Detection of Autofluorescent *Mycobacterium Chelonae* in Living Zebrafish

Christopher M. Whipps,¹ Larry G. Moss,² Dana M. Sisk,³ Katrina N. Murray,⁴
David M. Tobin,³ and Jennifer B. Moss²

Abstract

Mycobacterium chelonae is widespread in aquatic environments and can cause mycobacteriosis with low virulence in zebrafish. The risk of infection in zebrafish is exacerbated in closed-recirculating aquatic systems where rapidly growing mycobacteria can live on biofilms, as well as in zebrafish tissues. We have discovered a method of identifying and visualizing *M. chelonae* infections in living zebrafish using endogenous autofluorescence. Infected larvae are easily identified and can be excluded from experimental results. Because infection may reduce fertility in zebrafish, the visualization of active infection in contaminated eggs of transparent *casper* females simplifies screening. Transparent fish are also particularly useful as sentinels that can be examined periodically for the presence of autofluorescence, which can then be tested directly for *M. chelonae*.

Introduction

TRANSGENIC ANIMALS WITH endogenous fluorescent tags are increasingly being used in many model systems for understanding a range of biological phenomena. Embryonic zebrafish, *Danio rerio*, marked with cell-type-restricted fluorescent proteins are particularly useful for evaluating cell fate during development since these vertebrates are optically transparent and develop *ex utero*. Therefore, it is critical that fluorescence output reflects the underlying nature of the biological process of interest. We have employed fluorescence-tagged transient (MLC1a:GFP¹), stable (Ins:eGFP²), and conditional (Ins-NTRmCherry³) transgenic zebrafish as the basis for understanding development and disease. We recently transferred the *Ins:nfsB-mCherry* transgene⁴ into transparent *casper* zebrafish, generating *casper* Insulin-Nitroreductase-mCherry (*casper*INC) transgenics. Adult zebrafish incubated in water that contains the prodrug metronidazole is converted by the beta-cell-specific nitroreductase enzyme into a toxic compound that selectively destroys beta cells.³ Beta cell regeneration occurs during a 2-week time course, a process that does not occur in diabetic humans or aging murine diabetic models. During development of our zebrafish model system, we discovered an unusual *in vivo* fluorescence in the transparent *casper*INC adults

that could not be attributed to the transgenic construct. Through analysis of potential contributors to the aberrant fluorescence signal, we determined that zebrafish infected with the rapidly growing *Mycobacterium chelonae* contained foci of infection that generated a broad emission spectrum of green and red autofluorescence, leading to the exclusion of these infected fish from the study.

Mycobacteriosis is a recognized disease of laboratory zebrafish.^{5,6} Although infections of *Mycobacterium haemophilum*⁷ and *Mycobacterium marinum*⁸ are associated with significant mortalities, infections with less virulent species like *M. chelonae* may also have significant impact on research that relies on the zebrafish model. *M. chelonae* is not typically associated with mortalities, even when fish are experimentally exposed by intraperitoneal injection.⁹ The concern is that these underlying infections are more likely to go unrecognized and may influence the outcome of an experiment, especially where fish are evaluated over weeks and months.¹⁰ Complete eradication of all mycobacteriosis from a colony is a challenge yet to be met¹¹ and strict biosecurity is recommended to keep the more virulent species out of the aquatic system. However, the effort required for complete elimination of all mycobacterial species may exceed expectations for a typical facility. With this in mind, it becomes very important to recognize and evaluate the potential influence these infections have on zebrafish research.

¹SUNY-ESF, State University of New York College of Environmental Science and Forestry, Environmental and Forest Biology, Syracuse, New York.

²Sarah W. Stedman Center for Nutrition and Metabolism and Endocrine Division, Duke University Medical Center, Durham, North Carolina.

³Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, North Carolina.

⁴Zebrafish International Resource Center, University of Oregon, Eugene, Oregon.

Little is known about subclinical infections in zebrafish and their impact on zebrafish studies.¹² In many cases, the experimental endpoints do not include a histological or microbiological examination. If discovered, then an infected group of fish may be excluded from any further analysis as well as from the final publication. For example, when mycobacteria were observed histologically in two experimental groups, infected animals were excluded from the report.¹³ Underlying infections are more likely to be associated with mortalities in fish that are stressed.¹⁴ If a procedure involves frequent handling, crowding, or suboptimal husbandry conditions, then unanticipated mortalities may occur. When the medaka *Oryzias latipes* was infected with *M. marinum* and exposed to a carcinogen, a greater number of liver tumors were observed compared with uninfected controls.¹⁵ In addition, the zebrafish transcriptome is altered in response to *M. marinum*,^{16,17} highlighting the potential variation that infection can cause at the level of gene expression. These studies have provided an incomplete picture of the impacts of mycobacterial infections. Here we show that a simple method for detection of *in vivo* fluorescence due to *M. chelonae* infection of larvae or transparent adults can be used to identify mycobacterial infection.

Materials and Methods

Zebrafish care, maintenance, and strains

Zebrafish were raised in an eight-rack Aquatic Habitats integrated system using a single pump, reverse osmosis (RO) water reservoir and an Emperor Aquatics quad UV sterilization system. The particulate and charcoal filters were changed once every 6 weeks and sump filters were changed weekly. RO water filters and UV lights were changed every 6 months. Tanks were cleaned daily in a commercial cage wash after scrubbing in bleach. Salinity was manually maintained at 800 μ S with Instant Ocean and pH 7.6 was balanced using sodium bicarbonate. Temperature ranged between 27.5°C and 28°C. Light–dark cycle settings were 14 h on and 10 h off. Adult zebrafish were fed decapsulated artemia once daily after culturing for 48 h, supplemented with Zoe Marine vitamins. Aquatic Habitats Adult Zebrafish Diet was fed on weekday afternoons. New fish were introduced into the system as bleached embryos. After 5 days in dishes, larvae were transferred to the system in baskets at a density of 18–20 per basket, where they were fed a series of Zeigler larval food that ranges LD100, 150, and 250 during a 3-week period after which they were transferred to 3-L tanks at a density of 10–15 per tank or 10-L tanks at a density of 25–40 per tank. After breeding, adult zebrafish (4 months old) were maintained at a density of 6 per 3-L tanks, typically containing four males and two females. Ins:eGFP² transgenics were originally generated in our laboratory and are available from the ZIRC stock center [*Tg(1.0ins:eGFP)scI*]. The *casper* strain was a generous gift of Dr. R.M. White¹⁸ and has two mutations, one in the *mitfa* gene (nacre) and another in the *mpv17* gene (roy). The *AB strain was maintained in an independent zebrafish facility at Duke University. The Insulin-nitroreductase-mCherry plasmid (*Ins:nfsB-mCherry*),⁴ a generous gift from Mike Parsons, was injected into *casper* embryos using tol2-mediated transposition¹⁹ to create *casper*INC zebrafish. Transgenic adults were maintained as heterozygotes.

Imaging and histology

A Leica MZFI III microscope attached to a Hamamatsu Orca camera was used for imaging Tricaine-anesthetized²⁰ zebrafish. The mCherry Chroma filter set No. 49008 ET for Leica and Leica GFP-2 set from Chroma were used to detect red or green fluorescence, respectively. To evaluate mycobacterial infection, adult zebrafish were euthanized, fixed in Dietrich fixative, and placed on a laboratory rocker for a minimum of 24 h. Fixed fish were decalcified in 10% tri-fluoroacetic acid, processed for paraffin sectioning according to standard techniques, and stained with Ziehl–Neelsen acid-fast stain.²¹ Cultured bacteria were examined with Zeiss Imager M1 AX10 microscope using a 63 $\times$ water-dipping objective and GFP filter set. Images were captured with a Zeiss AxioCam HSM digital camera and processed using AxioVision software. Figures were prepared using Improvise Openlab and Adobe Photoshop software on Apple computers.

Mycobacterial culture and identification

Mycobacteria were isolated from zebrafish on two occasions. In the case submitted February 2, 2011, spleen and liver were removed from six INC fish and placed in a sterile 1.5 mL tube with 100 μ L of 0.9% saline. In the case submitted June 2, 2012, zebrafish were examined under fluorescence to determine infection status. A single fish was selected with an infection of the testes and the infected tissue was removed and placed in sterile saline as explained just now. Tissues were ground with a microtube pestle and 50 μ L of this suspension was plated onto Middlebrook 7H10 agar supplemented with OADC (BD) and incubated at 28°C for 5–7 days. Individual colonies were subcultured in Middlebrook 7H9 supplemented with OADC for 7 days. Aliquots were frozen and this strain was designated H11-05. DNA was extracted from a bacterial pellet using the UltraClean microbial DNA isolation kit (Mo Bio Laboratories). PCR was used to amplify part of the heat shock protein 65 (*hsp65*) gene as described.²² The same forward primer was used to sequence *hsp65* with the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 on an ABI PRISM[®] 3730 DNA Analyzer (Applied Biosystems).

Injection of independent *M. chelonae* strains into *AB wild-type embryos

The H11-05 strain or the independently isolated *M. chelonae* strain ZF178²³ was propagated in Middlebrook 7H9 liquid culture supplemented with 10% OADC and 0.25% Tween 80 (20% v/v) at 28°C. Embryos were collected from *AB wild-type adults located in an independent facility from the one where infected zebrafish were first identified, dechlorinated at 1 dpf, and maintained until 3 dpf in 1.5 $\times$ phenylthiourea in filtered fish water. On the day of infection, 1 mL of each liquid culture was centrifuged and resuspended in one-half-volume 7H9 media prior to homogenization with a 27½-gauge needle. To aid in visualization, the inoculum was prepared for injection by the addition of 0.125% phenol red. Prior to manipulation, embryos were anesthetized in pH 7-buffered MS-222 (Tricaine) at 150 ppm. Microinjections were performed with an Eppendorf Transjector 5246 into the caudal vein of 3 dpf embryos as previously described.²⁴

FIG. 1. *Mycobacterium chelonae* red and green autofluorescence in adult *casper*INC zebrafish. Living, transgenic adult and larval zebrafish were anesthetized and imaged with a Leica MZFL III microscope. (A) Eight-month-old male *casper* transgenic. mCherry-positive beta cells mark the pancreas (arrow), while additional red (left panel) and green (middle panel) fluorescence is due to mycobacterial autofluorescence (yellow granulomas; merge). (B) Mycobacterial infection in female *casper*INC zebrafish is apparent as red/green autofluorescence surrounding eggs (Merge-yellow). (C) Autofluorescence in infected, 6-day-old *casper* InsGFP larvae is both red and green (Merge-yellow). Arrow-head: swim bladder; arrow: GFP+ transgene expressed only in beta cells; *: gall bladder; bracket: yellow mycobacterial nodules.

FIG. 2. Rapidly growing *M. chelonae* infection in *casper* or **AB* zebrafish retains autofluorescence in culture. (A) Adult *casper* Female: granuloma (arrow) within the ovary (400 $\times$ magnification). (B) Adult *casper* Male: granuloma (arrow) containing acid-fast bacilli (purple) within abdominal fat (400 $\times$ magnification). (C) Wet mount of *M. chelonae* that expresses green autofluorescence in culture (630 $\times$ magnification). Bright-field image overlaid with fluorescence image and green autofluorescence alone. Scale bar=10 μ m (C). Red or green autofluorescence from regrowth of *M. chelonae* H11-05 and an independent *M. chelonae* strain ZF178 isolated and then cultured for injection into zebrafish embryos.

FIG. 3. Living 6-day-old *AB* wild-type zebrafish larvae infected with *M. chelonae* isolates. Hatched embryos were inoculated at 3 dpf with strain H11-05 or a previously isolated and characterized *M. chelonae* strain ZF178 from an independent source. Control or infected larvae were anesthetized and then imaged in the trunk region at 6 dpf. (A) Uninjected control: Red and green autofluorescence. Merge: overlay of red and green images indicates *M. chelonae* infectious nodules (yellow). (B) *M. chelonae* strain H11-05: red and green autofluorescence from *M. chelonae* isolated and characterized from zebrafish described in this report. (C) *M. chelonae* strain H11-05: red and green autofluorescence from an independent isolate. Merged autofluorescent signals appear yellow. Red and/or green fluorescent images were overlaid on bright-field images and indicate granulomas. Scale bars = 100 μ m.

Images were taken at 3 dpi. Bacterial burden was 150–250 CFU per fish as determined by plating onto 7H10 plates and maintained at 28°C until colonies were visible. Single bacterial colonies from H11-05 or ZF178 were mixed into 10 μ L sterile-filtered PBS. Smears were made on glass slides and then imaged for red/green fluorescence. Microscopy was performed using a Zeiss Axio Observer Z.1 equipped with an X-Cite 120 Fluorescence Illumination System (EXFO) utilizing a 120 W mercury lamp, Cy3 or GFP filters, and bright-field optics. The objectives used included a Zeiss 2.5 $\times$ Fluor and a 20 $\times$ LD Plan Neofluar.

Results

We previously developed a conditional ablation system in adult zebrafish where the destruction followed by expansion of pancreatic beta cell mass occurs within a 2-week period.³ Metabolic demands on animal physiology largely determine adult beta cell growth and function.²⁵ To evaluate changes in beta cell mass in adult zebrafish, we generated a transparent, transgenic zebrafish where mCherry-derived red fluorescence expressed only in beta cells was recorded in living adults as regeneration progressed. During the course of this study, we

observed spurious, red fluorescent nodules that were often associated with the gonads of male ($n=6$, Fig. 1A) or female ($n=8$, Fig. 1B) *casperINC* adults as well as casper InsGFP larvae (Fig. 1C). The nonpancreatic tissue that contains this anomalous red fluorescence was also positive for green fluorescence (Fig. 1, merge). The fish behaved and appeared normal and were housed in a single 10-L tank. We could not detect fluorescence in anesthetized, pigmented wild-type adults located in adjacent tanks, either because melanophores and iridophores blocked the view of internal organs or because they were not infected. Adult *casperINC* females presented with visible red and green fluorescence located within and around eggs (Fig. 1B). In addition, we observed red as well as green autofluorescence in InsGFP² larvae, suggesting that the aberrant fluorescence was not due to the transgene (Fig. 1). InsGFP transgenics express green (eGFP) instead of red (mCherry) fluorescence only in beta cells. Cords of red and green autofluorescence lining the swim bladder and projecting caudally along the gut were present in infected InsGFP larvae (Fig. 1C). Red/green fluorescent nodules were present whether the zebrafish expressed a red or green fluorescent protein. Larvae were euthanized after imaging and all dishes and pipets were soaked in bleach and

cleaned in a commercial cage wash to prevent contamination. On dissecting the red/green nodules present in *casper*INC adult male fish, we discovered a bright red-and-green fluorescent mass that could be excised from between the gonads and swim bladder. To determine whether the red/green fluorescence was due to an infectious agent, preserved male and female zebrafish were prepared and sent to ZIRC Health Services (<http://zebrafish.org/zirc/health/>) for histological analysis. Granulomatous nodules that contain acid-fast bacilli indicative of mycobacteriosis were found in both male paraffin sections (Fig. 2A).

Adult zebrafish that contain red/green fluorescent nodules were subsequently tested for their mycobacterial strain and culture characteristics. Touch imprints made from tissues dissected from zebrafish contained characteristic acid-fast rods (data not shown). A suspension of spleen and liver was plated on 7H10 media and incubated for 5–7 days at 28°C. Visible colonies were subcultured and designated H11-05. Extracted DNA was used as a template to amplify the mycobacterial *hsp65* gene, generating a fragment 650 base pairs in length. The fragment was sequenced and identified as *M. chelonae* using a BLAST search in GenBank (www.ncbi.nlm.nih.gov/genbank). DNA was also extracted directly from the spleen and liver suspension and PCR was used to amplify the *hsp65* gene. Sequencing yielded identical results to that of the isolated H11-05 colony, suggesting no evidence of a mixed infection. In another batch of infected fish, animals were examined for autofluorescence. Testes that exhibit green fluorescence and numerous granulomas were dissected. A touch imprint was made of this tissue, and the remainder was plated directly onto 7H10 media. DNA sequencing confirmed the identity of this species as *M. chelonae*. After 5 days of culture at 28°C, wet mounts were prepared from colonies and observed under a microscope. Characteristic 1.5–2- μ m rods were present (Fig. 2B) and many emitted green fluorescence. To further test the infectious nature of independent isolates in a wild-type zebrafish strain, we compared two different *M. chelonae* strains injected into *AB zebrafish embryos for red/green autofluorescence.

M. chelonae strain ZF178²³ or the *M. chelonae* H11-05 isolates exhibited growth in culture at 28°C after 7 days. Both strains exhibited red as well as green fluorescence in histological smears (Fig. 2C). Embryos from a mating of *AB wild-type adults maintained in a separate zebrafish facility were inoculated with either strain of *M. chelonae* (H11-05 and ZF178). On day 3 post-fertilization, homogenized inoculum was injected at ~200 CFU/anesthetized embryo. Green and red fluorescence was observed after 3 more days (Fig. 3). Granulomas could not be detected in the 6-day, uninjected control *AB larvae (Fig. 3A). In contrast, both the H11-05 strain (Fig. 3B) as well as the independently isolated ZF178 strain (Fig. 3C) produced red and green fluorescence in fish. Because *M. marinum* strains used by investigators in the facility where the injections were performed are negative for red/green fluorescence (data not shown), we conclude that *M. chelonae* generates a characteristic red-and-green fluorescence whether growing in culture or after infection of different zebrafish strains, irrespective of the presence of fluorescent transgenes.

Discussion

We discovered that *M. chelonae*-infected zebrafish can be screened and eliminated from aquatic systems or exper-

imental results after observation under a fluorescence-equipped microscope. We determined that a recognition of mycobacteriosis caused by the rapidly growing mycobacteria *M. chelonae* can be made directly in living zebrafish larvae or transparent adults. The observation of red/green autofluorescence from *M. chelonae* is a potential source of variation in experimental protocols as unrecognized infection could be misinterpreted as fluorescently labeled target tissue. In the event of an anomalous finding, a simple test for infected embryos or larvae could be used to observe both red and green fluorescence under a dissecting microscope with red (mCherry or Cy3) and green (GFP) fluorescence filters. These are commonly used filters in the zebrafish community. A potential application of this discovery might be to monitor infections in aquatic facilities using *casper* adults as sentinels for the presence of autofluorescent *M. chelonae*. If the goal is to screen zebrafish to minimize nonprotocol variation using autofluorescence, then further identification of species may not be required. However, it is still important to discriminate the strain of mycobacteria due to differences in severity of disease by species and the potential for zoonotic transmission.

Blue autofluorescence has previously been reported as a diagnostic tool for *M. tuberculosis* in humans.^{26,27} Patiño *et al.*²⁸ examined fluorescence emitted from additional mycobacterial species, including *M. smegmatis*, *M. fortuitum*, and *M. marinum*, using laser excitation at a wavelength of 405 nm with emission peaks centered at 470 nm. The source of this fluorescence may be due to the F420 and FO coenzymes important for mycobacterial electron transfer reactions, capable of generating a bright blue-green fluorescence that is enhanced by heat treatment.²⁸ Using a red exciter ET560/40X and emitter ET630/75m and/or a green exciter ET480/40X and emitter 510lp to analyze anesthetized *casper* adults or larvae, we visualized red/green nodules of *M. chelonae* *in vivo* and in isolated cultures of *M. chelonae*. These filter sets are commonly used for tracking tissue-specific expression in living embryos. Because many reporter transgenes are engineered to generate green or red fluorescence in zebrafish models, this rapid assay for the presence of *M. chelonae* can be used to evaluate unexpected results. We found that *M. chelonae* generates a characteristic red-and-green fluorescence whether growing in culture or after infection of either *casper* or *AB fish, irrespective of the presence of fluorescent zebrafish transgenes. Actions to eliminate infections typically rely on euthanization. Due to mycobacterial autofluorescence, any infected larvae or transparent *casper* adult sentinel can easily be eliminated. Because the virulence of *M. chelonae* in zebrafish colonies is low and often goes undetected,⁶ the ability to easily correlate infection with mycobacterial autofluorescence in living tissue provides a useful tool for eliminating spurious results or infected zebrafish. We have not examined animals other than zebrafish infected with this mycobacterial strain. However, it may be possible to identify granulomas in different species using the characteristic red/green autofluorescence we have attributed to *M. chelonae*.

We removed any zebrafish positive for red/green fluorescence because we were concerned about the potential for infection of other fish in the facility as well as the possibility of zoonotic transfer of mycobacteria to fish room workers. *M. marinum*, a nontuberculous mycobacterium that causes skin

infections in humans, is of particular concern because 84% of humans who were infected with this species had contact with home aquaria.²⁹ We have also linked identical strains of *M. marinum* in fish with a human infection.⁸ Humans with immune deficiencies are at risk and, although long-term antibiotic therapy eliminates infection, the treatment success relies on identification of the mycobacterial strain. *M. chelonae* has been documented as a human pathogen in immunocompromised patients and treated with antibiotics, primarily clarithromycin.³⁰ However, no link has been made between fish and humans for *M. chelonae* transmission, and the occurrence in both species may represent epidemiologically unlinked strains. Interestingly, a recent systematic survey for pedicure-associated nontuberculous mycobacterial outbreaks in humans determined that 91% of infections were caused by *M. chelonae*.³¹ Biofilms from footbaths were processed and precise isolation and speciation was successful in 50% of the samples. Cultured isolates were resistant to multiple antibiotics. However, 12 of 13 isolates from human samples were susceptible to clarithromycin. Treatment of zebrafish that have mycobacterial infections with antibiotics is not recommended. However, if it is considered as a means to maintain a particularly valuable line in order to spawn a next generation, then potential ramifications including antibiotic resistance and prolonged human exposure should be evaluated.

Acknowledgments

This work was supported by a grant from the Juvenile Diabetes Research Foundation to L.G.M. and J.B.M. The authors appreciate all the members of the Sarah W. Stedman Nutrition and Metabolism Center under the direction of Chris Newgard for continuing support. C.M.W. thanks Hadi L. Jabbar Al-Hasnawi for assistance in the initial characterization of the H11-05 isolate and Jeffrey D. Amack at SUNY Upstate Medical University for the use of the Zeiss microscope. C.M.W. was funded in part from a subaward from NIH-NCRR grant 5R24RR01386-07. The Zebrafish International Resource Center (ZIRC) is supported by the NIH Office of Research Infrastructure Programs.

Disclosure Statement

No competing financial interests exist.

References

1. Moss JB, Price AL, Raz E, Driever W, Rosenthal N. Green fluorescent protein marks skeletal muscle in murine cell lines and zebrafish. *Gene* 1996;173(1 Spec No):89–98.
2. diIorio PJ, Moss JB, Sbrogna JL, Karlstrom RO, Moss LG. Sonic hedgehog is required early in pancreatic islet development. *Dev Biol* 2002;244:75–84.
3. Moss JB, Koustubhan P, Greenman M, Parsons MJ, Walter I, Moss LG. Regeneration of the pancreas in adult zebrafish. *Diabetes* 2009;58:1844–1851.
4. Pisharath H, Rhee JM, Swanson MA, Leach SD, Parsons MJ. Targeted ablation of beta cells in the embryonic zebrafish pancreas using *E. coli* nitroreductase. *Mech Dev* 2007;124:218–229.
5. Kent ML, Whipps CM, Matthews JL, Florio D, Watral V, Bishop-Stewart JK, *et al.* Mycobacteriosis in zebrafish

- (*Danio rerio*) research facilities. *Comp Biochem Physiol C Toxicol Pharmacol* 2004;138:383–390.
6. Whipps CM, Lieggi C, Wagner RA. Mycobacteriosis in zebrafish colonies. *ILAR J* 2012;53:95–105.
7. Whipps CM, Dougan ST, Kent ML. Mycobacterium haemophilum infections of zebrafish (*Danio rerio*) in research facilities. *FEMS Microbiol Lett* 2007;270:21–26.
8. Ostland VE WV, Whipps CM, Austin FW, St-Hilaire S, Westerman ME, Kent ML. Biochemical, molecular, and virulence studies of select Mycobacterium marinum strains in hybrid striped bass (*Morone chrysops* x *M. saxatilis*) and zebrafish (*Danio rerio*). *Dis Aquat Org* 2007;79:107–118.
9. Watral V, Kent ML. Pathogenesis of Mycobacterium spp. in zebrafish (*Danio rerio*) from research facilities. *Comp Biochem Physiol C Toxicol Pharmacol* 2007;145:55–60.
10. Lawrence C, Ennis DG, Harper C, Kent ML, Murray K, Sanders GE. The challenges of implementing pathogen control strategies for fishes used in biomedical research. *Comp Biochem Physiol C Toxicol Pharmacol* 2012;155:160–166.
11. Kent ML, Buchner C, Watral VG, Sanders JL, Ladu J, Peterson TS, *et al.* Development and maintenance of a specific pathogen-free (SPF) zebrafish research facility for *Pseudoloma neurophilia*. *Dis Aquat Organ* 2011;95:73–79.
12. Kent ML, Harper C, Wolf JC. Documented and potential research impacts of subclinical diseases in zebrafish. *ILAR J* 2012;53:126–134.
13. Capps T MS, Rinhard JJ, Theodorakis CW, Blazer VS, Patino R. Exposure to perchlorate induces formation of macrophage aggregates in the trunk kidney of zebrafish and mosquito fish. *J Aquat Animal Health* 2004;16:145–151.
14. Ramsay JM, Watral V, Schreck CB, Kent ML. Husbandry stress exacerbates mycobacterial infections in adult zebrafish, *Danio rerio* (Hamilton). *J Fish Dis*. 2009;32:931–941.
15. Broussard GW, Norris MB, Schwindt AR, Fournie JW, Winn RN, Kent ML, *et al.* Chronic Mycobacterium marinum infection acts as a tumor promoter in Japanese Medaka (*Oryzias latipes*). *Comp Biochem Physiol C Toxicol Pharmacol* 2009;149:152–160.
16. van der Sar AM, Spaink HP, Zakrzewska A, Bitter W, Meijer AH. Specificity of the zebrafish host transcriptome response to acute and chronic mycobacterial infection and the role of innate and adaptive immune components. *Mol Immunol* 2009;46:2317–2332.
17. Hegedus Z, Zakrzewska A, Agoston VC, Ordas A, Racz P, Mink M, *et al.* Deep sequencing of the zebrafish transcriptome response to mycobacterium infection. *Mol Immunol* 2009;46:2918–2930.
18. White RM, Sessa A, Burke C, Bowman T, LeBlanc J, Ceol C, *et al.* Transparent adult zebrafish as a tool for *in vivo* transplantation analysis. *Cell Stem Cell* 2008;2:183–189.
19. Kawakami K. Transgenesis and gene trap methods in zebrafish by using the Tol2 transposable element. *Methods Cell Biol* 2004;77:201–222.
20. Westerfield M. The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (*Danio rerio*), 4th edition. Eugene: University of Oregon Press, 2000.
21. Murray KN, Bauer J, Tallen A, Matthews JL, Westerfield M, Varga ZM. Characterization and management of asymptomatic Mycobacterium infections at the Zebrafish International Resource Center. *J Am Assoc Lab Anim Sci* 2011;50:675–679.
22. Selvaraju SB, Khan IU, Yadav JS. A new method for species identification and differentiation of Mycobacterium

- chelonae complex based on amplified hsp65 restriction analysis (AHSPRA). *Mol Cell Probes* 2005;19:93–99.
23. Whipps CM, Matthews JL, Kent ML. Distribution and genetic characterization of *Mycobacterium chelonae* in laboratory zebrafish *Danio rerio*. *Dis Aquat Organ* 2008;82:45–54.
 24. Cosma CL, Swaim LE, Volkman H, Ramakrishnan L, Davis JM. Zebrafish and frog models of *Mycobacterium marinum* infection. *Curr Protoc Microbiol* 2006;Chapter 10:Unit 10B 2.
 25. Muoio DM, Newgard CB. Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. *Nat Rev Mol Cell Biol* 2008;9:193–205.
 26. Joshi P, Singh M, Bhargava A, Mehrotra R. Auto-fluorescence-an important ancillary technique for the detection of *Mycobacterium tuberculosis*: Revisited. *Diagn Cytopathol* 2013;41:330–334.
 27. Wright CA, van Zyl Y, Burgess SM, Blumberg L, Leiman G. Mycobacterial autofluorescence in Papanicolaou-stained lymph node aspirates: a glimmer in the dark? *Diagn Cytopathol* 2004;30:257–260.
 28. Patino S, Alamo L, Cimino M, Casart Y, Bartoli F, Garcia MJ, *et al.* Autofluorescence of mycobacteria as a tool for detection of *Mycobacterium tuberculosis*. *J Clin Microbiol* 2008;46:3296–3302.
 29. Aubry A, Chosidow O, Caumes E, Robert J, Cambau E. Sixty-three cases of *Mycobacterium marinum* infection: clinical features, treatment, and antibiotic susceptibility of causative isolates. *Arch Intern Med* 2002;162:1746–1752.
 30. Wallace RJ, Jr., Tanner D, Brennan PJ, Brown BA. Clinical trial of clarithromycin for cutaneous (disseminated) infection due to *Mycobacterium chelonae*. *Ann Intern Med* 1993;119:482–486.
 31. Stout JE, Gadkowski LB, Rath S, Alspaugh JA, Miller MB, Cox GM. Pedicure-associated rapidly growing mycobacterial infection: an endemic disease. *Clin Infect Dis* 2011;53:787–792.
- Address correspondence to:
Jennifer B. Moss, PhD
Sarah W. Stedman Center for Nutrition and Metabolism
and Endocrine Division
Duke University Medical Center
4321 Medical Park Drive #200
Durham, NC 27704
E-mail: jennifer.b.moss@duke.edu