use of fish in research. *Fisheries* 13(2):16-22.
- Hutter S., Penn D.J., Magee S. and Zala S.M. (2010) Reproductive behaviour of wild zebrafish (*Danio rerio*) in large tanks. *Behaviour* 147(5):641-660.
- Isayeva A., Zhang T. and Rawson D.M. (2004) Studies on chilling sensitivity of zebrafish (*Danio rerio*) oocytes. *Cryobiology* 49(2):114-122.
- Iwama G.K. (1992) Anesthesia, analgesia, and euthanasia in fish. In: *The Care and Use of Amphibians, Reptiles and Fish in Research*. (Schaeffer D.O., Kleinow K.M. and Krulish L., eds.). pp.167-174. Bethesda MD: Scientists Center for Animal Welfare.
- Iwama G.K., McGeer J.C. and Pawluk M.P. (1988) The effects of five fish anesthetics on acid-base balance, hematocrit, and blood gases, cortisol, and adrenaline in rainbow trout. *Canadian Journal of Zoology* 67:2065-2073.
- Iwama G.K. and Ishimatsu A. (1994) Cannulation of blood vessels. In: *Techniques in Fish Immunology – 3*. (Rowley A.F., Zeitkoff J.T., Kaatari S.L. and Smith S.A., eds.). Fairhaven NJ: SOS Publications.
- Jansen G.A. and Green N.M. (1970) Morphine metabolism and morphine tolerance in goldfish. *Anesthesiology* 32(3):231-235.
- Jenkins J.A. (2000) Infectious disease and quality assurance considerations for the transfer of cryopreserved fish gametes. In: *Cryopreservation in Aquatic Species* (Tiersch T.R. and Mazik P.M., eds.). pp. 343-363. Baton Rouge LA: World Aquaculture Society.
- Johnson G.R. (2000) Surgical techniques. In: *The Laboratory Fish* (Ostrander G.K., ed.). pp.557-567. San Diego CA: Academic Press.
- Jones K.S., Alimov A.P., Rilo H.L., Jandacek R.J., Woollett L.A. and Penberthy W.T. (2008) A high throughput live transparent animal bioassay to identify non-toxic small molecules or genes that regulate vertebrate fat metabolism for obesity drug development. *Nutrition and Metabolism* 5:23.
- Jones N.A.R., Mendo T., Broell F. and Webster M.M. (2019) No experimental evidence of stress-induced hyperthermia in zebrafish (*Danio rerio*). *Journal of Experimental Biology* 222(2): jeb192971.
- Kaushik S., Georga I., and Koumoundouros G. (2011) Growth and body composition of zebrafish (*Danio rerio*) larvae fed a compound feed from first feeding onward: toward implications on nutrient requirements. *Zebrafish* 8(2):87-95.
- Keck V.A., Edgerton D.S., Hajizadeh S., Swift L.L., Dupont W.D., Lawrence C. and Boyd K.L. (2015) Effects of habitat complexity on pair-housed zebrafish. *Journal of the American Association for Laboratory Animal Science* 54(4):378-383.
- Kent M.L., Bishop-Stewart J.K., Matthews J.L. and Spitsbergen J.M. (2002) *Pseudocapillaria tomentosa*, a nematode pathogen, and associated neoplasms of zebrafish (*Danio rerio*) kept in research colonies. *Comparative Medicine* 52:354-358.
- Kent M.L., Feist S.W., Harper C., Hoogstraten-Miller S., Law J.M., Sánchez-Morgado J.M., Tanguay R.L., Sanders G.E., Spitsbergen J.M. and Whipps C.M. (2009) Recommendations for control of pathogens and infectious diseases in fish research facilities. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 149(2): 240-248. *ILAR Journal* 53(2):126-134.

- Kent M.L., Spitsbergen J.M., Matthews J.M., Fournie J.W., Murray K.N. and Westerfield M. (2016) *Diseases of Zebrafish in Research Facilities*. Zebrafish International Resource Center (ZIRC), ZIRC Documents. Eugene OR: University of Oregon.
- Kestemont P. and Baras E. (2001) Environmental factors and feed intake: mechanisms and interactions. In: *Food Intake*. (Houlihan D., Boujard T. and Jobling M., eds.), pp. 130-156. Oxford UK: Blackwell Science.
- Khosla K., Wang Y., Hagedorn M., Qin Z. and Bischof J. (2017) Gold nanorod induced warming of embryos from the cryogenic state enhances viability. *ACS Nano* 11(8):7869-7878.
- Kinkel M.D., Eames S.C., Philipson L.H. and Prince V.E. (2010) Intraperitoneal injection into adult zebrafish. *Journal of Visualized Experiments* 42:e2126.
- Kinth P., Mahesh G. and Panwar Y. (2013) Mapping of zebrafish research: a global outlook. *Zebrafish* 10(4):510-517.
- Kistler C., Hegglin D., Würbel H. and König B. (2011) Preference for structured environment in zebrafish (*Danio rerio*) and checker barbs (*Puntius oligolepis*). *Applied Animal Behaviour Science* 135(4):318-327.
- Köhler A., Collymore C., Finger-Baier K., Geisler R., Kaufmann L., Pounder K.C., Schulte-Merker S., Valentim A., Varga Z.M., Weiss J. and Strähle U. (2017) Report of workshop on euthanasia for zebrafish — a matter of welfare and science. *Zebrafish* 14(6):547-551.
- Kreiberg H. (2000) Stress and anesthesia. In: *The Laboratory Fish*. (Ostrander G., ed.), pp. 503-511. San Diego CA: Academic Press.
- Krueger L.D., Thurston S.E., Kirk J., Elsaedi F., Freeman Z.T., Goldman D., Lofgren J.L. and Keller J. M. (2020) Enrichment preferences of singly housed zebrafish (*Danio rerio*). *Journal of the American Association for Laboratory Animal Science* 59(2):148-155.
- Kurtzman M.S., Craig M.P., Grizzle B.K. and Hove J.R. (2010) Sexually segregated housing results in improved early larval survival in zebrafish. *Lab Animal Europe* 10(7):23-33.
- Laale H. (1977) The biology and use of zebrafish *Brachydanio rerio* in fisheries research: a literature review. *Journal of Fish Biology* 10:121-173.
- Landgraf K., Schuster S., Meusel A., Garten A., Riemer T., Schleinitz D., Kiess W. and Körner A. (2017) Short-term overfeeding of zebrafish with normal or high-fat diet as a model for the development of metabolically healthy versus unhealthy obesity. *BMC Physiology* 17:4.
- Lange M., Neuzeret F., Fabreges B., Froc C., Bedu S., Bally-Cuif L. and Norton W.H.J. (2013) Inter-individual and inter-strain variations in zebrafish locomotor ontogeny. *PLoS ONE* 8(8): e70172.
- Lawrence C. (2007) The husbandry of zebrafish (*Danio rerio*): a review. *Aquaculture* 269:1-20.
- Lawrence C. (2011) Advances in zebrafish husbandry and management. *Methods in Cell Biology* 104:429-451.
- Lawrence C., Best J., Cockington J., Henry E.C., Hurley S., James A., Lapointe C., Maloney K. and Sanders E. (2016) The complete and updated "rotifer polyculture method" for rearing first feeding zebrafish. *Journal of Visualized Experiments* 107:e53629.
- Lawrence C., Best J., James A. and Maloney K. (2012) The effects of feeding frequency on growth and reproduction in zebrafish (*Danio rerio*). *Aquaculture* 368-369:103-108.

- Lawrence C., Eisen J.S. and Varga Z.M. (eds.) (2016) Health and Husbandry Issue. *Zebrafish* 13(S1): 1-170.
- Lawrence C., James A. and Mobley S. (2015) Successful replacement of *artemia salina nauplii* with marine rotifers (*Brachionus plicatilis*) in the diet of preadult zebrafish (*Danio rerio*). *Zebrafish* 12(5):366-371.
- Lawrence C. and Mason T. (2012) Zebrafish housing systems: a review of basic operating principles and considerations for design and functionality. *ILAR Journal* 53(2):179-191.
- Lawrence C., Sanders E. and Henry E. (2012) Methods for culturing saltwater rotifers (*Brachionus plicatilis*) for rearing larval zebrafish. *Zebrafish* 9(3):140-146.
- Learmonth C. and Carvalho A.P. (2015) Acute and chronic toxicity of nitrate to early life stages of zebrafish — setting nitrate safety levels for zebrafish rearing. *Zebrafish* 12(4):305-311.
- Lee C.J., Paull G.C. and Tyler C.R. (2018) Effects of environmental enrichment on survivorship, growth, sex ratio and behaviour in laboratory maintained zebrafish *Danio rerio*. *Journal of Fish Biology* 94:86-95.
- Lin C., Zhang T. and Rawson D.M. (2009) Cryopreservation of zebrafish (*Danio rerio*) blastomeres by controlled slow cooling. *Cryo Letters* 30(2):132-141.
- Lindsay S.M. and Vogt R.G. (2004) Behavioural responses of newly hatched zebrafish (*Danio rerio*) to amino acid chemostimulants. *Chemical Senses* 29:93-100.
- Liu L. and Lee K.-Y. (2014) Studies of *in vitro* embryo culture of guppy (*Poecilia reticulata*). *Development & Reproduction* 18(3):139-143.
- Liu J., Zhou Y., Qi X., Chen J., Chen W., Qiu G., Wu Z. and Wu N. (2017) CRISPR/Cas9 in zebrafish: an efficient combination for human genetic diseases modeling. *Human Genetics* 136(1):1-12.
- Lockwood N., Parker J., Wilson C. and Frankel P. (2017) Optimal anesthetic regime for motionless three-dimensional image acquisition during longitudinal studies of adult nonpigmented zebrafish. *Zebrafish* 14(2):133-139.
- Lopez-Luna J., Al-Jubouri Q., Al-Nuaimy W. and Sneddon L.U. (2017) Reduction in activity by noxious chemical stimulation is ameliorated by immersion in analgesic drugs in zebrafish. *Journal of Experimental Biology* 220(8):1451-1458.
- Mann K.D., Turnell E.R., Atema J. and Gerlach G. (2003) Kin recognition in juvenile zebrafish (*Danio rerio*) based on olfactory cues. *The Biological Bulletin* 205:224-225.
- Mansur B. deM., dos Santos B.R., de Mattos Dias C.A.G., de Sena Pinheiro M. and Gouveia A.Jr. (2014) Effects of the number of subjects on the dark/light preference of zebrafish (*Danio rerio*). *Zebrafish* 11(6):560-566.
- Manuel R., Gorissen M., Stokkermans M., Zethof J., Ebbesson L.O.E., van de Vis H., Flik G. and van den Bos R. (2015) The effects of environmental enrichment and age-related differences on inhibitory avoidance in zebrafish (*Danio rerio* Hamilton) *Zebrafish* 12(2):152-165.
- Marcon M., Mocelin R., Benvenuti R., Costa T., Herrmann A.P., de Oliveira D.L., Koakoski G., Barcellos L.J.G. and Piato A. (2018) Environmental enrichment modulates the response to chronic stress in zebrafish. *Journal of Experimental Biology* 221: jeb176735.
- Martins T., Diniz E., Félix L.M. and Antunes L. (2018) Evaluation of anaesthetic protocols for laboratory adult zebrafish (*Danio rerio*). *PLoS ONE* 13(5):e0197846.

- Mason T., Snell K., Mittge E., Melancon E., Montgomery R., McFadden M., Camoriano J., Kent M.L., Whipps C.M. and Peirce J. (2016) Strategies to mitigate a *Mycobacterium marinum* outbreak in a zebrafish research facility. *Zebrafish* 13(Suppl. 1):S77-S87.
- Matthews M., Trevarrow B. and Matthews J. (2002) A virtual tour of the Guide for zebrafish users. *Lab Animal* 31(3):34-40.
- Matthews M. and Varga Z.M. (2012) Anesthesia and euthanasia in zebrafish. *Laboratory Animal Research* 53(2):192-204.
- Matthews J., Murphy J., Carmichael C. and Varga Z. (2017) ZIRC E400/RMMB Sperm Cryopreservation & IVF Protocol. *ZIRC Protocols*. University of Oregon: ZIRC.
- Matthews J.L., Murphy J.M., Carmichael C., Yang H., Tiersch T., Westerfield M. and Varga Z.M. (2018) Changes to extender, cryoprotective medium, and *in vitro* fertilization improve zebrafish sperm cryopreservation. *Zebrafish* 15(3):279-290.
- McElligott M.B. and O'Malley D.M. (2005) Prey tracking by larval zebrafish: axial kinematics and visual control. *Brain, Behaviour and Evolution* 66:177-196.
- McMillan S.C., Géraudie J. and Akimenko M.A. (2015) Pectoral fin breeding tubercle clusters: a method to determine zebrafish sex. *Zebrafish* 12(1):121-123.
- Mellor D.J., Beausoleil N.J. and Stafford K.J. (2004) *Marking amphibians, reptiles and marine mammals: animal welfare, practicalities and public perceptions in New Zealand*. Wellington NZ: Department of Conservation.
- Meritet D.M., Mulrooney D.M., Kent M.L. and Löhr C.V. (2017) Development of quantitative real-time pcr assays for postmortem detection of *Mycobacterium* spp. common in zebrafish (*Danio rerio*) research colonies. *Journal of the American Association for Laboratory Animal Science* 56(2):131-141.
- Miller M., Sabrautzki S., Beyerlein A. and Brielmeier M. (2019) Combining fish and environmental PCR for diagnostics of diseased laboratory zebrafish in recirculating systems. *PLoS ONE* 14(9):e0222360.
- Miller S., Pollack J., Bradshaw J., Kumai Y. and Perry S.F. (2014) Cardiac responses to hypercapnia in larval zebrafish (*Danio rerio*): the links between CO₂ chemoreception, catecholamines and carbonic anhydrase. *Journal of Experimental Biology* 217(Pt. 19):3569-3578.
- Mochó J.-P., Martin D.J., Millington M.E. and Saavedra Torres Y. (2017) Environmental screening of *Aeromonas hydrophila*, *Mycobacterium* spp., and *Pseudocapillaria tomentosa* in zebrafish systems. *Journal of Visualized Experiments* 130:e55306.
- Monson C.A. and Sadler K.C. (2010) Inbreeding depression and outbreeding depression are evident in wild-type zebrafish lines. *Zebrafish* 7(2):189-197.
- Moorman S.J. (2001) Development of sensory systems in zebrafish (*Danio rerio*). *ILAR Journal* 42(4):292-298.
- Morris J.P.IV, Hagen A. and Kanki J.P. (2003) Zebrafish sperm cryopreservation with N,N-Dimethylacetamide. In: *Methods in Reproductive Aquaculture: Marine and Freshwater Species* (Cabrita E., Robles V. and Herráez, P., eds.), pp.329-337. Boca Raton FL: CRC Press.

- Morton D.B., Jennings M., Buckwell A., Ewbank R., Godfrey C., Holgate B., Inglis I., James R., Page C., Sharman I., Verschoyle R., Westall L. and Wilson A.B. (2001) Refining procedures for the administration of substances – Report of the BVVAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. *Laboratory Animals* 35:1-41.
- Murray K.N., Dreska M., Nasiadka A., Rinne M., Matthews J.L., Carmichael C., Bauer J., Varga Z.M. and Westerfield M. (2011) Transmission, diagnosis, and recommendations for control of *Pseudoloma neurophilia* infections in laboratory zebrafish (*Danio rerio*) facilities. *Comparative Medicine* 61(4):322-329.
- Murray K.N., Varga Z.M., and Kent M.L. (2016) Biosecurity and health monitoring at the Zebrafish International Resource Center. *Zebrafish* 13(Suppl. 1):S30-S38.
- Nasiadka A. and Clark M.D. (2012) Zebrafish breeding in the laboratory environment. *ILAR Journal* 53(2):161-168.
- National Research Council – NRC (1993) Nutrient requirements of fish. In: *National Academy of Sciences*, pp. 124. Washington DC: National Academy Press.
- National Research Council – NRC (2009) Effective pain management. In: *Recognition and Alleviation of Pain in Laboratory Animals*. Committee on Recognition and Alleviation of Pain in Laboratory Animals - National Research Council. Washington DC: National Academies Press.
- Nemetz T.G. and Shotts E.B.Jr. (1993) Zoonotic diseases. In: *Fish Medicine* (Stoskopf M.K., ed.), pp. 214-220. New York NY: WB Saunders.
- Noga E.J. (2010) *Fish Disease: Diagnosis and Treatment*, 2nd ed. Hoboken NJ: Wiley-Blackwell.
- Norris L.J., Watral V. and Kent M.L. (2018) Survival of bacterial and parasitic pathogens from zebrafish (*Danio rerio*) after cryopreservation and thawing. *Zebrafish* 15(2):188-201.
- O'Keefe T. (2000) Feed handling and storage. In: *Encyclopedia of Aquaculture* (Stockney R.W., ed.), pp.350-354. New York NY: John Wiley & Sons, Inc.
- Oliveira J., Silveira M., Chacon D. and Luchiari A. (2015) The zebrafish world of colors and shapes: preference and discrimination. *Zebrafish* 12(2):166-173.
- Olsson I.A., Nevison C.M., Patterson-Kane E.G., Sherwin C.M., Van de Weerd H.A. and Würbel H. (2003) Understanding behaviour: the relevance of ethological approaches in laboratory animal science. *Applied Animal Behaviour Science* 81:245-264.
- Ostrander G.K. (ed.) (2000) *The Laboratory Fish*. San Diego CA: Academic Press.
- Overstreet R.M., Barnes S.S., Manning C.S. and Hawkins W.E (2000) Facilities and husbandry (small fish models). In: *The Laboratory Fish* (Ostrander G.K., ed.). San Diego CA: Academic Press.
- Pagnussat N., Piato A.L., Schaefer I.C., Blank M., Tamborski A.R., Guerim L.D., Bonan C.D., Vianna M.R.M. and Lara D.R. (2013) One for all and all for one: the importance of shoaling on behavioral and stress responses in zebrafish. *Zebrafish* 10(3):338-342.
- Parichy D.M., Elizondo M.R., Mills M.G., Gordon T.N. and Engeszer R.E. (2009) Normal table of postembryonic zebrafish development: staging by externally visible anatomy of the living fish. *Developmental Dynamics* 238:2975-3015.

- Parker M.O., Millington M.E., Combe F.J. and Brennan C.H. (2012) Housing conditions differentially affect physiological and behavioural stress responses of zebrafish, as well as the response to anxiolytics. *PLoS ONE* 7(4): e34992.
- Paull G.C., Filby A.L., Giddins H.G., Coe T.S., Hamilton P.B. and Tyler C.R. (2010) Dominance hierarchies in zebrafish (*Danio rerio*) and their relationship with reproductive success. *Zebrafish* 7(1):109-117.
- Peeters B.W.M.M., Moeskops M. and Veenvliet A.R.J. (2016) Color preference in *Danio rerio*: effects of age and anxiolytic treatments. *Zebrafish* 13(4):330-334.
- Pelegri F. (2002) Mutagenesis. In: *Zebrafish – A Practical Approach* (Nüsslein-Volhard C. and Dahm R., eds.). Oxford UK: Oxford University Press.
- Perry S.F. and Reid S.G. (1994) Injection techniques. In: *Biochemistry and Molecular Biology of Fishes*, vol. 3 (Hochachka P.W. and Mommesen T.P., eds.), pp.85-92. Amsterdam Netherlands: Elsevier.
- Peterson T.S., Ferguson J.A., Watral V.G., Mutoji K.N., Ennis D.G. and Kent M.L. (2013) *Paramecium caudatum* enhances transmission and infectivity of *Mycobacterium marinum* and *Mycobacterium chelonae* in zebrafish (*Danio rerio*). *Diseases of Aquatic Organisms* 106(3): 229-239.
- Pritchard V.L., Lawrence J., Butlin R.K. and Krause J. (2001) Shoal choice in zebrafish, *Danio rerio*: the influence of shoal size and activity. *Animal Behaviour* 62:1085-1088.
- Qin Z., Lewis J.E. and Perry S.F. (2010) Zebrafish (*Danio rerio*) gill neuroepithelial cells are sensitive chemoreceptors for environmental CO₂. *Journal of Physiology* 588(5):861-872.
- Ramsay J.M., Feist G.W., Matthews J.L., Westerfield M., Varga Z.M., Kent M.L. and Schreck C.B. (2006) Whole body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. *Aquaculture* 258:565-574.
- Ramsay J.M., Feist G.W., Varga Z.M., Westerfield M., Kent M.L. and Schreck C.B. (2009a) Whole-body cortisol response of zebrafish to acute net handling stress. *Aquaculture* 297(1-4):157-162.
- Ramsay J.M., Watral V., Schreck C.B. and Kent M.L. (2009b) Husbandry stress exacerbates mycobacterial infections in adult zebrafish, *Danio rerio* (Hamilton). *Journal of Fish Diseases* 32(11): 931-941. *PLoS ONE* 8(9): e73773.
- Readman G.D., Owen S.F., Knowles T.G. and Murrell J.C. (2017) Species specific anaesthetics for fish anaesthesia and euthanasia. *Scientific Reports* 7:7102.
- Reddy P.K. and Leatherland J.F. (1998) Stress physiology. In: *Fish Diseases and Disorders*, vol. 2 (Leatherland J.F. and Woo P.T., eds.), pp.279-301. Wallingford Oxon: CABI.
- Reed B. and Jennings M. (2011) Guidance on the housing and care of zebrafish (*Danio rerio*). Horsham UK: RSPCA.
- Reilly S.C., Quinn J.P., Cossins A.R. and Sneddon L.U. (2008) Behavioural analysis of a nociceptive event in fish: comparisons between three species demonstrate specific responses. *Applied Animal Behaviour Science* 114: 248-259.
- Rey S., Huntingford F.A., Boltaña S., Vargas R., Knowles T.G. and Mackenzie S. (2015) Fish can show emotional fever: stress-induced hyperthermia in zebrafish. *Proceedings of the Royal Society B, Biological Sciences* 282: 2266.

- Rey S., Moiche V., Boltaña S., Teles M. and MacKenzie S. (2017) Behavioural fever in zebrafish larvae. *Developmental and Comparative Immunology* 67:287-292.
- Ribas L., Valdivieso A., Diaz N. and Piferrer F. (2017) Appropriate rearing density in domesticated zebrafish to avoid masculinization: links with the stress response. *Journal of Experimental Biology* 220:1056-1064.
- Rodriguezmoldes I., Manso M.J., Becerra M., Molist P. and Anadon R. (1993) Distribution of substance P-like immunoreactivity in the brain of the elasmobranch *Scyliorhinus canicula*. *Journal of Comparative Neurology* 335:228-244.
- Rosenthal G.G. and Ryan M.J. (2005) Assortative preferences for stripes in danios. *Animal Behaviour* 70:1063-1066.
- Royal Society for the Prevention of Cruelty to Animals – RSPCA (2008) *Sharing and archiving of genetically altered mice: opportunities for reduction and refinement*. Resource Sharing Working Group. Horsham UK: RSPCA.
- Ruhl N., McRobert S.P. and Currie W.J.S. (2009) Shoaling preferences and the effects of sex ratio on spawning and aggression in small laboratory populations of zebrafish (*Danio rerio*). *Lab Animal Europe* 9(9):19-30.
- Russell W.M.S. and Burch R.L. (1959) *The Principles of Humane Experimental Technique*. London UK: Special edition published by Universities Federation for Animal Welfare – UFAW, 1992.
- Samaee S.-M. (2015) Experimental assessment of the efficacy of five veterinary broad-spectrum anthelmintics to control the intestinal capillariasis in zebrafish (*Danio rerio*). *Zebrafish* 12(3):255-267.
- Sanders E. (2013) Considerations and reflections on design and construction of a centralized zebrafish facility. *ALN Magazine* January 3.
- Saszik S., Bilotta J. and Givin C.M. (1999) ERG assessment of zebrafish retinal development. *Visual Neuroscience* 16:881-888.
- Sassen W.A. and Köster R.W. (2015) A molecular toolbox for genetic manipulation of zebrafish. *Advances in Genomics and Genetics* 5:151-163.
- Schaefer J. and Ryan A. (2006) Developmental plasticity in the thermal tolerance of zebrafish *Danio rerio*. *Journal of Fish Biology* 69(3):722-734.
- Schilling T.F. (2002) The morphology of larval and adult zebrafish. In: *Zebrafish – A Practical Approach* (Nüsslein-Volhard C. and Dahm R., eds.). Oxford UK: Oxford University Press.
- Schmidt L.J., Gaikowski M.P., Gingerich W.H., Dawson V.K. and Schreier T.M. (2007) An environmental assessment of the proposed use of oxytetracycline-medicated feed in freshwater aquaculture. La Crosse, Wisconsin: U.S. Geological Survey.
- Schroeder P.G. and Sneddon L.U. (2016) Exploring the efficacy of immersion analgesics in zebrafish using an integrative approach. *Applied Animal Behavioural Science* 187:93-102.
- Schroeder P., Jones S., Young I.S. and Sneddon L.U. (2014) What do zebrafish want? Impact of social grouping, dominance and gender on preference for enrichment. *Laboratory Animals* 48(4):328-337.

- Sessa A.K., White R., Houvras Y., Burke C., Pugach E., Baker B., Gilbert R., Look A.T. and Zon L.I. (2009) The effect of a depth gradient on the mating behavior, oviposition site preference, and embryo production in the zebrafish, *Danio rerio*. *Zebrafish* 5(4):335-339.
- Sfakianakis D.G., Leris I., Mylonas C.C. and Kentouri M. (2012) Temperature during early life determines sex in zebrafish, *Danio rerio* (Hamilton, 1822). *Journal of Biological Research-Thessaloniki* 17:68-73.
- Shoji W. and Sato-Maeda M. (2008) Application of heat shock promoter in transgenic zebrafish. *Development, Growth & Differentiation* 50(6):401-406.
- Siccardi A.J.III, Garris H.W., Jones W.T., Moseley D.B., D'Abramo L.R. and Watts S.A. (2009) Growth and survival of zebrafish (*Danio rerio*) fed different commercial and laboratory diets. *Zebrafish* 6(3):275-280.
- Smith D.L.Jr., Barry R.J., Powell M.L., Nagy T.R., D'Abramo L.R. and Watts S.A. (2013) Dietary protein source influence on body size and composition in growing zebrafish. *Zebrafish* 10(3):439-446.
- Sneddon L.U. (2009) Pain perception in fish: indicators and endpoints. *ILAR Journal* 50(4):338-342.
- Sneddon L.U., Braithwaite V.A. and Gentle M.J. (2003) Do fish have nociceptors? Evidence for the evolution of the vertebrate sensory system. *Proceedings of the Royal Society, London Series B* 270:1115-1121.
- Spagnoli S., Sanders J. and Kent M.L. (2017) The common neural parasite *Pseudoloma neurophilia* causes altered shoaling behavior in adult laboratory zebrafish (*Danio rerio*) and its implications for neurobehavioral research. *Journal of Fish Diseases* 40(3):443-446.
- Speare D.J. (1998) Non-infectious disorders associated with intensive aquaculture husbandry. In: *Fish Diseases and Disorders*, vol. 2 (Leatherland J.F. and Woo P.T., eds.), pp. 303-313. Wallingford Oxon: CABI.
- Spence R., Fatema M.K., Reichard M., Huq K.A., Wahab M.A., Ahmed F. and Smith C. (2006a) The distribution and habitat preferences of the zebrafish in Bangladesh. *Journal of Fish Biology* 69(5):1435-1448.
- Spence R., Jordan W.C. and Smith C. (2006b) Genetic analysis of male reproductive success in relation to density in the zebrafish, *Danio rerio*. *Frontiers in Zoology* 3:5.
- Spence R., Magurran A.E. and Smith C. (2011) Spatial cognition in zebrafish: the role of strain and rearing environment. *Animal Cognition* 14:607-612.
- Spence R. and Smith C. (2005) Male territoriality mediates density and sex ratio effects on oviposition in the zebrafish, *Danio rerio*. *Animal Behaviour* 69:1317-1323.
- Spence R. and Smith C. (2006) Mating preference of female zebrafish, *Danio rerio*, in relation to male dominance. *Behavioural Ecology* 17:79-783.
- Spence R. and Smith C. (2007) The role of early learning in determining shoaling preferences based on visual cues in the zebrafish, *Danio rerio*. *Ethology* 113:62-67.
- Spence R., Fatema M.K., Ellis S., Ahmed Z.F. and Smith C. (2007) The diet, growth and recruitment of wild zebrafish (*Danio rerio*) in Bangladesh. *Journal of Fish Biology* 71(1):304-309.
- Spence R., Gerlach G., Lawrence C. and Smith C. (2008) The behaviour and ecology of the zebrafish, *Danio rerio*. *Biological Reviews* 83(1):13-34.

- Stephenson J.F., Whitlock K.E. and Partridge J.C. (2011) Zebrafish preference for light or dark is dependent on ambient light levels and olfactory stimulation. *Zebrafish* 8(1):17-22.
- Stevens E.D., Sutterlin A.M. and Cook T. (1998) Respiratory metabolism, oxygen dependency and swimming performance of growth hormone transgenic Atlantic salmon. *Canadian Journal of Fisheries & Aquatic Sciences* 55:2028-2035.
- Stickney R.R. (1994) *Principles of Aquaculture*. New York NY: John Wiley & Sons.
- Strykowski J.L. and Schech J.M. (2015) Effectiveness of recommended euthanasia methods in larval zebrafish (*Danio rerio*). *Journal of the American Association for Laboratory Animal Science* 54(1):76-79.
- Suriyampola P.S., Shelton D.S., Shukla R., Roy T., Bhat A. and Martins E.P. (2016) Zebrafish social behavior in the wild. *Zebrafish* 13(1):1-8.
- Tacon A.G.J. (1992) *Nutritional Fish Pathology, Morphological Signs of Nutrient Deficiency and Toxicity in Farmed Fish*. FAO Fish Technical Paper No. 330, 75pp. Rome: FAO.
- Tierney K.B. (2011). Swimming performance assessment in fishes. *Journal of Visualized Experiments* 51:e2572.
- Tort L. (2011) Stress and immune modulation in fish. *Developmental & Comparative Immunology* 35(12): 1366-1375.
- Turnell E.R., Mann K.D., Rosenthal G.G. and Gerlack G. (2003) Mate choice in zebrafish (*Danio rerio*) analyzed with video-stimulus techniques. *The Biological Bulletin* 205(2):225-226.
- Turner P.V., Brabb T., Pekow C. and Vasbinder M.A. (2011) Administration of substances to laboratory animals: routes of administration and factors to consider. *Journal of the American Association for Laboratory Animal Science* 50(5):600-613.
- Tye M.T., Montgomery J.E., Hobbs M.R., Vanpelt K.T. and Masino M.A. (2018) An adult zebrafish diet contaminated with chromium reduces the viability of progeny. *Zebrafish* 15(2):179-187.
- Valentim A.M., Félix L.M., Carvalho L., Diniz E. and Antunes L.M. (2016) A new anaesthetic protocol for adult zebrafish (*Danio rerio*): propofol combined with lidocaine. *PLoS ONE* 11(1):e0147747.
- Vandenberg L.N., Stevenson C. and Levin M. (2012) Low frequency vibrations induce malformations in two aquatic species in a frequency-, waveform-, and direction-specific manner. *PLoS ONE* 7(12):e51473.
- van den Hurk R. and Resink J.W. (1992) Male reproductive system as sex pheromone producer in teleost fish. *Journal of Experimental Zoology* 261:204-213.
- Vargesson N.A. (2007) Zebrafish. In: *Manual of Animal Technology* (Barnett S., ed.). Oxford UK: Blackwell Publishing.
- Vecino E., Pinuela C., Arevalo R., Lara J., Alonso J.R. and Aijon J. (1992) Distribution of enkephalinlike immunoreactivity in the central nervous system of the rainbow trout – an immunocytochemical study. *Journal of Anatomy* 180:435-453.
- Verheijen F.J. and Buwalda R.J.A. (1988) *Do pain and fear make a hooked carp in play suffer?* Utrecht Netherlands: Report of the Department of Comparative Physiology, University of Utrecht.

- Vignet C., Bégout M.L., Péan S., Lyphout L., Leguay D. and Cousin X. (2013) Systematic screening of behavioral responses in two zebrafish strains. *Zebrafish* 10(3):365-375.
- Villamizar N., Vera L.M., Foulkes N.S. and Sánchez-Vázquez F.J. (2014) Effect of lighting conditions on zebrafish growth and development. *Zebrafish* 11(2):173-181.
- Vliegenthart A.D.B., Starkey Lewis P., Tucker C.S., Del Pozo J., Rider S., Antoine D.J., Dubost V., Westphal M., Moulin P., Bailey M.A., Moggs J.G., Goldring C.E., Park B.K. and Dear J.W. (2014) Retro-orbital blood acquisition facilitates circulating microRNA measurement in zebrafish with paracetamol hepatotoxicity. *Zebrafish* 11(3):219-226.
- Wafer L.N., Jensen V.B., Whitney J.C., Gomez T.H., Flores R. and Goodwin B.S. (2016) Effects of environmental enrichment on the fertility and fecundity of zebrafish (*Danio rerio*). *Journal of the American Association for Laboratory Animal Science* 55(3):291-294.
- Wallace C.K., Bright L.A., Marx J.O., Andersen R.P., Mullins M.C. and Carty A.J. (2018) Effectiveness of rapid cooling as a method of euthanasia for young zebrafish (*Danio rerio*). *Journal of the American Association for Laboratory Animal Science* 57(1):58-63.
- Wang J., Liu C., Ma F., Chen W., Liu J., Hu B. and Zheng L. (2014) Circadian clock mediates light/dark preference in zebrafish (*Danio Rerio*). *Zebrafish* 11(2):115-121.
- Watts S.A., Lawrence C., Powell M. and D'Abramo L.R. (2016) The vital relationship between nutrition and health in zebrafish. *Zebrafish* 13(Suppl. 1):S72-S76.
- Wedemeyer G.A. (1972) Some physiological consequences of handling stress in the juvenile coho salmon and steelhead trout. *Journal of the Fisheries Research Board of Canada* 29(12):1780-1783.
- Wedemeyer G.A. (1985) *Development and Evaluation of Transport Media to Mitigate Stress and Improve Juvenile Salmon Survival in Columbia River Barging and Trucking Operations*. 70pp. Portland: Contract report to Bonneville Power Administration, #82-19.
- Wedemeyer G.A. (1996) *Physiology of Fish in Intensive Culture Systems*. New York: Chapman & Hall.
- Westerfield M. (2007) *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio)*, 5th ed. Eugene OR: University of Oregon Press.
- Whipps C.M., Lieggi C. and Wagner R. (2012) Mycobacteriosis in zebrafish colonies. *ILAR Journal* 53(2):95-105.
- Whipps C.M., Moss L.G., Sisk D.M., Murray K.N., Tobin D.M. and Moss J.B. (2014) Detection of autofluorescent *Mycobacterium chelonae* in living zebrafish. *Zebrafish* 11(1):76-82.
- Whitfield T.T. (2002) Zebrafish as a model for hearing and deafness. *Journal of Neurobiology* 53(2):157-171.
- Whitlock K.E. (2006) The sense of scents: olfactory behaviors in the zebrafish. *Zebrafish* 3(2):203-213.
- Wilson J.M., Bunte R.M. and Carty A.J. (2009) Evaluation of rapid cooling and tricaine methanesulfonate (MS222) as methods of euthanasia in zebrafish (*Danio rerio*). *Journal of the American Association for Laboratory Animal Science* 48(6):785-789.
- Wilson C.A., High S.K., McCluskey B.M., Amores A., Yan Y., Titus T.A., Anderson J.L., Batzel P., Carvan M.J.III, Schartl M. and Postlethwait J.H. (2014) Wild sex in zebrafish: loss of the natural sex determinant in domesticated strains. *Genetics* 198:1291-1308.

- Woodward M.A., Winder L.A. and Watt P.J. (2019) Enrichment increases aggression in zebrafish. *Fishes* 4(1):22.
- Wong D., von Keyserlingk M.A.G., Richards J.G. and Weary D.M. (2014) Conditioned place avoidance of zebrafish (*Danio rerio*) to three chemicals used for euthanasia and anaesthesia. *PLoS One* 9(2):e88030.
- World Organisation for Animal Health – OIE (2019) [Aquatic Animal Health Code](#) (accessed on 2020-10-02).
- Wynd B.M., Watson C.J., Patil K., Sanders G.E. and Kwon R.Y. (2016) A dynamic anesthesia system for long-term imaging in adult zebrafish. *Zebrafish* 14:1-7.
- Yang H., Carmichael C., Varga Z.M. and Tiersch T.R. (2007) Development of a simplified and standardized protocol with potential for high-throughput for sperm cryopreservation in zebrafish, *Danio rerio*. *Theriogenology* 68(2):128-136.
- Yanong R.P.E. (2012) *Fish health management considerations in recirculating aquaculture systems – Part 2: pathogens*. Gainesville FL: Institute of Food and Agricultural Sciences, University of Florida.
- Zaccone G., Fasulo S. and Ainis L. (1994) Distribution patterns of the paraneural endocrine cells in the skin, gills and the air ways of fishes determined by immunohistochemical and histological methods. *Histochemical Journal* 26:609-629.
- Zang L., Shimada Y., Nishimura Y., Tanaka T. and Nishimura N. (2013) A novel, reliable method for repeated blood collection from aquarium fish. *Zebrafish* 10(3):425-432.
- Zhdanova I.V. (2006) Sleep in zebrafish. *Zebrafish* 3(2):215-226.

APPENDIX 1

SUMMARY OF WATER QUALITY VARIABLES

VARIABLE	SUGGESTED MONITORING FREQUENCY		ACCEPTABLE RANGE
	SETTING UP A SYSTEM	ESTABLISHED SYSTEMS	
Temperature	Daily	Daily but could be extended depending on the system and long-term stability	26-29°C (Cartner et al., 2019) 25-30°C in general; 28.5°C for breeding (Lawrence, 2007) 26-28.5°C (Avdesh et al., 2012)
pH	Daily	Daily but could be extended depending on the system and long-term stability	7-8 (Cartner et al., 2019) 6.8-8.0 (Lawrence, 2007; Brand et al., 2002) 6.8-7.5 (6.0-8.5 tolerated) (Avdesh et al., 2012)
Conductivity/ salinity	Daily	Depending on stability of water source/ion dosing system*	Conductivity 200-3000µS (Cartner et al., 2019) Salinity < 5 g/L (Harper and Lawrence, 2010, cited in Lawrence and Mason, 2012) Salinity 0.5-1 g/L; Conductivity 300-1,500 µS (Avdesh et al., 2012)
Total hardness	Depends on stability of water source/ion dosing system*	Depends on stability of water source/ion dosing system*	>75-200 mg/L (ppm) (Cartner et al., 2019) 75-200 mg/L CaCO ₃ (Lawrence and Mason, 2012) 50-100 mg/L CaCO ₃ (Avdesh et al., 2012)
Total alkalinity	Depends on stability of water source/ion dosing system*	Depends on stability of water source/ion dosing system*	50-75 mg/L (ppm) (Cartner et al., 2019) 50-150 mg/L CaCO ₃ (Lawrence and Mason, 2012; Avdesh et al., 2012)
Total dissolved gases	Depends on the system*	Periodically*	Total gas pressure <100% (Lawrence and Mason, 2012) CO ₂ < 15-20 ppm, but keep as low as possible; DO 6-8 ppm (Cartner et al., 2019)

VARIABLE	SUGGESTED MONITORING FREQUENCY		ACCEPTABLE RANGE
	SETTING UP A SYSTEM	ESTABLISHED SYSTEMS	
Un-ionized Ammonia	Daily	Periodically*	< 0.05 mg/L (ppm) (Cartner et al., 2019) <0.02 mg/L (Avdesh et al., 2012)
Nitrate (NO ₃ ⁻)	Daily	Periodically*	< 50 mg/L (ppm) (Cartner et al., 2019) <50 mg/L (Avdesh et al., 2012)
Nitrite (NO ₂ ⁻)	Daily	Periodically*	< 0.1 mg/L (ppm) and as close to 0 as possible (Cartner et al., 2019) <0.1 mg/L (Avdesh et al., 2012)

***Note:** Cartner et al. (2019) state that monitoring of these variables should be performed weekly; however, it is challenging to be prescriptive as the required frequency varies with the system. Facilities need to use their experience of water quality results to determine the periodicity for their system. Measurements need to be taken often enough to ensure the system is stable and within acceptable parameters noted in the last column. If fluctuations occur, measurements need to be taken more frequently.

REFERENCES

- Avdesh A., Chen M., Martin-Iverson M.T., Mondal A., Ong D., Rainey-Smith S., Taddei K., Lardelli M., Groth D.M., Verdile G. and Martins R.N. (2012) Regular care and maintenance of a zebrafish (*Danio rerio*) laboratory: an introduction. *Journal of Visualized Experiments* 69:e4196.
- Brand M., Granato M. and Nüsslein-Volhard C. (2002) Keeping and raising zebrafish. In: *Zebrafish – A Practical Approach*. (Nüsslein-Volhard C. and Dahm R., eds.). Chapter 1. Oxford UK: Oxford University Press.
- Cartner S., Eisen J., Farmer S., Guillemin K., Kent M. and Sanders G. (eds.) (2019) *The Zebrafish in Biomedical Research: Biology, Husbandry, Diseases, and Research Applications*, 1st ed. American College of Laboratory Animal Medicine Series. San Diego CA: Academic Press.
- Harper C. and Lawrence C. (2010) *The Laboratory Zebrafish*. Boca Raton (FL): CRC Press.
- Lawrence C. (2007) The Husbandry of zebrafish (*Danio rerio*): a review. *Aquaculture* 269:1-20.
- Lawrence C. and Mason T. (2012) Zebrafish housing systems: a review of basic operating principles and considerations for design and functionality. *ILAR Journal* 53(2):179-191.

APPENDIX 2

SUMMARY OF RECOMMENDATIONS FOR WATER TEMPERATURE FOR HOUSING ZEBRAFISH

SOURCE	RECOMMENDATION STATED	RATIONALE (Where Provided)
Cartner et al. (2019)	26-29°C	
Howells and Betts (2009)	The ideal water temperature is 26-28°C.	
Vargesson (2007)	A temperature range of 27°C-28.5°C is necessary for optimal breeding conditions.	Temperatures below 25°C and above 30°C reduce the breeding capability of the fish and thus the numbers of embryos produced.
Matthews et al. (2002)	A widely used standard temperature for developmental studies is 28.5°C. A gradual drop in temperature to 22-23°C to lower zebrafish metabolic rate is acceptable in emergencies, such as water system mechanical failures.	
Brand et al. (2002)	Between 25°C and 28°C. The temperature is normally adjusted to around 26°C using several heaters placed into the filter basin. The room temperature should be set slightly higher (e.g., 27°C), which prevents condensation of water and growth of mold on the walls of the rooms. A drop in temperature to room temperature by failure of heaters is not dangerous for the fish.	Higher temperatures are uncomfortable for people working in the fish rooms and might also reduce the life span of the fish. The higher the temperature, the lower the oxygen content of the water.
Westerfield (2007)	28.5°C	Above 31°C and below 25°C, zebrafish probably will not breed and development will be abnormal.
Andrews (1999)	A steady temperature in the range 18-25°C (a little higher when breeding e.g., 28-29°C).	
Bilotta et al. (1999)	An ideal temperature for both breeding and development of the embryos is 28.5°C.	

REFERENCES

- Andrews C. (1999) Freshwater fish. In: *The UFAW Handbook on the Care and Management of Laboratory Animals*, 7th ed., volume 2 Amphibious and Aquatic Vertebrates and Advanced Invertebrates (Poole T., ed.). Oxford UK: Blackwell Science.
- Bilotta J., Saszi S., DeLorenz A.S. and Hardesty H.R. (1999) Establishing and maintaining a low-cost zebrafish breeding and behavioural research facility. *Behaviour Research Methods, Instruments and Computers* 31(1):178-184.
- Brand M., Granato M. and Nüsslein-Volhard C. (2002) Keeping and raising zebrafish. In: *Zebrafish – A Practical Approach*. (Nüsslein-Volhard C. and Dahm R., eds.). Chapter 1. Oxford UK: Oxford University Press.
- Cartner S., Eisen J., Farmer S., Guillemin K., Kent M. and Sanders G. (eds.) (2019) *The Zebrafish in Biomedical Research: Biology, Husbandry, Diseases, and Research Applications*, 1st ed. American College of Laboratory Animal Medicine Series. San Diego CA: Academic Press.
- Howells L. and Betts T. (2009) A beginner's guide to the zebrafish (*Danio rerio*). *Animal Technology and Welfare* 8(3):117-120.
- Matthews M., Trevarrow B. and Matthews J. (2002) A virtual tour of the Guide for zebrafish users. *Lab Animal* 31(3):34-40.
- Vargesson N.A. (2007) Zebrafish. In: *Manual of Animal Technology* (Barnett S., ed.). Oxford UK: Blackwell Publishing.
- Westerfield M. (2007) *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio)*, 5th ed. Eugene OR: University of Oregon Press.

APPENDIX 3

SUMMARY OF RECOMMENDATIONS FOR WATER VOLUME FOR HOUSING ZEBRAFISH

SOURCE	RECOMMENDATION STATED	RATIONALE (Where Provided)
Vargesson (2007)	<p>5 fish per litre in systems possessing filters and a biofilter, as long as there is good water exchange, good feeding regime and good water quality.</p> <p>For breeding purposes it is best to have fewer fish per tank (2-3 fish per litre).</p> <p>In a tank that does not have filters or a biofilter, the maximum number should be 1 or 2 fish per litre.</p>	
Brand et al. (2002)	In large-scale re-circulating systems, families of sibling adult fish are kept in serial tanks at densities of 5 adult fish per litre (60 fish/12 litres).	Zebrafish tend to be aggressive if few fish are kept together in small volumes of water.
Matthews et al. (2002)	<p>20 eggs/embryos per 100ml water.</p> <p>20 young larvae per 400ml up to juvenile stage.</p> <p>Growing juvenile fish and holding adults: 5 fish per litre.</p> <p>For breeding, a pair can be kept overnight in 1.5 litres, or 6 fish in 2.3 litres of water.</p>	
Westerfield (2007)	25 fish in 45 litres (~10 gallons).	

REFERENCES

- Brand M., Granato M. and Nüsslein-Volhard C. (2002) Keeping and raising zebrafish. In: *Zebrafish – A Practical Approach*. (Nüsslein-Volhard C. and Dahm R., eds.). Chapter 1. Oxford UK: Oxford University Press.
- Matthews M., Trevarrow B. and Matthews J. (2002) A virtual tour of the Guide for zebrafish users. *Lab Animal* 31(3):34-40.
- Vargesson N.A. (2007) Zebrafish. In: *Manual of Animal Technology* (Barnett S., ed.). Oxford UK: Blackwell Publishing.
- Westerfield M. (2007) *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio)*, 5th ed. Eugene OR: University of Oregon Press.

GLOSSARY

Acclimation – a persisting physiological, biochemical or morphological change within an individual animal during its life as a result of a prolonged exposure to an environmental condition such as a high or low temperature; generally, the changes are reversible.

Airstone – a device added to a system to gradually aerate the water.

Analgesia – decrease in response to noxious stimuli.

Anesthesia – a state caused by an external agent leading to loss of sensation and motor function.

Asepsis – absence of living germs, free from septic and poisonous putrefactive products.

Biofilter – a water filter containing live microorganisms that oxidize ammonia to nitrite and then to nitrate.

Chloramine – a chemical compound that contains chlorine and ammonia, often found in municipal water supplies.

Competency – competency refers to the ability to effectively perform a particular task in relation to the care, maintenance or use of the animals, while ensuring their welfare is protected as far as possible within the constraints of any approved studies that the animals are involved in. Focusing on competency rather than training acknowledges that there may be a variety of ways of acquiring the necessary knowledge and skills, and places emphasis on learning outcomes. See [*CCAC guidelines on: training of personnel working with animals in science*](#) (CCAC, 2015) for more details.

Conspecifics – animals belonging to the same species.

Distress – a state where the animal must devote substantial effort or resources to the adaptive response to challenges emanating from the environmental situation; it is associated with invasive or restrictive procedures conducted on an animal, or other conditions which significantly compromise the welfare of an animal, which may or may not be associated with pain.

Ectothermic – an animal that assumes the temperature of its surroundings.

Endpoint – predetermined criteria for intervening in a procedure to terminate, minimize or reduce an animal's pain and/or distress, which takes into account the welfare of the animal (welfare endpoint) and the goal of the experiment (scientific endpoint).

Environmental enrichment – enhancements to an animal's environment that go beyond meeting its basic species-specific needs and further improve overall quality of life.

Exsanguination – a procedure causing extensive loss of blood due to internal or external hemorrhage.

Fomites – non-living objects that can carry disease organisms (e.g., mops).

Genetically modified – a deliberate modification of the genome (the material responsible for inherited characteristics).

Genotyping – a process used to determine differences in the genetic makeup (genotype) of an individual animal by examining the individual's DNA sequence using biological assays and comparing it to another individual's sequence or a reference sequence.

Hypothermia – lower than normal body temperature.

Integument – the natural outer covering of an animal; the skin.

Lamellae – area of the gills where the exchange of gases and waste products occurs.

Lateral line – a sensory system running along the side of a fish that detects movement, vibration and pressure.

Morbidity – visible manifestation of a diseased state.

Mortality – loss of life; death.

Noxious stimuli – those stimuli that are damaging or potentially damaging to normal tissue.

Osmotic imbalance – nonoptimal concentration of salts.

Pain (in fish) – fish pain is a response to a noxious stimulus that results in a change in behaviour or physiology and the same noxious stimulus would be painful to humans (a working definition).

Personal protective equipment (PPE) – garments or equipment designed to protect personnel from injury, infection, or allergic reaction when working with animals; potential hazards include physical injury (bites, scratches, etc.), biohazards, and airborne particulate matter.

Progeny – offspring.

Quarantine – confinement of animals which may carry an infectious disease, for a specified period to allow for evaluation.

Sedatives – drugs which reduce an animal's agitation.

Sentinel fish – specific pathogen-free (SPF) fish known to be susceptible to an infectious agent that are placed in the area suspected of being contaminated, for example in a new shipment of fish under quarantine; the sentinel fish are then tested for infection or development of antibodies to the infectious agent.

Shoal – a group of fish swimming together.

Standard operating procedure – written documents that describe in step-by-step detail how a procedure should be carried out.

Supersaturation – a condition where the total gas pressure in a body of water exceeds the barometric pressure in the overlying atmosphere.

Stress – a state caused by factors external to an animal that displace homeostasis; stress can be beneficial (e.g., in triggering a flight response if the animal is threatened, thus helping it to cope with changes in its environment); however, prolonged stress can cause changes to an animal's endocrine system, leaving it less able to cope with its environment.

Three Rs – Replacement, Reduction and Refinement in animal-based science, as first explained by Russell and Burch in *Principles of Humane Experimental Technique* (1959).

Welfare – the physical health and mental well-being of the animal.

Zoonotic – relating to the transmission of a disease from a non-human species to humans.