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*T*he zebrafish, *Danio rerio* (also recognized as *Brachydanio rerio* and the zebra danio), is now one of the most widely used experimental animals in biological and biomedical research. This popularity is due to the zebrafish's external development and optical clarity during embryogenesis, reasonably fast generation time, and the vast array of genomic resources available, all of which allow for manipulations at the smallest scale. Today, zebrafish are mainly used in molecular and developmental biology, neurobiology and genetics research. Their use in the fields of cancer research, neurophysiology and drug discovery is becoming increasingly commonplace. Reasons for the rise of zebrafish in biomedical research include low maintenance costs reported to be less than 1/1000th of the cost of mice, and reproductive efficiency. Breeding adults can produce up to several hundred fertilized eggs from a single spawning each week which provides an abundance of data for screening mutations and for phenotypic analyses. Thus, the zebrafish has emerged as an excellent model organism for studies of vertebrate biology.

In this issue of the *BRL Bulletin*, we will highlight some key factors and considerations regarding the use of zebrafish in biomedical research. For additional information, the BRL has a policy available which provides guidelines related to this species.

The Aquatic System

Zebrafish housing systems are unique and, oftentimes, complex. Zebrafish are acutely sensitive to stress arising with alterations in water chemistry, flow, and temperature in addition to stressors associated with housing conditions such as stocking density, tank size and bottom substrate. Health of the animals is dependent not only on proper nutrition and animal selection, but also on the maintenance of proper water quality parameters.

A clean source of water is required for any fish facility. Water purified by reverse osmosis is the ideal source of water for a zebrafish facility. Once treated by reverse osmosis, water must be conditioned by reconstituting minerals and nutrients lost during the osmotic procedure. A commercial sea salt mixture can restore proper levels of salinity, and chemicals such as bicarbonate can be used to achieve the desired water alkalinity.

Temperature is a critical factor tied to survival, growth, and development. Zebrafish are poikilothermic animals having a body temperature that varies with the temperature of their surroundings. Water temperature should be maintained between 74-84ºF (23-39ºC) and preferably between 78-82ºF (26-28ºC). Small shifts in water temperature are usually well-tolerated; however, abrupt and large fluctuations in temperature can cause shock in addition to other physiologic derangements in metabolism and reproductive activity.

An important parameter related to temperature is dissolved oxygen. Dissolved oxygen is the amount of oxygen that is present in the water. If the dissolved oxygen drops below a critical level, it can result in asphyxiation of the zebrafish. When dissolved oxygen levels are too low, fish will avoid these areas in the tank, gather around aerators, and might appear to "gasp" or "gulp" at the water surface for air. Conversely, water can become supersaturated with dissolved oxygen (or other gases) resulting in "gas bubble disease", where fish appear disoriented, may have subcutaneous emphysema, and can present with air embolisms resulting in high mortality rates.

Zebrafish perform optimally in a water pH of 7-8, ideally at 7.8. The pH of water is also influenced by temperature. The pH of the water system has a direct influence on the susceptibility of fish to toxins such as heavy metals in the water, levels of unionized ammonia, and can upset the balance of nitrifying bacteria in biologic filters.

Zebrafish constantly excrete ammonia both across the gills and in the feces. This, along with floating decaying food particles, will foul the water and may have implications on fish health. A fully functioning biologic filter should be able to remove a large majority of toxic nitrogenous wastes (ammonia and nitrite) produced within a system. The biologic filter consists of two groups of nitrifying bacteria, one which converts ammonia to nitrite and a second which converts nitrite to nitrate. Ammonia levels should be kept at 0, nitrite between 0-25 ppm, and nitrate between 0-40 ppm.

General Husbandry

Tanks should always be clearly labelled with the genetic background and sex of the animals inside. Identification of individual zebrafish is not an easy task. When necessary, the preferred method is observation of natural marking patterns as this negates the need to handle and physically mark fish. Fin clipping is a common method used for identification and to obtain material for genotyping. This technique has limitations as the fish need to be anesthetized as the fins are innervated, and the fins rapidly regrow.

As with the mouse and other laboratory animal species, zebrafish must be provided with a complete and balanced diet. There is ongoing research to determine the precise nutritional requirements of zebrafish, but in most cases, laboratory zebrafish are fed a combination of live foodstuffs (zooplanktons, brine shrimp, etc.) and formulated diets. When using formulated diets, essential fatty acids are a nutrient requirement of particular importance. The ratio of omega 6:omega 3 fatty acids has an effect on both growth and reproduction with a higher demand for omega 6, an important consideration to keep in mind as many commercial diets are higher in omega 3.

Fish should be provided with enough food daily so all food placed in the tank is consumed within 3-5 minutes. Excess food can negatively affect both mechanical and biologic filters thus affecting water quality and pH. Adult zebrafish should be fed 1-3 times daily. For early stage larvae, more frequent feedings may be beneficial.

Zebrafish are a social species and as such, must be housed with conspecifics unless there is scientific or veterinary justification otherwise. In the laboratory setting, housing density has a profound impact on welfare. Zebrafish are territorial and establish hierarchies within the tank. Surprisingly, aggressive and territorial behavior is inversely proportional to housing density where aggression is highest when stocking density is at lower levels. Current studies are evaluating the use, risks, and benefits of enrichment devices in this species. If zebrafish are to be housed alone, enrichment devices such as small plastic plants must be provided. In group housed situations, zebrafish can also benefit from the use of enrichment devices. It has been shown that the use of plastic plants decreases aggression and provides a preferred location for egg deposition. The implementation of environmental enrichment should take into consideration the scientific goals of the study for which the animals are used. Performance standards should be applied taking into consideration health, welfare, and species-typical behavior.

The 8th edition of the *Guide to the Care and Use of Animals* states that zebrafish are to be maintained at densities of five adults per liter of water. In addition, a maximum limit of five adult fish per liter has been established by the Office of Laboratory Animal Welfare unless performance standards have been set at the institution. Per UIC policy, an adult fish is defined as a fish aged 90 days or older.

Animal Monitoring and Health

Zebrafish and the aquatic system must be monitored on a regular basis. At a minimum, water quality monitoring should meet standards put forth by the World Aquaculture Society. Temperature, pH, conductivity, dissolved oxygen, and chlorine must be monitored on a daily basis. Nitrate, nitrite, alkalinity, hardness, and carbon dioxide must be monitored weekly. As with other laboratory species, zebrafish health and activity must be monitored daily along with room temperature and lighting. Tanks should be cleaned regularly to ensure the fish are always visible, to decrease a potential reservoir for pathogens, and to maintain adequate water quality. Investigators and laboratories at UIC are required to develop and maintain their own standard operating procedures for maintenance, monitoring, and health of zebrafish colonies.

The health status of zebrafish colonies must be monitored on a regular basis and health testing is to be performed, at a minimum, on a semi-annual basis. The NIH Zebrafish International Resource Center (ZIRC) has developed a model sentinel fish program, similar to that used in rodent sentinel surveillance programs.

Anesthesia, Analgesia, and Euthanasia

Zebrafish are considered a vertebrate animal at the time of hatching, which typically occurs three days post fertilization (dpf). Upon hatching, a yolk sac is present which nourishes the animal until 5 dpf. By this time, the digestive tract is fully formed and functional allowing the zebrafish to utilize external food. Zebrafish develop the neuronal circuitry that allows them to experience pain or distress in response to noxious stimuli at 8dpf. Therefore, anesthetics and analgesics must be considered after this time point.

The most common anesthetic agent currently used is tricaine methanesulphonate (MS-222), and this agent is currently FDA approved (marketed as Tricaine-S). Anesthesia is induced by immersion in a buffered solution of MS-222. Advantages include a moderate safety margin, and rapid induction and recovery. Anesthesia in zebrafish is divided into four stages on the basis of locomotor activity, rate of opercular movement, reflex response, equilibrium, and other physiologic parameters. As zebrafish are poikilothermic, the water temperature during anesthesia will affect their metabolism. This in turn, influences the rate of absorption and excretion of the anesthetic agent and its subsequent effectiveness.

There is no compelling reason to consider fish any differently from other vertebrates in regards to pain; however, there are no published guidelines on adequate analgesia for zebrafish. Zebrafish respond to opioid and non-opioid analgesics with changes in behavior and metabolism, but these drugs have not been evaluated in focused nociceptive studies. It should be noted that zebrafish have opioid receptors, such as the mu opioid receptor, which has functional characteristics similar to the mammalian receptor. Buprenorphine added to water at concentrations of 5uM has been shown to significantly impact the response to aversive stimuli. Previous studies have demonstrated the analgesic effect of morphine in rainbow trout, goldfish and carp, but not yet in zebrafish. Similarly, receptors for NSAIDs are conserved evolutionarily between mammals and zebrafish, but have not yet been tested for analgesic properties in the zebrafish. NSAIDs are metabolized in a way resembling the process in mammals, but more studies are needed to assess analgesic efficacy in zebrafish.

Zebrafish should be euthanized by methods consistent with the American Veterinary Medical Association's *Guidelines for the Euthanasia of Animals*. The choice of method should consider the intended use of the fish after euthanasia, and the age of the fish measured in days post fertilization. The most common euthanasia techniques used in zebra fish greater than 8 days post fertilization (dpf) are ice bath immersion or an overdose of tricaine methane sulfonate (MS222). For zebrafish 4-7 dpf, submersion in ice water or exposure to a bleach solution are most commonly used for euthanasia, and for embryos less than 3 dpf exposure to bleach solution is recommended. A key consideration in appropriately euthanizing zebrafish using the techniques listed above for the respective life stage is the duration of exposure. For a more comprehensive description on specifics related to the ice bath slurry, MS222 euthanasia dosage, bleach concentration, and exposure times please refer to the UIC-OACIB Policy: Zebrafish Research.

Further Reading

Farrell, A.P., Stevens, E.D., Cech, J.J., Richards, J.G. *Encyclopedia of fish physiology: From genome to environment*. London: Academic Press, 2011

Guidance on the housing and care of zebrafish (*Danio rerio*) RSPCA, England, Wales, 2013

Guide for the Care and Use of Laboratory Animals. Washington, D.C.: National Academies, 2011.

Jenkins, J.A., Bark, H.L., Bowker, J.D., Bowser, P.R., Macmillan, J.R., Nickum, J.G., Rachlin, J.W., Rose, J.D., Sorensen, P.W., Warkentine, B.E., Whitledge, G.W. *Guidelines for use of fishes in research — revised and expanded.* Fisheries 39(9):415-16, 2014.

NIH Guidelines on the Use of Zebrafish in Research. 2014.

UIC-OACIB Policy—Zebrafish Research

Zebrafish International Resource Center http://zebrafish.org/home/guide.php

Announcements: Welcome Aboard

We are pleased to welcome Tracy Latalladi as our newest member to the BRL animal care supervisory staff. Tracy comes to the BRL with over 14 years of experience working with rodents at the University of Chicago. She will be providing supervisory oversight and service to the BRL's mouse colony and UIC mouse users, respectively.

We are also pleased to welcome Dr. Alexandra Smith as our newest veterinary fellow in the UIC laboratory animal training program. Dr. Smith is a graduate from the Royal Veterinary College in London. She will be joining our team August 1st.