Controlling Endemic Pathogens—Challenges and Opportunities

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Abstract

By most measures, the University of Utah Centralized Zebrafish Animal Resource is a successful zebrafish core facility: we house ~4000–5000 tanks for over 16 research groups; provide services and equipment for ~150 users; are currently undergoing an expansion by 3000 tanks; and have been praised by institutional and national regulatory agencies for the cleanliness and efficiency of our facility. In recent years, we have implemented new programs to improve the overall health of our colony and believe we have seen a reduction in apparently sick fish. However, there are still deficiencies in our monitoring and pathogen control programs. Our histopathology sample sizes have been insufficient to estimate prevalence, but our sentinel tank program reveals the presence of *Pseudoloma neurophilia* and myxozoan, presumably *Myxidium streisingeri*, in our facility. As we develop protocols to further reduce the burden of disease, we are focused on defining our baseline, establishing goals, and implementing methods to monitor our progress. The data generated by this approach will allow us to evaluate and implement the most cost-effective protocols to improve fish health.

Introduction

THE UNIVERSITY OF UTAH Centralized Zebrafish Animal Resource (CZAR) was established in 2001 and became a University of Utah Core Research Facility in 2002. Over 30 different research groups have used the CZAR during this time. The CZAR currently supports 16 different research groups with over 150 users investigating a wide range of topics, including developmental biology, cardiology, cancer biology, bacterial and fungal virulence factors, neurobiology, nerve regeneration, eye development, axon guidance, and behavior. The users range in experience from volunteer high school students to full professors. Some will use the facility for only 6 weeks during a graduate student rotation, while others have more than 30 years of experience. Our smallest laboratory has 24 tanks, while the largest has more than 600. This diverse group represents the wide range of users we serve with an equally wide range of knowledge and understanding of zebrafish husbandry issues and concerns.

The three most common endemic pathogens found in zebrafish facilities are the microsporidium (*Pseudoloma neurophilia*¹ and *Pleistophora hyphessobryconis*²), several species of mycobacteria,^{3–5} and a myxozoan recently identified as *Myxidium streisingeri*.⁶ Over the last few years, there has been increasing concern that these endemic pathogens may affect the outcomes or reproducibility of experiments in zebrafish.^{7,8} For

example, startle response assays are frequently used in the growing field of behavioral research,^{9–11} and it has recently been shown that *P. neurophilia* infection can alter zebrafish startle responses.¹²

Since past histopathology results indicated that our facility contains *P. neurophilia* and myxozoa (consistent with *M. streisingeri*)⁶ over the last 3 years, we implemented several new programs aimed at reducing the burden of disease in our facility. These programs (added to our existing pathogen control protocols) resulted in a more user-friendly overall cleaner facility that drew the praise of our Institutional Animal Care and Use Committee (IACUC) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) in their review of our program.

Despite these efforts, we continued to see sick fish in tanks and histopathology reports positive for *P. neurophilia* and myxozoa (presumably *M. streisingeri*). In this study, we describe the programs implemented, the results they generated, and the areas where we can make immediate improvements.

Materials and Methods

Facility description

The CZAR facility (Fig. 1) comprises two large fish rooms, two microinjection rooms, a pump room, a dish washing and

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FIG. 1. Line drawing showing the layout of the current CZAR and the expansion/renovation areas. The current CZAR (white areas) comprises large fish rooms (North and South), two microinjection rooms, a pump room, a dishwashing kitchen, fluorescent microscope room, a small quarantine room, and two offices for three full-time staff. The CZAR East expansion and renovated areas (stippled areas) provide an additional 21 racks for adult fish (CZAR East), a 15-rack nursery, an alternate light cycle room, a filtration/pump room, a third injection room, a rotifer room, mating tank setup areas, and a staging area to collect dirty dishes for transport to the kitchen. The renovation also includes upgrades to rooms near the kitchen to provide separate areas for shrimp cones, food preparation, a genotyping bench, and a room for *in vitro* fertilization (IVF) and sperm freezing. Stars indicate finger print access points. CZAR, Centralized Zebrafish Animal Resource.

food prep room, fluorescent microscope room, and two offices for three full-time staff. The two large fish rooms (CZAR North and CZAR South) contain 70 Pentair (Apopka, FL) racks divided into four independent recirculating water systems. There is an additional single-rack system in a small quarantine room (QX) for five systems total. A 2700-aquaria, 1923 square feet expansion, is currently under construction, directly across the hall to the east of the current facility. The new “CZAR East” will have two large fish rooms: a 21-rack adult fish room and a 15-rack nursery room. The expansion will also provide additional space to separate food prep and dish washing, as well as rooms for microinjections, a pump/filtration system, rotifer growing, and an alternate light cycle room with two single-sided racks to facilitate afternoon egg laying for injections. (See Supplementary Data; Supplementary Data are available online at www.liebertpub.com/zeb for a more complete description of the facility and the expansion plans.)

Access to facility

Card readers and fingerprint scanners are located in the hallway; access is restricted to CZAR users that have passed online AAALAS training and certification and completed an orientation for using the CZAR.

Pathogen control

Our major method of pathogen reduction is UV irradiation of the recirculating water. We do not treat with antibiotics or other medications or chemicals. Dirty tank components are soaked in a bleach solution (825 ppm sodium hypochlorite, pH 6.5–7.0) for at least 30 min and the chlorine is neutralized in sodium thiosulfate. The tanks are scrubbed on a Bar Maid (Bar Maid Corp., Pompano Beach, FL) with Dawn dish soap (Procter & Gamble, Cincinnati, OH) and then put through Hobart (Hobart, Troy, OH) dishwashers for a final high temperature rinse (180°C). Employees wear vinyl aprons, nitrile gloves, and protective eyewear when doing dishes. Gloves are not required in the facility while handling fish.

The effectiveness of the UV bulbs is tested monthly by using a sterile swab to spread post-UV water samples (two replicates/system) from each of the four systems on Tryptic Soy Bacto (TSB; Becton, Dickinson and Company, Franklin Lakes, NJ) agar plates. Water from the quarantine system and dishwashers are also tested monthly. The dishwashers and the UV bulbs are deemed to be working normally if there are fewer than 10 colonies on any given plate incubated at 30°C overnight. If more than 10 colonies are seen on any given plate, the samples for that system are repeated. Since most

plates show no growth, sump water samples serve as a positive control that the plates support growth.

Separate nets are used for each tank of fish to avoid spreading pathogens between tanks. The nets are soaked for at least 30 min in Virkon Aquatic (Du Pont, Wilmington, DE), rinsed well, and run through the dishwashers. They are placed on racks to dry completely before returning to the facility.

The colony diet includes a wide range of foods (Supplementary Data), some only used in the nursery. These include fresh-hatched *Artemia* (Inve, Salt Lake City, UT) and salt water rotifers (*Brachionus plicatilis*) (Reed Mariculture, San Jose, CA). While we recognize that live feeds are a potential source of new pathogens,¹³ we do not have a program in place to test for pathogen entry by these sources.

Quarantine program

We have a quarantine (QX) room with a stand-alone single-sided rack that holds up to 60 tanks. Users are required to house all fish from outside facilities (on or off campus) in the QX room. This also applies to fish that have been temporarily removed from the CZAR (brought to the users lab). No fish in the QX may ever be brought into the main facility. The room is equipped with its own set of tanks and mating tanks that may be used to set up fish. Only surface-sterilized embryos from QX fish may be brought into the main facility. Bleach and 50 mL tubes of embryo water (E3) (Cold Spring Harbor Protocols, 2011) are provided for users to make fresh bleach solution (100 ppm sodium hypochlorite, pH 6.5–7.0, for 5 min¹⁴) each time they need it.

Monitoring pathogens—sentinel tank program

From 2005 to 2012, two fish per system (a total of 8–10 fish/year) of approximate equal age, but not necessarily the same line, were sent to the Zebrafish International Resource Center (ZIRC) (<http://zebrafish.org/home/guide.php>) for histopathology evaluation. The number of samples testing positive each year for *P. neurophilia* ranged from 0% to 90%, while the number testing positive for myxozoa (presumably *M. streisingeri*) ranged from 0% to 65%. These results reflect the random variation seen with such small sample sizes and inconsistent sampling methods.

In 2012, we started a sentinel tank program. Each system has four tanks of fish that are all sibs from the mass breeding of a WT line. Two control tanks are supplied with water immediately downstream from the UV tubes, and two tanks are supplied with effluent water. Fish supplied with effluent water are exposed to all of the pathogens shed from the infected fish upstream. We expect the histopathology results from the effluent-fed tanks to reveal which pathogens are present, but at an exaggerated rate. The control tanks are a first-order demonstration that healthy fish can be raised in our systems.

Four fish from each of our systems, two from control tanks and two from sump tanks, were sent to ZIRC for histopathology evaluation in 2012 and 2013 (Table 1). The results indicated that our filtration and UV irradiation methods do reduce the transmission of disease. Neither the number of samples nor the sampling methods were designed to provide an estimate of pathogen prevalence in the colony. They were designed as a simple monitor of our systems and it has been effective at that.

Going forward, we recognize the importance of estimating a colony infection rate with a consistent method. Using the formula cited in Kent *et al.*¹⁵ to detect pathogens present in

TABLE 1. PATHOGENS IDENTIFIED IN CZAR FISH FROM SENTINEL TANKS

Year	2012	2013
Control tanks		
No. of fish submitted	N=8	N=8
Microsporidia (%)	0	0
Myxozoans (%)	13	0
Effluent tanks		
No. of fish submitted	N=8	N=8
Microsporidia (%)	86	50
Myxozoans (%)	29	0

Samples were submitted to ZIRC for histopathology evaluation. The table shows the number of fish submitted from control tanks versus effluent tanks for the four main systems. The two most abundant pathogens identified in our facility are *P. neurophilia* and myxozoa, presumably *M. streisingeri*. *Mycobacteria* (identified as *Mycobacteria cleone*) have only been detected in one fish out of the 154 fish tested since 2002.

≥5% at 95% confidence in our expanded colony of ~140,000 fish, we will need to submit at least 60 fish for analysis. This number jumps to 300 fish/year if we want to detect pathogens present in ≥1% of the population with 95% confidence. In balancing detection with available resources, we believe we can support detection at the ≥5% rate.

Results

Recent programs to reduce the burden of disease

It is well established in aquaculture that poor husbandry practices increase the risk of infection in fish.^{16–18} However, the need to maximize fish growth and reproduction also drives us to feed fish at levels that current tank designs cannot adequately clear debris from the bottom of tanks. This creates many dirty tanks with clogged baffles, poor water quality, and a rich biofilm that clogs water tubes. It becomes a balancing act between promoting rapid growth and maintaining clean water, since sick fish make poor research subjects.

Cleaner tanks and sick fish lists

In an effort to reduce the number of sick fish in our colony, 3 years ago, we instituted several programs to address these inherent problems related to water quality.

- (1) Twice a week, each tank is checked for slow or clogged tubes. Correcting the clogs ensures that clean oxygenated water is delivered to the tanks, and the improved flow rate promotes better waste removal from the bottom of the tanks.
- (2) Using a homemade “baffler,” every tank in the facility gets a stream of water sprayed under the baffle to clear it out every Saturday. This also facilitates effective waste removal and prevents fish from escaping from overflowing tanks.
- (3) The employees checking for clogged tubes also remove dead fish and make a sick fish list for the director. The director checks each of the tanks on the lists and either euthanizes the sick fish or refers it to the user for euthanasia.

- (4) We implemented a blue flag program. Once a month, the director places blue flags on all tanks that are dirty and need to be changed. The part-time employees receive one-on-one training in how to change out these dirty tanks, one at a time, so fish are never mixed up. Laboratories can opt out of having CZAR employees change their tanks and take responsibility for changing out any flagged tanks on their own. If these tanks are not changed by the end of the month, the director asks the PI or laboratory manager to remind their members to change their tanks. The blue flag program has eliminated most algae-covered tanks. Most tanks are changed out on average every 3 months as opposed to the previous norm of every 6 months.
- (5) Each week, one of the 26 biofilter panels holding Siporex (Sera; GmbH, Berlin, Germany) beads in the sumps is removed and the bacterial mats and biofilm are washed off. This ensures that each panel is cleaned at least once every 6 months, ensures available biofilter surfaces, and reduces biofilm mats that can harbor pathogens.

The goal of these programs was to reduce the loss of fish due to poor water exchange, poor tank hygiene, and disease transmission from sick fish or dead fish. The sick fish list removes a number of emaciated fish, twirling fish, and fish displaying the bloated phenotype known as dropsy. The experience of the director is that the number of fish noted with dropsy has declined from about 10–20/week 4 years ago to less than 5/week currently. However, many factors could contribute to these apparent declines, including changes in the demographics of the colony. Since the programs were implemented without a standard protocol for initial and regular data collection, we are unable to document their specific contributions.

Discussion

Pathogen control challenges

While it has been demonstrated that a specific pathogen-free (SPF) facility¹⁵ is possible for zebrafish, it required a high level of training and restricted access to achieve. For most facilities like the CZAR, constrained space, time, and resources limit how much can be done to eradicate entrenched pathogens. Fish and tanks are moved freely between our four main water systems. Fish graduating from the nursery on system 2 go to every other system in the facility. When the expansion is completed, the nursery will move across the hall to CZAR East, and fish will move back and forth with the users. To maximize room for fish racks, the CZAR's kitchen and food prep areas will also serve CZAR East. Dishes and food will need to travel back and forth across a hall that cannot be isolated. For all practical purposes, any pathogen resident in the current facility will soon find its way across the hall to CZAR East.

Given these challenges, what are reasonable goals for reducing our burden of pathogens? How will we know we have reached those goals? The following is a brief description of a few areas that are likely to reduce the pathogen load in the CZAR:

(1) *Mating tanks*—Under our current protocols, users rinse out mating tanks after each use and then these are stacked to

dry before being used again. During the first week of each month only, used mating tanks are run through the heat cycle of our dishwashers. It has been shown that *P. neurophilia* spores can be shed into the tank water along with eggs from infected females during mating.¹⁹ Since our tanks are not washed for 75% of each month, it is quite likely that this has contributed to the spread of this pathogen throughout the facility. In preliminary experiments, we have seen that mating tanks that have been through the hot water cycle of the dishwashers show a drastic reduction ($>10^3$) in the number of colony-forming units (cfu) on TSB plates (data not shown). If we assume that cfu are a reasonable proxy for our fish pathogens, then washing mating tanks 100% of the time should greatly reduce the burden of new infections due to this particular mode of pathogen transmission. We will use the polymerase chain reaction (PCR) methods described in Sanders *et al.*²⁰ to evaluate the level of *P. neurophilia* spores present in multiple mating tanks before and after dishwashing, and/or bleaching. These data will let us find the balance between effort and efficacy that best meets our goals within our limits.

(2) *Bleaching all embryos*—Another step that could reduce our current burden of disease would be to request that users bleach all embryos going into the nursery incubator. This would include those originating from within the facility as well as those from quarantine. Even though bleach will not kill intraovum¹⁹ *P. neurophilia*, Ferguson *et al.*¹⁴ demonstrated that buffered bleach at 100 ppm for 5 min will kill over 90% of exposed *P. neurophilia* spores. This should help reduce the transmission cycle of this pathogen drastically. We will use the PCR methods described in Sanders *et al.*²⁰ to document the impact on the transmission rates of *P. neurophilia* in bleached versus nonbleached embryos taken from the same starting pool of wild-type embryos. These data will provide evidence and a rationale to request our users to make this change to their protocols.

(3) *Prescreening of QX embryos*—While all outside fish must go into the quarantine room and may never be brought into the main facility, they go directly onto the QX recirculating system without an observation period in isolation. Users can set up matings with new fish anytime, bleach the resulting embryos, and then place them directly into the main nursery incubator. This leaves us vulnerable to new pathogens in two respects. First, it leaves our QX colony susceptible to any pathogen brought in with new fish. A minimal improvement would be the addition of an isolation tank where incoming fish could be kept for observation for several weeks, while their feces is examined for *Pseudocapillaria tomentosa* eggs.²¹ Because we do not have the means to house fish long-term off of the system, treating their infection is not a viable option.²² Fish testing positive for *P. tomentosa* would need to be mated, their eggs bleached for the nursery incubator, and the adult fish euthanized as soon as possible. However, these measures would not prevent other pathogens such as *Pleistophora hyphessobryconis*² or the various *Mycobacterium* spp.^{3,4,17,23–25} from infecting our QX colony.

Second, this also leaves our main colony vulnerable to pathogens with bleach-resistant eggs or spores. Visual examination of bleached embryos may allow us to detect some pathogens (i.e., intraovum *P. neurophilia*),¹⁹ but this is far from sensitive. Kent *et al.*¹⁵ described the measures needed to create an SPF facility that could protect a colony from bleach-

resistant pathogen eggs or spores, but such measures are beyond our constraints. We continue to seek viable alternate approaches to this problem.

Conclusions for moving forward

As our experience demonstrates, pathogen control in a zebrafish facility is still a work in progress; there are still many potential avenues for pathogen introduction into our facility. As the field of zebrafish husbandry grows and develops, both the challenges and opportunities increase. As facility managers gain new insights into the consequences of various husbandry practices, the sharing of information through the Zebrafish Husbandry Association (ZHA); (www.zhaonline.org/index.html), publications, and other online resources, helps drive the development of new solutions.

Since these challenges and solutions impact facility users and staff, managers need to provide convincing evidence that justifies changing standard operating procedures (SOPs). As our recent intervention programs illustrate, effort without data may be praiseworthy and improve the general environment, but we do not have hard data to show that our efforts reduced the pathogen load in our facility. If we had designed these interventions with data collection in mind, we would be in a better position to justify the time and expense. So our plans for the future include the following considerations:

Establish a baseline. We have set up a sentinel fish program that shows us what pathogens are present, but this doesn't give us a quantitative measure of prevalence for our various pathogens. We will begin screening 60 randomly selected fish from the general colony each year to identify pathogens with $\geq 5\%$ prevalence.¹⁵ Randomly selected fish will allow us to include fish frequently exposed to mating stress, which is expected to increase their susceptibility to diseases.^{16,17} In addition, we will use 16–20 of our effluent fish and add an equal number of moribund fish (since it is reasonable to expect that these fish will generally have a larger pathogen burden), to better detect which pathogens are present in our colony.

Evaluate new SOPs before proposing changes. To evaluate the effectiveness of new SOPs, we will collect pilot data to determine what works and how well it works. Our current pilot studies include washing mating tanks after each use and evaluating the impact that bleaching *all* embryos has on the burden of disease in the facility. We will recommend new SOPs when we have data that the proposed changes improve fish health and/or reduce the burden of disease.

Present the data. Once we have data in hand, we will seek feedback from our peers, either by presenting the data to our users, through ZHA, or by submission of articles for review. Currently, we give at least one talk a year to the Zebrafish Interest Group on campus. This allows us to provide updates on new information available regarding zebrafish husbandry. As more data accumulate that demonstrate how pathogen loads may affect research outcomes, this venue provides an open forum where the impact of these data can be discussed with the users. Follow-up meetings with our board of directors are held to discuss and vote on proposed changes in protocols that will impact the users.

Our goal is to improve animal health in support of reproducible research outcomes for the users. It has been extremely helpful to us to be part of a husbandry community that continues to develop a body of literature that shares protocols, outcomes, and information.

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