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Development and maintenance of a specific pathogen free (SPF) zebrafish research facility for *Pseudoloma neurophilia*

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Abstract

Pseudoloma neurophilia (Microsporidia) is very common in zebrafish research facilities. A new zebrafish facility was established at the Sinnhuber Aquatic Resource Laboratory (SARL) at Oregon State University, and thus we used this as an opportunity to establish a Specific Pathogen Free (SPF) colony of zebrafish for this microsporidium. Progeny from 10 zebrafish lines (n = 2,203) were initially transferred to the SARL facility in 2007 following PCR screening of broodstock and a subpopulation of progeny (258/1,000 fish from each family). Screening of fish within the facility was conducted as follows: 1) Moribund or dead fish were examined by histology 2) Each line was regenerated on a 4 mo. rotation, and for each of these major propagations, a subsample was screened for *P. neurophilia* by PCR, in which 60 fry from were collected randomly at 10 days post hatch and screened by PCR for *P. neurophilia* in pools of 10 3) Adult fish from each line were retired and euthanized at approximately 1 year of age. Twenty of these fish were examined by histology and the brains and spinal cords of 60 fish were combined in pools of five and screened by PCR 4) Sentinel fish were held in 4 tanks receiving effluent water from all tanks in the facility (20 fish/tank). Twenty fish were examined by histology and the brains of another 60 fish (in pools of five) were screened by PCR for *P. neurophilia* and 5) 760 4 mo old fish from a toxicology study conducted within the laboratory were examined by histology. To date, we have evaluated 2,800 fish by PCR and 1, 222 fish by histology and have not detected *P. neurophilia*. Thus we have established 9 lines of zebrafish SPF for *P. neurophilia*. However, 26 fish exhibited mycobacteriosis, diagnosed by the presence of acid fast bacteria visualized in tissue sections. Forty-nine other fish exhibited chronic inflammatory lesions, including egg associated inflammation and hyperplasia, in which acid-fast bacteria were not detected. Eight exhibited hepatic megalocytosis or hepatocellular pleomorphism, and three exhibited neoplasia (cholangiocellular carcinoma, and two with seminoma). One of the seminomas occurred in a female, and was classified as ovo-testes.

Keywords

Danio; Disease control; Microsporidian

INTRODUCTION

A corner stone for control of infectious diseases in laboratory animals, particularly with mice, is the use of animals that are specific pathogen free (SPF). The concept of working

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with SPF animals is gradually being accepted by laboratory researchers using zebrafish. Zebrafish research was first focused on developmental genetics using embryonic stages of the fish (Fishman 2001). Now they are used in post-embryonic stages in toxicology, immunology, transplant studies, infectious disease research, and as a platform for high through-put chemical screening (Sumanasa and Lin 2004; Traver et al. 2004; Zon and Peterson 2005; Lieschke and Currie 2007; Sullivan and Kim 2008; Kanther and Rawls 2010).

With all of these research areas there is a need to use fish that are SPF or at least have a comprehensive disease and pathogen history (Kent et al. 2009). For example, Vojtech et al. (2009) stated that they used SPF zebrafish for studies with *Francisella*, but did not state for which pathogens they were SPF. The concept of certification of pathogen-free stocks has been a mainstay in the salmonid industry, particularly with transport of fish between geographic regions (Kent and Keiser 2003; Stead and Laird 2002). This is facilitated by using SPF eggs when moving fish into new facilities. Indeed, egg laying fish provide an advantage over live bearing fish and mammals in that pathogen exposure to the next generation can be reduced by separating eggs from their parents and disinfecting them before introduction into another facility. The general rearing practice for zebrafish in research is to introduce new stocks via chlorine surface disinfected eggs. This allows for the possibility of applying the same principles and methods routinely used in salmonid aquaculture for establishing SPF stocks, such as screening broodstock, eggs, sex products, and rearing fry in an environment completely separate from potentially infected fish, including broodstock.

The most important and widespread pathogens of zebrafish in research facilities are *Pseudoloma neurophilia* and *Mycobacterium* spp. The former is the most common pathogen in zebrafish facilities. Based on over 10 years of data from the Zebrafish International Resource Center diagnostic service (<http://zebrafish.org/zirc/health/index.php>), the parasite has been found in over 50% of the zebrafish facilities. Various lines of evidence suggest that the microsporidium is maternally transferred, either within or outside eggs. Spores are found within the ovary (Kent and Bishop-Stewart 2003) and we have also found it within developing follicles. The spores of this parasite are resistant to chlorine used at the levels for treating zebrafish eggs and fry are very susceptible to the infection (Ferguson et al. 2007). Also, there are neither treatments nor vaccines available for this infection. We therefore decided that *P. neurophilia* would be a good candidate pathogen for establishing SPF populations at a new zebrafish laboratory at the Sinnhuber Aquatic Research Laboratory, Oregon State University.

MATERIALS AND METHODS

Development of SPF fish

In 2007, a new zebrafish facility was built at the Sinnhuber Aquatic Research Laboratory (SARL), Oregon State University. As this facility had never held zebrafish, it provided the appropriate situation to attempt to establish a SPF colony of zebrafish. Moreover, the source (brood fish) for the new facility were held in R. Tanguay's laboratory in another building on the OSU campus (Weniger Hall). Most of the lines were transported to OSU from University of Colorado Health Sciences Center, Denver, Colorado with R. Tanguay when he moved to the former location in 2003. All lines were originally obtained from the researchers who developed them as cited below. Our previous testing of various fish from his facility showed no *P. neurophilia* infections. Therefore, they provided an ideal source for establishing SPF populations. The protocol for screening and handling brood and their progeny fish destined for SARL was as follows:

Brood Fish—Adult fish were spawned as individual pairs or in small group using traditional spawning containers with plastic grid inserts to collect eggs (Westerfield 2007). The goal was to obtain at least 1,000 eggs from the pairs or groups. This often required spawning the same fish 2 or 3 times. In this case, brood fish were held in separate aquaria and spawned at 2–3 week intervals.

Eggs and Fry—Eggs were collected, surface disinfected with chlorine at 50 ppm for 5 min, and transferred to hatching water that had been sterilized with an autoclave and had methylene blue at 0.5 µg/L (Westerfield 2007). Larvae were held in static water in 500 ml beakers in a temperature controlled (28 °C) isolation room that had no other fish. Each group of 1,000 fry derived from the same parents, even if from multiple spawns, was considered a separate family. Fry were fed paramecium for the first 2 wk, then brine shrimp nauplii and commercial diet. Using terminal procedures, 258 fry (whole fish) from the family at 10 days post fertilization and the brains of all the parents were screened for *Pseudoloma* infection using the PCR test developed by Whipps and Kent (2006). We were unable to obtain sufficient numbers of AB brood fish from our source laboratory. Therefore, we purchased 1,000 embryonated eggs of this strain from the Zebrafish International Resource Center. A total of 258 fry were screened by PCR (Whipps and Kent 2006) and the surviving fry were transferred to the SARL.

Our sample size selection allows for detection of infection at 1% prevalence or greater with a 95% confidence (Simon and Schill 1984; AFS-FHS 2007). If families were smaller than 1,000, we adjusted the sampling size to maintain this detection limit using tables based on the following formula:

$$n = \left[1 - (1-p)^{\frac{1}{d}} \right] \times \left[N - \left(\frac{d}{2} \right) \right] + 1$$

where: n = required sample size, N = Population size, p = probability of finding one infected fish, usually set at 95% ($p = 0.95$), d = minimum number of infected fish expected given a presumed prevalence (P) so that $d = P \times N$. If all samples were negative, then the remaining fry were transferred to the SARL.

Husbandry and Biosecurity at the SARL

Facility Design—The SARL utilizes a recirculating water system. The water source is well water, charcoal filtered, particulate filtered, and UV treated at target 30,000 µWs/cm². The incoming water is then filtered by reverse osmosis (RO), and discharged to the sump of the recirculating system. All waste water is filtered through a particulate bead filter, a fluidized biofilter, and a 10 µm bag filter and then passed through biological UV treatment (100,000 µWs/cm²) before it is returned to aquaria holding zebrafish. Hardness in the system is 80 to 120 ppm, conductivity is 575 to 625 microsiemens/cm, and temperature is maintained at 28 C. Fish are housed in one room and maintained in 13 racks with a capacity of approximately 1300 tanks (Aquaneering, San Diego, CA).

Diet—Larval fish were initially fed paramecium (*Paramecium caudatum*) as a first diet but were switched to a completely artificial diet regime starting 15 October 2009. With paramecium, 5 days-post-fertilization (dpf) fish were fed 100 ml of paramecium and 1 scoop of Ziegler Larval AP100 < 100 µm (Ziegler Ziegler Bros., Inc, Gardners, PA) 3 times/day until 10 dpf. Fish reared without paramecium were fed as follows: 5 dpf fish, diet consisting of equal parts of Ziegler Larval AP100 < 100 µm, Golden Pearls 100–200 µm (Brine Shrimp Direct, Ogden, UT) and Freeze Dried Rotifers 100–200 µm (Brine Shrimp Direct, Ogden,

UT). Then at 10–21 dpf fish were fed artemia naupli and powdered diet (50% Ziegler Larval AP100 <100 μm and 50% Ziegler Larval AP100 150–250 μm) 3 times/day. A 22–45 dpf fish were fed artemia nauplii and powdered diet (50% Ziegler Larval AP100 150–250 μm and 50% Ziegler Larval AP100 250–450 μm) 3 times/day. At 45–60 dpf were fed as 22–45 dpf in the morning and mid-day and are fed artemia nauplii and adult flake in the evening.

Personnel Access and Staff Procedures—To avoid potential transmission of water borne pathogens between tanks, separate nets are used for each tank. Nets are disinfected by washing in the lab dishwasher using Neodisher detergent and neutralizer (Chemische Fabrik Dr. Weigert Gm Co. Hamburg) and the nets are then hung until completely dry before the next use. Staff, visitors, etc. are allowed into the facility for specific reasons and only with prior approval by the laboratory manager and/or Dr. Tanguay. Staff working in the main facility are not allowed to have contact with other zebrafish populations. All staff must wash their hands thoroughly before entering room. Protective foot wear (disposable booties or designated non-disposable shoes or boots must be worn). No one is allowed in the animal room without strict supervision until they have been trained and have shown proficiency with each component of the biosecurity program. Staff are given a written copy of the biosecurity protocol, and senior staff oversees new staff and students to assure they are competent at implementing biosecurity rules. Retraining occurs as needed based on observation of senior staff. Visitors and staff are not allowed into the animal room if they have come in contact with other live fish the same day. If they must visit/work in other labs, this is done after working in the SPF facility and staff are not allowed to return to the main facility that day.

Health Monitoring

Fish at the SARL were routinely monitored for the presence of *P. neurophilia* using both PCR and histology. Histology was also used to document other infections and histological changes. For histology, fish were preserved in Deitrich's fixative and processed for routine histology. Midline sagittal sections were prepared so that a large portion of the brain and spinal cord was present. Slides were then stained with hematoxylin and eosin or Accustain Gram stain (Sigma-Aldrich, St. Louis). Additional sections were stained with Fite's Acid Fast for any fish that exhibited histological changes suggestive of mycobacteriosis.

For PCR, either whole fish (larvae) or brains (juveniles and adults) were frozen, and screened using a conventional PCR test (Whipps and Kent 2006). Starting 1 November 2008 we switched to a new, qPCR test recently developed in our laboratory (Sanders et al. 2011). The program for these examinations was as follows:

Moribund or Dead Fish—Dead or moribund fish were saved for histology. External, macroscopic changes were noted. To assure proper fixation, the body cavity was exposed by a cut in abdominal wall and then fish were placed in individual tubes.

Routine screening of existing lines—Each line at the SARL is regenerated on a 4 mo. rotation. For each of these major propagations, a subsample was screened for *Pseudoloma*. Sixty fry from the large pool (e.g., 500 – 2,000 fry) were collected randomly at 10 days post hatch. Fry are pooled in groups of 10 and screened by PCR for *P. neurophilia*

Retired populations—Brood fish from each line are retired and euthanized at approximately 1 year of age. Twenty fish were examined by histology and the brains and spinal cords of 60 fish were combined in pools of five and screened by PCR.

Sentinel Program—The sentinel program at the SARL consists of holding 80 4 month-old 5Ds in 4 sentinel tanks (20 per tank) and 80 ABs in 4 sentinel tanks (20 per tank). These tanks are placed so that they receive effluent water from all tanks prior to filtration in the main room. Fish are fed daily as in main room. Fish are exposed for 3–4 mo. and then replaced with the next generation of 5Ds and AB's. Twenty fish are examined by histology and the brains of another 60 fish (in pools of five) are screened by PCR for *P. neurophilia*.

RESULTS

Between April 2007 and July 2009 we successfully screened and transferred 9 populations, representing the following lines: Tg(isl1:GFP) Higashijima et al. (2000); Tg(HuC:GFP) Higashijima et al. (2003); Tg(NBT:MAPT-GFP (Gift from Chien Laboratory, University of Utah); Tg(fli1a:EGFP)y1 Lawson and Weinstein (2002); AB (developed at University of Oregon); 5D, an out bred stock developed from fish that we obtained from 5D Tropical Plant City Florida; Tg(-8.4neurog1:GFP) Blader et al. (2005); Tg(hsp70:tcf3-GFP)w26 Lewis et al. (2004); and MPO (Tg(mpx:GFP)i113) Renshaw et al. (2006). This represented 2,203 fish in total transferred to the SARL and were designated F₀. During our screening, we found positive fish in one population of Islets from the source laboratory (Weniger Hall) for *P. neurophilia* by PCR. Brood fish from this population were spawned May 2007 and 4 of 7 fish were also positive by histological examination. F₁s from this group, also held at the source laboratory (Weniger Hall), were also positive by both PCR and microscopy. Fortunately, another population of Islets held at Weniger Hall was available. Thirteen of these fish were spawned in a group spawn (Lawrence 2007; Westerfield 2007) and their fry were screened, found to be negative, and were transferred to the SARL following the protocol described above.

Adults and progeny of another line from the source laboratory, called Blingless (purchased from Znomics, Portland, Oregon) were positive for *P. neurophilia*. Unfortunately, other populations were not available, and thus this line was not established at the SARL. The various lines at Weniger came from Dr. Tanguay's laboratory at the University of Wisconsin and were transferred to Weniger in 2002, except that the Blingless and Islets came from two other laboratories.

Pathogens and Diseases at SARL

As of 15 July 2010, we have evaluated 2,800 fish by PCR and 1,222 fish by histology from subsequent generations at the SARL that were clinically normal, moribund or deformed fish from the sentinel program or a toxicology study. This represents 25 retired fish groups, 3 sentinel groups, 10 moribund fish, and about over 700 clinically normal fish from a toxicology study conducted at SARL. The toxicology study represented fish from two separate experiments in which larval fish were exposed to continuously to a toxicant starting at 12 days post hatch and reared for 4 mo. Controls and fish exposed to various levels of the undisclosed toxicant were examined by histology at the end of each experiment.

No fish exhibited histological changes consistent with *P. neurophilia* and all fish that we examined by PCR were negative for the parasite. The AB line was the first group transferred to this new facility in April 2007. These fish exhibited chronic mortality and survivors grew poorly. Histological examination of 10 moribund fish that had been on the system for over month exhibited severe hepatic megalocytosis (Fig 1). As this lesion is associated with toxicant exposure, it was surmised that a toxicant from the plastics in the new system was leaching into the water. Therefore, a charcoal filter was added to the recirculating system and subsequent examinations of both moribund and sentinel fish showed no more dramatic liver changes. Subsequently, one individual fish from six different groups of retired fish and one sentinel fish exhibited mild to moderate hepatic megalocytosis (Fig 1).

Mycobacteriosis was detected in a few fish from all categories (Table 1). Aside from the moribund fish, all fish with this disease appeared clinically normal. Histological examination of these fish showed a variety of lesions consistent with mycobacteriosis, including chronic coelomitis, aerocystitis, or oophoritis. Multiple granulomas or more diffuse, chronic inflammatory lesions were observed in the liver, kidney, pancreas, swim bladder or ovaries (Fig. 1). Diagnosis was confirmed by observation of acid fast bacteria in sections stained with Fite's acid fast. Mycobacteriosis was significantly higher in the retired fish (3.8%) than those in the toxicology experiments (0.7%) (Fisher's Exact Test, $p > 0.001$).

Several other fish exhibited chronic inflammatory lesions in the mesenteries or visceral organs in which acid fast bacteria were not visualized. In addition, egg associated inflammation and fibroplasia (EAIF), characterized by chronic oophoritis was seen in several females (Fig. 1). One moribund Islet fish had a cholangiocellular carcinoma that replaced almost the entire liver parenchyma (Fig 1). A spermatocytic seminoma was observed in one retired male Fli strain fish, and one retired female also had a large spermatocytic seminoma that arose within pancreatic tissues. This female fish had a well-developed ovary and follicles that are undergoing normal maturational development, and hence the diagnosis was ovo-testes.

DISCUSSION

After 3 years in operation, *P. neurophilia* has not been detected in this facility based on the examination of over 3,000 fish composed of multiple generations of moribund, sentinel, fry, retired broodstock, and fish used in experiments. While morbidity and mortality has been minimal, we have detected some lesions in fish. Hepatic megalocytosis in fish is associated with exposure of both natural and anthropogenic toxicants. We concluded that these liver lesions seen in the first group of AB line were caused by toxicants released from the new plastic tanks, pipes, and possibly a soft plastic bladder in the system, etc. Following addition of a charcoal filter severe hepatic megalocytosis was not observed. However, it was not known if this corrected the problem or if the putative toxicant was removed over time merely by leeching of the plastics. Mild forms of hepatic megalocytosis and other related toxicopathic lesions are actually rather common in zebrafish facilities. Microcystin, produced by freshwater cyanobacteria, causes similar changes in fish (Andersen et al. 1993) and it is possible that a natural toxin produced by bacteria, fungi or algae may be the cause of these lesions. Spitsbergen and Kent (2003) noted that hepatic megalocytosis was particularly common in recirculating systems using fluidized sand filters.

Mycobacteriosis was observed in a few fish. This is the second most common disease in zebrafish facilities (Astrofsky et al. 2000; Kent et al. 2004). Unlike *P. neurophilia*, which is an obligate intracellular parasite, some species *Mycobacterium* spp. (e.g., *M. chelonae*) are ubiquitous in freshwater and proliferate outside of the host. Hence it would be more difficult to establish an SPF zebrafish colony for *Mycobacterium* spp. in general. The infection was only diagnosed by histopathology and thus the species infecting these fish was not determined. There was over five times the prevalence of mycobacteriosis in the retired fish than in the toxicology experiments. The retired fish were about 1 year old, while those from the toxicology study were euthanized at when they were 4 mo. old. Incidents of mycobacteriosis increase with age in other animals, including fish (Gauthier et al. 2008), which provides an explanation for this difference. Therefore, one approach to reduce mycobacteriosis in zebrafish colonies would be to retire brood stock at an earlier age when feasible. Acid fast bacteria were not observed in several fish with lesions that were consistent with mycobacteriosis, such as chronic coelomitis, aerocystitis or oophoritis. All of these lesions have been associated with mycobacteriosis in zebrafish (Kent et al. 2004; Whipps et al. 2008), but can be due to other causes. For example, chronic oophoritis,

referred to as egg associated inflammation (EAIF) is thought to be caused by egg retention in fish that are not spawned on a regular basis. Conversely, absence of detection of acid fast bacteria in tissue sections does not confirm absence of *Mycobacterium* spp. (Canale et al. 2000).

It should be easier to establish SPF fish in zebrafish research facilities compared to larger aquaculture endeavors as they use pathogen-free incoming water, utilize high doses of UV in recirculating systems, and introduce fewer fish to main facilities at a given time. One negative factor with many zebrafish facilities is that, unlike mouse colonies, breeding colonies are housed in the same rooms as research fish and often staff (including inadequately trained students) have unrestricted access to both.

Appropriate education of personal staff is essential as many of the staff responsible for zebrafish care have little formal training in control of infectious diseases. These individuals also often maintain home aquaria and thus have contact with other fish species. The recent observation of *Pleistophora hypnessobryconis* (Microsporidia) in zebrafish research colonies (Sanders et al. 2011) exemplifies the risk of aquarium fish pathogens to zebrafish in a laboratory setting. The zebrafish community should consider protocols used by the mice community for controlling and avoiding pathogens. For example, most SPF mouse facilities do not allow technicians working with the mice to be in contact with rodents outside the facility, including home pets. Perhaps a protocol this extreme is not required with zebrafish, as presumably fish pathogens are not as easily transmitted as those in a terrestrial environment. However, it is reasonable to require staff and visitors to thoroughly wash their hands before entering facilities and assure the existence of other biosecurity measures to prevent the movement of water or other wet material into a restricted laboratory.

Pleistophora hypnessobryconis may be another candidate to screen for using the methods described here. To date, no viral diseases have been detected in zebrafish facilities but once they are identified, a similar approach as we have employed here could be implemented. Now we are distributing limited numbers of these SPF fish through a collaboration with the Zebrafish International Resource Center, University of Oregon, Eugene.

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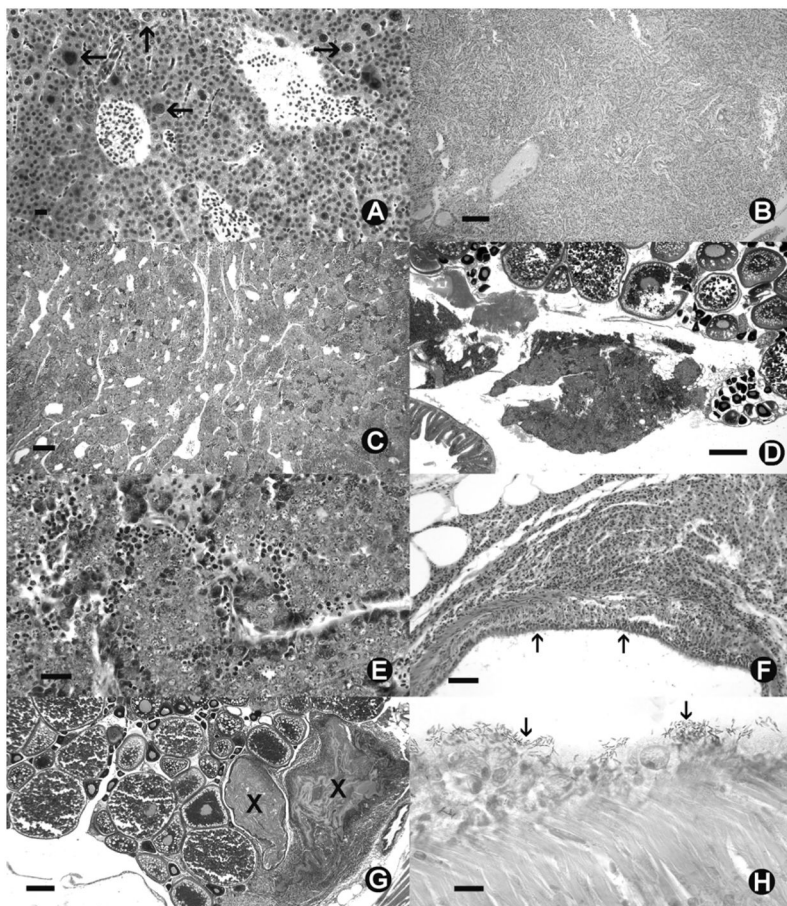


Fig 1. *Danio rerio* Histological sections of zebrafish from SPF colony. (A) Severe hepatocytic megalocytosis, with enlarged nuclei (arrows) and hepatocytes. (B) Cholangiocellular carcinoma replacing entire liver parenchyma. (C) Spermacytic seminoma. (D) Ovotestes. S = seminoma in mesenteries adjacent to mature ovaries. (E) High magnification of seminoma from fish with ovotestes. (F) Chronic aerocystitis. (G) Oophoritis (egg associated inflammation and fibroplasia) X = raft of eosinophilic yolk debris and fibroplasia. (H) Swimbladder surface with acid bacteria (arrows). (A, H) = 10 μ m. (B, C) = 100 μ m. (F) = 50 μ m. (E) = 25 μ m (D, G) = 500 μ m. (A–E) = Hematoxylin and eosin. (H) = Fite's acid fast.

Danio rerio Diseases and lesions observed in retired sentinel and moribund fish and fish examined by histology from a toxicology experiment at the Sinhuber Aquatic Research Center. EAIF = chronic oophoritis, egg associated inflammation and fibroplasia. Liver = hepatocellular pleomorphism or hepatic megalocytosis. Tox = toxicology experiments. Chronic inflammation = chronic inflammatory lesions in visceral organs or mesenteries in which acid fast bacteria were not detected.

Table 1

	Number of Groups	Number of Fish	Mycobacteriosis	Chronic Inflammation	EAIF	Liver	Neoplasia
Sentinels	3	58	1	0	0	1	0
Retired Fish	25	397	15	7	8	6	2
Moribund	7	10	4	0	0	0	1
Tox 1	5	363	1	3	25	0	0
Tox 2	5	394	4	6	9	1	0