Zebrafish

History

The zebrafish (*Danio rerio*) is a tropical freshwater fish belonging to the minnow family (*Cyprinidae*) of order *Cypriniformes*. Native to the Himalayan region, it is a popular aquarium fish, frequently sold under the trade name zebra danio. The zebrafish is also an important vertebrate model organism in scientific research. It is particularly notable for its regenerative abilities, and has been modified by researchers to produce several transgenic strains. *Danio rerio* (formerly *Brachydanio rerio*) is one of approximately 45 *Danio* species worldwide. They are part of the Cyprinidae family that includes carp and minnows. Understanding the behavior and biology of experimental animals is crucial to improving both animal welfare and the quality of scientific research. Relatively little is known about the natural behavior or biology of zebrafish and few studies have been conducted on wild populations. However, some of the work that has been undertaken suggests the conditions under which zebrafish are often kept in laboratories conflict with their natural preferences.

Appearance

The zebrafish takes its name from the stripes on the side of the body, which all extend to the anal fin and onto the caudal fin rays of the tail. Five alternating blue-black stripes contain two types of pigment cells, melanophores and iridiophores, and silvery-yellow stripes contain xanthophores and iridophore.

The onset of pigmentation in normal strains occurs shortly after 24-hours post-fertilization. Pigment formation can be suppressed by incubating the embryo/larvae in 1-phenyl-2-thiourea (PTU). This will allow for prolonged visualization of the internal organs.

Zebrafish adapt their pigmentation levels to blend in with the background as a camouflage response. Blind 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. Like all minnows, zebrafish have a single dorsal fin and no adipose fin.

Manipulation of the breeding of individuals of this species has produced other varieties including the long fin, golden and albino strains. The golden or albino strains are hypo pigmented which means stains such as vital dyes, fluorescent tracers, antibodies and riboprobes are more visible.
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).

When alive, though similar in size and coloration, the sexes can be fairly reliably distinguished 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 color than females, and have more yellow coloration in the anal fin. Zebrafish are usually less than 5cm in length

**Use of zebrafish in research and testing**

**The zebrafish as a ‘model’ - past and present**

The use of zebrafish in research began to increase as the field of molecular biology progressed during the 1960s. However, the real expansion in their popularity as a mainstream genetic model occurred after George Streisinger and colleagues at the University of Oregon established the methods critical for allowing the eventual genetic manipulation of the species.

Their use burgeoned steadily through the 1980s, and boomed post-1996 after genetic screens identifying over 4000 mutations were completed and published in the journal *Development*. The zebrafish genome sequence is now publicly available.

As the biology of the zebrafish becomes better understood, an increasing number of advantages and applications are claimed for their use in research. Some have stated zebrafish to be 'the ideal organism' for studying the function of human genes, whilst the National Institutes for Health (USA) has ranked the zebrafish as the third most important experimental organism.

Today, zebrafish are mainly used in molecular biology, developmental biology, and neurobiology and genetics research. They have also recently been brought into fields of study such as cancer research, nervous system physiology and drug discovery. One reason for this is probably that maintenance costs of zebrafish are less than 1/1000th of the cost of mice.

As new research applications emerge, the number of zebrafish facilities worldwide continues to grow. There are now estimated to be around 5,000 researchers working with zebrafish in around 450 laboratories worldwide. The majority of these laboratories are university-based. There also appears to be a trend for establishments to move towards large-scale use of zebrafish, with facilities often holding tens of thousands of zebrafish across hundreds or thousands of tanks.

**Lighting**

Appropriate lighting facilitates good breeding success and minimizes stress.

**Photoperiod**
Light triggers zebrafish to breed, so periods of darkness are important for allowing animals to rest. 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. However, they appear able to recover from the effects of early rearing in abnormal lighting if they are subsequently housed under normal cyclic conditions. Being kept in constant darkness delays general development of embryos, with hatching still not being observed by 7 days post-fertilization.

A cycle of 14 hours light, 10 hours dark has been advised, and would appear to be common practice. A brightening and dimming period can also be arranged to avoid startling the fish, rather than switching lights abruptly on and off.

**Spectrum**

Adult zebrafish appear to have the necessary mechanisms for color vision, but no specific requirements with regard to 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. Elimination of those light wavelengths proven to encourage algal growth within the tank should be considered as this can help with tank hygiene.

**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 particular regime can confidently be considered most beneficial or best practice.

**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. The sensitivity of these fish to sounds like talking or music is uncertain.

**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.

**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.

25 adult zebrafish per 45 liters tank is the most recommended housing or tanks with 60cmx30cmx30cm are to be used.

**Quantity and temperature**

**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.

It has been recommended that as long as tanks have relatively large surface area’ water depth does not have to exceed 25cm. 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. 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.

**Volume and population density**

Keeping zebrafish in ‘crowded’ conditions is detrimental to their welfare. Adults kept at high densities have been observed to show a four-fold increase in whole body cortisol levels and reduced egg production. Development is also affected, with zebrafish maintained at higher densities growing slower than those maintained at lower densities.

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

**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. This is confirmed by studies in the laboratory that have shown that wild-type zebrafish have a maximal thermal tolerance range13 of 6.2°C - 41.7°C.
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. 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.

**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. Though some generally useful principles exist, ideal parameters are neither broadly agreed nor defined. 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 bio filter is healthy.

**PH level**

Systematic studies detailing growth and reproductive performance of zebrafish at different levels of pH have not been conducted. However, field studies have observed zebrafish to be present in waters between 5.9 and 8.1. Most laboratory facilities aim for maintaining pH between 7.0 and 8.0 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).

**General hardness and other water quality parameters**

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

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.
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.

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.

**Cleaning**

The cleanliness of the aquaria and filters is a very important factor in keeping fish in healthy breeding conditions. Zebrafish constantly excrete ammonia (across the gills and to a lesser extent in faces) 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 is 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.

**Tank housing**

**Tank material**

Tanks used to hold zebrafish are usually made of polycarbonate, high-quality glass or acrylic. 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.

**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. 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.

**Lids and drain covers**

Zebrafish can jump 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. 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. Tank drains should be covered to prevent the fish escaping the tank.

**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. Indeed, the most important social interactions occur during shoaling and spawning.

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. 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. Displays by territorial males are usually brief and serve only to deter others from approaching the spawning site.

Zebrafish kept together for breeding should have some means of escape from more aggressive fish. Providing extra space will help, but if the tank contains plant-like materials or structures 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 on a daily basis and that social grouping influenced egg production.

**Catching and handling**

The majority of 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. 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. 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.

**Food type and feeding regime**

**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. 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.

**Feeding requirements of zebrafish**

A quality diet specifically developed for zebrafish should be used. Some commercial feeds claim to offer a nutritionally complete food. However, the precise nutritional requirements of zebrafish have yet to be determined.

**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. Poor water quality will increase the chances of disease, and along with overfeeding (causing fish to become fat) can reduce breeding performance.

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

**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. 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.

Although there is still debate over the extent to which zebrafish benefit from environmental enrichment, and what form it should take.

**Environmental complexity**

It has been suggested that zebrafish appear indifferent to environmental enrichment. 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
- zebrafish prefer to spawn in sites associated with aquatic vegetation

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.

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

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.

**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. Fish should be observed at least daily for indicators of poor health. Sick fish should be removed from the tank as quickly as possible and veterinary advice sought.

**Analgesia and anesthesia**

As with other species, zebrafish require appropriate anesthesia when used in potentially painful procedures. Anesthesia is also needed in most instances for handling these animals in order to reduce stress and minimize the risk of damage due to escape behaviors.

**Analgesia**

No literature which made suggestions for provision of analgesia for zebrafish subjected to invasive and potentially painful procedures (such as fin clipping). Little work has been carried out to determine the safety and efficacy of potential analgesics, routes of administration and dose rates. To date, just a few analgesics, such as morphine and ketoprofen have been evaluated - but only in fish species other than zebrafish.

**Anesthesia**

The most common anesthetic agent currently used is probably tricaine methanesulphonate (e.g. MS222) in an aqueous solution. Anesthesia is induced rapidly following immersion in a buffered solution containing MS222 at 100-200 mg/L. A concentration of MS 222 at 168mg/L has been used at the University of Oregon (2001). Where maintenance anesthesia is required (which is rare) the dose is lower (50-100 mg/L).

Volumes for anesthesia should be carefully calculated and the solution should be made up freshly on each occasion. Where several fish are anaesthetized serially in the same baths, handlers should ensure adequate oxygenation of the water.

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

For surgery, fish are usually kept on a moist cloth. Other than for brief anesthesia, care should be taken to irrigate the gills with aerated water containing the anesthetic. Recovery should occur within around 10 minutes of a return to clean, well-aerated water.

**Considerations**
Since fish are ectoderms, 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.

Although MS222 is commonly used for inducing anesthesia in fish, concerns have recently been raised that it may be aversive to at least some species, and may in fact be acting as a neuromuscular blocking agent rather than an anesthetic.

**Humane killing**

If animals are to be killed, it should be done with the minimum of pain, fear and distress. Killing has the potential to cause substantial pain and distress if it is done incompetently or using an unsuitable method. Staff carrying out humane killing should therefore be appropriately trained and competent in the approved method deemed to cause the minimum stress or pain to the animal.

When deciding upon the method of humane killing, the following points should be considered:

- does the method require the handling of fish (a stressor in itself) or can they be euthanized in the home tank?
- is the method aversive - if so, how can this be minimized?
- Does loss of consciousness ensues rapidly?
- Following loss of consciousness, does death occur rapidly?
- Does induction of unconsciousness occurs without causing pain?
- Is the method reliable and does it ensure that the animal does not regain consciousness?
- Is the method simple to carry out, with little room for error?

The most appropriate method should be determined on a case by case basis, giving animal welfare high priority.

**Euthanasia**

Euthanasia of zebrafish must be carried out by one of the following methods:

1. For zebrafish ≥8dpf the following methods are acceptable for euthanasia:
   - Immobilization by submersion in ice water (5 parts ice/1 part water, 0-4° 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.
• Anesthesia with tricaine methane sulfonate (MS222, 168 mg/l) followed by rapid freezing in liquid nitrogen.

2. For zebrafish 4-7 days post-fertilization (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 20 minutes to ensure death by hypoxia.
• Addition of bleach solution (sodium hypochlorite 6.15%) to the culture system water at 1 part bleach to 5 parts water. They should remain in this solution at least five minutes prior to disposal to ensure death. Pain perception has not developed at these earlier stages so this is not considered a painful procedure.

3. Recent observations have indicated that zebrafish larvae up to at least 15 dpf can survive anesthetic overdose and rapid chilling. Despite prolonged absence of heartbeat they can revive once returned to water that is within their normal environmental parameters. When these methods are used on larvae up to 15 dpf, an adjunct method such as adding sodium hypochlorite as described in B2 above should be used after the heart beat has stopped in order to ensure death.

4. For embryos ≤ 3dpf, development should be terminated using bleach as described in section 2 above.

5. Additional methods can be used if required by experimental design and approved by the IC Institutional ACUC committee:
• Decapitation with a sharp blade by a trained individual.
• Anesthetic overdose or rapid chilling followed by fixation 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 insure rapid freezing and death.
• Maceration using a well maintained macerator designed for the size of the fish being euthanized.

Zebrafish carcasses from any of these methods should be disposed of as Medical Pathological Waste according to NIH policies. 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.

Zebrafish advantages
As vertebrates, zebrafish have more in common with humans than either flies or worms, other lab animals heralded for being cheap and easy to work with. They have hearts divided into chambers, a retinal structure similar to humans, and a liver, pancreas, kidneys and intestines.

Unlike humans, their eggs develop outside the mother's body and are translucent, allowing easy study of all aspects of development.

Zebrafish also hold advantages over species closer to humans - such as rodents - for other reasons. They are tiny, easy to care for, and breed rapidly. To researchers, they are a model species.

In science, lab animals, known as model species, perform the critical function of allowing researchers to understand basic processes common to all species.

A native of the tropical freshwaters of India and Myanmar, zebrafish had long been popular in aquarium displays. Then in the 1970s, a University of Oregon scientist discovered the fish were excellent for studying development and genetics.

Zebrafish, it seemed, just might be the next lab rat.

In the subsequent decades, the fish gained popularity, chiefly among developmental scientists.

In recent years, technological advances as well as a growing acceptance have led scientists in other fields, like Baraban, to consider the humble zebrafish.