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Note: Text has been edited for clarity.

Contents:

Zebrafish 101 for IACUCs

Speakers:
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George E. Sanders, DVM, MS, University of Washington
George Babcock, PhD, University of Cincinnati and OLAW
Doreen Bartlett, OLAW

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View Recording: http://youtu.be/hu9aVjCIUpo (YouTube).

Slides 2 (Zebrafish 101 for IACUCs)
>>*Babcock*: Today is March 12, 2015. I am Dr. George Babcock and it is my pleasure to welcome Monte Matthews and George Sanders to OLAW Online Seminars to present **Zebrafish 101 for IACUCs.**

Dr. Sanders holds a Veterinary degree from Louisiana State University and a Master of Science degree in Comparative Medicine from the University of Washington. In 2003, he was certified as a fish pathologist by the American Fisheries Society, Fish Health Section. Dr. Sanders is currently the Aquatic Animal Program director at the University of Washington in Seattle.

Mr. Matthews holds a BA from the University of Oregon [UO], where is currently the Director for Animal Care Services. He is also Co-Director, Vice President, and a core faculty member of the IACUC 101 series program and a founding member of the Certified Professional IACUC Administrator, or CPIA, program of PRIM&R.

Slide 3 (Zebrafish 101 for IACUCs)
>>*Matthews*: Thank you for inviting us to participate in this webinar. My part of this presentation will focus on strategies for training and educating IACUC's about zebrafish management and care, under what circumstances are institutions required to have a protocol, and the requirements and expectations for counting zebrafish.

Slide 4 (Institutional Oversight: Recommendations for Selecting IACUC Members)
The most common mistake I find at institutions that are struggling to integrate zebrafish into their animal care and use programs is not having zebrafish researchers be members on their IACUC. The concept of self-regulation, largely through the establishment of IACUCs, is the hallmark of the US system of animal care and use oversight. Self-regulation is required by federal law and provides us a great deal of strength and flexibility in creating each of our programs of animal care and use. I strongly recommend appointing zebrafish researchers to the IACUC, and you may even want to consider having senior zebrafish animal care personnel appointed as either voting or non-voting members. At the University of Oregon, we have adopted the criteria shown on the slide for selecting IACUC members. These criteria go beyond the PHS Policy requirements for IACUC membership.

Slide 5 (How to Foster a Team Approach to Zebrafish Oversight)
Here are some mechanisms, other than making them members of the IACUC, we use to integrate zebrafish researchers into our animal care program, to include them as part of the team.

- Solicit their input in developing and reviewing husbandry and veterinary care standard operating procedures, such as standardization of anesthesia and euthanasia techniques, transportation of animals, policy for single housing of zebrafish, and scientific criteria for assessing pain, distress and discomfort.
- Invite new zebrafish Principal Investigators to IACUC meetings to establish rapport with the IACUC.
- Incorporate experienced zebrafish researchers into your training program. For certain technical procedures they are often the most appropriate to conduct the training.
- During semiannual inspections we have found it extremely helpful to ask questions, such as, how can the IACUC help support your research? We have found researchers more than willing to share their thoughts on how to improve our processes and even garner help from the administration for financial support to help with facility improvements.

Slide 6 (IACUC Training and Education Resources)
At the University of Oregon, we have developed a specific IACUC member handbook that includes standard materials, such as the PHS Policy, the *Guide*, the AWA Regulations, our own PHS Assurance, AAALAC Program Description, institutional IACUC policies, our zebrafish animal use application or protocol template form, and the new *AVMA Guidelines for the Euthanasia of Animals* (2013) which includes a lot more information on aquatic animals that George will be addressing shortly.

In addition we have some zebrafish specific references, such as the Zebrafish Book developed at the University of Oregon. This book is freely available on the Web and includes numerous experimental techniques that IACUC members can refer to when the investigator lists specific procedures in their protocol. You can find the Zebrafish Book and

other zebrafish specific SOPs by going to the Wiki link provided on this slide.

There are on-line training modules on zebrafish husbandry and care offered by both CITI and the AALAS Learning Library. There are also several day-long workshops on aquatics including zebrafish husbandry and veterinary care offered by MDI Biological Laboratory in Bar Harbor, Maine and the Zebrafish course offered by Gadsden State Community College and the University of Alabama at Birmingham.

Slide 7 (Zebrafish-specific Training and Education Resources)
There are several good general references on zebrafish health and husbandry, including The Laboratory Zebrafish by Dr. Claudia Harper and Christian Lawrence, volume 53, number 2 issue of the ILAR Journal, and several articles in Lab Animal dedicated to zebrafish care and use. These citations will be listed in the reference section at the end of the webinar.

Slide 8 (Training and Education)
We are required to follow the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training. These 9 basic, yet elegant principles are the ethical foundation for the work we do with all live vertebrate animals. These principles can be found on page 4 of the PHS Policy and pages 199-200 of the *Guide*.

Slide 9 (Training and Education: Principle IV)
For example, Principle number IV states: “Proper use of animals, including the avoidance or minimization of discomfort, distress, and pain when consistent with sound scientific practices, is imperative. Unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in other animals.”

Several years ago at the University of Oregon, our IACUC was reviewing one of our procedures for mutagenizing zebrafish with ENU, ethyl nitrosourea. ENU has been used on mice and fish for many years to make mutations and establish mutant lines for studying gene function. At that time, administering ENU to male zebrafish resulted in about 50% mortality. These male zebrafish seemed to go through a hypersensitive phase after treatment, which is when we were observing this mortality.

Back then, and even to this day, there are no current scientific studies that have demonstrated whether or not ENU treatment causes pain, distress, or discomfort in zebrafish. Whether or not fish can experience pain, distress or discomfort is still being debated today, but most of the current scientific studies being done now demonstrate that fish do have the anatomical, physiological and behavioral characteristics for sensing pain, distress, or discomfort. This capacity to sense painful, distressful or discomforting stimuli may not be qualitatively the same as it is for humans or mammals, but nevertheless, we gave the benefit of the doubt to the fish.

So we asked ourselves, if we as humans were subjected to this ENU procedure and 50% of us were dying, then we could reasonably assume that there would be some degree of pain, distress or discomfort involved. So now we had a duty, an imperative, to try and find a way to avoid or minimize any pain, distress or discomfort for these male zebrafish undergoing ENU treatment. We suggested MS-222, a common anesthetic used for fish, be used during treatment. There was some discussion about whether or not the use of MS-222 would interfere with the mutagenic properties of ENU. So what did we do? Well, we conducted a pilot study and found that using a low dose of MS-222, about 10mg/L, and making several husbandry adjustments to minimize disturbance of the fish undergoing treatment, increased our survival rate from 50% to over 90%.

Slide 10 (Training and Education)
This pilot study eventually became a part of the publication in Lab Animal [Vol. 40:11 (2011)] on ENU mutagenesis in zebrafish by Dr. Bill Trevarrow who at that time was from the University of Oregon and who worked out most of the procedures for conducting successful ENU treatments. This is a pragmatic example of how we were successful in utilizing a general, ethical principle to help improve animal welfare as well as the science.

Slide 11 (Is a Protocol Necessary? Definition of Animals)
When is a protocol necessary? The PHS Policy defines animal as “any live, vertebrate” and the *Guide* gives further guidance on the use of aquatic animals. As a vertebrate animal, the use of zebrafish for PHS-supported research, testing or teaching requires a protocol be reviewed and approved by the IACUC.

Slide 12 (Is a Protocol Necessary?)
But does this requirement for a protocol include all developmental stages of zebrafish? Neither the PHS Policy nor the *Guide* specifically addresses this. Fortunately OLAW has published guidance on this topic and their guidance states that all larval forms are covered, which for zebrafish, occurs when the embryo hatches from its chorion, usually around 3 days post fertilization. We usually abbreviate this as dpf. This timeframe varies somewhat depending on the strain of zebrafish and the temperature used for raising the fish. [OLAW FAQ A5]

Slide 13 (Early Larval Stage Characteristics)
Here are some early larval stage developmental characteristics that occur after hatching, and are additional reasons why we are concerned about their care:
The swim bladder is inflated by 4-6 days and they are almost free swimming. Active feeding begins to occur around day 4, and both startle and optokinetic responses are present.

Slide 14 (Is a Protocol Necessary?)
At the University of Oregon, we cover all stages of zebrafish development, including embryos, for the following reasons:

- The use of zebrafish post hatching is clearly covered under PHS Policy and requires an IACUC approved protocol. The number of adults required for breeding will depend on the number of embryos needed for experiments. Hence, to adequately justify the number of adults used for breeding should require estimation and justification for the use and number of embryos needed for the experiments.
- Embryos turn into larvae, which turn into juveniles, etc. If you already have an IACUC approved protocol in place for embryos, then you won't find yourself out of compliance if all of a sudden you went beyond the embryonic or hatching stage.
- If you are out of compliance without a valid protocol, then the consequences can be severe. Not only is this now a reportable event, but you also could potentially be required to give money back to the funding agency.
- We consulted with university counsel and their recommendation was to cover embryos.
- What about husbandry standards for the embryo? E.g., embryo media, water temperature, water quality, etc. Who will be reviewing those standards of care if you don't have a protocol, or at least standard operating procedures?
- UO has a standardized zebrafish protocol template, which makes it relatively easy for investigators to fill out by incorporating standardized procedures for working with embryos and other stages of zebrafish development.
- The utilization of zebrafish embryos represents a replacement of not only a higher vertebrate animal with a lower one, but the use of an early developmental stage. Documenting these studies with IACUC approvals that are protocol specific, that describe "…their relevance to human or animal health, the advancement of knowledge, or the good of society" [US Government Principle II], is an important message that should be documented and communicated to the general public and the funding agencies.

Slide 15 (Is a Protocol Necessary?)
If your institution doesn't cover the embryonic stages of development, that is less than 3 days post fertilization or prior to hatching, then it is a good idea for you to have a policy or procedures in place in cases where there may be animals that hatch unexpectedly so that your institution isn't out of compliance with OLAW's guidance.

Slide 16 (Is a Protocol Necessary for Field Studies?)
This is a photo of one of our researchers, with his collaborator, collecting zebrafish in the wild. Is a protocol necessary for this field study? If the activities are PHS-supported and involve vertebrate animals then the IACUC is responsible for oversight. IACUCs must know where field studies will be located, what procedures will be involved, and be sufficiently familiar with the nature of the habitat to assess the potential impact on the animal subjects. Studies with the potential to impact the health or safety of personnel or the animal's environment may need IACUC oversight, even if described as purely observational or behavioral. The IACUC must also ensure compliance with the requirements of pertinent state, national and international wildlife regulations. [OLAW FAQ A6]

Slide 17 (Is a Protocol Necessary?)
And what about field studies that are funded by the National Science Foundation [NSF]? Proposed projects involving use of any vertebrate animal for research or education must be approved by the IACUC. The institution should also follow recommendations specified in the *Guide* and the taxon specific guidelines approved by the American Society of Ichthyologists and Herpetologists. [NSF Grant Proposal Guide, Chapters II.D.7. and VI.B.3.]

Slide 18 (Is a Protocol Necessary for Field Studies in Foreign Countries?)
Is a protocol necessary for conducting PHS or NSF supported activities in a foreign country? The answer is yes. If your institution receives support from PHS and/or NSF that includes conducting research in a foreign country, then your institution is responsible and there should be IACUC approval. [OLAW FAQ D13]

Slide 19 (Is a Protocol Necessary for Collaborations?)
Is a protocol necessary for collaborating institutions? Yes, although if both institutions have a domestic, PHS Assurance, there is no federal requirement for both institution's IACUC to review and approve the protocol. IACUCs may choose which IACUC will perform the review. However, it is recommended that both committees maintain documentation of the review.

There should also be a formal written agreement that defines which institution is responsible for the care of the animals, who owns the animals, and which IACUC has the responsibility for protocol review and program oversight. Institutions should have a policy that defines how they will address collaborations. [OLAW FAQ D8]
□
Slide 20 (Counting Zebrafish)
Finally, what about counting zebrafish? PHS Policy requires that protocols specify a rationale for the approximate number of animals to be used. It also requires that investigators limit the number to obtain valid results. Institutions have some flexibility in how they track animal usage. At the University of Oregon we have a database that tracks how many zebrafish we have at any given time and the approximate numbers of animals to be used are described in the protocol.

Slide 21 (Zebrafish 101 for IACUCs)
Now it is my pleasure to turn the podium over to my colleague, Dr. George Sanders.

Slide 22 (Zebrafish are NOT Aquatic Mice)
>>*Sanders:* Please understand that zebrafish are not aquatic mice and should not be treated as such!

Slide 23 (Occupational Health and Safety)
For my portion of this webinar, I will discuss occupational health and safety and

veterinary medical care as it relates to anesthesia and euthanasia of zebrafish.

Per Chapter 2 of the *Guide for the Care and Use of Laboratory Animals*, the identification of hazards and the assessed risk of those hazards, facility design, operation, and use of engineering controls, should drive the decisions about which and what type of personal protective equipment is necessary for use in the zebrafish animal housing and procedural areas. We need to understand that "…personnel should be trained, maintain good personal hygiene, be knowledgeable about the hazards in their work environment, understand the proper selection and use of equipment, follow established procedures, and use the PPE provided."

Slide 24 (Personal Protective Equipment (PPE))
With this in mind the following are some general recommendations for PPE use in zebrafish housing and procedural areas. The use of dedicated outer garments protects against splashes and sprays and reduces the risk of transfer of potential pathogens or allergens outside of the area in question. The use of non-powdered gloves of adequate length protects the user conducting specific tasks. Performance of and following good hygienic practices are recommended, like frequent hand washing with non-scented or non-antibiotic soaps, with adequate rinsing both before and after handling fish, husbandry equipment or after removal of damaged protective gloves.

And also the use of closed toe, non-slip shoes, shoe covers over appropriate foot ware, or foot baths that are appropriately maintained. We must understand that this is not all or nothing but that special safety equipment should be used in combination with appropriate management and safety practices.

Slide 25 (Disposable PPE)
Disposable or single use PPEs should be used as such and not re-used! Again the PPEs in use should be determined by a complete and adequate risk assessment of the identified hazards within the zebrafish housing and procedural areas by the occupational health and safety program.

Slide 26 (Launder Reusable PPE on Site)
Reusable PPE, like scrub tops and bottoms, lab coats, etc. must be laundered on a regular schedule and should be done on site or via a commercial clothing service vendor. Reusable PPE should not be laundered via the personal washing equipment of employees to prevent the exposure of potential pathogens or allergens to individuals not enrolled in the institutions occupational health and safety program for animal users.

Slide 27 (Additional Important Safety Considerations)
Other important safety considerations for zebrafish animal housing and procedural areas include: potential slip and fall hazards, electrocution hazards, traumatic injury hazards, ergonomic related injury hazards including the risk of developing repetitive movement disorders (remember water is heavy!) and potential chemical exposure hazards. The goal

is to mitigate and minimize these hazards within the zebrafish housing and procedural areas as much as possible.

Slide 28 (Important Safety Reminder)
Please understand the re-capping of needles without the use of appropriate engineering safety equipment, like needle re-capping devices, is unsafe and should not be done. Unfortunately, needle stick injuries is still a common finding by institutional occupational health and safety programs or by IACUCs conducting semiannual inspections.

Slide 29 (Zoonotic Diseases and Allergies)
Zoonotic diseases are those that can be transmitted between animals and people. As it relates to zebrafish, the most common types of zoonotic disease agents are bacterial in nature and include, but are not limited to, primarily acid-fast (mycobacteria) and gram negative bacteria. These organisms can be readily transmitted by traumatic injuries including needle stick, acute skin lacerations, and of water or fish exposure to previously damaged skin and, in rare cases, through mouth pipetting. Primary method of protection includes the appropriate use of PPEs and the use of good standard hygienic practices. Parasitic and fungal agents listed are normally only transmitted via the fecal oral route or through the consumption of undercooked tissues of infected fish species.

The institutional occupational health and safety program as well as the individual's primary care physician should be knowledgeable of allergies, potential immunosuppressive medical conditions, or ongoing immunosuppressive medical treatment that could put the individual at greater risk for infection by a zoonotic disease agent while working in zebrafish housing or procedural areas. Again the goal here is to minimize and mitigate hazards identified and potential risks by varying and sometimes multiple methods.

Slide 30 (*Mycobacterium marinum*)
This is an example of a documented occupational health exposure that caused the development of multifocal to diffuse, raised, nodular, epidermal lesions that progressed over a three month period from the hand to the elbow of this individual. The diagnosis of these epidermal lesions was *Mycobacterium marinum,* confirmed by culture. This individual was an employee from a pet shop who was assigned the task of cleaning fish tanks without the use of protective gloves. The individual was successfully treated with four-month course of two antibiotics. This is an important case and people should consider this because zebrafish facilities have had documented zoonotic cases of *Mycobacterium marinum* within their facilities. [Nguyen, C. NEJM 350:9 (2004)]

Slide 31 (Veterinary Care: Anesthesia and Euthanasia)
According to the *Guide*, "the selection of appropriate [analgesics and] anesthetics should reflect professional veterinary judgment as to which best meets clinical and humane requirements as well as the needs of the research protocol. …Most anesthetics cause a dose-dependent depression of physiologic homeostasis and the changes can vary

considerably with different agents. The level of consciousness, degree of antinociception (lack of response to noxious stimuli), and status of the cardiovascular, respiratory, musculoskeletal, and thermoregulatory systems should all be used to assess the adequacy of the anesthetic regimen. Interpretation and appropriate response to the various parameters measured require training and experience with the anesthetic regimen and the species."

Slide 32 (Anesthesia of Zebrafish)
Sedation is used when minimization of movement is required for procedures that cause at most momentary pain or distress such as physical examination, conduction of weight and length measurements. Anesthesia is required for procedures in which movement must be reduced to prevent trauma associated with the procedure and when more then momentary pain or distress is expected such as during fin clipping for genotyping, conducting individual identification procedures (like visible elastomers, chips and tags), physical removal of eggs and sperm, and preforming surgical procedures.

Slide 33 (Stages of Anesthesia)
It is necessary to understand the stages of anesthesia in zebrafish for those that will be conducting procedures that require sedation and anesthetic use. The rate at which zebrafish progress through these stages correlates directly with the dose of anesthetic administered or the length of time the zebrafish is exposed to the anesthetic. For example, the lower the dose of anesthetic, the slower the rate of progression.

For procedures requiring only sedation, stage II or stage III plane 1 should be reached. For procedures requiring anesthesia, stage III plane 2 should be reached and zebrafish should be monitored and anesthesia adjusted to ensure that they do not progress into stage IV, unless the procedure is terminal (results in euthanasia). This is critically important when using chemical agents like MS-222 or Benzocaine hydrochloride because these agents are significantly affected by the pH of both the resulting anesthetic solution and the pH of the system water used to house the zebrafish. It is best practice to manually test, using a calibrated electronic meter and probe OR pH test strip paper of the appropriate range. After testing, adjust the pH of the final anesthetic solution to match that of the system water. You should not be relying on the addition of equal (1:1) or unequal (1:2) ratios of anesthetic and buffer in the water alone.

Slide 34 (MS-222 (Tricaine Methanesulfonate) Preparation)
In the powdered form, MS-222 is a respiratory hazard and should be used within a certified fume hood, in a well-ventilated area (e.g., if outdoors you should be upwind of your work area), or personnel should be wear the correct type of protective mask or respirator. MS-222 is available commercially in both pharmaceutical grade and non-pharmaceutical grade sources. According to OLAW guidance, pharmaceutical-grade MS-222 should be used.

The dosages and preparation of stock and working solutions of MS-222 are described on this slide. It is important to understand that stock solutions are not buffered because this may result in the precipitation of the drug out of solution into a non-active form. All solutions of MS-222 should be labeled with a creation date and expiration date. These solutions are light-sensitive and must be stored appropriately to prevent rapid degradation.

Slide 35 (Anesthetic Administration)
The typical dose and administration route of MS-222 for zebrafish is described on this slide. Depending upon the type of procedure being conducted and the anticipated length of the procedure, the dose may need to be adjusted. MS-222 is provided via immersion in an adequately buffered solution to maintain pH between 7 and 8. When zebrafish are removed from the anesthetic solution, their skin and gills must be constantly exposed to the anesthetic solution to maintain the appropriate level of anesthesia for the procedure being conducted. This can be accomplished by the manual regular application of anesthetic solution to the skin and gills or via the provision of a constant flow of anesthetic solution over the gills via a flow-through or recirculating anesthesia system.

Slide 36 (Zebrafish Euthanasia)
This slide provides several references on zebrafish euthanasia. Per the *Guide*, euthanasia is the act of humanely killing animals by methods that induce rapid unconsciousness and death without pain or distress. Unless a deviation is justified for scientific or medical reasons by the IACUC, euthanasia methods should be consistent with the *AVMA Guidelines for the Euthanasia of Animals* (AVMA Guidelines). When necessary, standardized methods of euthanasia that are predictable and controllable should be developed and approved by the attending veterinarian and IACUC. Euthanasia should be carried out in a manner that minimizes and avoids animal distress.

- *AVMA Guidelines for the Euthanasia of Animals – 2013 Edition* *https://www.avma.org/KB/Policies/Documents/euthanasia.pdf*
- *Guidelines for Use of Zebrafish in the NIH Intramural Research Program* *http://oacu.od.nih.gov/arac/documents/zebrafish.pdf*
- *Matthews, M. and Varga, Z.M. 2012. Anesthesia and Euthanasia in Zebrafish: Institutional Animal Care and Use Committee Considerations. ILAR 53(2): 192-204. http://ilarjournal.oxfordjournals.org/content/53/2/192.full.pdf+html*
- *Blessing, J. J., J. C. Marshall, and S. R. Balcombe. 2010. Humane killing of fishes for scientific research: a comparison of two methods. J Fish Biology 76, 2571–2577.*
- *Wilson, J.M., R.M. Bunte, and A.J. Carty. 2009. Evaluation of Rapid Cooling and Tricaine Methanesulfonate (MS222) as Methods of Euthanasia in Zebrafish (Danio rerio). JAALAS 48 (6): 785–789.*
- *Strykowski, J.L. and J. M. Schech. 2015. Effectiveness of Recommended Euthanasia Methods in Larval Zebrafish (Danio rerio). JAALAS 54 (1) 81-84.*

Slide 37 (Euthanasia Methods of Adult Zebrafish)
Primary methods for euthanasia of adult zebrafish include anesthetic overdose with a buffered solution of either MS-222 or Benzocaine hydrochloride, eugenol (clove oil) or CO_2 from a compressed gas cylinder. Less commonly used euthanasia methods for adult zebrafish, due to their small size, are physical methods like cranial concussion followed by decapitation or decapitation followed by double pithing. Depending upon the institutional requirements, the use of physical methods may require IACUC approval and training of personnel performing these procedures to ensure they are done properly.

Slide 38 (Zebrafish Euthanasia: Rapid Chilling)
The AVMA 2013 Guidelines approved rapid chilling or hypothermal shock as a method to euthanize zebrafish and other similar, small, warm water tropical fish species. This procedure involves rapid chilling in water between 2 – 4 degrees C and continued maintenance within chilled water until 20 minutes after the loss of orientation and cessation of operculum movements. This must be followed by either verifying death or use of an adjunctive method to ensure that death occurs.

Slide 39 (Rapid Cooling: Hypothermal Shock)
This is a picture that depicts the addition of the appropriate amount of ice to the tank (on the right) to euthanize zebrafish via hypothermal shock. In this configuration, it is important to add the fish to the water before the ice is added to the tank to ensure that the fish do not come into direct contact with the ice. The use of physical barriers like spawning tank inserts or screens are also useful to separate the fish from the ice during hypothermal shock procedure.

Slide 40 (Euthanasia of Embryos and Larvae)
The euthanasia of zebrafish larvae or embryos is a bit more complicated by the fact that immersion anesthetics or rapid chilling is not reliably irreversible for these younger stages. When used alone, rapid chilling requires a much longer contact time and anesthetics require a much higher concentration to euthanize larval zebrafish and embryos. In addition, it is very difficult to visually confirm death after euthanasia by cessation of opercular movements and/or heartbeat of these smaller stages of zebrafish.

Slide 41 (Euthanasia of Embryos and Larvae with Bleach)
As a result, only zebrafish embryos and larvae can be euthanized by direct immersion in a 5% sodium hypochlorite solution. This euthanasia method must not be used on juvenile or adult zebrafish. After completing this procedure an adjunct method is used to ensure death has occurred.

Slide 42 (Verification of Death after Euthanasia)
According to the AVMA Guidelines, (pp. 11 & 16), death must be confirmed before disposal of any animal remains. Therefore when using some euthanasia procedures, an adjunctive method is required to ensure death. For zebrafish these adjunctive methods include: exsanguination, freezing, rapid decapitation, or maceration using a well-

maintained system (e.g., reliably sharp blades). As with primary physical euthanasia methods, the use of some adjunctive methods may also require approval and proficiency training by the IACUC to ensure they are performed properly.

Slide 43 (Zebrafish Carcass Disposal)
Zebrafish carcass collection and disposal should be done a described in the facility's standard operating procedure and must comply with the institutional rules and regulations on the disposal of animal carcasses. Typically zebrafish carcasses (adults, juveniles, embryos) are stored frozen in the local housing or procedural area until enough are present for transfer to a larger carcass collection area and are sorted appropriately by bio-hazardous, medical, or other waste category prior to disposal.

Slide 44 (Additional References)
Here are some additional references for this discussion.

- *National Research Council. 2011. Guide for the Care and Use of Laboratory Animals 8th ed.*
- *Harper C, Lawrence C. 2011. The Laboratory Zebrafish. Boca Raton: CRC Press.*
- *Collymore, C. A. Tolwani, C. Lieggi, and S. Rasmussen. 2014.Efficacy and Safety of 5 Anesthetics in Adult Zebrafish (Danio rerio). JAALAS 53:(2) 198-203*

Slide 45 (Zebrafish Husbandry Association Webinar)
For those interested, this slide lists information on an upcoming webinar in which I will be discussing how to prepare your facility for IACUC Inspections.
[http://www.zhaonline.org/webinar-series.html]

Slide 46 (Question 1)
>>*Babcock:* Thank you George and Monte that was a wonderful presentation. Now we will address some questions that we have received prior to this broadcast. Doreen Bartlett will be joining us from OLAW.

This first question is for Doreen: OLAW's FAQ A5 asks, "Does the PHS Policy apply to larval forms of amphibians and fish? OLAW's answer is, "Yes, larval forms of fish and amphibians have vertebrae and are covered by the PHS Policy. As noted in FAQ A4, the PHS Policy applies to the offspring of egg-laying vertebrates only after hatching. Zebrafish larvae, for example, typically hatch 3 days post fertilization." Our IACUC would prefer not to oversee the welfare of zebrafish larvae until they are feeding, free swimming fish, at 5 days post fertilization. How do you justify your guidance?

Slide 47 (Answer 1)
>>*Bartlett:* Thank you George. The PHS Policy on Humane Care and Use of Laboratory Animals defines an animal as "any live, vertebrate animal used or intended for use in research, research training, experimentation, or biological testing or for related purposes." OLAW oversees the welfare of live vertebrates and interprets the PHS Policy as applicable to egg-laying vertebrates after hatching. Zebrafish hatch at approximately 3

dpf, when the zebrafish is no longer protected by its chorion and has developed features defining it as a vertebrate including the notochord, neural tube, pharyngeal arches, somites and posterior tale. Therefore, OLAW defines the hatched zebrafish as a vertebrate animal.

There are several ways your IACUC could meet the requirement for oversight of zebrafish larvae. Some institutions, such as Monte described, cover all live stages of zebrafish in their research protocols. Other institutions have a core protocol that covers larvae and fry until they are transferred to the research protocol at the required stage needed (i.e., post 3 dpf). And then many other institutions have protocols that cover all zebrafish beginning at 3 dpf. As Monte discussed, OLAW expects Assured institutions to have policies and procedures in place that address the care or euthanasia of animals that hatch unexpectedly.

Slide 48 (Question 2)
>>*Babcock:* Thank you, Doreen. The next question is for George: Do Mycobacterium and other microbial agents come from the fish or are they environmental? If they come from the fish, do they also grow in the water or on the sides of the tank?

Slide 49 (Answer 2)
>>*Sanders:* Mycobacteria and other agents are found in and on the fish and are also in the aquatic environment in which the fish are housed.

As to the second question, yes, these agents also grow in the water and on the side of the tank.

Slide 50 (Question 3)
>>*Babcock:* Question 3: How is decontamination of the tank and water accomplished given the sensitivity of zebra fish to their environment? Could you briefly discuss tank cleaning? What about contamination on fishnets and other equipment you use?

Slide 51 (Answer 3.1)
>>*Bartlett:* Zebrafish users must always be hyperaware of anything coming into contact with their zebrafish systems. Decontamination of tanks can be done by several different methods and is often determined by the capabilities of the facility or area in which the zebrafish are housed. Tanks can be cleaned with mechanical methods such as dishwashers, cagewashers, or manual gross organic debris removal by hand or with the use of an in -sink glass washer; high heat such as autoclaves; or disinfectants such as bleach, net soak, 1% Virkon, etc.

Slide 52 (Answer 3.2)
Tank screens, baffles, plugs, and lids can be scrubbed thoroughly to remove any feed detritus and then can be processed in the same manner as the tanks. Nets can be autoclaved, sanitized in a mechanical washer, and/or bleached (1% solution) which is

followed by soaking in a dechlorination solution. Be aware that autoclaving will definitely reduce the useful life of the nets. I can't stress enough that it is absolutely essential for thorough dechlorination before use.

Slide 53 (Answer 3.3)
Any use of chemicals must be carefully considered and used with extreme care. Water is decontaminated with the system's recirculation filtration process as described by Dr. Sanders.

Slide 54 (Question 4)
>>*Babcock:* Dr. Sanders described the various forms of PPE including the importance of gloves and hand washing. But in Mr. Matthews' slides of field studies, the investigator was not wearing PPE or gloves. Is there a risk to the fish or the investigator? Should PPE be used in the field?

Slide 55 (Answer 4)
>>*Matthews:* Good catch, George, and yes, adequate PPE should be used in the field and gloves are typically worn when working with fish and potentially contaminated equipment. Interestingly, I have another picture of this same investigator wearing appropriate gloves while handling a fish net.

Slide 56 (Question 5)
>>*Babcock:* Dr. Sanders advised that the stability of MS-222 is 3 months and recommended storage at -20 C. Is this the stability in the frozen state or after MS-222 has been thawed?

Slide 57 (Answer 5)
>>*Sanders:* The stability of MS-222 begins to degrade once either the stock or working solution are made. Freezing or refrigeration only slows down the degradation process.

Slide 58 (Question 6)
>>*Babcock:* Here's one for OLAW, Doreen. Since there are only two sources of MS-222, can the non-pharmaceutical-grade MS-222 be used solely for the purpose of euthanasia of zebrafish?

Slide 59 (Answer 6)
>>*Bartlett:* Since pharmaceutical grade MS-222 is available, that is the one that must be used. The use of a non-pharmaceutical-grade euthanasia agent must meet the same criteria that OLAW and USDA require for other substances used in animals. For further guidance, please refer to FAQ F4 at URL http://grants.nih.gov/grants/olaw/faqs.htm#662

Slide 60 (Question 7)
>>*Babcock:* This is a follow up question for you, Doreen. Our institution uses pharmaceutical-grade MS-222. Can we use expired pharmaceutical-grade MS-222 for euthanasia?

Slide 61 (Answer 7)
>>*Bartlett:* Expired pharmaceutical-grade MS-222 should not be used for euthanasia. Euthanasia, anesthesia and analgesia agents should not be used beyond their expiration date, even if a procedure is terminal. For further guidance, please refer to FAQ F5 at URL http://grants.nih.gov/grants/olaw/faqs.htm#663

Slide 62 (Question 8)
>>*Babcock:* How is respiration observed or measured in sedated zebrafish in water?

Slide 63 (Answer 8)
>>*Sanders:* Respiration is observed or measured in sedated zebrafish in water by visual observation of the movement of the fish's opercula while under sedation or anesthesia.

Slide 64 (Question 9)
>>*Babcock:* Is a garbage disposal an acceptable macerator for the euthanasia of zebrafish?

Slide 65 (Answer 9)
>>*Bartlett:* AVMA guidelines describe a required macerator as a "well maintained macerator designed for the size of finfish being euthanized." The AVMA guidelines also state that "flushing of finfish into sewer, septic, or other types of outflow system is unacceptable for many reasons." The development and application of performance standards and measures would determine whether a garbage disposal proposed for use as a macerator for the euthanasia of zebrafish meets the AVMA Euthanasia Guidelines and is, therefore, acceptable for use.

Slide 66 (Question 10)
>>*Babcock:* Since pain management is limited in zebrafish, is it possible to have fish in column D? Could technique, use of MS-222, housing, and euthanasia at certain endpoints be considered appropriate measures to relieve pain and distress? In this specific case, the institution uses the USDA categories for all animals on their campus even though they understand that the fish are not USDA-regulated.

Slide 67 (Answer 10)
>>*Matthews:* Many institutions choose to use the USDA pain categories for all species, not just USDA regulated animals. Hence, Category D is defined as, "Number of animals upon which experiments, teaching, research, surgery, or tests were conducted involving accompanying pain or distress to the animals and for which appropriate anesthetic, analgesic, or tranquilizing drugs were used." So in this example, MS-222 is proposed, as

well as a modification in housing, such as keeping them in an area with a lower light intensity and noise level for the purpose of minimization of pain, distress or discomfort. The development of humane endpoints would also be appropriate.

Slide 68 (Question 11)
>>*Babcock:* When are adjunct or secondary methods of euthanasia required with zebrafish?

Slide 69 (Answer 11)
>>*Babcock:* The use of adjunct or secondary methods of euthanasia are required to ensure that zebrafish are dead when death cannot be confirmed by observation alone.

Slide 70 (Question 12)
>>*Babcock:* Is environmental enrichment a consideration for zebrafish?

Slide 71 (Answer 12)
>>*Sanders:* Yes, according to the *Guide*, the use of environmental enrichment for zebrafish must be considered by the IACUC, investigators, and attending veterinarian.

>>*Babcock:* At this point we have time to take a couple of live questions from the audience. However, I'd like to remind everybody to send in your questions so we don't have time to get to and our panelists will post their answers on the OLAW website in a timely manner. We received some tips from experienced zebrafish managers and we thank them for these and we'll share them with you now.

Slide 72 (Comment 13)
System design is specifically geared towards rearing the intended species. One would need to know the optimum living parameters (water chemistry; tank dimensions, shape and possibly color; noise, vibration, light intensity) of the animal to be raised and the intended use of the animal. In the case of zebrafish, it would be egg production. In the agricultural fish industry, it would be meat production. Then, one would build a system to accommodate each. Each system will be unique and operate and be maintained according to the target need. For instance, in zebrafish production, keeping genetic lines isolated is critical, so installing tank anti-jump mechanisms and fine filters to act as fish exclusion devices is necessary.

Slide 73 (Comment 14)
It is important to understand that when zebrafish get sick, it is likely caused by an environmental issue (e.g., poor water quality, disrupted light cycle, etc.) that is causing stress leading to disease. In the case of an outbreak, although understanding the causative pathogen is important, the top priority should be isolating the environmental change that is causing the stress.

Slide 74 (OLAW Online Seminar)

>>*Silk:* We have some good questions coming in to the OLAW office where I'm located. This is Susan Silk. So I'm going to read these questions now. Doreen, this first question is for you. [Question 15] What criteria is used to determine the appropriate amount of algae growth in tanks? Is “as needed” an appropriate answer?

>>*Bartlett:* Actually, algae is not considered to be a need or requirement of zebrafish. In reality, algae is often a problem in tanks when the holding room has a high level of light intensity. This growth can develop so that the required daily observations of zebrafish are not possible. An SOP should be developed as to when a tank should be changed due to too much algae growth. One institution that I know of requires tanks to be changed with algae growth obscures one half of the front of the tank.

>>*Silk:* Here's one for you, Dr. Sanders. [Question 16] For buffering MS-222, can sodium hydroxide be used instead of sodium bicarbonate?

>>*Sanders:* Sodium hydroxide or other buffers can be used as long as you're very comfortable with how they're going to affect the pH of the resulting solution. Some of them have a greater propensity to overshoot the pH that you're shooting for so care must be taken when using other types of buffers besides sodium bicarbonate, TRIS, etc.

>>*Matthews:* Susan, this is Monte. I'd like to add a little bit to what Doreen said about the algae growth. It's also important to remember that not all algae species are the same. And in addition to that, because some could be problematic for potentially harboring various pathogens and in addition to that, you know, why would you want to introduce in your facility another variable where you have some tanks covered with algae growth and others not? So again just in terms of providing consistent care and reducing variability in the science is another good reason.

>>*Silk:* Thanks, Monte. And I encourage all of our speakers to chime in when you have something to add to an answer. The next question has been directed to George, but you're all invited to contribute. [Question 17] Can you please comment on IACUC semiannual inspection of fish housing areas -- unsealed wood, degree of algae growth in tanks, water quality records, etc.?

>>*Sanders:* So there is a good JLAS journal article on this that covers that and we could provide that reference to you later, but as it relates to untreated wood, untreated wood can be problematic in that you can't disinfectant and it's porous so it absorbs things. So those should be treated with something to make it nonporous so you can sanitize it on a regular basis. As it relates to -- what was the other part of the question?

>>*Silk:* Semiannual inspection of fish housing areas; unsealed wood, degree of algae growth in tanks, water quality records.

>>*Sanders:* The degree of algae growth as we have already discussed can be problematic if you can't visually observe the fish or light can't get to the fish. So things should be done to basically rectify those situations as necessary. Water quality records should be available for review by whoever needs to review them so that a veterinary staff, the facility manager for the facility as well as the PIs that might have concerns about fluctuations in water quality that can affect the animals in those systems.

>>*Matthews:* This is Monte again. When we conduct our semiannual IACUC inspections we're looking for records that will record in some cases daily and in other cases weekly various water quality parameters such as pH, ammonia, if hopefully not present, nitrates, nitrites, dissolved oxygen, salinity, etc. We actually look for those records when we do our semiannual inspections just like you would looking at a mammalian facility when making sure that people are documenting observations of animals, cleaning, sanitizing frequencies of rooms and cages, etc.

>>*Babcock:* This is George Babcock. Going a little further with what George Sanders said about the untreated wood, another problem we see occasionally is a lot of rust on metal racks supporting tanks. And rust can interfere with or bind to several of the commonly used disinfectant agents or it can actually make it more severe. So this is something we feel should be avoided too wherever possible.

>>*Sanders:* And to chime in on that, the rust, especially on structural elements, need to be careful because rust can corrode materials. Rust is corrosion. And if that corrosion is severe enough it can affect the structural integrity of support systems. You want to make sure that the rust you're seeing is just surface, it's not actually deeper than that because that can be problematic structurally.

>>*Matthews:* That's a really good point, George. This is Monte again. And in fact, we had some racks that contained many, many tanks and these racks were like the old erector set style racks that you put together. And they became so severely rusted that we defined it at one point as a significant deficiency because it could potentially impact the health or safety of those animals. So we actually used that deficiency to help us in our semiannual report documenting that, and using that as a means for obtaining a G20 grant to get new racks. So you can actually turn those deficiencies in to positive results.

>>*Silk:* Doreen, [Question 18] has OLAW received reports of animal welfare issues related to zebrafish larval mortality? And if so, what was the response of OLAW?

>>*Bartlett:* No, we haven't, Susan. We have received reports of fish mortality due to water quality problems, equipment failure; but not larval mortality issues.

>>*Silk:* [Question 19] What types of cleaning items can be used to care for zebrafish without harm?

>>*Bartlett:* Anything you introduce into a tank could be potentially harmful for the zebrafish. Normally for removing feed detritus on the bottom of tanks, you might pipet it out. On the outside of the tank, you might be doing spray-downs of the tanks and the racks themselves, being careful not to spray anything into the tanks or into the water systems if at all possible. Dilute bleach solutions for floors or Virkon and just good old plain elbow grease and mechanical abrasion is the best cleaner.

>>*Sanders:* And please understand the addition of chemical agents to the recirculating system can and will affect not only the biofilm in the system but also the biological filtration system which can be adversely impacting the animals housed in those systems.

>>*Silk:* [Question 20] Can tank cleaning properly be done with 70% ethanol?

>>*Bartlett:* I have not heard of this personally. Monte or George, have you heard of this being done?

>>*Sanders:* For cleaning or disinfection? Is the question cleaning or disinfection?

>>*Silk:* The question said cleaning.

>>Bartlett: Cleaning.

>>*Sanders:* Cleaning can be -- you can use ethanol to help break up the algae on the tanks and then clean it and do disinfection by a different mechanism. Alcohol is not a very good and efficient disinfectant. It usually takes a very long contact time to actually do any disinfection properly because of its low disinfectant properties. That can make it difficult to disinfect anything with 70% ethanol that has a lot of organic material that has yet to be removed.

>>*Williams:* We have a fairly large facility and we use a cage rack washer system similarly to a lot of rodent facilities out there. And we've tested the cleaning agents, there are various companies now that will manufacture for aquatic species cleaning solutions that do a good job of removal. And again, you want to make sure that all of that is rinsed off very well in your final rinse cycle. And the use of 70% ethanol, as George states, we don't use it as a cleaning agent, but as a disinfecting for tabletops and areas where there's work that's done or water that's spilled in the facility.

>>*Sanders:* Right. That's what it is mostly used for.

>>*Silk:* Monte, the listener specified this question for you. [Question 21] Besides the method of using a database to track and count zebrafish, what other methods are recommended?

>>*Matthews:* Again for us, because we require all stages, including the embryonic stages, for investigators to utilize a protocol and most of the majority of cases our investigators are going out to post-hatching up to 4, 5, 6 days old, etc., in which they would be required to have a protocol anyway. We don't require the investigators right now to count every single early larval stage up to 6 days post fertilization animal that they're using, however we do prospectively ask them to estimate the number of larval that they will be using in the protocol before the experiment begins.

>>*Silk:* George Sanders, [Question 22] is there a known stability of MS-222 at 4 degrees?

>>*Sanders:* A known stability? You mean how long it stays, exactly?

>>*Silk:* How long it maintains its stability at 4 degrees.

>>*Sanders:* Stability at 4 degrees is as recommended. Usually it's about a month, depending on a working or a stock solution and also depending upon how much light exposure to that specific container.

>>*Silk:* How would they know that?

>>*Sanders:* The degradation has been tested upon the vendors that make this product. So that's part of the recommendations listed on the insert of MS-222.

>>*Matthews:* And we also require people to, obviously, write down on the stock solution the date it was prepared.

>>*Sanders:* Right. You could also test it. Some people have found that in their use, depending on the concentration of working solution that they make, it is still effective beyond that month and they can document that, and that's fine for the IACUC, as for the veterinary staff, but just putting it in there and guessing it something a little bit different. So the standard or the recommended is usually about -- as we talked about before. If you want something more specific than that, you can test it on your animals and your situation and then you might be able to increase the length of those times based on the recommendations that you find.

>>*Matthews:* And that sounds like a perfect opportunity for institutions for their specific setups to conduct a performance-based approach. So as George mentioned, go ahead and test it for yourself and look at the performance. So looking at whether or not the fish are being anesthetized equally the same, whether it's 1, 2 days out, 1, 2 weeks out, 1 month out, etc.

>>*Silk:* We're going to run a few minutes over today. We understand if you have to leave. We've got these wonderful experts collected. So I'm going to read the next 3

questions which are all on the same topic. We invite all of our experts to continue and then George Babcock, I will give the podium back to you after I read these 3 questions: [Question 23] What is zebrafish enrichment? Please give an example of zebrafish enrichment. What are suggested enrichments for zebrafish? What type of environmental enrichment is used for zebrafish.

>>*Sanders:* So for enrichment for zebrafish there is a lot of research still ongoing. People have used plastic plants as separators to basically separate the less active or the less aggressive animals from the more aggressive animals during breeding or other social interactions. People have used different color marbles. People have used several different things as enrichment. One thing that a lot of people don't think about is the use of live prey items. So live prey items actually enrich the zebrafish by activating their normal hunting behavior and so are able to chase down their feed and consume it, which is also an enrichment that can be used.

>>*Matthews:* And most facilities will also have multiple zebrafish in a tank and so that they provide a social enrichment for them as well and some could look at that as environmental enrichment. We're really at the very beginning stages to understand what is appropriate environmental enrichment for zebrafish and just recently at the zebrafish husbandry meeting associated with the aquaculture meeting in New Orleans last month, there was a presentation from someone who looked at the differences between plastic grass and I think leaves being used. It's not published yet, but it might be available through the zebrafish husbandry association website in which they found that grass was actually exhibited better species specific behavior and seemed to work better than leaves. Not all environmental enrichment plastic shapes or configurations result in similar environmental enrichment behaviors.

>>*Sanders:* When you use enrichment you have to be careful that whatever you're using doesn't cause problems for the animals, i.e., bleach, any pseudoestrogenic compounds, or anything that breaks down by some of the disinfectants used to clean the equipment. And you also don't want pieces or segments of it breaking off, entering the recirculating system and causing problems, blocking tanks and blocking pipes, etc. You do have to be careful with that. It's not something you can use willy-nilly.

>>*Matthews:* That's a really good point, George. We test anything that's novel, that hasn't been tested aquatic safe. We have a whole testing regimen that we test these new plastics with, whether it's for environmental enrichment, or a new type of net, or whatever it happens to be.

>>*Sanders:* I believe at ZIRC [Zebrafish International Resource Center] or at Oregon in Monte's facility they cut up the pieces of things they want to use, they'll expose them to larval stages of the zebrafish and actually see if there are any effects. And then they'll be able to determine what type of gloves, what type of enrichment, what type of nets, whatever, plastics, etc., might be harmful to the research that they are doing. And that's

something that people should do, in general. I think that ZIRC has put that on their website so you can get an idea of those methods used as tests, that type of equipment.

>>*Matthews:* I encourage folks when they're looking at designing or testing these different plastics is to include that in some kind of facility SOP or protocol in which you allow for those activities to be conducted and then the IACUC is aware of that.

>>*Babcock:* I would like to thank George, Monte and Doreen for the presentation. I would like to remind the listeners who still have questions, to submit them, and then our speakers will address them and it will be posted to the OLAW website. Thank you.

>>*Silk:* So join us for our next online seminar on June 4th, 2015 when we'll be talking about non-pharmaceutical-grade substances. Thanks and good-bye everyone.

Additional Submitted Questions Not Addressed During the Webinar

[Question 24] **Would food grade, stabilized 7% hydrogen peroxide products be an acceptable sanitizer in sanitizing tanks, fish nets, etc.? There would be no chlorine byproducts to deal with.**
>>We are not familiar with these products and have not used them for husbandry sanitation. These products may be effective but gross organic debris should be removed from equipment before the product is applied. The potential corrosive effects on more delicate equipment like nets and those constructed of various metals is not known. The use of the fry test to establish its safety and the establishment of a performance plan would be recommended to confirm sanitization effectiveness.

[Question 25] **Can an institution's IACUC approve the use of non-pharmaceutical-grade MS-222 for zebrafish euthanasia in the same manner as approval for non-pharmaceutical-grade sodium pentobarbital?**
>>Regardless of the specific non-pharmaceutical-grade substance proposed, the process of IACUC approval remains the same. For guidance, please refer to the *Guide* (p. 31) and OLAW FAQ F4.

[Question 26] **Where can we get pharmaceutical-grade MS-222?**
>>MS-222, branded as TRICAINE-S, may be obtained from Western Chemical, Inc., Ferndale, WA.

[Question 27] **Doesn't MS-222 also change color when it gets too old?**
>>MS-222 is a light sensitive chemical and should be kept in a dark container or in a cabinet/drawer. If exposed to light, it may develop a brown tinge (The Laboratory Zebrafish). Per manufacturer's instructions (PDF), "Store TRICAINE-S solutions in a cool place away from light."

[Question 28] **Can you recommend a method to track and count zebrafish breeding numbers and colony size?**
>>There are several methods that can be employed to accomplish this task. Many systems in use were created in house but there are database vendors that are willing to work with an institution to customize their products. We would recommend working with the zebrafish facility manager, investigators, veterinarians, and IACUC to find a method that works well for your institution.

[Question 29] **Please speak to the importance of maintaining and accessing water quality standards when using zebrafish and other aquatic organisms. We seek best practices and practicality in application.**
>>Maintenance of stable water quality is essential to zebrafish health and productivity. The continuous monitoring and optimization of water quality is needed for the health and productivity of the zebrafish, the efficiency of the biological filtration process, and to minimize the negative impact of water quality fluctuation on research. The frequency of testing depends on the age and stocking density of the system, and/or any changes introduced to a system (e.g., new feed). Redundancy in the testing method should be incorporated. Water quality parameters should be accurately measured and recorded on a regular basis in either an electronic or paper format that is reliably accessible over time. This data must be easy to retrieve and should be reviewed by knowledgeable staff on a regular basis (e.g., daily). Any abnormal trends or irregularities noted should be promptly communicated to the appropriate person (e.g., facility manager) immediately for further evaluation. For guidance, please refer to the *Guide* (p. 78) and The Laboratory Zebrafish (pp. 109-119)

[Question 30] **We have researchers that say the only enrichment needed is co-housing as zebrafish are a shoaling species. What other forms of enrichment could be used?**
>>Appropriate co-housing with con-specifics is the basis for social housing. The provision of additional enrichment should be considered. The decision should be based on the potential effects of the enrichment to the fish housing, husbandry, and research outcomes. “The primary aim of environmental enrichment is to enhance animal well-being… according to species-specific characteristics.” (*Guide*, p. 52) While co-housing is probably the main form of environmental enrichment, the feeding of live food and the use of plastic plants has become increasingly accepted. Caution must always be used when introducing anything into the system that could inadvertently also introduce a pathogen to the fish or a chemical that could affect the fish health or fecundity. The provision of enrichment items should not be done solely on the basis that it makes people "feel" better. The zebrafish facility manager, investigative staff, veterinarians, and IACUC should work together to evaluate past and current literature on enrichment in fish and determine what is best for the animals in question. “Enrichment programs should be reviewed by the IACUC, researchers, and veterinarian on a regular basis to ensure that they are beneficial to animal well-being and consistent with the goals of animal use. They should be updated as needed to ensure that they reflect current knowledge.” (*Guide*, p.

53) For guidance, please also refer to the *Guide*, pp. 82-83.

[Question 31] **Should there be a limited time that fish are singly housed?**
>>Single housing should always be the exception in any social species unless scientific, welfare, or veterinary justification is provided. Any time limits during which fish are singly housed should be developed by the zebrafish facility manager, investigative staff, veterinarians, and IACUC.

[Question 32] **What is the expectation of fish health recordkeeping?**
>>Fish health recordkeeping will vary with the situation. At a minimum, the morbidity and mortality should be monitored on a regular basis. This information, along with additional information collected about the colony (e.g., colony population, spawning success rates, larval fish survival rates, fish health diagnostic test results, import/export records) is used to evaluate the overall health of the colony over time. For additional guidance, please refer to the *Guide* (pp. 87-88) and The Laboratory Zebrafish (pp. 66-69)

[Question 33] **Can you address the components of an adequate zebrafish sentinel health monitoring program?**
>>Sentinel or health monitoring programs will also vary with the situation. Sentinel or health monitoring programs are useful in understanding what organisms are present in your system and may also prove useful in the assessing the effectiveness of the system's filtration and the change in organisms over time. The Zebrafish International Resource Center (ZIRC) utilizes quarterly testing of fish that have been exposed to the dirty system water and clean system water (Sentinel Fish Program – PDF). This is but one component of a sentinel or health monitoring program and other means should also be incorporated, such as the regular evaluation of sick and dead fish. For additional guidance, please refer to the *Guide* (pp. 109-113) and The Laboratory Zebrafish (p. 171).

[Question 34] **In term of per diem, what is the average per diem for zebrafish?**
>>Per diems will vary from facility to facility depending on the administrative structure of the institution and facility. Without knowing how the per diems are developed, calculated, what they include, how the facility is funded, etc. it is very difficult to compare one per diem rate to another.

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