Guidance on the housing and care of Zebrafish (Danio rerio)
Notes regarding this Reference Resource:

The Council identified four points requiring clarification with this Reference Resource:

Environmental Enrichment:

The document states on p. 37, "Providing artificial plants or 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."

While AAALAC International acknowledges the use of environmental enrichment may be beneficial to the zebrafish, its implementation should take into consideration the scientific goals of the study for which the animals are used. Performance standards should be applied taking into consideration the health, welfare and species-typical behavior.

Temperature:

The document states on p. 24, "A water temperature of 28.5° C is widely cited as the optimum temperature for breeding zebrafish. There has however, been little research to investigate the full implications of constantly keeping fish at this very specific temperature."

While the document recommends a very specific temperature of 28.5° C as the optimum for breeding zebrafish, it provides a number of references with a range of 25-29° C and acknowledges that more research is needed to determine temperature preference and implication of maintaining fish at warmer temperatures for an extended period of time. Performance standards should be applied with consideration of health, welfare and species-typical behavior.

Ice Cold Water:

The document states on p. 51, "There is also debate relating to whether submersion in ice water can be considered humane (Robb & Kestin 2002). Some advised against its use (e.g., AVMA 2007), but recent studies appear to suggest that inducing hypothermal shock in adult zebrafish by placing them in ice slush (4° C or less) may actually be more humane than using MS222 (Wilson et al 2009)."

AAALAC International acknowledges that the document refers to the 2007 AVMA Guidelines on Euthanasia of Animals; however, it is superseded by the 2013 AVMA Guidelines which determined that rapid chilling by immersion in 2° to 4°C (36° to 39°F) water is acceptable for zebrafish euthanasia.

MS222:

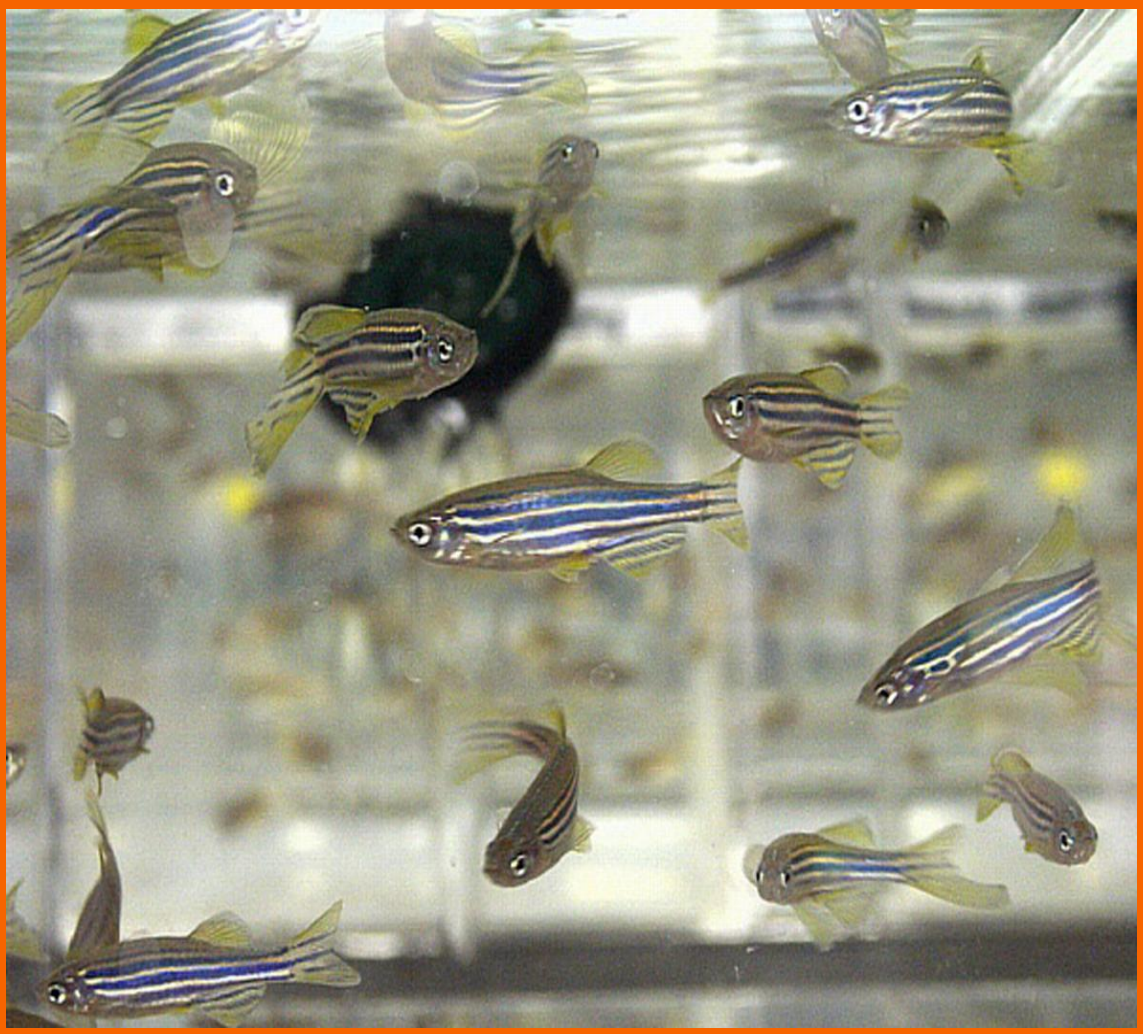
The document states on p. 49, "Although MS222 is commonly used for inducing anesthesia in fish, concerns have recently been raised that it may be aversive to at least some species, and may in fact be acting as a neuromuscular blocking agent rather than an anesthetic. Research work is ongoing to assess the humaneness of MS 222 and so researchers should keep themselves informed of the latest findings."

While the document recommends ongoing research into the humaneness of tricaine methanesulphonate (MS222), AAALAC International acknowledges that MS222 has been approved by government regulatory agencies to anesthetize and tranquilize fish and other ectothermic animals.

(Reference Resource begins next page...)

Guidance on the housing and care of Zebrafish

Danio rerio



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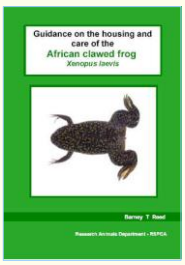
Note:

The views expressed in this document are those of the authors and do not necessarily represent those of the persons named above or their affiliated organisations.

Other resources in this series

- Guidance on the housing and care of the African clawed frog, *Xenopus laevis*

  This resource can be downloaded at: www.rspca.org.uk/xenopus



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About the RSPCA

The RSPCA adopts a constructive, practical approach to the use of animals in experiments, judging every issue individually, critically questioning the necessity and justification for animal use and striving to reduce the conflict between the interests of animals and science as far as possible. Our ultimate aim is the replacement of animal experiments with humane alternatives. Until this can be achieved, we work to help ensure that the minimum numbers of animals are used and that they experience the minimum suffering and have the best possible quality of life. This resource is part of the Society's work on refinement.

CONTENTS page

1 Introduction

The refinement of all aspects of the husbandry, care and use of laboratory animals is important for legal, ethical, scientific and animal welfare reasons. The last two are inextricably linked, since it is increasingly accepted that good animal welfare is essential to produce good scientific results (OIE 2010, Nuffield Council on Bioethics 2005).

Specific husbandry requirements for zebrafish are still poorly understood (Wilson 2009). Protocols for feeding, grouping, and breeding these animals, plus environmental factors such as water quality, can vary from laboratory to laboratory. There has historically been little investigation into the natural ecology of the zebrafish or its environmental preferences, and there is a possibility that some may be being housed under sub-optimal conditions. Efforts to better define ideal standards relating to the husbandry, care and use of zebrafish are only now beginning (Obenschain & Aldrich 2007).

As zebrafish use increases, there is a desire for a fuller understanding of the behaviours and requirements of these animals and a clear need to identify factors that may affect their welfare.

Aims of the report

This report aims to improve the welfare of zebrafish by:

- facilitating understanding of zebrafish behaviour and thus a better appreciation of their requirements;
- highlighting current potential welfare and ethical concerns relating to the breeding, supply, housing and care of zebrafish;
- arriving, where possible, at consensus based on available evidence and sound scientific argument for appropriate environmental and care conditions for keeping zebrafish in the laboratory environment;
- providing recommendations for improving health, welfare and egg quality, for reducing the potential for stress and suffering, and for reducing the number of animals used;
- in areas where current knowledge is sparse or inconclusive, stimulating discussion and research to identify 'good practice'.

Where relevant, a number of the more common experimental procedures involving zebrafish will be discussed, although experimental techniques associated with research involving zebrafish eggs and embryos are not addressed, as they are covered comprehensively elsewhere (e.g. Westerfield 2000, Nüsslein-Volhard & Dahm 2002).

This report is based on information obtained from:

- relevant legislation and guidelines from the UK, EU and elsewhere;
- the scientific literature;
- observations from visits to research establishments;
- discussions with animal research regulators, animal users, veterinarians and animal technologists and care staff.

2 Background information on zebrafish

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 (Mann et al 2003, Spence et al 2006a, Engeszer et al 2007). 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 (Delaney et al 2002). The following section therefore includes details of the anatomy, physiology and behaviour of zebrafish that need to be taken into consideration when designing and implementing laboratory housing and husbandry regimes.

Natural geographic range and habitat

The exact current natural geographic range of the zebrafish is still far from clear. Engeszer et al (2007) state that data from original collections of these fish suggests a range extending from Pakistan in the west to Myanmar (Burma) in the east, and from Nepal in the north to the Indian state of Karnataka in the south. However, they caution that given the historical nature of many of these records, along with the possibility that specimens may have been misidentified (particularly with regard to their presence in the most extremes of this distribution) this suggested range may not be an entirely accurate reflection of their true present day distribution.

They can typically be found in standing or slow-moving bodies of water, such as pools, ponds, lakes, ditches or rice paddies and would appear to be a floodplain rather than a true riverine species (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).

***Figure 1* -**
The natural habitat of the zebrafish

Photo credit:
R. Spence
University of St Andrews

Species characteristics

Taxonomy

- *Danio rerio* (formerly *Brachydanio rerio*) is one of approximately 45 *Danio* species worldwide (Fang 2003).
- They are part of the Cyprinidae family that includes carp and minnows.

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 iridophores (Schilling 2002).
- The onset of pigmentation in normal strains occurs shortly after 24-hours post-fertilisation. Pigment formation can be suppressed by incubating the embryo/larvae in 1-phenyl-2-thiourea (PTU). This will allow for prolonged visualisation 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 (Goldsmith & Solari 2003).
- Like all minnows, zebrafish have a single dorsal fin and no adipose fin (Schilling 2002).
- 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 hypopigmented which means stains such as vital dyes, fluorescent tracers, antibodies and riboprobes are more visible (Whitfield 2002).
- 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).
- 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 colour than females, and have more yellow coloration in the anal fin (Ruhl et al 2009, Schilling 2002).
- Zebrafish are usually less than 5cm in length (De Tolla et al 1995).

Activity

- They 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 2005).
- When zebrafish become aware of an actual or perceived threat, behaviours displayed may include: shoal cohesion; either agitated swimming or freezing on the substrate; decrease in feeding rate; increase in aggression (Spence et al 2008).

Life-span

- In the laboratory, zebrafish have a maximal recorded life-span of 5½ years, though an average of 3½ years has been reported (Gerhard et al 2002).
- In laboratories, these animals are routinely only kept for 18 months to two years, after which they are considered to be of lower reproductive value.
- In the wild, there is little evidence that individuals survive more than a year or two. This may be due to predation or parasites (Spence 2007).
- Spinal curvature has been observed in both domesticated and wild type zebrafish kept in the laboratory after their second year in captivity. This is not seen in wild populations as it is likely that fish die before the condition develops (R. Spence, personal communication).

Senses (general)

- Zebrafish possess all of the classes of senses: taste, touch, smell, balance, vision and

hearing (Moorman 2001).

- 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 recognisable two days after fertilisation. 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 movements of, and vibrations within, the water and helps guide behaviours such as shoaling, prey capture, and predator and obstacle avoidance (Whitfield 2002, Moorman 2001).

Hearing

- They do not possess outer or middle ears but have a fairly typical vertebrate inner ear which, together with visual cues, is used to maintain balance (Whitfield 2002).
- They possess four small bones (the Weberian ossicles) linking the swim bladder to the inner ear which enhance hearing. This is a characteristic of Ostariophysan fish - which are also known as 'hearing specialists'. These fish may be sensitive to a frequency range between 100Hz and 5000Hz (Nelson 1994, Bang et al 2001, Fay and Simmonds 1999 in Whitfield 2002)[1].

Olfaction

- Zebrafish can respond to external chemical cues within 24 hours of hatching - just four days after fertilisation (Lindsay & Vogt 2004).
- Zebrafish use olfactory cues to distinguish between kin and non-kin (Mann et al 2003). They show a preference to associate with kin during the larval and early juvenile stage, but this changes to avoidance (and preference for non-kin) once they reach sexual maturity (Gerlach and Lysiak 2006).

Vision

- The zebrafish visual system appears to be similar to other vertebrates (Bilotta & Saszik 2001).
- Visual behaviour is displayed very early and visual acuity appears to improve with age (Easter and Nicola 1996 in Bilotta & Saszik 2001, Bilotta 2000).
- Behavioural experiments have revealed that zebrafish first see changes in light intensity at approximately 68 hours after fertilisation. By 72 hours, the eye is believed to be emmetropic (able to adjust itself well for all distances) and able to transmit both visible and ultraviolet wavelengths, since the adult is ultimately responsive to ultraviolet wavelengths. They can also make eye movements that track the stripes on a rotating drum, thus providing evidence for pattern vision. This response improves over the next day to achieve adult levels of performance at just 96 hours after fertilisation (Hughes et al 1998, Moorman 2001).
- The retina is duplex, consisting 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 photopigments, including an ultraviolet photopigment with a peak sensitivity of 362nm. The peak sensitivities of the remaining cone types in the zebrafish are 415, 480 and 570 nm (Bilotta 2000).

Shoaling behaviour

- In the wild these fish have been observed in small shoals of 2-30 individuals (Spence et al, unpublished).
- It has been proposed that stripes are a shoaling cue in *Danio* fishes (Rosenthal & Ryan 2005).
- Pattern preference is learned rather than innate, with individuals preferring to

[1] NB: the range of hearing in healthy young humans is said to be between about 20Hz and 20,000Hz (Cutnell and Johnson 1998).

associate with shoals of the colour pattern with which they have been raised (Spence & Smith 2007).

Diet and feeding

- The zebrafish is omnivorous. Its natural diet consists primarily of zooplankton and insects, although phytoplankton, filamentous algae and vascular plant material, spores and invertebrate eggs, fish scales, arachnids, detritus, sand and mud have also been reported from gut content analysis (Spence et al 2008).
- Whilst it is thought that these fish essentially feed within the water column, it is also suggested that they feed at the surface and from the substrate (Spence et al 2008).
- Larvae are capable of independent feeding by 5 days - this is necessary as yolk supplies are largely depleted by the end of the first week (Vargesson 2007, Lindsay & Vogt 2004, Jones et al 2008).
- Zebrafish lack teeth in the jaw, and instead they have pharyngeal 'jaws', with tooth rows that grind food in the back of the throat. These teeth are usually fused to a modified pharyngeal bone of the most posterior gill arch (Schilling 2002).

Breeding

- 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 will preferentially use this (Spence et al 2006a).
- A female generally produces around 100 transparent eggs in a single spawning (though this number can range between a just few eggs to over 1000). There is no parental care of the offspring post-laying.
- Eggs have a diameter of about 1.0 - 1.5 mm (Matthews et al 2002).
- Unlike many other fish species, zebrafish do not require a seasonal change in their 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[2] to two or three days, and all mature ova being released during a single hour (Matthews et al 2002, Spence et al 2006a).
- Some male zebrafish are territorial during mating and a single male may aggressively attempt to control rivals' access to a spawning site and access to females (Spence & Smith 2005).
- Females are thought to be able to distinguish between the sexes based on body shape alone, and appear to show a preference for males with a larger body (Turnell et al 2003, Pyron 2003).
- However, male body size does not appear to be correlated to either dominance rank or the clutch size of eggs laid by females. Indeed, the female preference may be over-ridden as dominant males do not allow the females to access other males (Spence & Smith 2006).
- The mating behaviour of zebrafish seems to be 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 gonadal hormones in the water (Delaney et al 2002).
- Females spawn smaller clutches at high population densities (Spence et al 2006a).
- It is thought that when selecting mates, males may rely more on olfactory cues than visual cues (Turnell et al 2003).
- Courtship in zebrafish involves the male swimming quickly in close proximity to the female, often touching her flanks with his snout, circling tightly in front of her while

[2] Though a female who lays daily will not produce a large quantity or good quality of eggs.

attempting to lead her to a spawning site. Once over the spawning site, the male swims alongside the female, in close contact but slightly behind her, sometimes oscillating 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).

- 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).

Development

- The different stages of the zebrafish life cycle have been broadly established as follows (Fleming 2007):
  0-72 hours post-fertilisation - *Embryos*
  72 hours to 13 days post-fertilisation - *Early larvae*
  14 days to 29 days post-fertilisation - *Mid larvae*
  30 days to 3 or 4 months - *Juveniles*
  When sexually mature - *Adults*
- However, the rate an individual develops can be affected by both genetic and environmental factors. For this reason, others (such as Parichy et al 2009) have established indicators other than age, including size and various anatomical changes, to identify specific milestones in the stages of zebrafish development.
- After fertilisation, the basic body plan of the animal develops within 24 hours. This is equivalent to about 9 days in the mouse (Lardelli 2000).
- Newly hatched 'early' larvae (3 days post-fertilisation) are largely inactive and negatively buoyant, lying immobile on the bottom, although occasional tail flicks can be observed. On, or just before day 5 (usually related to water temperature), the larvae inflate their gas bladders by swimming up and gulping air at surface. After this point, they are neutrally buoyant and are capable of continuous swimming and maintaining their position within the water column.
- Swimming involves regular but discontinuous beating of the tail which provides the motive force for swimming and is characteristic of the method of swimming observed in these animals as they continue to grow and age (e.g. Lindsay & Vogt 2004).
- The period of metamorphosis from larvae, through juvenile, to adult includes events such as 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).
- After the first three months post-hatch, growth starts to decrease and approaches zero by about 18 months. It has been suggested that growth rates of domesticated strains in the laboratory are higher than that for wild fish (Spence et al 2007, 2008).
- Standard laboratory strains of zebrafish have been found to have a faster growth rate, more 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). This variation is presumably caused by adaptation to the laboratory environment and is consistent with the effects of domestication in other fish species such as salmonids (Robison, no date).

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 (The Wellcome Trust 2003). 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 (Streisinger et al 1981).

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* (Haffter et al 1996, Driever et al 1996). The zebrafish genome sequence is now publicly available[3].

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 (e.g. Nüsslein-Volhard 2000), whilst the National Institutes for Health (USA) has ranked the zebrafish as the third most important experimental organism (see Goldsmith & Solari 2003).

Today, zebrafish are mainly used in molecular biology, developmental biology, 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 (Goldsmith & Solari 2003).

As new research applications emerge, the number of zebrafish facilities worldwide continues to grow (Astrofsky et al 2002). There are now estimated to be around 5,000 researchers working with zebrafish in around 450 laboratories worldwide (Westerfield 2008). The majority of these laboratories are university-based (Marine Biotech 2005). 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.

The dramatic increase in the use of zebrafish for research purposes can be illustrated by a review of the number of scientific papers produced each year in the journals covered by the PubMed database - see *Figure 2*.

[3] See: i) www.ensembl.org/Danio_rerio/Info/Index and, ii) www.sanger.ac.uk/Projects/D_rerio

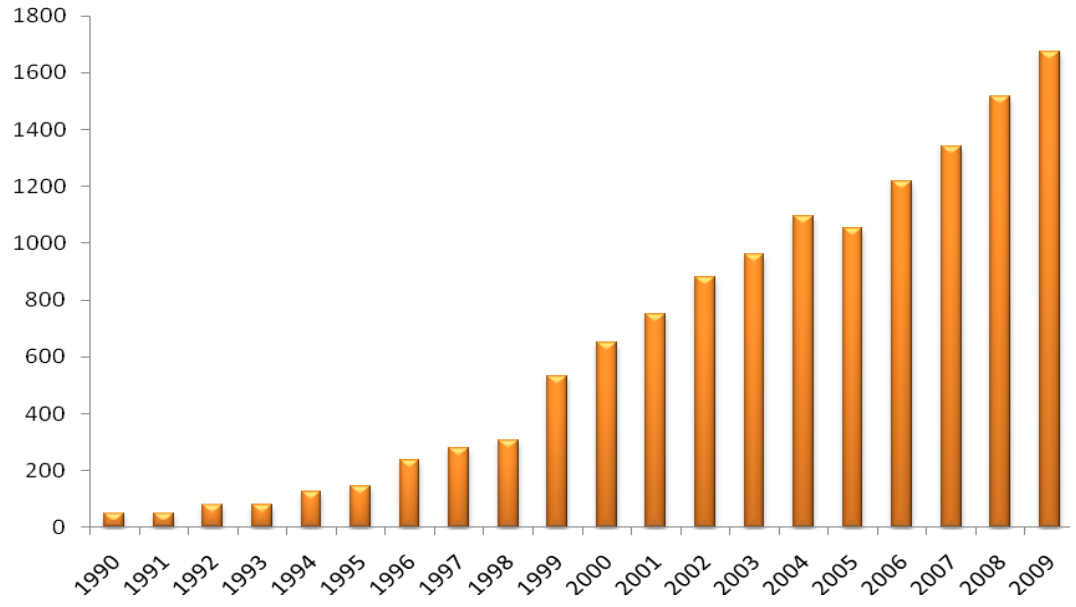

Figure 2:
The number of references found for each year of publication on the PubMed database (www.pubmed.gov) **of the US National Library of Medicine, using the keyword 'zebrafish'.**

Numbers of zebrafish used

It is impossible to gain an accurate global figure for the number of zebrafish used in scientific research and testing, but it is certain to be in the millions (or even possibly hundreds of millions depending upon the developmental stage at which these fish are considered to be 'animals').

In the **UK**, although it is recognised that the zebrafish is the main species of fish used, it is not possible to determine the exact number. Home Office figures simply show 397,464 'fish' were used during 2009 (Home Office 2010).

There are further obstacles to discovering the full extent of the numbers used or maintained in UK laboratories:

- The *Animals (Scientific Procedures) Act 1986* only regulates fish *from the time at which they become capable of independent feeding*[4]. This means that the majority of experiments on zebrafish, which involve embryonic and early larval (less than 6 days post-hatching) stages, would not be covered and reported.

[4] This is widely agreed to be at around 5 days post-fertilisation when the gut is open end to end, there is little or no yolk sac remaining, and ingestion is reported (Fleming 2007).

- Some practices do not require reporting in the annual Home Office statistics as they do not fall under the *Animals (Scientific Procedures) Act 1986.* This includes: the humane killing of zebrafish by an approved method in order to obtain tissues, organs, sperm or eggs; and the use of zebrafish in breeding programmes (unless they are genetically modified).

Consequently, current UK figures are likely to significantly underestimate the actual number housed in research and testing establishments, which is likely to be hundreds of thousands - the majority of which are used for breeding purposes.

The most recent figures for the **European Union**, produced for 2008 (EC 2010) from data provided by 27 member states, are also likely to be an underestimate of the total number kept in laboratories, for similar reasons. 1,087,155 'fish' are listed as having being used in 'scientific procedures with the potential to cause pain, suffering, distress or lasting harm'. It is likely that a significant number of these were zebrafish. In particular, zebrafish probably account for a large percentage of the 440,852 fish used in fundamental biological research.

Outside the EU, it is even harder to estimate the numbers of zebrafish used. In **Canada** for 2008, 499,445 'fish' were used in research (CCAC 2009) and in **New Zealand** for 2008, the figure was 41,057 (NAEAC 2009), but in both cases the species is not given. The **USA** official reporting does not even categorise the total number of 'fish' used[5], let alone the numbers of individual species (e.g. USDA 2008).

[5] No poikilothermic vertebrates are included in the USA's Animal Welfare Act.

3 Supply and transport

Factors associated with the sourcing of animals can affect both zebrafish health and welfare and the quality of scientific data.

Source

For reasons of animal health, welfare and quality of science, it is better that animals are obtained from captive colonies bred specifically for research purposes. For example, the stress of collecting, handling and transporting fish from the wild can make them very susceptible to disease (Fabacher & Little 2000). There is also anecdotal evidence that wild fish can be much more nervous in the captive environment (and more disposed to jumping), and that populations of wild zebrafish are declining - at least in Bangladesh (C. Smith, personal communication)[6].

Legislation is now beginning to reflect these concerns. For example, the new European Directive 2010/63/EU on the protection of animals used for scientific purposes[7] requires that zebrafish used in research be purpose bred.

As zebrafish can be bred and reared easily under laboratory conditions many establishments already routinely breed their own. In addition, a large range of specific zebrafish mutants, plus wild type lines, can be obtained from commercial suppliers (fish hatcheries), other laboratories, or stock centres (Matthews et al 2002). Even a modest facility can generate millions of embryos per year (Goldsmith & Solari 2003) and many people or places will provide fertilised eggs of strains they hold, free of charge or for a nominal fee[8].

Purpose bred animals should be used in preference to animals collected from the wild - a researcher should be able to provide compelling scientific justification for the need to use wild-caught individuals.

Establishments using zebrafish are encouraged to build up liaisons with others to facilitate the sharing of strains.

[6] Possible reasons include a consequence of the loss of aquatic habitats to agriculture and flood control, the widespread use of pesticides, and the release of non-native fishes from aquaculture.
[7] See: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:EN:PDF (Article 10)
[8] e.g. see the Zebrafish International Resource Centre (ZIRC) www.zebrafish.org

Transport considerations

Animals bred off-site and transported to the place of use may have to endure a long journey, often to another country or continent; zebrafish are regularly imported into Europe from North America or Asia. Long journeys have the potential to cause health and welfare problems for animals, for example, the stress of transportation can adversely affect the immune system (De Tolla et al 1995). For this reason, suppliers usually implement a variety of measures to ensure the health of the zebrafish in transit (see below). When all steps are taken to safeguard welfare, long-distance flights should not pose serious risks to zebrafish, and in most instances animals seem to travel well and appear healthy on arrival.

The factors to consider are the same as for other species. Relevant codes of practice such as the European Convention on the Protection of Animals during International Transport (1968: CETS 65) should be adhered to, together with the recommendations of the International Air Transport Association and the Animal Air Transport Association. More information on general principles for the transport of laboratory animals can be found in the report of the LASA Transport Working Group (2005).

To avoid the potential risks to health and welfare associated with transport, zebrafish should be bred at the establishment where they will be used.

Where this is not possible, the transport of eggs or early larvae (which will arrive at the receiving establishment before they reach 7-days post-hatch) is recommended in preference to fish.

Packing and insulation

When transporting zebrafish, the potential to cause **injury** or **stress** must be minimised. 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 to a half gallon (1.9 litres) of water.
- The bag should be about two-thirds full of air or oxygen.
- Food should be withheld for a day before shipping so that fish will produce less ammonia while confined.
- AmQuel® (a commercially available ammonia sequestrator) can be added to bind any ammonia that is produced.

Eggs and larvae

- At a density of no more than 1-2 per ml of sterile water (or preferably a sterile embryo media) bleached eggs are packed in a 250-500 ml tissue culture flask.
- The container should be filled with 50% water or embryo media (which allows air to form the remaining 50% of the space).
- Methylene blue (0.5mg/litre or 0.5 ppm) can also be added to the solution to reduce fungal growth.
- The packing box should be insulated and any extra space filled with packing chips.

Arrival

Sufficient preparation and communication between supplier and buyer regarding transport itinerary and conditions, breeding history of fish, dietary background and health status should take place before shipment. This should also occur at the establishment receiving the animals, to ensure that suitably trained and informed animal care staff are on hand to receive the shipment, and check the health and welfare of the animals upon arrival.

It is also important to know the water temperature and water quality conditions during transport, so that the fish can be acclimated to the water temperature and water quality conditions in the laboratory (Ostrander 2000).

Upon arrival, the unopened transport bag should be floated in water of the same temperature as the receiving water. Once the temperatures have equilibrated, the bag can be opened, the bag water poured through a net and the fish transferred to the receiving water.

Some have suggested opening the transport bag and gradually adding small amounts of the receiving water whilst it is floating in the receiving tank. However, this may potentially compromise welfare if ammonia levels in the transport bag are high[9], as chemical changes (ammonia becomes more toxic) in the water can occur immediately the bag is opened.

[9] as may happen where fish have travelled in a high density or for a prolonged period.

Information relating to transport itinerary and conditions, breeding history of fish, dietary background and health status should be communicated between supplier and receiver before shipment of fish takes place.

The health and welfare of the fish must be checked upon arrival by suitably trained and informed animal care staff.

Quarantine

The importance of quarantining newly arrived fish away from those already present in an establishment in order to reduce the opportunity for transmission of infection or disease is widely agreed upon. However, the period of quarantine is debated, with suggestions ranging from two 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 supply, their life history, and the housing system in use at the receiving establishment. Many establishments choose never to integrate brought in fish with existing colonies, but will treat the eggs of these animals and introduce these instead.

Matthews et al (2002) offer the following protocol:

- 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 (35mg/litre sodium hypochlorite for five minutes). Only these sanitised embryos will be introduced into the main aquarium facility.

Ideally, fish should come from a 'known health status' source.

The health and welfare of zebrafish must be checked on arrival by an appropriately trained and competent person (see 'Assessment of health and disease prevention' in *Section 4*)**. Fish should then be closely monitored and quarantined away from any existing resident colonies to avoid introducing disease.**

As a precaution, establishments may choose to only integrate bleached eggs to their existing general population rather than imported adult fish.

4 Housing and care

In order 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-fertilisation) (*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.

This section outlines and assesses current practice, guidance and research in relation to the environmental parameters that need to be considered, with the aim of developing consensus on good practice.

Lighting

Appropriate lighting facilitates good breeding success and minimises stress.

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 behaviour, 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-fertilisation (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).

Spectrum

Adult zebrafish appear to 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. Until any such needs have been established, it is suggested that standard fluorescent lighting is acceptable (Matthews et al 2002).

Elimination of those light wavelengths proven to encourage algal growth within the tank should be considered as this can help with tank hygiene (Matthews et al 2002).

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 minimising algal growth in tanks. Further investigation is required before any particular 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.

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).

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 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).

Quantity and temperature

i) 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.

ii) Volume and population density

Keeping zebrafish in 'crowded' conditions is detrimental to their welfare. Adults kept at high densities[10] have been observed to show a four-fold increase in whole body cortisol levels[11] and reduced egg production[12] (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 3: **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	

[10] e.g. 40 fish/L versus 0.25 fish/L

[11] though this effect was not seen in fish that had recently been fed.

[12] 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.

	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.

iii) 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[13] 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[14] (see *Figure 4*). 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

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

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

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 4:
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.

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 minimised. 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).

i) 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 colormetric test kit or preferably, a precise electronic meter (which should be regularly calibrated).

ii) 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.

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/desalinised. Water should be dechlorinated before use[15].

The pipes used for transporting water into and around an aquatic system should not be galvanised 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 other emergency.

iii) Cleaning

The cleanliness of the aquaria and filters is a very 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 faeces) 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 minimising 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.

[15] 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.

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).

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 sterilised 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.

Careful use of cleaning agents

Although the majority of 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 also 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 minimise disturbance and distress to the fish. Disinfectants should be used with extreme caution.

Tank housing

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).

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).

Colour 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 colours can lead to hygiene problems since contamination may not be obvious (The Berlin Workshop 1994). A container colouration 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 coloured surfaces (Brand et al 2002).

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 minimised.

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 (for more on this, see *Section 5*).

Figure 5:
Methods of identification and some points to consider regarding their suitability

Taking into account both animal welfare and scientific requirements, the 'pros' ■ , 'cons' ■ and 'other factors needing consideration' ■ are highlighted for each method.

Method	Points to consider (with reference)	Most preferred method
Observation of natural marking patterns	Where fish are kept in groups of up to four or five, it is possible to reliably distinguish individuals based on colour patterns alone (R. Spence 2007, personal communication). ▪ Negates the need to handle and mark fish.	✓
Elastomer marking[16]	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. ▪ Fish will need to be anaesthetised during the procedure.	
Freeze branding	A blunt ended needle is cooled to below 0°C and held against the skin to mark the fish with a 'dot' in different positions for each fish. ▪ Potential to cause tissue necrosis. ▪ Fish will need to be anaesthetised during the procedure.	

[16] e.g. see: Northwest Marine Technology: www.nmt-inc.com/products/vie/vie.htm (accessed 10/08/2010)

Removal of specific scales	▪ Sire et al (2000) comments that such removal does not increase fish mortality in laboratory breeding conditions and that regenerated scales are easily distinguishable from non-regenerated ones. ▪ Fish will need to be anaesthetised during the procedure.	
Fin clipping	Small clip(s) from different fins or at different positions can be used to identify individuals within tanks. ▪ Fins can regrow quite rapidly. ▪ Fins are innervated so could be painful. ▪ Fish will need to be anaesthetised during the procedure.	
Dorsal fin tagging	Fish may be tagged individually behind the dorsal fin with tags all bearing a different colour. ▪ Standard floy fish tags are quite large and therefore may be considered to be unsuitable for zebrafish. Cutdown or microfloys may be better. ▪ Floy tags may cause ulceration or infection. ▪ They seem not to affect the behaviour of zebrafish (e.g. Delaney et al 2002).	

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.

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 behaviour 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 behaviour, 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[17] 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 (see *Section 5*).

Also see the comments on water quality in *Section 4.*

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 minimise aggressive behaviour.

Catching and handling

The majority of zebrafish in research facilities are the descendents of many generations of captive bred animals. Although they appear to exhibit reduced 'nervousness' or predator avoidance behaviours, 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

[17] 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.

hours (Wedemeyer 1972, in Kreiberg 2000). For this reason it is advisable to minimise 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 sterilised between uses.

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

Food type and feeding regime

Natural behaviour 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.

Feeding requirements of zebrafish

Francis (2008) suggests that a quality diet[18] specifically developed for zebrafish should be used. Some commercial feeds claim to offer a nutritionally complete food[19]. However, the precise nutritional requirements of zebrafish have yet to be determined (C. Lawrence, personal communication).

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

[18] which also contains information relating to production and use before dates.
[19] e.g. the Irradiated Adult Zebrafish Diet from Harlan.

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 (see: *Section 4*).

Figure 6 provides a summary of recommendations that have been made for feeding zebrafish.

***Figure 6*:**
Summary of recommendations for food type and feeding regime for 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 between brine shrimp and flake.	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 increase the chances of disease.	In general, fish should be fed twice a day - once in the morning and once in the early evening.

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 behaviour of the animals. If automatic feeders are used then additional opportunities for observing the fish need to be built in to the management system.

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 behaviours 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.

Environmental complexity

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[20].

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.

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 with regard to possible treatments, and animal carers should be made aware of any requirements for, or restrictions on, the use of medicines.

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 recognised 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,

[20] Refuges are also used by males to avoid aggressive encounters with other males.

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 7*). Sick fish should be removed from the tank as quickly as possible and veterinary advice sought.

***Figure 7*: Some key signs of ill health in zebrafish**

	Possible cause											
Clinical signs	**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 behavioural indicators to look for include: failure to feed; swimming in an abnormal position in the tank; or rubbing their bodies on the tank side.

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

Alarm behaviours

When zebrafish become aware of an actual or perceived threat, behaviours 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 behaviours may indicate a chronic welfare problem.

Responses to acute noxious stimuli

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

A good understanding of zebrafish biology and behaviour, including diseases, clinical signs and treatments, is necessary to minimise suffering or death.

Zebrafish should be regularly monitored for signs of ill health.

This section has only considered a small number of the most common diseases and infections found in zebrafish. More detailed information can be found in:

- **Zebrafish International Resource Center** - **Disease Manual**
  http://zebrafish.org/zirc/health/diseaseManual.php
- **Laboratory Animal Medicine (2002)** (Second edition)
  American College of Laboratory Animal Medicine Series
- **The Laboratory Fish (2000)** - Gary K. Ostrander (editor)
  Academic Press, San Diego

5 Scientific procedures

Procedures commonly carried out on zebrafish include egg harvesting, the induction of anaesthesia, and humane killing. All have the potential to cause pain, suffering or distress so opportunities for refinement need to be explored and implemented. Suitable humane endpoints should be identified and agreed for procedures causing pain or distress. This section sets out background information on the purpose of some common procedures, together with recommendations for refinements that will help minimise and ideally avoid suffering.

Capacity to experience pain

A review of the literature provides no compelling reason to consider fish any differently from other vertebrates. Indeed, there is as much evidence that fish feel pain and can suffer as there is for birds and mammals - and more than there is for human neonates and preterm babies (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 2003, 2009). Although there has been little specific study involving zebrafish, given the above, zebrafish should be given the benefit of any doubt, and invasive, potentially painful procedures should be subject to appropriate ethical review, and accompanied (where appropriate) by anaesthesia and peri-operative care including analgesia.

Researchers and care staff should be well informed regarding any adverse effects that zebrafish may experience as a result of experimental procedures. This includes awareness of the responses zebrafish are likely to show, likely effects of scientific interventions, the need to appropriately monitor fish, what the humane endpoints of the project are, and what to do when these are reached.

Egg harvesting

Research in developmental biology, embryology and genetics, generate a high demand for a constant supply of zebrafish eggs.

Egg quality

Obtaining good quality eggs from the animals is important, not only for helping to realise research objectives, but also to keep the number of animals used to a minimum.

Some establishments obtain better quality eggs than others. The number of eggs laid is less important than the quality of the eggs; for example, a hundred good-quality eggs are far preferable to thousands of poor quality eggs. Since the majority of zebrafish eggs produced at research establishments are for experimentation, not reproduction, 'quality' generally refers to the ability of the eggs to remain viable after experimental manipulation, such as pharmacological treatment or microinjection. For most of the academic areas in which zebrafish eggs are used (e.g. developmental biology, anatomy, genetics studies) it is particularly important that a high percentage (i.e. > 80%) of the eggs are fertilised successfully, that the eggs undergo a clean and even first cleavage, and that they remain normally developed at gastrulation. Healthy eggs have a translucent, yellowish appearance (Pelegri 2002).

Numerous husbandry parameters are often manipulated in an attempt to improve egg quality. Such factors include diet, lighting, water salinity, water flow, frequency of cleaning, tank size, type of hormonal stimulation, frequency of egg collection, and age of females. Hygiene is especially important as zebrafish eggs are highly susceptible to fungal infections (Overstreet et al 2000).

Egg quality can have a direct effect on the quality of scientific data so researchers should seek to share and publish information regarding factors affecting egg quality.

Methods of egg collection

There are a number of techniques associated with the procurement of eggs. The main ones are:

- natural mating;
- manual expression ('squeezing') of eggs from females for *in vitro* fertilisation;

Natural mating

Zebrafish are normally kept under laboratory conditions designed to replicate perpetual summer. Depending upon food availability and temperature they can breed all year round (Spence et al 2006) with females generally producing eggs once every one to three days. Darkness allows the zebrafish to rest and the return of light will trigger fish to breed (Vargesson 2007). A layer of marbles, closely spaced rods, or mesh can be used to cover part or the whole of the bottom of the tank to prevent the fish eating their eggs once laid (Matthews et al 2002).

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 & Smith 2006). A good clutch consists of between 70 and 300 eggs, of which at least 80% are fertilised (Brand et al 2002).

It has been reported that keeping females together (as opposed to on their own) for four days prior to being separated and mated with a single male significantly suppresses the numbers of eggs produced (Delaney et al, unpublished data).

Ruhl et al (2009) observed that eggs were significantly more likely to be absent in tanks in which aggressive interactions had occurred between fish[21].

Zebrafish sex 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 outnumber females unless inbreeding is eliminated by continually introducing a few individuals from other sources into the fish breeding stock (Overstreet et al 2000).

Induction of ovulation and mating behaviour

Ovulation is thought to be induced via the presence of male gonadal pheromones in the surrounding water (van den Hurk and Resink 1992).

Kurtzman et al (2010)[22] write that past research has shown that population dynamics influence zebrafish clutch size. Per capita egg production is typically highest in small shoals. However, group shoaling for breeders is not practical in most laboratory settings where 1:1 pairings are frequently required (e.g. heterozygote screens, developmentally time-critical studies or gene-knockdown studies). Most facilities mate groups of two to four fish.

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

- a small (typically <1L) plastic mating 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 box in the evening;
- when the fish spawn (usually the following morning), the fertilised eggs fall through the 'floor' of the inner box (which means the fish are prevented from eating them).

[21] either between competing males or between the male and the female.
[22] widely citing the work of Ruhl et al (2009).

Manual expression of eggs from females

For certain procedures[23], researchers may want to manually express eggs from females. If undertaking this procedure, fish should first be anaesthetised.

Pelegri (2002) illustrates the typical 'expression' process. Once gill movement has slowed, fish can be removed from the water using a plastic spoon and placed onto a paper towel to dry briefly. The fish can then be transferred to a small plastic dish. Fingers should be slightly dampened. One finger can be placed on the dorsal side of the fish. A finger of the other hand can be 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. If gentle pressure fails to produce eggs then it is important not simply to squeeze harder, as **extra squeezing may injure the fish.**

To help protect the health of the fish, strong consideration should also be given to wearing unpowdered, non-textured gloves when handling these animals.

It is imperative that only people experienced and trained in the 'manual expression' procedure attempt this process. Injury can be caused through incorrect or too heavily applied pressure.

Obtaining sperm from males

Sperm collections can be made either by shredding the testes of humanely killed dissected males, or by collecting milt from live, anaesthetised males.

Pelegri (2002) provides protocols for both methods, suggesting that the first method is both more reliable and less laborious. It would also appear that it requires fewer males[24], and the potential for individual animal suffering is less, as single or repeated recovery from anaesthesia is not required. Pelegri suggests that to provide enough sperm to fertilize about 40 egg clutches, 40-60 males would be needed if expressing the sperm (using forceps and a pipette) from anaesthetised males, whilst just 10 would be required if using the shredded testes of dissected males.

[23] For example, when producing haploid or clonal diploid animals; when using with reconstituted frozen sperm; where synchronous fertilisation is a requirement of the study; or where females are not engaging in mating.

[24] A larger volume of sperm can be obtained from the dissected testis of a male zebrafish than can be squeezed from an anaesthetised animal.

Frequency of egg collection

Though zebrafish females are capable of spawning on a near daily basis (Lawrence 2007), a female which lays eggs daily is very unlikely to produce a good quantity or quality of 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 2010). Given the likelihood that such a regime places a significant metabolic cost on the females, some suggest they should not be bred from more than once a week (e.g. Kunkel 1998). Elsewhere it is stated that in order to maintain a healthy, fecund breeding population, large breeding facilities generally breed no more frequently than once every 1-2 weeks (Kurtzman 2010). Maintaining this baseline breeding frequency, even when demand for embryos is low, may also help avoid the visceral cavity of gravid females becoming inflammed due to excessive egg retention (Kent et al 2007).

It is likely that the frequency at which eggs of good quality can be collected from females, and the impact of this process on this animals, is heavily influenced by parameters such as water quality and nutrition.

The health and welfare effects of encouraging females to spawn at a range of time intervals needs to be more fully evaluated.

On the basis of current knowledge, a minimum interval of a week should usually be allowed between episodes of breeding in females.

Age of females

It is possible for both males and females to reach sexual maturity within three months of hatching. Although establishments may begin using fish for breeding from this age (Kurtzman 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 fish between 6 and 18 months of age (Vargesson 2007).

Kurtzman (2010) states that males are maintained in breeding stocks until they are around 1 year old and then replaced with younger breeders (even though they are capable of producing sperm - albeit at reduced levels - beyond 1 year of age).

Transgenesis

Transgenic zebrafish can be created by a variety of methods. The most common is to inject DNA directly into the fertilised egg (Whitfield 2002). However, injection of DNA at a high concentration can be lethal to embryos or cause abnormalities in their

development. For more information on manipulating gene expression in the zebrafish, see Gilmour et al (2002).

Mutagenesis

Mutagenesis 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 screen[25], since this determines the efficiency of mutation induction. Efficient induction will minimise the number of fish involved.

The type of mutagen also influences the type of mutation - and hence potential harms - induced. In addition, 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[26]. Fish can also become very nervous during ENU treatment. It is therefore essential to ensure that refinements such as reduction in external stimuli (noise, light) which may disturb the animals are implemented, to help reduce potential suffering. This also improves survival rates (Pelegri 2002).

Genotyping

Zebrafish genetic composition 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. Obtaining these 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 part of the caudal fin from an anaesthetised fish using a sterile razor blade or scalpel. Only the minimum amount of tissue necessary (2-3mm is sufficient) should be taken as the caudal fin is innervated and clearly important for locomotion. Matthews et al (2002) state that with practice the fin clip procedure can be undertaken very rapidly and should cause no bleeding. 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 (500ml) individual tanks containing clean water from the holding tank should be set up for anaesthesia 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 them (see *Section 4*). If single housing is required this should be for the minimum possible period of time.

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

[26] e.g. one-hour treatments above 3mM, or two-hour treatments at lower concentrations, induce more than 50% lethality (Pelegri 2002).

Cryopreservation

Cryopreservation of sperm, eggs and embryos has been highlighted as a useful tool for archiving genetically altered (GA) animal lines until they are required and for providing a strategic reserve in case of genetic contamination or 'drift', pathogenic infection and natural disasters. When used for GA mice for example, cryopreservation can avoid the 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 GA line (RSPCA 2008). The cryopreservation process is currently being investigated with a view to applying the technology to zebrafish.

Sperm

A small number of cryopreservation and thawing protocols are available which result in a degree of success in the preservation of viable sperm, but these methods are not yet well developed or widely used and more resource investment is required to further develop them (Johnson et al, no date). A review of these methods can be found in Yang and Tiersch (2009).

Two methods are currently in general use. A simple method uses 10% N,N-dimethylacetamide (DMA) diluted in buffered sperm motility-inhibiting solution (BSMIS) as a cryoprotective medium. A 14% fertilisation rate[27] has been observed using 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. Recently, several adaptations have been made to this protocol that show good potential (e.g. Draper & Moens 2009, Yang et al 2007).

Given the work ongoing in this area, rates of successful fertilisation using preserved and thawed sperm look set to increase and so keeping informed of the latest developments is essential.

Eggs

Isayeva et al (2004) demonstrated that zebrafish eggs are highly sensitive to chilling, with survival after chilling depending on exposure temperature, exposure time period, developmental stage, and 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.

[27] Which compares to a >95% fertilisation rate for non-cryopreserved sperm (Draper and Moens 2009).

Embryos

Cryopreservation of embryos has not previously been possible due to the problems associated with high egg yolk content and low membrane permeability which stops water removal from the cell, and CPA penetration, which result in chilling injury. However, there has been recent success with freezing both zebrafish blastomeres, and yolk-removed embryos, and research continues in this field (Lin et al 2009, Higaki et al 2010).

Blood collection

As there is so little available for collection, it is not practical to obtain blood from live zebrafish as a survival procedure since it generally involves causing irrevocable harm.

Blood collection should be carried out under anaesthesia followed by euthanasia.

Injections

There is little information available on the refinement of procedures for the administration of substances specifically relating to zebrafish, other than limited information on injection techniques in Morton et al (2001). General principles should include taking care to try and inject between the scales, and to minimise any damage to the fish.

Analgesia and anaesthesia

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

Analgesia

The authors could find 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 (National Research Council 2009).

Anaesthesia

The most common anaesthetic agent currently used is probably tricaine methanesulphonate (e.g. MS222) in an aqueous solution[28]. Anaesthesia is induced rapidly following immersion in a buffered[29] solution containing MS222 at 100-200 mg/L (Matthews et al, 2002). A concentration of MS 222 at 168mg/L has been used at the University of Oregon (2001). Where maintenance anaesthesia is required (which is rare) the dose is lower (50-100 mg/L).

Volumes for anaesthesia should be carefully calculated and the solution should be made up freshly on each occasion. Where several fish are anaesthetised 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 anaethesia (Matthews et al 2002).

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

Considerations

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

- Although MS222 is commonly used for inducing anaesthesia 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 anaesthetic. Research work is ongoing to assess the humaneness of MS222 and so researchers should keep themselves informed of the latest findings.

Researchers should ensure they stay informed as to latest thinking on what appropriate anaesthetic regimes are for fish. This should include the effectiveness of current dosing recommendations for MS222 in terms of its ability to induce anaesthesia or relieve pain without causing distress.

[28] Though some researchers are now also looking at the potential for using other agents, such as alfaxalone.

[29] A solution of MS222 in water is acidic and should therefore be buffered to neutral pH using sodium bicarbonate before fish are immersed.

Appropriate analgesic agents and doses for zebrafish need to be determined.

For more guidance relating to anaesthesia in aquatic species generally, see:

Anaesthetic and Sedative Techniques for Aquatic Animals (3rd Edition) **(2008)** Lindsay Ross and Barbara Ross: Wiley-Blackwell.

Humane killing

The principle

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 euthanased in the home tank?
- Is the method aversive - if so, how can this be minimised?
- Does loss of consciousness ensue rapidly?
- Following loss of consciousness, does death occur rapidly?
- Does induction of unconsciousness occur without causing pain?
- Is the method reliable and does it ensure that the animal does not regain consciousness?
- It 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.

Methods for humane killing of zebrafish

Personnel need to check and comply with relevant legislation. In the UK for example, under the *Animals (Scientific Procedures) Act 1986*, acceptable methods for fish are currently[30]:

[30] Also see: Appendix IV of European Directive 2010/63/EU on the protection of animals used in scientific procedures: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:EN:PDF

- overdose of an anaesthetic using a route and an anaesthetic agent appropriate for the size and species of animal, followed by one of the stated confirmatory methods[31];

- concussion of the brain by striking the cranium (with destruction of the brain before the return of consciousness).

It should be noted that **the most humane method of killing for zebrafish has not yet been determined** (Wilson et al 2009).

As mentioned in the previous section on anaesthesia, although MS222 is widely used as a method of killing zebrafish, concerns have recently been raised that it may be aversive to at least some species of fish, it could in fact be acting as a neuromuscular blocking agent, and it may not actually always be reliable.

There is also debate relating to whether submersion in ice water can be considered humane (Robb & Kestin 2002). Some advise against its use (e.g. AVMA 2007), but recent studies appear to suggest that inducing hypothermal shock in adult zebrafish by placing them in ice slush (4°C or less) may actually be more humane than using MS222 (Wilson et al 2009).

Research work is currently ongoing to assess the humaneness and efficacy of MS222 as a method of killing, and also that of clove oil, rapid cooling and electrical stunning and killing, so researchers should endeavor to keep informed of the latest findings.

[31] These are: confirmation of permanent cessation of the circulation; destruction of the brain; dislocation of the neck; exsanguination; confirming the onset of *rigor mortis*; or, instantaneous destruction of the body in a macerator.

6 Training of animal care staff and users

The importance of staff having a good understanding of the animals that they work with cannot be underestimated. Persons not suitably trained can harm animals when performing procedures such as capture, or marking (particularly in the case of small aquatic species), and will have a reduced ability to recognise adverse effects or implement the 3Rs. Inappropriate fish care, or behavior towards them, will have a direct impact on animal welfare and scientific quality; the achievable benefits of the work will be reduced and the potential harms increased (Orlans 2001).

The general principles of training in good laboratory animal care apply, but additional species-specific training is essential. Personnel should have a detailed species-specific knowledge of the natural history, behaviour and requirements of the zebrafish in their care. They should be up to date with the latest thinking and publications on good practice with regard to housing and care and, where appropriate, with advances in the refinement of scientific procedures. A sound understanding of the importance and practical aspects of the prevention, recognition and alleviation of ill health, pain and stress is also essential.

Training for those persons caring for and using animals should be species-specific. Focused courses addressing the behaviour and requirements of zebrafish should be developed for those using and caring for this species.

7 Concluding comments

This report sets out many factors that need to be considered by those using, and caring for zebrafish. Existing guidelines and recommendations have been summarised for consideration, with perceived good practice highlighted where possible. Further research is clearly necessary to establish the most appropriate housing and care conditions for zebrafish, and the report highlights the main areas where further information to determine good practice is required. This includes better definition of environmental needs, nutritional requirements, and the most appropriate methods for analgesia, anaesthesia and humane killing - but there are also many other areas where there is a need for further studies and discussion in order to develop a science based consensus as a sound basis for future recommendations.

It is important from the perspectives of ethics, animal welfare and science that effort is focused on defining and implementing *optimal* protocols for housing and care, not simply those that zebrafish will tolerate.

The exchange of information between laboratories housing zebrafish is very important in facilitating this. With this in mind, the following resources will be of use to those using and caring for zebrafish:

- **Zebrafish Husbandry Association (ZHA)**
  www.zhaonline.org

- **British Association for Zebrafish Husbandry (BAZH)**
  www.bazh.co.uk

- **Zebrafish Information Network - the zebrafish model organism database (ZFIN)**
  http://zfin.org/zf_info/dbase/db.html

- **Zebrafish International Resource Center (ZIRC)**
  http://zebrafish.org/zirc/home/guide.php

And the following book will also be of interest:

- **The Laboratory Zebrafish (2010)**
  Claudia Harper & Christian Lawrence; CRC Press, Boca Raton, USA.

More general guidance relating to the care and use of fish in research can be found in:

- **The UFAW Handbook on the Care and Management of Laboratory and Other Research Animals** (8th edition) **(2010)** Chapter 49 - Fish, by James Turnbull and Iain Berrill: Wiley-Blackwell: Oxford, UK.

- **Do fish feel pain? (2010)**
  Victoria Braithwaite: Oxford University Press, Oxford, UK.

- **Harmonisation of the care and use of fish in research**
  Norecopa (*conference proceedings from 2005 and 2009*)
  www.norecopa.no

- **Pain and distress in fish (2009)**
  ILAR Journal (50)4: National Research Council of the National Academies, USA.

- **Guidelines on the care and use of fish in research and testing (2005)**
  Canadian Council on Animal Care (CCAC)
  www.ccac.ca

- **The Laboratory Fish (2000)**
  Gary K. Ostrander (editor): Academic Press, San Diego, USA.

- **Textbase**
  A database of information on current textbooks within the field of laboratory animal science.
  http://oslovet.veths.no/textbase

- **NC3Rs** - information on fish
  www.nc3rs.org.uk/fishhousing

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