Policy on Zebrafish Research

Ministry of Public Health
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Table of Contents

Background

Zebrafish makes an ideal animal model for research. No other vertebrate model organism's popularity has grown as quickly as that of the zebrafish. The reputation of this small tropical fish was firmly established during the nineties of the previous century when big genetic screening projects in the US and Europe led to the discovery of hundreds of mutants that gave novel insights into the functions of essential human developmental genes. These and other genetic studies confirmed that the zebrafish is a good predictive model for human development and disease. The second reason for the rapid expansion of zebrafish colonies in research labs is the optical transparency of zebrafish embryos. This property makes them ideally suited for microscopic imaging of life processes. Fluorescent markers can be used to 'light up' different cells and organs in the zebrafish embryo. Microscopic imaging of such fluorescent cells in different zebrafish mutants helps understanding how the normal functions of cells can be affected during disease processes. The zebrafish genome is sequenced, and researchers have already identified strains containing mutations in genes of interest to human disease.

Policy Scope

This policy outlines the current practice and guidance concerning the ethical and environmental parameters that need to be considered on conducting Zebrafish research.

In developing this policy, The Research Regulation Department at Qatar Ministry of Public Health has consulted with international regulations for the humane Use of Zebrafish in research.

1. Introduction

The embryonic development of zebrafish and humans is similar. Helpfully, the zebrafish embryo is transparent and grows outside of the fish, with the complete "body plan" laid down within 24 hours. A single pair of zebrafish can produce 100 to 300 embryos in a week and this allows statistically significant analyses at a minimal cost. It is also advantageous for high throughput screening of drugs and therapies. Consequently, to maintain a healthy colony and stimulate good quality egg production throughout the year, fish should be kept under *optimal* conditions. Little research has been conducted to evaluate what these conditions are and how they can be judged. It has been suggested that the suitability of an environment can be judged by the survival of eggs and embryos, aiming to achieve at least 80-95%, together with growth over a standard period (1.0 - 1.5cm by 21-days post-fertilization) (*anon*). However, these are not the only criteria to consider - good reproductive performance may be a useful indicator of health but may or may not reflect optimal welfare.

2. Lighting

Appropriate lighting facilitates good breeding success and minimizes stress.

2.1 Photoperiod

Light triggers zebrafish to breed, so periods of darkness are important for allowing animals to rest (Vargesson 2007, Brand et al 2002). Francis (2008) states that one of the fastest ways to ensure fish will not lay eggs, is to leave the lights on all the time.

Zebrafish larvae reared in constant light have been observed to show severe deficits in visual acuity and behavior, though not anatomical abnormalities (Bilotta 2000). However, they appear able to recover from the effects of early rearing in abnormal lighting if they are subsequently housed under normal cyclic conditions (Bilotta 2000). Being kept in constant darkness delays general development of embryos, with hatching still not being observed by 7 days post-fertilization (Bilotta 2000).

A cycle of 14 hours light, 10 hours dark has been advised, and would appear to be common practice (Matthews et al 2002, Brand et al 2002). A brightening and dimming period can also be arranged to avoid startling the fish, rather than switching lights abruptly on and off (The Berlin Workshop 1994).

2.2 Spectrum

Adult zebrafish appear to have the necessary mechanisms for color vision (Saszik et al 1999), but no specific requirements regarding the light spectrum of their environment have been determined. Until any such needs have been established, it is suggested that standard fluorescent lighting is acceptable (Matthews et al 2002).

2.3 Intensity

It would appear that little, if any, research has been carried out to determine the effect on zebrafish health and welfare of different lighting intensities. Matthews et al (2002) have cited quite a broad range of 54-324 lux as being appropriate at the surface of the water. Some establishments maintain a low intensity of lighting with the aim of minimizing algal growth in tanks. Further investigation is required before any regime can confidently be considered most beneficial or best practice.

- *A lighting regime of 14 hours light and 10 hours dark is recommended.*
- *Continuous 24-hour light, or dark, regimes should not be used.*
- *Ideally, where artificial lighting is use, a gradual brightening/dimming period of around 20-30 minutes in the morning/evening can be incorporated.*

3. Noise and other disturbances

Zebrafish can appear to grow accustomed to their surroundings and as such, may apparently habituate to certain vibrations - from a pump in the room for example. But they can also react strongly to sudden loud noises or novel vibrations so steps should be taken to avoid such disturbances. Ideally, any vibration causing equipment should not be kept in the same room. It has also been suggested that spawning in these fish may be affected if it is very noisy or if there is a lot of nearby movement or activity (Vargesson 2007). The sensitivity of these fish to sounds like talking or music is uncertain (Matthews et al 2002).

4. Humidity

From an animal welfare perspective, there is little need to control humidity levels in rooms with tanks holding aquatic animals. In any case, such control is difficult in rooms with open tanks as the humidity at the water's surface is likely to be different from that elsewhere in the room.

5. Water provision

Tanks need to be of sufficient size to accommodate the physical and behavioral 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).

6. Quantity and temperature

6.1 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).

It has been recommended that as long as tanks have a 'relatively large surface area' water depth does not have to exceed 25cm (Brand et al 2002). Elsewhere it has been suggested that for spawning, just 10cm water depth in a 50-litre tank should be provided for three adult males and two females (Andrews 1999). However, given the findings of Spence et al, it should not be assumed that only providing water to these shallow depths is appropriate for long term housing.

6.2 Volume and population density

Keeping zebrafish in 'crowded' conditions is detrimental to their welfare. Adults kept at high densities[1] have been observed to show a four-fold increase in whole body cortisol levels[2] and reduced egg production[3] (Ramsay et al 2006, Goolish et al 1998). Development is also affected, with zebrafish maintained at higher densities growing slower than those maintained at lower densities (Vargesson 2007).

Stocking density also influences the male: female ratio of offspring, with a female bias shown at low densities (Vargesson 2007).

Figure 1: Summary of recommendations made for water volume for housing zebrafish

Source	Recommendation stated	Rationale (where provided)
Matthews et al (2002)	20 eggs/embryos per 100ml water. 20 young larvae per 400ml up to juvenile stage. Growing juvenile fish and holding adults - 5 fish per litre. For breeding, a pair can be kept overnight in 1.5 litres, or 6 fish in 2.3 litres of water.	
Vargesson (2007)	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. For breeding purposes it is best to have less fish per tank (2-3 fish per litre). In a tank that does not have filters or a biofilter, the maximum	

	number should be 1 or 2 fish per litre.	
Brand et al (2002)	In large-scale re-circulating systems, families of sibling adult fish are kept in serial tanks at densities of five adult fish per litre (60 fish/12 litres).	Zebrafish tend to be aggressive if few fish are kept together in small volumes of water.
Westerfield (2000)	25 fish in 45 litres (~10 gallons)	

Fish should not be kept in ‘crowded’ conditions. Keeping 5 fish per litre is common, although further research is required to ascertain preferred space requirements from a welfare perspective.

6.3 Temperature

Zebrafish are classified as eurythermal which means that they can tolerate a wide temperature range. In their natural habitat, zebrafish have been observed to survive temperatures as low as 6°C in winter to over 38°C in summer (Spence et al 2008). This is confirmed by studies in the laboratory that have shown that wild-type zebrafish have a maximal thermal tolerance range[4] of 6.2°C - 41.7°C (Cortemeglia & Beitinger 2005). However, the temperature range at which an animal can survive is different to its *preferred* temperature range. Maintenance at sub-optimal temperatures will have a metabolic cost that may affect breeding, development and welfare.

A water temperature of 28.5°C is widely cited as the optimum temperature for breeding zebrafish[5] (see *Figure 2*). Whilst practical experience suggests that zebrafish generally maintained at this temperature grow and breed satisfactorily, there may be welfare concerns with keeping fish at this temperature all year round. Fish may spawn continuously, which is unnatural and places a high metabolic cost on the animal. There has however, been little research to investigate the full implications of constantly keeping fish at this very specific temperature.

Whatever the system of water exchange used, incoming replacement water should be the same temperature as the water it is replacing.

Figure 2: Summary of recommendations for water temperature for housing zebrafish

Source	Recommendation stated	Rationale (where provided)
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.	
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.

Bilotta et al (1999)	An ideal temperature for both breeding and development of the embryos is 28.5°C.	
Howells and Betts (2009)	The ideal water temperature is 26-28°C.	
Andrews (1999)	A steady temperature in the range 18-25°C (a little higher when breeding e.g. 28-29°C).	
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 mould 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 (2000)	28.5°C	Above 31°C and below 25°C, zebrafish probably will not breed and development will be abnormal.

On the basis of users' experience, a water temperature of around 28.5°C is suggested for zebrafish when breeding, though more research is required to understand the exact temperature preferences of zebrafish and implications of maintaining them at this water temperature longer term.

7. Water quality

Water quality is the most important factor 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). Though some generally useful principles exist, ideal parameters are neither broadly agreed nor defined (Obenschain & Aldrich 2007).
Levels of contaminants need to be minimized. 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).

7.1 pH level

Systematic studies detailing growth and reproductive performance of zebrafish at different levels of pH have not been conducted (Lawrence 2007). However, field studies have observed zebrafish to be present in waters between 5.9 and 8.1 (Engeszer et al 2007). Most laboratory facilities aim for maintaining pH between 7.0 and 8.0 (Lawrence 2007). Brand et al (2002) suggest aiming for between 6.8 and 7.5 (and not lower than 6 or higher than 8).
It is important to monitor the pH of the water in the tanks regularly, using a colorimetric test kit or preferably, a precise electronic meter (which should be regularly calibrated).

7.2 General hardness and other water quality parameters

Fish require ions such as calcium and magnesium, plus iron and selenium, to maintain health and function. These can be provided through the diet or environment.

Matthews et al (2002) suggest an adequate dissolved oxygen content of 6.0 ppm (mg/L).

If a large increase in ammonia or nitrite is detected a large water exchange must be carried out. This is because high levels of ammonia and nitrite levels can cause damage to the fish. For instance, nitrite is absorbed through the gills and interferes with the ability of fish to absorb oxygen, resulting in death (Vargesson 2007).

It is important to have a full knowledge of the origin and properties of the water used for maintaining zebrafish. Properties (e.g. fluoride content) will vary widely depending on whether water is obtained from municipal sources (e.g. tap water), or natural sources (springs, lakes or rivers), and whether it is distilled/desalinized. Water should be dechlorinated before use6.

The pipes used for transporting water into and around an aquatic system should not be galvanized or copper, since heavy metals can leach from such pipes and may be toxic (Wolfensohn and Lloyd 2003).
Water quality and pH level should be routinely monitored. Contingency plans should be made in case of system breakdown or another emergency.

7.3 Cleaning

The cleanliness of the aquaria and filters is a particularly important factor in keeping fish in healthy breeding conditions (Brand et al 2002). Zebrafish constantly excrete ammonia (across the gills and to a lesser extent in feces) into the surroundings. This, along with floating decaying food particles, will foul the water and may have implications for fish health where space and animal movement are limited, as in a laboratory tank. Consideration must therefore be given to how best to maintain the quality of the water, whilst at the same time minimizing disturbance to the animals.

Zebrafish are routinely housed either in tanks of standing water (partly or fully 'dumped and refilled' every day or few days) or more commonly, in tanks where a drip-through system continuously and slowly changes the water. In drip-through systems the water coming in may be new or treated and cleaned re-circulating water. Static systems require frequent cleaning of tanks and/or for fish to be kept at lower stocking densities but have the benefit of enabling disease outbreaks to be more easily controlled. This can be harder in re-circulating systems.

All recommendations for cleaning practices will be influenced not only by the tank or system design in place, but also by the feeding regime and quality of water entering the system.

7.3.1 Standing water tanks
Tanks maintained by manual water changes can be equipped with filtration units that will continually remove undesirable material from the water (Matthews et al 2002). If a third of the water is replaced each

day by siphoning up debris from the bottom of the tank, a separate tank filtering system should not be necessary. If a filter is used, around half the water will need to be changed at least once a week (Westerfield 2000).

7.3.2 Drip-through water systems

In drip-through systems, levels of toxic waste are kept low and solid waste (in suspension) can be drained continuously. The downside of these systems is that they use a lot of water (if not re-circulating) and the quality of the input water must be monitored constantly which often means a significant capital investment. 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).

In the wild, zebrafish can be found in slow-flowing waters (Spence et al 2008). As they sense water movement through a highly developed lateral line system, the position of in- and out- flowing taps in the tanks and the rate of water flow should be set so water turbulence or motion is not excessive.

7.3.3 Careful use of cleaning agents

Although most tanks holding zebrafish are now made of polycarbonate, most establishments do not autoclave them (Francis 2008). If a cage washer is used to clean polycarbonate tanks, they should be thoroughly rinsed as residues in the aquatic environment may be easily absorbed into the bodies of zebrafish causing illness and possibly death. Bleaches and detergents must be used with considerable caution. Brand et al (2002) suggest using a sponge soaked in 5% acetic acid to wipe the walls of the tanks, and then the same process using a sponge soaked in 3% hydrogen peroxide in 0.1% NaOH. After using such cleaning agents, tanks should be rinsed thoroughly several times with clean, cold, dechlorinated tap water before they are used.

Avoiding placing lights right over the racks will help reduce algal growth (Francis 2008). Some institutions also try to keep algae growth at bay by keeping fish together with snails (e.g. Florida freshwater snails, Planorbella spp.), that clean the walls of algae and eat any surplus food (Brand et al 2002). However, extreme care should be taken when introducing snails as they can be a source of infection so should only be introduced if it is certain they are disease-free. Snail spawn can be bleached in the same way as fish embryos (Brand et al 2002).

Cleaning strategies should be designed to minimize disturbance and distress to the fish. Disinfectants should be used with extreme caution.

8. Tank housing

8.1 Labelling

Tank housing should always be clearly labelled with the genetic background and sex of the animals inside. If the fish are currently being used in a project, the reference to that research (and who is responsible) should be clearly identifiable and staff should know where to find relevant information relating to the project. This is so that all relevant personnel are aware of the experimental procedures involved, the objectives of the work, the potential adverse effects the animals may experience and the agreed humane endpoints (where applicable).

8.2 Tank material

Tanks used to hold zebrafish are usually made of polycarbonate, high-quality glass or acrylic (Matthews et al 2002). Care should be taken to ensure that all other materials used in setting up the aquarium, such as tanks, pipes, plastic connections, tubing, siphons and pumps, do not leak toxic compounds into the water (Brand et al 2002).

8.3 Color and transparency

Glass and other transparent-walled containers have the advantage of allowing easy observation and monitoring of the fish, but a disadvantage in that movements of staff and equipment outside the tank can disturb them. On the other hand, opaque, or very dark colors can lead to hygiene problems since contamination may not be obvious (The Berlin Workshop 1994). A container coloration of medium blue is probably best. Consideration should be given to placing tanks on a dark surface which will prevent light emanating from below, as it is suggested that fish prefer this to light colored surfaces (Brand et al 2002).

8.4 Lids and drain covers

Zebrafish can jump (Brand et al 2002) so all tanks should be provided with a cover. A translucent lid, which allows light in whilst reducing the risk of alarm to the fish from movements of staff working nearby, is the most suitable (The Berlin Workshop 1994). If tank lids have a small hole, no larger than 1cm in diameter, then feeding can be carried out using a squirt bottle without having to open the lid thus reducing disturbance (Brand et al 2002). Tank drains should be covered to prevent the fish escaping the tank.
Tank design and material should ensure that the impact of staff movements and disturbance outside the tank are minimized.

9. Identification and marking techniques

Marking techniques can affect animals and their wellbeing 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). Tagging or marking small species such as zebrafish is not an easy task so the need for individual or group identification must first be critically assessed. If identification of individual animals is necessary, then only the most humane methods must be used.

The method of identification employed 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;
- be easy and quick to read/identify.

Note that current evidence suggests fish should be given the benefit of the doubt and assumed to perceive pain in a way analogous to mammals.
Careful consideration should be given to whether identification of individual animals is necessary. If so, the least invasive method should be used.

Non-invasive methods of identification, for example, based on observed and recorded differences in natural markings are preferred where practical.

10. Group housing

Zebrafish are highly social animals. They prefer to shoal with other fish, regardless of shoal composition or even species, rather than to be on their own (Ruhl et al 2009). Indeed, the most important social interactions occur during shoaling and spawning (Spence & Smith 2007).

Aggressive behavior is usually limited to the spawning period, about one hour after lights come on in the laboratory setting, whilst at other times of day fish frequently shoal together peacefully (Spence & Smith 2005). Aggressive territoriality is a normal feature of zebrafish spawning behavior, and although fish do not usually inflict physical harm on one another, chasing and sometimes 'biting' may be observed which can 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 & Smith 2005).
In the laboratory setting, males appear to display different rates of aggression depending upon how many other males are nearby. At low densities, territorial males follow and actively court females, periodically returning to the spawning site. In contrast, at high densities, territorial males confine their activities to within a few body lengths of the spawning site, vigorously defending the area from other males (R. Spence, personal observation). However, genetic analysis of male reproductive success has shown that under high-density conditions in the laboratory, males with territories are no more successful that those without (Spence et al 2006b).

Zebrafish kept together for breeding should have some means of escape from more aggressive fish (Matthews et al 2002). Providing extra space will help, but if the tank contains plant-like materials or structures[7] then these can be used as hiding places.

Delaney et al (2002) reports that females avoid staying alone and under normal conditions might live with one or two males but separated from other females. Ruhl et al (2009) observed that single males also apparently preferred shoaling with single females rather than groups of three. These authors also observed that females preferred to shoal with a group of three individuals rather than with a single individual, regardless of the sex of the other fish. Females can behave aggressively towards each other and develop a dominance hierarchy. This probably explains why, they were observed to spend only 5% of the time in female-only groups. The study also showed that males seemed to change female partners daily and that social grouping influenced egg production.
Zebrafish should not be kept on their own without scientific or veterinary justification.

Tanks should contain carefully considered structures that the fish can use as hiding places, to help minimize aggressive behavior.

11. Catching and handling

Most zebrafish in research facilities are the descendants of many generations of captive bred animals. Although they appear to exhibit reduced 'nervousness' or predator avoidance behaviors, as a prey species, being handled represents a potentially dangerous stressor. 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). For this reason, it is advisable to minimize handling of zebrafish.

In small-scale facilities, some people use containers rather than nets to scoop fish out of holding tanks - so the animal does not experience the stress of being removed from water. This may also reduce the potential for scales to be lost due to abrasions caused by the transfer net (Ruhl et al 2009). However, it may mean it takes longer to isolate and catch each animal.

For hygiene reasons, each tank should have its own dedicated handling equipment, or the equipment should be routinely sterilized between uses.

Handling should be kept to a minimum and precautions taken to avoid causing stress or injury.

12. Food type and feeding regime

12.1 Natural behavior in the wild

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

Adult zebrafish usually feed on small crustaceans, insect larvae and, to some extent, algae.

12.2 Feeding requirements of zebrafish

Francis (2008) suggests that a quality diet[8] specifically developed for zebrafish should be used. Some commercial feeds claim to offer a nutritionally complete food[9]. However, the precise nutritional requirements of zebrafish have yet to be determined (C. Lawrence, personal communication).

12.3 Food content and frequency

Current practice is to feed fish of mid-to-late juvenile stage and beyond, twice (once in the morning and early evening) or three times a day. For early stage larvae and those undergoing metamorphosis, more frequent feedings may be beneficial.

Adults can tolerate a few days without food but require daily feeding for optimal egg production (Matthews et al 2002). Poor water quality will increase the chances of disease, and along with overfeeding (causing fish to become fat) can reduce breeding performance (Vargesson 2007).

It is good practice for housing system designs to incorporate or allow for an effective mechanism for removing any solids after the last feeding.

Figure 3: provides a summary of recommendations that have been made for feeding zebrafish.

Source	Food type/content	How much	How often
Westerfield (2006)	Feed manually ground dry or moist trout pellets (Ranger 1/4 inch brood food or Oregon wet pellets) as well as dry flake food like Tetra brand (available at most pet stores). The best possible food for breeding adults is live adult brine shrimp.	Add enough food to each tank so that all the fish get some and all the food is eaten within 5 minutes.	Feed adults at least twice a day although multiple light feedings allow the fish better opportunity to utilise the food.
Vargesson (2007)	Although crushed flake food is suitable for zebrafish it is not recommended to feed this alone as it will reduce breeding efficiency. It is a good idea to alternate	All of the food should be consumed within 10-15 minutes of being fed. It is important not to overfeed the fish as it will cause them to become fat, reduce breeding, will lead to poor water quality and will	In general, fish should be fed twice a day - once in the morning and once in the early evening.

	between brine shrimp and flake.	increase the chances of disease.	
Brand et al (2002)	Dry food alone is not sufficient to keep fish in good breeding conditions. Therefore, it is necessary to supplement it with live or frozen food. The most commonly used additional live food is *Artemia nauplii*. Alternatively, or in addition to *Artemia*, *Drosophila* larvae or different types of frozen food that are available from aquaculture supply stores can be used. Live or frozen food (e.g. tubifex, *Daphnia* and *Chironomus* larvae) that has been harvested from freshwater systems that also harbour fish, should be avoided, as it may be a source of pathogens. On the other hand, salt-water-dwelling articulates are safe (e.g. frozen adult *Artemia* and krill).	When feeding it is important to take the number of fish in a tank into account and not to overfeed them. It is good practice to check whether all the food has been eaten within about 10min.	A typical feeding regimen is to feed adult fish tanks twice a day (once at weekends). Adult fish that have to be kept for longer periods of time without breeding require very little feeding (e.g. twice a week, preferably with live food). Two weeks of rich feeding will bring them back into breeding condition again.
Andrews (1999)	As they become free swimming, fry should be fed newly hatched brine shrimp nauplii, sieved culture *Daphnia*, and a fine dried fry food.		
Matthews et al (2002)	Newly hatched zebrafish can eat *Paramecium* (800µm x 80µm), as well as a variety of prepared foods, infusoria and rotifers. As they grow larger, zebrafish hatchlings can add to their diet larger items such as vinegar eels, microworms, or larger prepared foods. Eventually they are large enough to eat *Artemia nauplii* (newly hatched brine shrimp), which have a high protein content, can be hatched on demand, but can be expensive.		

	Adult-size fish can be fed adult prepared foods (tropical fish flake foods, tropical fish micropellets, and ground trout meal) and live brine shrimp.		
Howells and Betts (2009)	Once fish reach one month of age: flake food supplemented with live food such as *Artemia*. Adult fish being prepared for breeding: live food		Twice a day and once daily at weekends. Twice a week. Reverting to daily feeding will help bring them into breeding condition.

Although two of the statements in the above table suggest that it may be possible to maintain fish whilst only feeding them twice a week, many people believe it is not preferable to feed fish any less than daily. Similarly, suggestions for feeding only once at weekends are usually due to staff availability within an establishment rather than the feeding requirements of the fish.

Feeding time is often used as an opportunity to observe the health and behavior of the animals. If automatic feeders are used, then additional opportunities for observing the fish need to be built in to the management system.

13. Environmental enrichment

Environmental enrichment is a means of enhancing the quality of captive animal care by identifying and providing the environmental stimuli necessary for optimal psychological and physiological wellbeing (Shepherdson 1998). Allowing animals to have a degree of control over their surroundings and exhibit a range of species-typical behaviors can improve welfare and reduce stress. This is also important for scientific reasons as animals whose wellbeing is compromised (e.g. by being placed in unsuitable social groupings or an inadequate environment) are often physiologically and immunologically compromised, which can have an adverse impact on the quality of scientific data.

Providing appropriate environmental enrichment for fish should be considered the norm with compelling arguments required for leaving tanks bare (ASPI 2006) - although there is still debate over the extent to which zebrafish benefit from environmental enrichment, and what form it should take.
It has been suggested that zebrafish appear indifferent to environmental enrichment (Matthews et al 2002). However, field and laboratory-based studies have shown both wild and captive-bred zebrafish prefer habitats with vegetation. For example:

- in the wild, the vast majority of sites where zebrafish were observed had submerged or overhanging vegetation (Engeszer et al 2007);

- zebrafish prefer to spawn in sites associated with aquatic vegetation (Spence et al 2008);

- when a laboratory tank was split into 16 areas, of which 7 contained artificial plants, zebrafish could be found in those 7 squares 99% of the time (Delaney et al 2002).

Weed is also an important refuge, especially for females to allow the avoidance of males[10].

Providing artificial plants or other structures that imitate the zebrafish habitat allow animals a choice within their environment. It should be strongly considered - especially for breeding tanks or where fish are kept at low density - although any enrichment provided should not allow fish to become entangled.
Before introducing enrichment objects to the tank, careful planning and consideration should also be given to the method and frequency of cleaning the object, the potential for chemicals to leach into the water, and the ability of animal care staff to observe and assess the wellbeing of the fish.

Consideration should be given to providing zebrafish with environmental enrichment. Tanks can include structures that provide fish with refuge opportunities.

14. Assessment of health and disease prevention

An animal's welfare can be compromised by poor health. This section addresses the identification of discomfort or clinical signs of illness, and the treatment of common diseases in zebrafish.
Before fish are acquired, a veterinarian (with specific knowledge of zebrafish if possible) should be consulted to agree a programme for assessing the health status of the incoming animals, how animals will be monitored, and the potential use of preventive medicine and treatment strategies. A veterinarian should again be consulted about possible treatments, and animal carers should be made aware of any requirements for, or restrictions on, the use of medicines.

14.1 Diagnosis of ill health

Significant reductions in the numbers of animals used can be achieved when animals are kept healthy and when early signs of disease are recognized and appropriate veterinary care is provided.

It is not uncommon for a fish to appear healthy one day, only to die on the next (ASPI 2006). This suggests more work needs to be done to improve knowledge regarding definition and recognition of clinical signs and the assessment of welfare. Indeed, Matthews et al (2002) acknowledges that whilst it is accepted that fish have the capacity to experience pain, their responses can be difficult to interpret (Matthews et al 2002). Fish should be observed at least daily for indicators of poor health (see Figure 4). Sick fish should be removed from the tank as quickly as possible and veterinary advice sought.

Figure 4: Some key signs of ill health in zebrafish

Clinical signs	Possible cause Bacterial infection	Viral infection	Parasites	Chemical or environmental irritation	Toxicity	Environmental stress	Gas supersaturation	Oxygen depletion	Hormonal influences	Baroregulatory failure	Mechanical trauma	Starvation
Changes in body colour	*	*		*		*			*			*
Clamped fins			*	*								
Emaciation	*		*									*
Exophthalmos	*		*				*					
Improper buoyancy	*		*							*		
Lethargy	*	*	*		*	*		*				*
Opercular flaring	*		*			*	*	*				
Petechiation or haemorrhage	*	*	*		*		*				*	
Scale loss	*		*								*	
Sloughed mucus	*		*	*								
Sudden death	*	*	*	*	*	*	*					
Surface breathing			*	*		*	*	*				

(Table information taken from Astrofsky et al 2002)

Other behavioral indicators to look for include: failure to feed; swimming in an abnormal position in the tank; or rubbing their bodies on the tank side.

14.2 Common diseases

Clinical signs of common conditions in zebrafish and some suggestions in the literature for their treatment are detailed in the table below. A veterinarian should be consulted if any of the clinical signs are observed.

Pseudoloma neurophilia (or microsporidiosis)

Background

- This microsporidian is common in laboratory colonies (Spitzbergen & Kent 2003). It is likely that the parasite is transmitted from parents to progeny, even when eggs are surface cleaned with chlorine because: the parasite is abundant in ovaries; larvae are extremely susceptible to the infection; and chlorine levels used to treat eggs is not entirely effective for killing the spores (Kent 2007).

Clinical signs

- It infects the central nervous system, cranial and spinal nerves, and skeletal muscle of zebrafish, causing chronic emaciation (or 'skinny disease'), reduced growth, ataxia and spinal malformations.

Proposals for prevention/treatment

- Although there is no known effective treatment, UV light sterilisation of the water has proven to be reasonably helpful in reducing its incidence (Kent 2007).
- PCR-based tests can be used to screen for carriers (such that *Pseudoloma*-free facilities may be established and maintained) but the process required is particularly laborious (C. Lawrence, personal communication).

Fish tuberculosis (or mycobacteriosis)

Background

- This bacterium is frequently present in aquaria but by keeping a clean, well-watered system and the fish healthy, this infection should not pose a problem (Vargesson 2007). There is a high risk of infection between fish (Vargesson 2007). In addition, there is some evidence that fish tuberculosis can be spread to humans so, if dealing with infected fish, gloves must be worn to avoid any chance of cross-contamination (Vargesson 2006). Several mycobacterium species have been implicated, including *M. chelonae*, *M. peregrinum*, *M. marinum* and *M. haemophilum*. Observations from outbreaks and experimental transmission studies indicate that the latter two are of the most concern, while *M. chelonae* usually causes opportunistic infection (Kent 2007).

Clinical signs

- Fish may look unwell e.g. they may have open sores, be lethargic, have raised scales or appear emaciated (Vargesson 2007).

Proposals for prevention

- Some level of disease control can be obtained by removing sick fish, by routinely sterilising tanks and all equipment that comes into contact with the fish or the tank water, and by reducing stress caused by moving fish between tanks or by changes in temperature, water flow, or feeding regimen (Westerfield 2006).
- UV lamps can be incorporated into the circulation system, which kills 99% of all *Mycobacterium tuberculosis* when delivered at a dose of at least 10 000 W/s/cm^2 (Brand et al 2002).

Recommendations for treatment

- There is currently no known successful treatment for this disease (Vargesson 2007).

Velvet disease

Background

- Zebrafish are highly susceptible to the very contagious 'velvet disease' caused by *Oodinium pillularis*, a parasitic dinoflagellate alga. This oval-shaped parasite attaches to the fish near the fins, especially the dorsal fin, and around the gills (Westerfield 2006).

Clinical signs

- Rubbing behaviour, lethargy, fins (particularly the dorsal fin) held close to the body, parasites near fins and gills.

Proposals for prevention/treatment

- This disease can be cured with minimal damage to the fish using a 3-day treatment of Atabrine (Quinacrine hydrochloride). The following treatment has been taken from Westerfield (2006):

  Day 1
  Turn off incoming water.
  Slowly drip 2 litres of sea salts into an infected 10-gallon (38 litre) tank.
  Add 3.3 ml of the Atabrine stock solution
  Day 2
  Add 3.3 ml Atabrine stock.
  Day 3
  Add another 3.3 ml Atabrine stock for a total of 9.9 ml.
  At the end of the 3-day period, clean the bottom of the tank thoroughly and slowly dilute out the salt and the Atabrine with fresh water.
  Continue cleaning the bottom of the tank daily for several days.
  Solutions:
  Atabrine Stock: 10 mg/ml dH2O. Store in light tight bottle.
  Salt Stock: 20 tablespoons (280 g) Instant Ocean Sea Salts (Aquarium Systems, Inc.) dissolved in 2 litres of distilled water.

Some other factors relevant to fish welfare and its assessment

14.3 Alarm behaviors

When zebrafish become aware of an actual or perceived threat, behaviors displayed may include: shoal cohesion; either agitated swimming or freezing on the substrate; decrease in feeding rate; increase in aggression (Spence et al 2008). Regular occurrence of such behaviors may indicate a chronic welfare problem.

14.4 Responses to acute noxious stimuli

Signs of pain or distress in zebrafish may include: escape behavior; frantic movements; significant reduction in activity; increased respiration (rapid movement of opercula); and blanching of color (Matthews et al 2002, Reilly et al 2008).

A good understanding of zebrafish biology and behavior, including diseases, clinical signs and treatments, is necessary to minimize suffering or death. Zebrafish should be regularly monitored for signs of ill health.

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1 e.g. 40 fish/L versus 0.25 fish/L.

2 though this effect was not seen in fish that had recently been fed.

3 in this case, significant reductions in mean egg production were observed in fish when the volume of water supplied for 2 males and 4 females was reduced to 200ml or 100 ml.

4 the specific figure slightly varies depending on the temperature at which the groups of fish had previously been acclimated.

5 though anecdotal reports suggest breeding can appear unaffected at temperatures down to 24°C.

6 This can be achieved by exposure to air (for at least 24 hours) in standing tubs or by running the water through a carbon filter.

7 The introduction of any enrichment items should be carefully assessed, taking into consideration the potential for trapping fish, the method and frequency of cleaning introduced objects, the potential of chemicals leaching into the water, and the ability of care staff to view and check the health of the fish.

8 which also contains information relating to production and use before dates.

9 e.g. the Irradiated Adult Zebrafish Diet from Harlan.

10 Refuges are also used by males to avoid aggressive encounters with other males.

15. Embryo/Larvae Research

15.1 Stages and definitions

The Zebrafish lifecycle is defined as follow:

≤72 hours (3 days) post fertilization – embryos
4-5 days post fertilization – larvae (IACUC review is not required)
6-29 days post fertilization – larvae (IACUC review is required)
30-89 days post fertilization – juveniles
>90 days post fertilization – adults

15.2 Policy and Rationale:

From the moment zebrafish become free-feeding larvae, any intervention on them must follow MOPH regulation on the humane use of animals. This means that any procedure done in Zebrafish from about 5 days after fertilization must comply with the MOPH regulations on the protection of animals used for scientific purposes. Zebrafish embryos that are manipulated before the 5 days following fertilization limit and are euthanized before that time are not regulated by the IACUC.

Zebrafish embryos survive on nutrients from the yolk sack from days one through five. Although this may vary, depending on such factors as temperature, It has been decided that, in a laboratory situation, temperature variability would be minimized and would approach ideal conditions that allow the larvae to develop to a free feeding state by 5 days. Additionally, the only practical way to apply policy was to select a period of time after fertilization from which larval numbers could be realistically counted, or at least estimated. Based on these considerations, a tie point of 5 days for viability of zebrafish has been established.

Consequently, the Institutional-IACUC must evaluate all experiments on zebrafish that can develop greater than 5 days post fertilization. Any experiments performed on zebrafish larvae that are older than 5 days must be described in an IACUC approved animal use protocol and the number used should also be reported in the protocol.

16. Euthanasia Guidelines

Recent observations indicate that zebrafish up to at least 15 dpf can survive anesthetic overdose and rapid chilling even after prolonged absence of heartbeat. They can revive if returned to water that is within their normal environmental parameters. An adjunct method such as sodium hypochlorite treatment should be used to ensure death in embryos <15 dpf.

Similarly, embryos less than 3 dpf that are being disposed should be treated with sodium hypochlorite to prevent further development.

Euthanasia of zebrafish must be carried out by the following methods.

1. For zebrafish ≥15 dpf the following methods are acceptable for euthanasia:

• Immobilization by submersion in ice water (5 parts ice/1 part water, 0-4º C) for at least 10 minutes following cessation of opercular (i.e., gill) movement. In any fish where it is difficult to visualize opercular movement, fish should be left in the ice water for at least 20 minutes after cessation of all movement to ensure death by hypoxia.

• Overdose of tricaine methane sulfonate (MS222, 200-300 mg/l) by prolonged immersion. Fish should be left in the solution for at least 10 minutes following cessation of opercular movement. MS-222 solution should be buffered with sodium bicarbonate to a neutral pH before immersing fish. Non-buffered MS-222 is acidic and causes an aversive reaction in unanesthetized fish.

• Anesthesia with tricaine methane sulfonate (MS222, 168 mg/l) followed by rapid freezing in liquid nitrogen.

2. For zebrafish larvae up to 8-15 dpf: a secondary method must be used to ensure death. Use of the ice water or MS-222 method as above should be used as a method of anesthesia/immobilization. An acceptable secondary method is the addition of bleach solution (sodium hypochlorite 6.15%) to the culture system water at 1 part bleach to 5 parts water. The larvae should remain in this solution at least five minutes prior to disposal to ensure death.

3. For embryos ≤ 7 dpf, development should be terminated using bleach as described above. Pain perception has not developed at these earlier stages so this is not considered a painful procedure.

4. Additional methods can be used if approved by the IC Institutional ACUC committee:

• Clove Oil (Eugenol, Isoeugenol) as an alternative to MS-222. AVMA Guidelines recommend that products with standardized, known concentrations of essential oils (eugenol, isoeugenol) be used so that accurate dosing can occur. Clove oil and eugenol products are described in the AVMA Guidelines as “acceptable agents of euthanasia for finfish.” They are not available in an FDA approved form but there is at least one commercial form available in the U.S. (Aqui-S) as an Investigational New Animal Drug.

• Decapitation with a sharp blade by a trained individual.

• Anesthetic overdose or rapid chilling by submersion in ice water followed by fixation in paraformaldehyde or other fixative

• For embryos <8 dpf: immersion in paraformaldehyde or other fixative.

• For embryos <8 dpf: rapid freezing in -70 freezer. Embryos should be contained in a minimum amount of water to ensure rapid freezing and death.

• Maceration using a well-maintained macerator designed for the size of the fish being euthanized.

These methods ensure death provided the timeframes above are followed. The ice water method should not be extrapolated to other aquatic species without first confirming the effectiveness for that species. Aquatic species, native to a colder environment than zebrafish, may be more resistant to hypothermic shock and may recover subsequently.

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