

Einstein Zebrafish Facilities Manual

Table of Contents

ZEBRAFISH FACILITIES & SYSTEM DESCRIPTION	1
Chanin Facility	1
Chanin Quarantine Facility	4
Ullmann Facility	5
System Maintenance & Record Keeping	6
ZEBRAFISH CARE & HUSBANDRY	7
Adult Zebrafish Maintenance	7
Fish Identification / Tank Labels	7
Routine Observations / Activities	7
FEEDING	8
Food Storage / Labeling	8
Powder Food Recipes	9
Feeding Schedules	10
CLEANING & EQUIPMENT SANITIZATION	12
BREEDING	14
Setting Up Crosses	14
Collecting Crosses	14
Raising Progeny	14
Identification of New Stocks	15
Bleaching Protocol.....	16

QUARANTINE	17
ISOLATION FOR STUDIES WITH CHEMICAL & BIOLOGICAL AGENTS	17
HEALTH MONITORING (VETERINARY CARE)	19
Euthasia	20
Disposal of Deceased Fish	26
APPENDIX 1. SUMMARY OF HUSBANDRY / MAINTENANCE / CLEANING SCHEDULES	27
APPENDIX 2. GENOTYPING PROTOCOL	28
APPENDIX 3. EMERGENCY PLAN FOR ZEBRAFISH FACILITIES ...	31
APPENDIX 4. FACILITIES DIAGRAMMATIC FLOORPLANS	33
Chanin Facility Capacity/Map	
Chanin Quarantine	
Ullman Facility Capacity/Map	

ZEBRAFISH FACILITIES & SYSTEM DESCRIPTION

There are two main zebrafish housing facilities (Chanin and Ullmann) and one quarantine facility (Chanin 521) on the EINSTEIN campus. These systems are closed recirculating systems wherein multiple aquaria (tanks) are served by a common pump, filtration bank, and reverse osmosis water supply reservoir.

Chanin Fish System-

Distilled water first enters into a Reverse Osmosis (R/O) unit; a composite membrane, carbon filter, and sediment filter to provide 150 gallons of water per day [or 6.25 gallons per hour]. Maximum operating pressure is 175 psi and minimum pressure is 35 psi. **Note: Filters are routinely changed every other month and as needed to restore system pressure.**

Purified water is then fed into a reservoir tank (160 gallons). A float switch in the tank regulates water from the R/O unit. When the water reaches its maximum level, the switch turns the R/O unit off. Conversely, as the water level decreases the switch falls and activates the R/O unit. The water in the reservoir has a pump to circulate the water.

The system has automatic conductivity and pH adjustment capacity. Sodium bicarbonate and salt-dosing system are manually maintained in separate tanks. If the system pH or conductivity falls below the set point a relay activates the dosing pumps. The dosing pumps pump a solution of sodium bicarbonate (pH) from one tank and a solution of sea salt (conductivity) from the other dosing tank into the sump of the main system where they are mixed with the system water.

After leaving the reservoir water is then entered into the sump tanks in the system. The water passes through a biological filter to remove nitrates and ammonia. The water then passes through 50 micron mechanical filters and carbon filters to further remove solid waste and ammonia. **Note: The mechanical and chemical filters are**

changed every other month, or as needed to restore system pressure. If filters are replaced too often the filters will stifle beneficial bacteria that consume ammonia and nitrites.

The water then passes through an air blower, which provides oxygenation, degassing, and water mixing. Diffusers (air stones) diffuse air coming from the air pump and increase oxygen transfer to the water.

The water is then further cleaned against bacterial waste with UV bulb filters. UV bulbs are changed when needed based on UV indicator light. The water is then circulated to the fish tanks.

Water from individual tanks is leeched from the animal area through baffles at the back of each tank into troughs behind the tanks. The water then passes through a filter pad to collect solid waste. Filter pads accumulate solid waste over time and are visually inspected daily. The filter pads are changed regularly to prevent their saturation with waste. The water then enters the sump tank where it passes through filters (particle and carbon) before it is recirculated.

Quarantine Facility - Chanin 521

The system is a self-contained recirculating system similar to that in the main facility. A Reverse Osmosis (R/O) unit processes “house” distilled water; composite membrane, carbon filter, and sediment filter. The filters are changed every other month, or as needed to maintain system pressure. The purified water is held in a 200L reservoir. A float switch in the tank regulates water from the R/O unit. When the water reaches its maximum level, the switch turns the R/O unit off. Conversely, as the water level decreases the switch falls to activate the R/O unit. The water in the reservoir has a pump to circulate the water.

The water enters into the sump tanks where it passes the biological filters for ammonia and nitrate waste. The water then enters 50 micron mechanical and carbon filters. The mechanical and

chemical filters are changed every other month, or as needed to maintain system pressure.

The water is further clarified to remove bacteria and waste via UV bulbs. The bulbs are replaced as indicated by the electronic system maintenance module.

The water is then directed to the tanks. Tank water is slowly leeched off through baffles at the back of each tank and passes down through a filter pad to filter out solid waste. The water then enters the sump tank and is recirculated through the filter system.

Conductivity and pH are monitored with the electronic system maintenance module. Changes to pH and conductivity are made manually.

Ullman Facility-

Distilled water passes through a Rios Millipore Reverse Osmosis filtration system, prepack-no separate filters. The filters are changed based on manufacture indication through the electronic monitoring system. Water is then held in a 750L reservoir.

After the reservoir water then passes into the sump tanks. Conductivity and pH are electronically monitored and adjusted automatically to maintain optimal salt and pH. The sodium bicarbonate (pH) tank and a solution of sea salt (conductivity) tanks are manually maintained.

From the sump tanks the water is aerated with an air blower to oxygenate and degas the water. The water then passes through pedestal mechanical and carbon filters. The filters are changed monthly based on manufacture recommendation.

The water is clarified to remove bacterial and waste via UV

bulbs. The bulbs are changed when needed as indicated by electronic monitoring.

The system water is then fed into the tanks. Tank design allows water to be continually leached out. The water then is passed through a filter pad to remove solid wastes. The water then enters into the sump tanks and is circulated through the filtration system.

System Maintenance Schedule & Record keeping

Mechanical and Chemical Filters are routinely changed every other month and as needed to restore system pressure. The ultraviolet bulbs are replaced as indicated by the electronic system maintenance module.

Records of filter changes and scheduled filter changes are kept in each fish room (facility) at all times. Back-up filters are stored in each fish room.

System pressure, conductivity, pH, temperature are recorded bi-daily, and visual inspections of all necessary areas also are documented bi-daily. These records are maintained in each fish room.

ZEBRAFISH CARE & HUSBANDRY

Adult Zebrafish Maintenance

Up to 20 adult fish (> 1 month old) may be maintained in individual 3 L aquaria.

When larval fish or fry are first introduced to the system at 5 days post fertilization, a '300' screen, the smallest of the three sizes, should be used. Tanks with screens this size are monitored twice daily for signs of clogging. At a minimum the '300' screen is replaced two weeks after the fish have been entered into the facility or more frequently if clogging is noted.

At approximately 1 month, fish are large enough in size to receive a larger screen the '1000' screen, and at 1.5 months, the blue baffle should be used to ensure optimal water flow. A "flow chart" (APPENDIX 5), which includes specific check dates for each line (stock) is posted in each system to monitor progress of the larvae and juveniles. Stock numbers and information pertaining to "birthdates" and line information (i.e. parents, genetic background, allele, or transgene) are included on the tank labels (below) and recorded in the stocks database.

Fish Identification /Tank Labels

Every tank containing fish must be labeled. Every label should be dog-eared and include the following:

- 1) AE# - Every new stock receives a new AE#. (Check the Stock database for the next available number.)
- 2) Allele
- 3) Number of fish
- 4) Plate number
- 5) "Birth" date
- 6) Principle investigator
- 7) Source

All of this information (as well as additional details about the source of the stock and any relevant notes) are entered into the stock database log sheet to prevent duplication of stock numbers.

Routine Observations & Activities

Fish are checked and fed twice daily (see below). LOOK AT THE FISH WHILE YOU ARE FEEDING. Remove any dead or obviously ill fish and record in the deceased fish log. Make sure that water is flowing to all tanks and that the outlets are not clogged (the tank water levels will be higher than usual if the baffle is clogged).

Screens/Baffles should be checked regularly (daily) to prevent algae buildup and clogging of tanks. When changing screens and baffles, fish should be transferred to a clean tank entirely (with a clean screen or baffle of the appropriate size). Do not simply change the baffle as it will quickly clog. The old tank should be thoroughly washed and placed on the cart for sanitization.

After you finish feeding, check to make sure that there are no leaks or hoses out of the tanks in the system (i.e. no water on the floor). The most common cause of flooding is an open water tube that is not directed to a tank. When you fill up breeder cages with system water make sure that you close the orange valve completely (other potential cause of a flood). An open valve or tube will drain the system reservoirs; so, it is imperative that the source of any water on the floor is identified.

If the water level in a tank is too high, you need to adjust the water flow (if it is too high) or exchange the baffle (if the water flow is normal but the water level is high) to prevent fish from escaping from the tank.

FEEDING

Food Storage / Labeling

All prepared feed, other than live brine shrimp, (e.g. pellets, dry mix, flakes etc) must be stored in plastic sealed (vermin proof) containers and kept at the manufacturer's recommended temperature. All feed containers must be labeled clearly to indicate the name of the contents, manufacturer, milling date (if available) and /or expiration date (if available) and /or date of preparation. To preserve nutrient quality, all feed should be used within 90 days of preparation, unless otherwise shown to be stable per manufacturer's literature [which should be available for inspection].

Powdered Food Recipes

Mast Dry Mix:

Ingredients

AquaTox Flake (Zeigler Brand)- 150g
Cyclopeez- 12.5g
Hikuri Micropellet- 22.7g
Golden Pearl 300 to 500 micron- 34.1g
Spirulina Flakes- 21.6g

Crush Cyclopeez. Add Aquatox and crush into Cyclopeez. Add Golden Pearls and Hikuri micro pellets. Add Spirulina flakes. Shake to mix and mix by hand.

Powdered fry food:

Golden Pearls Larval Food 5-50 micron

Brine Shrimp:

Fresh brine shrimp are cultured from Premium Grade Brine Shrimp Eggs (from Brine Shrimp Direct) - 90% hatch rate.

Brine culture preparation

387g Sea Salt Mix
12.5L Distilled water
25g Shrimp eggs

Water, salt and eggs are added to a dedicated culture funnel with active air bubbler. The shrimp eggs are cultured for ~28hrs to allow for hatching (e.g.

the shrimp cultures are set up at 9-10am and are harvested the following day at 2pm).

Shrimp are harvested by removing the air tube from the funnel to allow the hatched eggs to settle to top of the funnel, the unhatched eggs float (5min).

Feeding Schedule

Weekday (Monday – Friday) Feeding:

Fish are fed twice per day as follows:

AM: Mast Dry Mix food to all adults in 3L and 10L tanks, Mast Dry Mix food to juveniles (2-3 weeks post fertilization and older), and powdered fry food (Golden Pearls) for fry.

PM: All adults are fed brine shrimp. Juveniles are fed brine shrimp and dry food. Larvae are fed powdered larvae food.

NOTE: Do not feed fish in the 1.5L tanks dry food. 1.5L tanks are fed a small pinch of flakes, 1/8 tsp for 3L tanks, and ¼ - ½ for 10L tanks.

Weekend Feeding:

On weekends fish are fed once per day according to the PM parameters from the weekday schedule and detailed protocol in APPENDIX 6.

CLEANING & EQUIPMENT SANITIZATION

Nets

Nets are washed after each use by soaking in net cleaning solution (labeled containers are on the counter of each fish room). A separate net should be used for each family (i.e. AE #), to avoid potential contamination.

Tanks / Lids

Plastic tanks should allow easy observation of the fish. Growth of algae on the tank walls is expected and a small amount is tolerable, however, excessive growth impedes observation of the fish. Florid algal growth may also indicate a water quality issue, such as an excess of nutrients (nitrogen, phosphorous). Furthermore, the algal mass contributes to the biofilm, which may harbor pathogenic microbes and parasites as well as harmless environmental organisms.

Fish tanks should be inspected during feeding and changed whenever filter pads require changing or when visible algal growth covers 10-20% of the tank surface.

Otherwise, all tanks (even unoccupied ones) should be rotated out of service and sanitized on a regular quarterly schedule (every 3 months).

Tank lids accumulate food residue quickly, which serves as a resource for vermin. Tank lids must be hand cleaned daily as needed and rotated out of service for cleaning along with the tanks.

Sanitization methods

Hand washing (spot cleaning, as needed) – hot water (**no detergents**) and dilute bleach for screens.

Laboratory Dishwasher – cross boxes are hand washed and then washed sanitized with 80 C rinse water daily. Tanks are routinely sanitized in this manner when fish are removed for breeding or fin clipping. Additionally, tanks not regularly removed from the system will be cleaned on a rotating schedule (once every 3 months) in the laboratory dishwashers (80 C wash and rinse) for

sanitization. Each rack of a system (including shelves) will be scheduled for cleaning once every 6 months.

Room Cleaning & Sanitization schedule.

Fish rooms should be free of clutter and debris. Room should be swept as needed and mopped weekly.

BREEDING

Setting Up Crosses

Crosses should be set up as late as possible (not before 3pm) in the dedicated cross containers. Place an insert in the cross box and fill with system water (about $\frac{3}{4}$ full or just above the first “step” of the insert). Place a male and female fish in the box, and label using your colored tape with corresponding stock numbers (Female AE# x Male AE#). If fish are to remain separate overnight (to ensure that synchronous clutches are obtained), place a divider in the cross box to separate the fish.

Cover the cross box and place on the shelf – crosses should not be left on the counters to keep the space clear for others to work. Any fish in the tank that are not being crossed should be transferred to a clean tank. Wash the “dirty” tank to prevent algae buildup and place it on the cart for sterilization.

Note: A separate net should be used for each family (i.e. AE #), to avoid potential contamination. Nets are washed after each use by soaking in net cleaning solution (labeled containers are on the counter of each fish room).

Collecting crosses

Use plastic petri dishes and 1X egg water or 1X Methylene Blue Embryo Medium to collect eggs from crosses. Prepare labels for all successful crosses (one label should stay with the fish and the second is for the petri with the eggs). Collect one cross at a time carefully in order to prevent any mix-ups. After you have collected your crosses, wash the cross boxes with warm water and place on the cart for sterilization. If the cart is full, take it to the kitchen. All crosses should be taken apart no later than 1PM to ensure adequate time for the cross boxes to be sterilized and returned to the facility.

Raising progeny

After the embryos have been screened, sort them into plates of 40, bleach the embryos according to the bleaching protocol (pg), and place them in the incubator until they are ready to be introduced to the fish facility. Create a new AE number and label, which includes the strain information, genotype, date of collection (“birth date”) and number of fry. Add this new information to the Zebrafish Stocks database.

Fry should start receiving Tetra AZ food on day 5. Fill 3L tanks (with a '300' screen) about 1-2 inches full with Methylene Blue Embryo Medium or system water (you will need one tank per plate of embryos). Insert the plate into the embryo medium, ensure that all of the larvae are transferred, then transfer the stock label to the tank and cover it. These tanks should then go in the dedicated 'Fry/Baby Area' of the facility; so, that they receive the appropriate diet and care.

Do not start water flow to the tanks – this will disrupt the coat of powdered food on the water surface and decrease the efficiency and duration of feeding for the fry. Over the next week, gradually raise the water by about an inch/ 2 days to provide fresh water to the fry. When the fry are 10 days to 2 weeks (depending on their size) start the drip and switch the label to add shrimp to their diet. When the fry are 1 month old (depending on their size i.e. when they are larger than the holes in the screen), transfer them to a clean tank with a "1000" baffle to ensure adequate water flow. At this time, tanks with 40 fry should be split into two tanks of 20 to ensure optimal growth.

Identification of new stock

Every tank containing new stock must be labeled. Every label should be dog-eared and include the following:

- 1) AE# - Every new stock receives a new AE#. (Check the Stock database for the next available number.)
- 2) Allele
- 3) Number of fish
- 4) Plate number
- 5) "Birth" date
- 6) Principle investigator
- 7) Source

All of this information (as well as additional details about the source of the stock and any relevant notes) should be entered into the stock database log sheet to prevent duplication of stock numbers.

Bleaching Protocol

Embryos to be raised should be bleached during gastrula stages (will still hatch on their own) or by day 1 (you will need to remove chorions by treating with pronase or manually) of development follows:

1. Prepare bleach solution.

500 mL Egg Water
350 uL Bleach

Label bottle with date prepared. Bleach solution can be kept for up to one week.

2. Soak embryos in Bleach Solution for exactly 5 minutes. Trying to bleach more than two lines at once will make it difficult to be consistent and accurate.

-Pour off original egg water and add bleach solution until petri dish is at least half filled.

-Gently swirl dish so that the embryos are evenly separated.

3. Rinse embryos in Egg Water for 5 minutes.

-Pour off bleach solution.

*****Be careful to remove all the bleach by using a pipette*****

-Add Egg Water until dish is at least half filled.

-Gently swirl dish so that embryos are evenly separated.

4. Repeat steps #2 and #3 so that embryos are bleached and rinsed a total of TWO times.

5. Rinse embryos two more times in Egg Water for 5 minutes.

6. Divide embryos into dishes of no more than 40. Manually dechorionate or treat with pronase if you bleached on day 1 as they can no longer free themselves. (Add 10 uL of 30mg/mL pronase (Thaw pronase from -20C and mixed as it does settle) to each petri dish and place into the incubator. Make sure embryos are not

clumped together, which causes retardation, rather spread them throughout the dish. Keep pronase on ice, as it will digest itself as well as lose activity.

EMBRYOS WILL NOT HATCH WITHOUT PRONASE IF BLEACHED AFTER TAILBUD STAGE. CHECK TO MAKE SURE THAT YOUR EMBRYOS HATCH AND REMOVE THE PRONASE BEFORE PUTTING THE BABIES INTO THE FACILITY

QUARANTINE

Fish brought in from the outside facilities are isolated in the quarantine room to reduce the risk of contaminating the existing stocks with infectious disease or parasites that may be present. Quarantine is used to hold fish imported from other institutions pending rederivation via breeding and collection and bleaching of embryos. Quarantine is carried out in a specifically defined zebrafish space, in Chanin 521 according to the standard operating procedure recommended by the zebrafish research community.

Once eggs are obtained from quarantined adults, the eggs are bleached according to standard published protocols (above) and then transferred to the regular fish room to grow to adulthood.

ISOLATION FOR STUDIES WITH CHEMICAL & BIOLOGICAL AGENTS

(Toxicologic, Infectious Disease, Oncology, Transplantation studies)

Because the standard aquatic housing systems share recirculated water through a common filter bank, experimental treatment of individual groups of adult fish in regular housing systems is not possible.

Therefore to isolate individual cohorts for treatment, they must be kept in individual aquaria that are not connected to the system. Isolation tanks can be static or actively filtered using small one tank filter set ups. If fish are maintained in static tanks, the water must be changed daily.

A designated area in the laboratory or quarantine system should be used to hold isolation aquaria and appropriately signed to indicate isolation for chemical and/or biological agent exposure. Volume of water in treated tanks should be minimized to reduce the amount of water that must be decontaminated or held for disposal.

Tanks containing treated water or fish must be appropriately labeled with the chemical agent and date(s) as well as PI and contact information. MSDS and other safety information about the chemical in use, must be readily available in the room or designated area. Safety information must include proper method of storage, deactivation and disposal of the agents in aqueous solution.

Tanks containing fish exposed to BSL-2 agents (infectious agents or human tissues) must be labeled with the agent and date(s) as well as PI and contact information. Readily available Safety information must include proper method of storage, deactivation and disposal of these agents in aqueous solution.

Note: Methods for handling of chemical / biological agents used in fish, proper personal protective equipment and practices, methods for treatment / disposal of contaminated water, holding times for fish that have been exposed to biological/chemical agents, and methods for decontaminating / cleaning aquaria used in these studies must be reviewed and approved by EH&S and the IACUC.

HEALTH MONITORING & VETERINARY CARE

Aquatic organisms and aquatic systems (biofilm) may harbor fish parasites and microbes that may impact / interfere with research outcomes, affect animal health (morbidity and mortality), and may even pose a risk to humans. For example, of specific concern are the pathogenic and zoonotic mycobacteria. There has been significant progress in understanding the potential adventitious pathogens that affect zebrafish and diagnostic panels are available for monitoring (Appendix 8).

The most effective means of identifying pathogens and adverse environmental conditions to prevent spread of disease and limiting morbidity is to systematically remove fish exhibiting behavioral or physical abnormalities of unknown etiology. Fish are monitored several times per day, during each feeding, during cleaning, setting up of crosses, or whenever individuals are in the fish room. If a fish showing behavioral or physical abnormalities (See Appendix 7) is encountered, the location of the “sick” fish is marked and the fish is removed immediately or as soon as possible. Log and report all morbidities and mortalities to an animal technician, Dr. Marlow, or the veterinarian immediately. All morbidities and mortalities, the system/location of the associated phenotypes (e.g. any symptoms observed, sporadic jumpers/ fish found on the floor (these fish are euthanized), or if the fish was euthanized for experimental purposes are recorded in the Morbidity and Mortality Log maintained in Ch523 (Appendix 7).

While sporadic losses may be expected in any large fish facility mass or clustered morbidity and mortality events should be investigated thoroughly. Unexpected morbidity and mortality must be investigated. However, even when morbidity and mortality is an expected outcome of an experimental protocol, initial cases should be examined to confirm the cause is as expected.

Dead fish should be removed from the tanks and frozen for disposal or fixed in neutral buffered formalin for pathological examination. Sick and symptomatic fish should be removed from the tanks, isolated. Log and report all morbidities and mortalities to an animal technician, Dr. Marlow, or the IAS veterinary services immediately. Once abnormal behavior (abnormal swimming behavior, elevated scales, bloated abdominal region, skin lesions, or skin sores not caused by a recent mating) has been noted, the fish may be euthanized and

presented for post mortem examination. Fresh (iced) or neutral buffered formalin fixed specimens may be presented for veterinary services. To get the most useful information from dying fish, it is best to present live or fresh (iced) animals to the veterinary services for examination. Freezing distorts histologic structure; however, tissues from fresh specimens can be frozen for molecular diagnostics (PCR).

On a routine basis (biannually), a sample of fish (culls) from each separate system should be submitted for diagnostics, including PCR, and histopathology screening. The number of samples from each system will be developed based on findings, as the power of detection and the required sample size varies with prevalence. Initially, a pooled sample of 10 individual adults from each system will be tested. Environmental samples may also be collected from the filter media and used tanks.

PCR based diagnostics will be conducted in house for the known zebrafish pathogens as specified in Appendix 8. Alternatively, when in-house capabilities are down, samples will be submitted to ZIRC. See (<http://zebrafish.org/zirc/health/index.php>) and (<http://zebrafish.org/zirc/documents/protocols.php#ZIRC%20Health%20Monitoring%20SOPs>) for their health monitoring SOPs. ZIRC also offers full service histopathology training and support to investigators conducting in house monitoring programs (<http://zebrafish.org/zirc/documents/fees.php#pathology>). Cost of testing will be billed to the Core.

The purpose of this biannual testing is to establish a knowledge base of what potentially impactful infectious agents are present in our facilities and to maintain surveillance against any new introductions. Although it is difficult to eradicate agents that are established in existing systems, this information can serve to guide planning and decisions either when new aquarium systems are established or when there is an opportunity to depopulate, break down, and reestablish an existing system.

In addition to routine health monitoring (above) the veterinary services are available to help investigate and diagnose causes of unexpected fish losses.

Euthanasia

All sick fish, those showing abnormal swimming behavior, elevated scales, bloated abdominal region, skin lesions, or skin sores, any fish that is healthy but no longer reproductively fit, or a fish for experimental purposes (e.g. dissection) are euthanized according to NIH guidelines found at <http://oacu.od.nih.gov/ARAC/documents/Zebrafish.pdf> and are inserted below (pgs 11-15):

Guidelines for Use of Zebrafish in the NIH Intramural Research Program

Scientific Background

These guidelines are predicated on the need to minimize suffering and distress in zebrafish. Suffering requires that the animal have both the neural apparatus for detecting noxious stimuli as well as the mental ability to interpret such stimuli as aversive (1). Many studies have demonstrated that adult zebrafish show evidence of higher order cognition, being responsive to a variety of learning protocols (e.g. 2, 3,4, 5), including learning to avoid aversive stimuli (6, 7, 8, 9). Thus while the ability of adult fish to experience suffering remains controversial in the scientific literature [for recent reviews reaching conflicting opinions see (10) and (11)], there is sufficient evidence to take a cautious approach in adult zebrafish by instituting guidelines that ensure rapid euthanasia.

In contrast there is no evidence of higher order cognition in zebrafish during the first week of development. Developmental studies examining learning (12), reward (13), social (14, 15) and fright (16) behaviors have found that these functions become operational only in older fish. During the first week of development, embryonic movements are simple reflexes that do not provide evidence for a capacity for suffering. Thus during the first week, zebrafish larvae can respond to simple stimuli but have not reached the point in brain development where stimuli can be experienced as aversive.

Zebrafish larvae during the first week resemble early mouse embryos in that they are chiefly sustained by nutrients derived from the yolk. The criterion of nutritional independence for developmentally immature animals is subject to empirical verification and has found support in international regulations for the welfare of immature vertebrates (17). While the capacity for suffering is the primary criterion for establishing a threshold for 8 days post fertilization (dpf) for euthanasia in zebrafish, the criterion of independent feeding also supports this age.

Hatching occurs at approximately 72 hours (which would be at the end of day 3 post fertilization), although hatching is not an accepted staging index in zebrafish (18).

Zebrafish larvae are not able to feed upon hatching and are chiefly sustained by nutrients derived from yolk which is not depleted until 7 dpf (19). Only after 7 dpf do zebrafish larvae manifest signs of ill health in the absence of external feeding (20).

Active feeding can not commence at hatching because brain structures required for detecting and catching prey have not developed. At hatching, larvae lack taste buds (21, 22), have poor visual acuity (14), and cannot swim effectively as they lack a swim bladder and have deficient motor control (23, 24). Therefore in zebrafish the period between hatching and nutritional independence at 8 dpf is essentially an extension of the early embryonic stage during which the fish continues to develop sensory and motor functions required for the independent larval stage.

Thus during the first week of development, zebrafish remain in an immature state consuming yolk for nourishment and responding to stimulation with simple reflexive movements. As larvae become nutritionally independent during the second week of life, they also acquire more sophisticated cognitive abilities. 2

Euthanasia Guidelines

Euthanasia of zebrafish must be carried out by one of the following methods. Although not described in the AVMA Euthanasia Guidelines, these procedures have been shown effective for euthanizing zebrafish (25).

A. For zebrafish ≥ 8 dpf the following methods are acceptable for euthanasia:

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

2. Overdose of tricaine methane sulfonate (MS222, 200-300 mg/l) by prolonged immersion. Fish should be left in the solution for at least 10 minutes following cessation of opercular movement.

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

4. Decapitation with a sharp blade by a trained individual when its use is required by the experimental design and approved by the IC Institutional Animal Care and Use Committee (26).

B. For zebrafish 4-7 dpf the following methods are acceptable for euthanasia (27):

1. Immobilization by submersion in ice water (5 parts ice/1 part water, 0-4° C) for at least 20 minutes to ensure death by hypoxia.

2. Addition of bleach solution (sodium hypochlorite 6.15%) to the culture system water at 1 part bleach to 5 parts water. They should remain in this solution at least five minutes prior to disposal to ensure death. As detailed above in the scientific

background section, pain perception has not developed at these earlier stages so this is not considered a painful procedure.

3. For embryos \leq 3dpf, development should be terminated using bleach as described in section B2 above.

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

General Guidelines

Current OLAW interpretation of PHS policy considers aquatic species as "live, vertebrate animals" at hatching. Although this is an imprecise stage for zebrafish it can be approximated at 72 hours post fertilization. For purposes of accountability all stages of development greater than three days of age should be described in an approved Animal Study Proposal. Thus an estimate of the number of larval zebrafish from day 4 - 7dpf should be included in Animal Requirements (Section B in the NIH ASP form).

Since these early stages (4-7dpf) do not feel pain or distress, it is preferable that their numbers be separated from zebrafish ≥ 8 dpf. This number can be listed as Column C in the Pain and Distress Category (Section H) of zebrafish ASPs as a separate number from zebrafish ≥ 8 dpf.

The pain and distress categorization of the ≥ 8 dpf fish should be determined by the investigator based on the specific procedures described in the protocol. The number of animals used may need to be provided as an estimate, particularly with these young larvae, considering their size and normal housing conditions. Estimated numbers may still be used after they have matured to adults if they are group housed.

References

1. Sneddon LU, Braithwaite VA, & Gentle MJ (2003) Do fishes have nociceptors? Evidence for the evolution of a vertebrate sensory system. *Proc. R. Soc. Lond. B* 270(1520):1115-1121.
2. Darland T & Dowling JE (2001) Behavioral screening for cocaine sensitivity in mutagenized zebrafish. *Proc Natl Acad Sci U S A* 98(20):11691-11696.
3. Colwill RM, Raymond MP, Ferreira L, & Escudero H (2005) Visual discrimination learning in zebrafish (*Danio rerio*). *Behavioural Processes* 70(1):19-31.
4. Risner ML, Lemerise E, Vukmanic EV, & Moore A (2006) Behavioral spectral sensitivity of the zebrafish (*Danio rerio*). *Vision Res* 46(17):2625-2635.
5. Braubach OR, Wood HD, Gadbois S, Fine A, & Croll RP (2008) Olfactory conditioning in the zebrafish (*Danio rerio*). *Behav Brain Res* doi:10.1016.
6. Pradel G, Schachner M, & Schmidt R (1999) Inhibition of Memory Consolidation by Antibodies against Cell Adhesion Molecules after Active Avoidance Conditioning in Zebrafish. *Journal of Neurobiology* 39(2):197-206.
7. Pradel G, Schmidt R, & Schachner M (2000) Involvement of L 1. 1 in memory consolidation after active avoidance conditioning in zebrafish. *Journal of Neurobiology* 43(4):389-403.

8. Rawashdeh O, de Borsetti NH, Roman G, & Cahill GM (2007) Melatonin Suppresses Nighttime Memory Formation in Zebrafish. *Science* 318(5853):1144.
9. Shcherbakov D, et al. (2005) Magnetosensation in zebrafish. *Current Biology* 15(5):161-162.
10. Braithwaite VA & Boulcott P (2007) Pain perception, aversion and fear in fish. *Diseases of Aquatic Organisms* 75(2):131.
11. Rose JD (2007) Anthropomorphism and 'mental welfare' of fishes. *Proc. R. Soc. Lond. B* 75(2):139-154.
12. Williams FE, White D, & Messer WS (2002) A simple spatial alternation task for assessing memory function in zebrafish. *Behavioural Processes* 58(3):125-132. 4
13. Bretaud S, et al. (2007) A choice behavior for morphine reveals experience-dependent drug preference and underlying neural substrates in developing larval zebrafish. *Neuroscience* 146(3):1109-1116.
14. Clark DT (1981) Visual Responses in Developing Zebrafish (*Brachydanio rerio*). Ph.D. (Oregon, Eugene).
15. Engeszer RE, Da Barbiano LA, Ryan MJ, & Parichy DM (2007) Timing and plasticity of shoaling behaviour in the zebrafish, *Danio rerio*. *Anim Behav* 74(5):1269-1275.
16. Whitlock KE (2006) The Sense of Scents: Olfactory Behaviors in the Zebrafish. *Zebrafish* 3(2):203-213.
17. United Kingdom (1986) Guidance on the Operation of the Animals (Scientific Procedures) Act.
18. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, & Schilling TF (1995) Stages of embryonic development of the zebrafish. *American Journal of Anatomy* 203(3):253–310.
19. Jardine D & Litvak MK (2003) Direct yolk sac volume manipulation of zebrafish embryos and the relationship between offspring size and yolk sac volume. *Journal of Fish Biology* 63(2):388-397.
20. Goolish E & Okutake K (1999) Lack of gas bladder inflation by the larvae of zebrafish in the absence of an air-water interface. *Journal of Fish Biology* 55(5):1054-1063.
21. Kotrschal K, Krautgartner WD, & Hansen A (1997) Ontogeny of the Solitary Chemosensory Cells in the Zebrafish, *Danio rerio*. *Chem Senses* 22(2):111-118.
22. Lindsay SM & Vogt RG (2004) Behavioral Responses of Newly Hatched Zebrafish (*Danio rerio*) to Amino Acid Chemostimulants. *Chem Senses* 29(2):93-100.

23. Robertson GN, McGee CA, Dumbarton TC, Croll RP, & Smith FM (2007) Development of the swim bladder and its innervation in the zebrafish, *Danio rerio*. *Journal of Morphology* 268(11):967.
24. Muller U & van Leeuwen J (2004) Swimming of larval zebrafish: ontogeny of body waves and implications for locomotory development. *Journal of Experimental Biology* 207(5):853-868.
25. University of Oregon (2008) Final Report to OLAW on Euthanasia of Zebrafish.
26. Fan, S. F. & Yazulla, S. Electrogenic hyperpolarization-elicited chloride transporter current in blue cones of zebrafish retinal slices. *J Neurophysiol* 77, 1447-1459 (1997).
27. National Institutes of Health (2009) Final Report to OLAW on Euthanasia of Zebrafish

Disposal of Deceased Fish

Zebrafish carcasses should be disposed of as Medical Pathological Waste according to NIH policies. Deceased fish intended for disposal, not intended for pathological evaluation, are temporarily placed into a biohazard bag in the freezer, and subsequently delivered to the INSTITUTE FOR ANIMAL STUDIES Ullmann, room 1008 cooler for disposal.

APPENDIX 1. SUMMARY ACTIVITY SCHEDULE

ACTIVITY	FREQUENCY				
	Twice daily AM / PM	Daily	Weekly	Bimonthly	Quarterly
Observe fish	X				
Feed	X				
Check / record water quality (pH, Conductivity), system pressure	X				
Check tanks water flow, leaks, filter pads	X				
Sweep/clean floors, surfaces			X		
Change R/O filter cartridges (Mechanical & Chemical filters)				X	
Change Mechanical filters				X	
Change UV bulbs				X (as indicated)	
Clean / sanitize aquaria and lids					X
Collect culls for health monitoring					X

APPENDIX 2. GENOTYPING PROTOCOL

DAY ONE:

Anesthetize fish in Tricaine solution according to fish book protocol. Add 9 mLs of stock solution (stored in -20) per 200 mLs of fish system water. (We've setup plastic beakers for these specific measurements.)

Place adult fish in Tricaine solution until the fish is asleep – the fish will not be swimming and will be on its side at the bottom of the container, but its gills will still be moving slowly. The fish will not try to escape when touched. Place about 5 fish in Tricaine solution at a time.

Using a plastic spoon, remove fish from Tricaine solution and gently blot dry on a paper towel. Place in a large plastic petri dish.

Lay fish on its side and remove the tail tissue with a razor blade. Place the fish into a labeled single box container. The label should include the stock number and the position of the fish in the PCR plate. For example: AE12-1

A1, AE12-2 A2 (designating row and column position)

The fin goes into a PCR plate in the corresponding row and column position and is fixed in 100% MeOH. The fins can be stored in the -20 freezer overnight or up to several years (for screen purposes).

To obtain genomic fin clip DNA, digest the fin overnight at 55 degrees (can do this in the PCR machine in Lysis buffer).

First remove MeOH and allow fins to sit for 10-15 minutes to ensure that no MeOH liquid remains.

10X RAPD PCR buffer (100 mLs):

10 mL 1M Tris pH8.7

50 mL 1M KCl

1.50mL 1M MgCl₂

100mg Gelatin

10mg/mL BSA

38.5mL ultra-pure water (autoclaved)

(1 mL aliquots are stored in -20)

Lysis Buffer:

10mL 1X RAPD PCR buffer
0.3% tween 20
0.3% NP40
1 ul Prot. K solution

Add 50ul to each sample then digest overnight in the PCR machine at 55 degrees (PCR program "55")

DAY TWO:

Heat kill the reaction by incubating at 94 degrees for 10 minutes, then incubate on ice or store in -20 freezer until ready to use. (PCR program "94")

PCR (For 48 Reactions)

100ul 10X PCR buffer
610ul ultrapure water
100ul 2mM dNTP
25ul Forward primer
25ul Reverse primer
5ul Taq polymerase (choice Taq)

18ul of the above mix + 2ul genomic DNA

Use PCR program "Genotype"

94 degrees 15 sec
57 degrees 15 sec
70 degrees 30 sec
Repeat 30 times
70 degrees 3 min
4 degrees hold

Run Metaphor Gel. Prepare metaphor gel at least 2 hours ahead of time with at least 1 hour in the cold room prior to running gel. Can also prepare the gel the night before. Make sure to wrap the gel very well with saran wrap too prevent drying out.

Large Metaphor gel:

500ml 1X TBE

7.5g Metaphor agarose

7.5 g ultrapure agarose

20ul Ethidium Bromide

*Add agarose gradually or it will not go into solution!

**It's very important to pour without creating air bubbles!

Load gel with the multichannel pipet and run at 250 for about 2 hours. Once the genotype is certain, fish can go be grouped in tanks according to their AE#, genotype.

APPENDIX 3. EMERGENCY PLAN FOR SATELLITE ZEBRAFISH FACILITIES

The Zebrafish Facilities are included as satellite housing facilities under the Einstein Animal Care & Use Program and are covered under the general “**Disaster Plan for Einstein’s Animal Care & Use Program**”. This disaster plan is published as an appendix in the “**Einstein College of Medicine of Yeshiva University Emergency Procedures Manual**”.

Overview: The IAS Director (Attending Veterinarian) is a member of Einstein’s Emergency Response Group (ERG) and can thus directly communicate with and advise the Emergency Response Commander, the Institutional Official, and other senior administrators regarding animal program related emergencies as well as impacts of non-animal related emergencies on the animal care & use program and the research programs that depend on animals. All emergencies occurring in the zebrafish facilities or potentially impacting these facilities should be reported immediately to the Director of the Zebrafish Core facilities and to the IAS Director.

General Contingency Plan: In the event that any disaster is anticipated to cause or actually results in significant damage to specific facilities, the IAS Director and Zebrafish Core Director will develop a plan to relocate fish (aquaria) to safer housing locations within animal facilities on campus. If appropriate alternate locations cannot be identified, the Emergency Response Commander will be contacted and Emergency Response Group will be tasked with developing a plan to move animals to temporary shelter elsewhere on campus or off-site. In the worst case scenario where fish cannot be rescued from conditions that are a significant risk to their well-being, health, and survival as determined by the IAS Director (Attending Veterinarian) in consultation with the Zebrafish Core Director, those fish would be euthanized (provided that conditions are safe enough for humans to carry this out).

Assessment of risk for Einstein’s zebrafish facilities

Highest potential risks include general Power failure and HVAC failure resulting in loss of water circulation (filtration and aeration), as well as environmental (water) temperature regulation. Other emergencies, such as

severe storms and transit strikes, may prevent staff from coming to work to perform husbandry duties. Water recirculation equipment (pumps, filtration, etc) is on emergency backup power. Portable air conditioning units and portable heaters are available for deployment in the event of HVAC failure. Emergency staffing of the fish facility to perform minimal maintenance and feeding duties would be coordinated by the Core Director and IAS director.

Contact information:

Zebrafish Core Director: Dr. Florence Marlow (718) 430-4208

Institute for Animal Studies Director: Dr. Lawrence Herbst (718) 839-7135

APPENDIX 4. FACILITIES DIAGRAMMATIC FLOORPLANS

Chanin Facility Capacity/Map

Chanin Facility System

A60	B60	C60	D60
A50	B50	C50	D50
A40	B40	C40	D40
A30	B30	C30	D30
A20	B20	C20	D20
A10	B10	C10	D10

E60	F60	G60
E50	F50	G50
E40	F40	G40
E30	F30	G30
E20	F20	G20
E10	F10	G10

09H	09I	01I
05H	05I	01H
04H	04I	02H
03H	03I	02I
02H	02I	03H
01H	01I	03I
09H	09I	01I

K60	L60	M60	N60	O60
K50	L50	M50	N50	O50
K40	L40	M40	N40	O40
K30	L30	M30	N30	O30
K20	L20	M20	N20	O20
K10	L10	M10	N10	O10

Ullman Facility Capacity/Map

.....

Ullmann 504 Facility System

2E	Ozbudak	1E	Ozbudak
2D	Ozbudak	1D	Ozbudak
2C	Ozbudak	1C	Ozbudak
2B	Ozbudak	1B	Ozbudak
2A	Ozbudak	1A	Ozbudak

14 small tanks per row

20 small tanks per row

	VE	4A
	BE	4B
	CE	4C
	DE	4D
	EE	4E

20 small tanks per row

14 small tanks per row

5E	GE
5D	GD
5C	Common WT
5B	Common WT
5A	6A

20 small tanks per row

14 small tanks per row

	7A	8A	9A
	7B	8B	9B
	7C	8C	9C
	7D	8D	9D
	7E	8E	9E

14 small tanks per row

14 small tanks per row

14 small tanks per row

10E	11E	12E
10D	11D	12D
10C	11C	12C
10B	11B	12B
10A	11A	12A

14 small tanks per row

14 small tanks per row

14 small tanks per row

	13A	Babies	20 small tanks per row
Juveniles	13B	Babies	20 small tanks per row
Juveniles	13C	Babies	20 small tanks per row
Juveniles	13D	Babies	20 small tanks per row
Juveniles	13E	Babies	20 small tanks per row

14 small tanks per row

14 small tanks per row

14 small tanks per row

14 small tanks per row

14 small tanks per row

APPENDIX 6: Detailed protocol for weekend feeding.

1. Start a new shrimp culture for the next days feeding: 7.5 liters of sea water + 15g shrimp. Label the new culture with the date and time the culture was started.
2. Remove air tube to allow the hatched shrimp to settle to the bottom of the funnel for harvesting as described above.
3. Meanwhile, feed fry and juveniles a pinch of AZ powder (use 5ml pipette to disperse the powder and coat the surface with a thin film of food). Larvae on an active drip and juvenile days 15-21 are fed both AZ and brine shrimp.
4. Collect the bottom fraction of the funnel where the hatched shrimp are located (orange layer) into a beaker (Do not collect the transparent or upper layer (brown) where the floating hatched eggs are located as the fish cannot digest brine cysts/shells), drain through a shrimp net, wash with dH₂O and rinse back into the beaker and bring the volume to 500mL with dH₂O. To dilute the shrimp for feeding transfer to 500ml squeeze bottle, add shrimp to fill ¼ of the bottle and top off with distilled water.
5. Dispense shrimp from squeeze bottle into each tank in the juvenile and adult areas and in tanks with larvae with active water flow (approximately 500uL for 3-liter tank containing 6-12 fish, a few drops (100-200uL) for fewer (1.5 L) or 1mL for fish in 10L tanks).
6. Rinse shrimp hatchery with distilled water, scrub with brush and allow to dry.
7. Perform a system parameters check and fill out and initial the system parameter logs posted in each system (see appendices x-y below).

APENDIX 7: Zebrafish International Resource Center Table of behavioral and physical abnormalities

From:

http://zebrafish.org/zirc/documents/protocols/pdf/health_monitoring/daily_monitoring_fish_morbidity.pdf

Behavioral Abnormalities	Physical Abnormalities
Fish at surface or near water inlet Rapid breathing/opercular movements Sluggish movements/lethargy Flashing/rubbing on tank surfaces Circling, twirling, spinning Loss of equilibrium	Color change Weight loss Exophthalmia/pop-eyes Distended abdomen Skeletal deformity Mass/swelling Hemorrhage/redness Gas bubbles Protruding scales Fin erosion or lesion Skin ulceration or lesion

APPENDIX 8: Einstein Facility Morbidity and Mortality Log

APPENDIX 9: Zebrafish Pathogen Profile assays and references:

Pathogen	Type of Screen	Primers	Product Size	Source
Pseudoloma neurophilia	PCR assay	PNA_03: 5' TGA AAT GTG GTG ACC CGT TTA GG 3'	441bp	ZIRC P. neurophilia
		PNA_04: 5' TCC TTG ACC CAT CCT TCC TGT G 3'		
Mycobacterium	PCR Assay	T39 (5'-CGAACGGGTGAGTAACACG-3')	900bp	Zebrafish Mycobacteriosis. Kent et al.
		T13 (5'-TGCACACAGGCCACAAGGGA-3')		
Pleistophora hyphessobryconis	PCR Assay	V1F (5'-CAC CAG GTT GAT TCT GCC TGA C-3')	1224bp	P. hyphessobryconis. Saunders et al.
		PleistR (5'-TCT CGC TTG TTC GCG CCT GA-3')		
Ichthyophthirius multifiliis	PCR Assay	IMRf1 (5'- AGTGACAAGAAATAGCAAGCCAGGAG-3')	190bp	I. multifiliis. Jousson et al.
		IMRr1 (5'-ACCCAGCTAAATAGGCA- GAAGTTCAA-3')		
ISKNV	PCR Assay	(5'-GCCACGCCACCACCTTCTATAAC-3')		ISKNV Xu et al.
		(5'-TGTCGCTTGCCCAAACAATCTTC-3')		
		(5'-ATCCCTCCATCACATCCAGCAAG-3')		
		(5'-CATgCAggCgTTCCAgAAgTCAAg-3')		
Piscinoodinium pillulare	IHC			ZIRC P. pillulare
Pseudocapillaria tomentosa	IHC			ZIRC Capillariasis

- *Mycobacterium*
 - Mycobacteriosis in zebrafish (*Danio rerio*) research facilities. Kent et al.
 - <http://dx.doi.org/10.1016/j.cca.2004.08.005>
 - <http://www.sciencedirect.com/science/article/pii/S1532045604001413>
- *Pseudoloma neurophilia*
 - Polymerase Chain Reaction Detection of *Pseudoloma neurophilia*, a Common Microsporidian of Zebrafish (*Danio rerio*) Reared in Research Laboratories. Whipps et al.
 - <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1435373/>
- *Pseudocapillaria tomentosa*
 - Kent, M. L., Bishop-Stewart, J. K., Matthews, J. L., and Spitsbergen, J. M. (2002b). *Pseudocapillaria tomentosa*, a nematode pathogen, and associated neoplasms of zebrafish (*Danio rerio*) kept in research colonies. *Comp. Med.* 52, 354-358
- *Piscinoodinium pillulare*
 - Noga, E. 2010. *Fish disease: diagnosis and treatment*. Mosby Electronic Publishing. St. Louis
- *Pleistophora hyphessobryconis*
 - Pleistophora hyphessobryconis (Microsporidia) infecting zebrafish *Danio rerio* in research facilities. Saunders et al.
 - <http://www.int-res.com/articles/dao2010/91/d091p047.pdf>
- *Ichthyophthirius multifiliis*

- Non-invasive detection and quantification of the parasitic ciliate *Ichthyophthirius multifiliis* by real-time PCR Jousson et al.
- <http://www.int-res.com/articles/dao2005/65/d065p251.pdf>
- ISKNV (Infectious spleen and kidney necrosis virus)
 - A zebrafish (*Danio rerio*) model of infectious spleen and kidney necrosis virus (ISKNV) infection. Xu et al.
 - <http://www.sciencedirect.com/science/article/pii/S004268220700829X>
 - <http://dx.doi.org/10.1016/j.virol.2007.12.026>