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The challenges of implementing pathogen control strategies for fishes used in biomedical research[☆]

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Abstract

Over the past several decades, a number of fish species, including the zebrafish, medaka, and platyfish/sword-tail, have become important models for human health and disease. Despite the increasing prevalence of these and other fish species in research, methods for health maintenance and the management of diseases in laboratory populations of these animals are underdeveloped. There is a growing realization that this trend must change, especially as the use of these species expands beyond developmental biology and more towards experimental applications where the presence of underlying disease may affect the physiology animals used in experiments and potentially compromise research results. Therefore, there is a critical need to develop, improve, and implement strategies for managing health and disease in aquatic research facilities. The purpose of this review is to report the proceedings of a workshop entitled “Animal Health and Disease Management in Research Animals” that was recently held at the 5th Aquatic Animal Models for Human Disease in September 2010 at Corvallis, Oregon to discuss the challenges involved with moving the field forward on this front.

1. Introduction

The rise of a number of fishes, especially the zebrafish (*Danio rerio*), medaka (*Orzyias latipes*) and various species of the genus *Xiphophorus*, over the past several decades to prominence as animal models of human development and disease is well documented (Fishman, 2001; Grunwald and Eisen, 2002; Furutani-Seiki and Wittbrodt, 2004; Meierjohann and Scharl, 2006; Lieschke and Currie, 2007). This growth has been quite rapid, and like the laboratory mouse during its advent, advances in performance-based husbandry and health management for these species have lagged well behind developments in science and technological innovation (Kent et al., 2009).

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There is a growing realization that this trend must change, particularly as the use of fish (the zebrafish in particular) has moved beyond developmental genetics to diverse applications in many different fields of biology. An increasing number of study designs may evaluate fish for weeks or months, so defining the health status of the colony is critical in order to prevent the introduction of confounding variables or inducing pathological changes associated with underlying infections. A workshop entitled “Animal Health and Disease Management in Research Animals” was held at the 5th Aquatic Animal Models for Human Disease in September 2010 at Corvallis, Oregon. Here we summarize topics discussed at this workshop. Issues relating to zebrafish are emphasized, but most topics pertain to other fish species used in biomedical research.

2. Need for disease free experimental animals

Low to moderate level mortalities, reduced growth and even reduced fecundity (Ramsay et al., 2009b) appear to be acceptable to many researchers using aquatic models. A far less appreciated, but potentially more serious, problem are the effects that subclinical disease may be having on the physiology, immunity, histology and genetics of research subjects. Experimental animals are known to vary from one another depending on age, sex, genetics, health, immune status and biology (Lipman and Perkins, 2002). Microbial organisms are frequently encountered confounding variables in murine research and significant efforts are invested in producing, distributing and maintaining rodents free of microbial pathogens (Lipman and Perkins, 2002). Although the presence of microbes can cause disease, morbidity and mortality, the most common problem encountered by researchers working with rodents carrying organisms that do not cause obvious disease include changes in the histology or biochemistry, which can complicate data analysis and potentially lead to misinterpretation of results (Lipman and Perkins, 2002). As a consequence, the research and veterinary community have established standard exclusion lists for most, if not all, commonly used biomedical in vivo research models. At a minimum, these include specific viruses, bacteria, fungi and parasites. Consequently, it is standard practice for rodent users to ensure that animals used in experiments do not carry any underlying pathogens that have the potential to alter host biochemistry and physiology. Indeed, the identification and characterization of pathogens, as well as methods and technologies to exclude them from animal colonies, is integral to any successful laboratory animal science program, and there is a concurrent global industry to support it.

Factors such as health, microbes, age, sex and genetics have all been shown to contribute to variation in most, if not all, biomedical research models. It would be naïve to assume that fish with opportunistic, commensal or pathogenic microbes would not also be affected in similar ways, potentially making them less suitable as research subjects for certain kinds of studies. Therefore, it is now clear that pathogen control strategies employed for small fish models must become more sophisticated if they are to be used beyond their traditional applications in embryology and developmental genetics. Whereas the occurrence of underlying diseases causing low mortalities may be acceptable for production aquaculture or with ornamental fishes, they should be minimized in species (as with any laboratory animal) used in research. While the lessons learned during the development of the modern mammalian laboratory animal sciences will undoubtedly prove instructive, the intrinsic biological differences between fishes and mammals must be considered when devising solutions to these problems for zebrafish, medaka, and other fish models.

The process will not be easy, as the obstacles to implementing pathogen control measures in aquatic research facilities are numerous and complex. A thorough and realistic understanding of these factors is critical for the success of any such effort. The purpose of this review, derived from discussions in the Workshop, is to provide a basic overview of the

most critical challenges involved in this important new phase in the maturation of biomedical fish model systems.

3. Animal procurement

Since microbes are known to frequently confound research findings, the mammalian laboratory animal science community has developed and implemented microbial pathogen control programs to produce, distribute and maintain animal research colonies free of selected pathogens. One of the hallmarks of pathogen control is the oversight of the trafficking of research animals into and out of institutional facilities. Indeed, the concept of certification of pathogen-free stocks and documentation of pathogens has been a mainstay in the salmonid industry, particularly with transport of fish between geographic regions (Stead and Laird, 2002; Kent and Kieser, 2003). While these types of approaches have not yet been implemented for biomedical fish models, the transfer of different animal strains between collaborating laboratories is essential to research. This is particularly the case for both the zebrafish and medaka, which are particularly amenable to mutagenesis (Doyon et al., 2008) and transgenesis (Suster et al., 2009). Current methods now allow for the rapid and relatively facile generation of stable mutant and transgenic lines, to the point, for example, where a single zebrafish laboratory may generate hundreds of different strains of fish, many of which turn out to be extremely valuable tools for investigators in other laboratories (e.g. Winn et al., 2000; Ellingsen et al., 2005; Pogoda et al., 2006; Baraban et al., 2007; Jin et al., 2007; Scott et al., 2007; Asakawa et al., 2008; Moens et al., 2008; Covassin et al., 2009). Indeed, the zebrafish and medaka research communities are characterized by a vibrant exchange of fish strains between collaborating laboratories in different institutions across the globe.

However, the movement of animals also carries with it a significant risk of pathogen transmission. Consequently, any animal transfer program should require that the pathogen status of incoming and outgoing animals be defined such that infected animals (and therefore pathogens) are not transferred from one facility or colony to another (NRC, 2010). For example, in rodent colonies, this problem is dealt with on a number of levels. First, and most importantly, there are well-defined standards for the care and management of mice and rats that most, if not all institutional animal programs adhere to. One critical component of rodent program management is the implementation of some form of sentinel health monitoring so that the disease status of animals in a given colony is known. Secondly, all rodent commercial vendors that generate and distribute rodents for the use in research produce, distribute, and maintain rodents with a known microbial flora and also generate specific pathogen free (SPF) animals that are made available to the research community. If an investigator requires a given strain of mouse from another laboratory at another institution for their research, the strains in question are either quarantined at the receiving institution after the health status of the originating colony and facility has been assessed, or they are rederived by a commercial vendor and then imported into the receiving facility.

Unfortunately, these options are limited for researchers using fish for biomedical research studies. There are no established commercial sources of SPF fish species bred and reared for research. The only entity that currently approaches this level of sophistication is the NIH-supported Zebrafish International Resource Center (ZIRC) at the University of Oregon. Whereas this resource is certainly valuable, the number of strains available for distribution to the community is limited, and fish are not certified as SPF. Specific populations for shipment are not screened for pathogens, but documentation of pathogens (*Pseudoloma neurophilia* and *Mycobacterium chelonae*) at ZIRC as seen in their sentinel fish is available online (http://zebrafish.org/zirc/documents/health_report.php). Hence, even if the animals originate from ZIRC, it is still possible that they may be carrying commensal, opportunistic,

or pathogenic organisms that could be transmitted to a receiving colony. Recently, SPF zebrafish for the microsporidium *P. neurophilia* have become available to the research community (Kent et al., in press). These populations are being maintained at the Sinhubber Aquatic Resource Center, Oregon State University, and are available through ZIRC (<http://zebrafish.org/zirc/documents/fees.php>).

Although ZIRC is a major supplier of zebrafish, and stock centers for medaka and *Xiphophorus* are also actively supplying those species to researchers, much of the traffic of fish models around the world involves the exchange of animals between collaborators at various academic institutions. This presents a major problem for the establishment and maintenance of pathogen control in fish research facilities for several reasons. First, there are few universally accepted standards for the care and management of fish in biomedical research settings, including the zebrafish (Lawrence, 2007) and regulatory oversight of aquatic programs is generally insubstantial (Lawrence et al., 2009). This means that conditions (including diets, water quality, genetic pedigree, etc.) may be and often are quite variable from one institution to another. It also means that it is often unclear as to whether or not management strategies essential for defining the status of animals in a colony (health monitoring programs, formalized and centralized animal transfer procedures, etc.) are actually in place at a given institution. For example, while it is not known how many existing zebrafish research facilities implement some form of sentinel or health monitoring program, it is possible that relatively few programs actually have such a practice in place. Based upon the information from the zebrafish information network (ZFIN), which listed 667 registered laboratories as of early 2011 (www.zfin.org) in comparison to the historical diagnostic submissions to ZIRC's pathology services (Table 1), one could surmise that this service is either being under-utilized, other diagnostic services are being utilized, or minimal diagnostic evaluations are occurring within these facilities. In addition, although the majority of cases submitted to the ZIRC Pathology Service are described as “health check”, it is often unclear whether submissions are from designated pre-and post-filtration sentinel tanks originally stocked with young fish of known pathogen status sampled at a pre-determined date versus a random sampling of asymptomatic and moribund fish. Therefore, it is likely that the great majority of transfers of zebrafish from one institution to another are defined by a general lack of understanding of the concepts of health status evaluation of the animals in question by both parties participating in the transaction. This trend probably also holds true for the other fish models.

4. Staffing challenges

Standard disease control measures for animal facilities require investments in time, money, and expertise. These can be substantial, especially in instances where selected pathogens are actively excluded from colonies. Such approaches require specialized and dedicated infrastructure and housing for animals, intensive screening of animals, routine monitoring of health status, implementation of strict quarantine methods — in addition to dedicated and specifically trained personnel to manage the operation (Yanabe et al., 2001; Watanabe et al., 2005; Morton et al., 2008; Ramsay et al., 2009a; Wolf et al., 2010). Relatively basic components of an animal health program, such as health monitoring, still require dedicated equipment, routine monitoring of animals, and regular oversight (Barthold, 1998; White et al., 1998; Nicklas et al., 2002; Gourdon, 2004; Shek, 2008). Indeed, even the most basic element of pathogen control – the maintenance of a stable, favorable, and well-defined environment (both macro- and micro-) in a facility – requires full-time, dedicated personnel with demonstrated expertise in the biology and husbandry of the animals in question. Because of the size and scope of the physical infrastructure and staff necessary to maintain in an “optimum” facility, it is recognized that these recommended institutional services will most likely be available at the larger institutions where there are economies of scale to

afford them. It is therefore also incumbent upon fish health specialists to identify affordable but effective management practices that can be economically scaled-down to the point where they can be readily employed in the small research facilities most typical in the fish research community.

While the commitment of these kinds of resources for maintaining animal colony health is considered to be an *indispensible* component of the management equation in a mammalian laboratory animal facility, this is not the case for fishes in research. Staffing practices are at the root of this problem. Many zebrafish and other aquatic research facilities are understaffed, particularly those that are under the direct management of investigators using the fish in their research, which still is the predominant situation in the field. In these settings and especially at the smaller research institutes, it is rare for an aquatic research facility to employ personnel that are *completely* dedicated to the care and management of fish. In many instances, the staff officially charged with these responsibilities are also required to work in the laboratory on research projects. When personnel's time is actually dedicated, it is usually in short supply, and so is often just devoted to and consumed with the most basic of tasks: feeding and cleaning. Even in facilities that employ personnel dedicated to animal care who may recommend removal of sick and old fish, the ultimate decision is often still made by the researcher using those fish. The results of these kinds of staffing practices are pervasive, and have far-reaching and negative implications for animal health and pathogen control. Although the researchers have a vested interest in the good health of their research animals, facilities operated in this fashion are often hard pressed to satisfy even the most elemental requirement of pathogen control – the maintenance of a stable, favorable, and well-defined environment – let alone to meet the more advanced demands of a professionally run health management program. And so while it is certainly possible to implement health management strategies in zebrafish research facilities, the requirements for doing so are so well beyond typical staffing levels and funding allocations that it is simply not realistic to expect that it can be achieved in most circumstances.

5. Gaps in information on infectious agents in zebrafish colonies

The understanding of diseases in zebrafish and other fish models has improved markedly over the past decade, particularly with respect to the identification and characterization of the most prominent agents, including *Pseudoloma neurophila* (Matthews et al., 2001; Kent and Bishop-Stewart, 2003; Whipps and Kent, 2006; Broussard and Ennis 2007; Ferguson et al., 2007; Watral et al., 2007) and *Mycobacterium* spp. (Kent et al., 2004; Watral and Kent, 2007; Whipps et al., 2008; Hegedus et al., 2009; Ramsay et al., 2009a; Cui et al., 2010). The central hub of information on diseases and pathogens in zebrafish is ZIRC, which operates a diagnostic service that has been compiling data on pathogens in zebrafish colonies since its inception in 1998. ZIRC has developed an extensive database as a result of the many institutions around the globe that have used the center's diagnostic services during this period for analysis as part of their health monitoring and/or quality control programs. This information is transmitted to the community in the form of a continuously updated, web-based manual on zebrafish disease (Kent et al. 2007).

However, knowledge of infectious agents in aquatic animal facilities across the community is incomplete since most subclinical and clinical cases are not investigated. Furthermore, there is a significant shortage of diagnostic resources and funds that would enable screening zebrafish, medaka and other fish models for microbial organisms. Nevertheless, only a small fraction of the many research laboratories using zebrafish have submitted samples to the ZIRC diagnostic laboratory. The reasons for the lack of effort and interest in diseases in affecting aquatic research animals in a research setting are numerous and complicated. They can largely be traced back to the aforementioned lack of universally adhered to standards for

care and management of these species. Indeed, the general manner in which many research facilities are managed is a major factor in this information gap. Regulatory oversight of most zebrafish and other aquatic operations has historically been inconsistent in terms of its ability to encourage systematic improvements in the way they are managed (Lawrence et al., 2009). While this is due in no small part to the fact that there are still very few published *comprehensive* references for fish husbandry, the end result of this dynamic is that level of care and oversight in many fish research colonies around the world is considerably below that typically found in a traditional rodent facility. One important consequence of this is that the health status of many of these colonies is very much in question. For example, the number of zebrafish facilities in existence greatly exceeds the those that have at least sent fish to ZIRC or other diagnostic laboratories. It stands to reason that some of the most serious cases are likely in operations with no professional husbandry staff, poorly controlled environmental conditions and few to no measures for limiting the entry of or maintaining and controlling disease in their colonies, and it is likely that these same laboratories are also not sending fish to ZIRC or other diagnostic services for routine health evaluation. Furthermore, it is also most likely that many of these same laboratories are still actively engaged in research collaborations, and transferring fish to other laboratories around the world.

Consequently, it is also very likely that there are pathogens present in zebrafish research facilities that have yet to be discovered. For example, we recently documented the occurrence of a microsporidium *Pleistophora hyphessobryconis* in three zebrafish facilities (Sanders et al., 2010). More serious viral pathogens, such as infectious spleen and kidney necrosis virus (ISKNV), are known to be pathogenic to zebrafish (Jeong et al., 2008) and present in pet fish populations. These are just two examples of potential pathogens that might cause serious problems in zebrafish facilities, particularly because some researchers still introduce zebrafish from pet stores or pet fish wholesalers directly into research facilities with no pathogen screening or evaluation.

This presents a major challenge for the control of diseases in the research community because it is impossible to effectively manage colonies for agents not yet recognized to be present. This problem will not be alleviated until data-driven standards for care and management are developed and ultimately adopted by the majority of fish research facilities worldwide. Of particular importance here is the practice of health monitoring. Widespread implementation of these programs will serve to identify new pathogens and evaluate the risks that they pose to fish colonies. Some of the impetus for change should come from the researchers as well because these pathogens have the potential to negatively impact anatomic, physiologic, and behavior data gathered from these models, which could influence research objectives.

6. Pathogen control vs. productivity

A necessary step in the process of moving the fish research field forward towards pathogen control is making the case to fish users that doing so is necessary *and* beneficial for research. Indeed, such strategies will only be widely adopted in the community if they are not perceived by investigators to be obstacles to doing the science, particularly because there will be considerable costs associated with implementation. The time and funds required to carry out this commitment will usually have to be borne directly by the researchers but not from their direct costs from grants, etc., as few academic institutions provide this infrastructure or support. It is important to consider another important possibility that the integration of strict pathogen control measures in aquatic facilities could have a negative impact on fish productivity (growth and reproductive performance), at least in the short term. A number of factors may contribute to this fact. One important issue is the relationship

between the natural “preferences” of the fish and productivity in cultured conditions. For example, in the wild, zebrafish are known to consume a wide variety of available food items, with zooplankton and aquatic insects being preferred (McClure et al., 2006; Spence et al., 2007). The medaka and platyfish also have similar dietary preferences (Arthington, 1989; Li and Fu, 2009a). It is due in part to this fact that live zooplankton, such as rotifers (*Brachionus plicatilis*), ciliates (*Paramecium* sp.), and especially brine shrimp (*Artemia* sp.) nauplii and metanauplii, are widely used and important components of the diets for these and other similar fish at all life stages and applications. The performance of zebrafish on live feeds is typically superior to those fed processed diets, especially during the larval stages (Carvalho et al., 2006; Best et al., 2010). At the same time, live diets are also a major potential source of pathogens and other contaminants (Harper and Lawrence, 2010). In addition, these feeds may also display considerable variability in nutritional profiles (Siccardi et al., 2009), a movement towards processed, semi-purified artificial diets is necessary to help close the loophole in pathogen control that the application of live feeds represents. However, progress on processed diets for zebrafish has been slow, primarily because precise nutritional requirements have not yet been carefully delineated (Lawrence, 2007). The situation in medaka is similar, as some efforts have been made to develop standard, purified diets (DeKoven et al., 1992), but as with the zebrafish, live diets are still very commonly utilized. Consequently, currently available feeds of this type do not appear to support the same productivity that live feeds do, at least when they are used exclusively (Goolish et al., 1999; Carvalho et al., 2006). Until these feeds have been improved, many researchers, particularly those that use the zebrafish, will be reluctant to completely eliminate *Artemia*, rotifers, and other live zooplankton from diets and the risk of pathogen entry via these prominent potential vectors will remain.

The zebrafish and medaka also appear to thrive in eutrophic conditions, especially during early development. This is not altogether unsurprising given their natural history; in the wild the fish are thought to spawn in shallow margins of flooded water bodies, depositing fertilized eggs where they will develop in “nursery” areas rich with nutrients, organic waste, and live prey (Spence et al., 2006; Engeszer et al., 2007; Li and Fu, 2009b). While this general model is supported by only a limited number of studies, it is bolstered by observations made in the laboratory, as larval zebrafish have been shown to display high rates of growth and survival in water containing levels of nitrogenous wastes shown to be toxic to other cultured fish species (Best et al., 2010). The same has also been observed for medaka (Best and Lawrence, unpublished observations). In spite of this, it stands to reason that such “natural” conditions may not be totally conducive to pathogen control. Indeed, these conditions are also favorable to the growth of opportunistic bacteria and other microorganisms that can pose a pathogenic risk to the fish. So while the levels of waste in fish tanks may be readily eliminated or reduced by increasing rates of water exchange, doing so may result in reduced growth and survival of larvae. Given all the concerns above, it should be noted that mortality due to infectious diseases or other causes not related directly to experiments has been extremely low in zebrafish at the Sinnhuber Aquatic Research Facility at Oregon State University, which is managed to be free of *P. neurophilia* (Kent et al., in press). Fish are closely monitored at this facility, and records showed mortality for 2010 to be less than 0.3%/year in post-larval zebrafish. While not empirical, this observation suggests that elimination of *P. neurophilia* has had a positive impact on overall survival.

Another point to consider relative to the relationship between disease control and fish productivity is the impact that some control methods are likely to have on the genetic diversity of stocks. For example, a promising new technique for generating and maintaining fish colonies SPF for *Pseudoloma neurophila* (Kent et al., in press) necessitates that founding animals at each generation are screened by molecular tests (e.g. PCR) and histology for the presence of the pathogen. While the employment of this practice will

exclude this important pathogen from a given colony, it also has the potential for genetic bottlenecks, because only a relatively small number of “clean” founders are used for propagation of new generations. This dynamic will only serve to accelerate the ongoing loss of genetic diversity associated with the maintenance of small closed populations (Stohler et al., 2004), and increases the potential that problems related to inbreeding depression (Monson and Sadler, 2010), including reductions in reproductive output, growth and survival, will occur.

7. Is strict pathogen control necessary to do the science?

The use of fishes in biomedical research, lead by the zebrafish, has grown exponentially during the last several decades. This has led to a number of important discoveries in a number of different fields, including the genetics of human skin pigmentation (Lamason et al., 2005), blood stem cell development (North et al., 2007), retinal biology (Alvarez-Delfin et al., 2009), and organ regeneration (Jopling et al., 2010). All of this has occurred with limited pathogen control measures incorporated in the great majority of fish biomedical research facilities where this type of research is done. Consequently, the concept of disease control is not on the “radar screen” of many fish investigators. Whereas it is a relatively simple exercise to point out the theoretical reasons why this needs to change in order for the science being done with the fish models to be improved, it is still necessary to convince investigators why this should be the case. This is particularly challenging in light of the fact that implementation of pathogen control measures will be expensive and logistically challenging to achieve, compared to the alternative of doing little to nothing about it. Moreover, there is a general attitude with many researchers that in, contrast to mammals, ongoing low or even moderate levels of mortality is acceptable and “normal”.

There is the possibility that specific fish models will be changed or altered by the removal of pathogenic agents. As an example, several rodent immunology models were altered once the most common mouse viral pathogen, mouse hepatitis virus (MHV), was identified and subsequently removed from rodent colonies from several facilities worldwide (GV-SOLAS Report, 1999; Felix and Homberger, 1997). MHV infection and subsequent disease (both clinical and subclinical) causes acute death, immunodepression and/or immunostimulation, altered immune cell function, modified hepatic enzyme levels, protein synthesis and response to injury/chemical carcinogenesis, bone marrow cellular dyscrasia, interference with tumor transplantation studies, and reduced/potentiated susceptibility to other viral or bacterial diseases (Barthold, 1986a,b; Compton et al., 1993).

Indeed, much of the underpinning of the argument for implementing improved control of diseases in aquatics comes from numerous examples in other laboratory animal model species. And while there are several published studies that characterize the relationship between infection by chronic diseases and husbandry in zebrafish (Ramsay et al., 2009a, b), there has up until very recently been a general paucity of data in the literature on how these pathogens may be affecting the overall physiology of the fish. New studies of this type are finally beginning to emerge, due in part to the continuous improvements being made in technology. For example, advances in high-throughput deep sequencing technology have allowed for the analysis of the zebrafish transcriptome response to infections by various pathogens, including *Mycobacterium marinum*. (Hegedus et al., 2009; van der Sar et al., 2009), and *Salmonella* strains Stockhammer OW et al. (2009). Interestingly, while infections by *M. marinum* are less severe in medaka than in zebrafish, infected medaka did show altered gene expression profiles in more than 700 genes, including the down regulation of retinoblastoma gene (*Rb*) which has been linked to increased cancer risk in a number of terrestrial and aquatic vertebrates (Broussard and Ennis, unpublished observations; Broussard et al., 2009). The results of these and similar studies, which demonstrate genome-

wide differences in gene expression in infected vs. non-infected animals, provide important information that can be used to better characterize how these underlying diseases impact host physiology and other endpoints that may be responsible for non-protocol induced variation in experiments.

One particularly illustrative example of the effects that chronic infections may have on animals used for experiments comes from the medaka. Broussard and colleagues found that medaka with chronic *Mycobacterium marinum* disease were more likely to develop hepatocellular lesions than uninfected fish when exposed to a chemical carcinogen (Broussard et al., 2009). The fact that chronic disease by *Mycobacterium* spp., which is also common in zebrafish (Watrall and Kent, 2007), may act as a tumor promoter in medaka has important implications for the field, especially for those using either species as a model for human cancer. This is precisely the type of data that is needed to convince researchers that more advanced methods of pathogen control are worth the effort and investment it takes to implement them. However, studies like this are too few in number, and at this time too underappreciated, by the research community to have a measurable effect on changing approaches to pathogen control. More data of this nature and improvements in the manner in which it is communicated to the community is required to generate the necessary momentum in the field to improve health management in fish research facilities. This will be particularly relevant for the zebrafish community, as the effect of chronic diseases and pathologies that may be subclinical in juvenile or young adult fish will become increasingly important as an increasing number of studies incorporate adults and genetically modified animals.

8. Entrenchment of diseases

A necessary consequence of the continued emergence of the zebrafish and other fish models as animal research subjects is that many aquaculture facilities have been built around the world to support them. The precise number of such facilities in existence is somewhat difficult to ascertain, but a reasonable estimate can be made from data kept at the zebrafish information network (ZFIN), which listed 667 registered laboratories as of early 2011 (www.zfin.org). While this is undoubtedly an underestimate, it is reasonable to conclude that there are hundreds of such facilities in operation across the globe, ranging from small and informal setups, such as a few glass tanks on a lab bench, to industrialized entities containing thousands of tanks in dedicated, specialized rooms.

Whatever the number actually is, a reality that must be confronted by the proponents of pathogen control is that the fish populations in many of these facilities are likely to harbor both known and unknown infectious agents or diseases, some of which are extremely difficult, if not impossible to eradicate, especially in the context of continuously maintaining active research programs. The combined approach of using appropriate disinfection agents and equipment (e.g. ultraviolet sterilizers), sound husbandry/environmental management, strict quarantine procedures would be of significant assistance in minimizing the future introductions and spread of disease throughout facilities. However, once the fish become infected, it is unclear as to whether or not these organisms may be completely eliminated from the animals or the facility without sacrificing the entire population, dismantling/sterilizing the equipment, and starting over with new, clean animals.

However, this drastic approach is not a viable option in many large facilities. The most reasonable alternative is the implementation of strict measures going forward, with a goal of reduction, but not complete elimination of such pathogens. For example, *P. neurophilia* is currently the most commonly diagnosed pathogen in zebrafish submitted to the ZIRC Pathology Service. Presently there is no therapeutic treatment that can be used to eliminate this parasitic agent from zebrafish stocks. *P. neurophilia* was first diagnosed in sentinel

animals at the ZIRC in pre-filtration sentinel fish in 2002. The ZIRC employs standard biosecurity measures including UV sterilization of post-filtration water at zap dose rates between 45,000–75,000 microwatts per square centimeter per second (mW/cm²/s), quarantine of all imported animals, allowing only surface disinfected embryos to enter the main fish facility, and disinfection of tanks and tank husbandry equipment between uses. The health monitoring program includes quarterly sampling of pre- and post-filtration sentinel fish, removal and diagnostic testing of moribund fish, and studies that involve random and targeted sampling of large stocks to establish disease prevalence. The health monitoring system has been key to understanding the prevalence of the pathogen within the facility and in particular stocks. With this background the ZIRC has been able to decrease the amount of *P. neurophilia* in the facility, as detected in the pre-filtration sentinel fish. Exposure time before detection of the pathogen in pre-filtration sentinels has increased from 3 months to 6 months, to at times being undetectable after even after 6 months (http://zebrafish.org/zirc/documents/health_report.php; Murray et al., in review). As discussed above, the prospects of this occurring at the level of the fish research field itself are dependent upon the increase in prevalence in such practices to the point where the great majority of colonies in the community are managed in this fashion.

The attainment of the goal of pathogen reduction in fish research facilities will also be highly dependent upon the manner in which new, yet to be built facilities, are constructed and subsequently managed once they become operational. It is imperative that the same errors in design and practice that have contributed to the present state of pathogen prevalence in the community are not perpetuated going forward.

9. Conclusions and recommendations

There are numerous and complex challenges that must be dealt with in order to achieve the goal of implementing pathogen control in fish research facilities. However, significant progress can be made on this front if the following occur:

1. Data driven standards for husbandry and care are developed and subsequently employed in facility management practices.
2. Processed diets that are precisely formulated to support fish growth, survival and reproductive performance, standardized in their ingredients, and free of pathogenic agents and other contaminants, are developed and made available to the community.
3. Screening of live diets for the presence of specific pathogens.
4. Routine health monitoring and formalized animal transfer programs become a standard, necessary element of fish research facility management.
5. Diagnostic tests for common pathogenic agents of zebrafish and other fish models are developed and/or expanded upon where already available and routinely utilized by fish facilities as part of the animal care program.
6. Successful treatments for pathogens that commonly infect zebrafish, medaka, and other model fish are developed.
7. Methods for the generation and maintenance of specific pathogen free facilities continue to be developed and improved upon where currently available.
8. Studies documenting the relationship between chronic diseases from various pathogens and the physiological state of fish used in research are conducted, published, and widely disseminated to the community.

9. The means to develop and distribute specific pathogen free, research quality animals are improved and expanded.
10. Develop appropriate paradigms to provide facilities with adequate staffing levels of knowledgeable and trained fish care personnel.

10. Uncited references

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Table 1

Submissions to the ZIRC (Zebrafish International Resource Center) pathology service.

Year	# submitting facilities (U.S.)	# submitting facilities (outside U.S.)	# of diagnostic cases submitted
2010	21	2	39
2009	24	5	44
2008	25	5	46
2007	15	8	47
2006	21	7	35