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Transmission of a common intestinal neoplasm in zebrafish by cohabitation

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Abstract

Intestinal neoplasms are common in zebrafish (*Danio rerio*) research facilities. These tumours are most often seen in older fish and are classified as small cell carcinomas or adenocarcinomas. Affected fish populations always contain subpopulations with preneoplastic lesions, characterized by epithelial hyperplasia or inflammation. Previous observations indicated that these tumours are unlikely caused by diet, water quality or genetic background, suggesting an infectious aetiology. We performed five transmission experiments by exposure of naïve fish to affected donor fish by cohabitation or exposure to tank effluent water. Intestinal lesions were observed in recipient fish in all exposure groups, including transmissions from previous recipient fish, and moribund fish exhibited a higher prevalence of neoplasms. We found a single 16S rRNA sequence, most similar to *Mycoplasma penetrans*, to be highly enriched in the donors and exposed recipients compared to unexposed control fish. We further tracked the presence of the *Mycoplasma* sp. using a targeted PCR test on individual dissected intestines or faeces or tank faeces. Original donor and exposed fish populations were positive for *Mycoplasma*, while corresponding unexposed control fish were negative. This study indicates an infectious aetiology for these transmissible tumours of zebrafish and suggests a possible candidate agent of a *Mycoplasma* species.

KEYWORDS
intestinal, *Mycoplasma*, neoplasia, transmission, zebrafish

1 | INTRODUCTION

Spontaneously and naturally occurring diseases in laboratory animals provide unique opportunities to investigate mechanisms of disease aetiology in a highly controlled model system. For over 15 years, spontaneous intestinal neoplasia has been observed in zebrafish (*Danio rerio)* submitted to the Zebrafish International Resource Center (ZIRC) diagnostic service. In a retrospective study of the ZIRC data base, we documented such tumours in 2% of about 10,000 fish from 18 zebrafish laboratories submitted between 2000 and 2012 (Paquette et al., 2013), and we continue to see these tumours in diagnostic cases from various zebrafish facilities. The tumours are most often seen in fish older than 1 year, and fish from the same facilities often exhibit preneoplastic changes in the intestine, including hyperplasia, dysplasia, as well as chronic enteritis. The neoplasms appear to be of epithelial origin based on morphology and immunohistochemistry (Paquette et al., 2015) and are consistent with either small cell carcinomas or more rarely adenocarcinomas. The aetiology of these common zebrafish tumours is unknown, but several potential mechanisms are unlikely. The high tank to tank variability in disease prevalence across fish held on shared recirculating water systems (where water is circulated through multiple tanks repeatedly) suggests that a water-borne chemical agent is unlikely to be responsible, as such an agent would be expected to quickly disperse

across all tanks in the system. When the diet from a laboratory with a high incidence of the disease was fed to zebrafish reared at a different laboratory where no tumours had ever been reported, no pathology was observed in the test fish, and the tumours have been observed in multiple different zebrafish genetic backgrounds (Paquette et al., 2013). Hence, water-borne or dietary carcinogens or zebrafish genetics are all unlikely causes.

These combined findings suggest the possibility of an infectious aetiology. Numerous neoplastic diseases of fishes have been shown to be caused by transmissible agents and are frequently associated with viruses (Anders & Yoshimizu, 1994; Coffee, Casey, & Bowser, 2013; Getchell, Casey, & Bowser, 1998; Schmale, 1995; Schmale, Gibbs, & Campbell, 2002). The origins of these neoplasms are usually skin epithelium, lymphoid or soft tissue (e.g., sarcomas), but to date no gastrointestinal cancers in fishes have been linked to viruses (Bowser & Casey, 1993; Getchell et al., 1998). Some parasites that cause chronic inflammation have also been linked to cancer in fish (Samaras, Rafailidis, Mourtzoukou, Peppas, & Falagas, 2010), with several parasites implicated in several cancers, including gastrointestinal neoplasia (Dvir, Clift, & Williams, 2010; Peterson & Weidner, 2011). For example, the nematode *Pseudocapillaria tomentosa*, a relatively common zebrafish parasite, causes profound chronic inflammation of the intestine (Kent, Bishop-Stewart, Matthews, & Spitsbergen, 2002; Kent, Harper, & Wolf, 2012), and zebrafish exposed to both DMBA and *P. tomentosa* demonstrate a higher prevalence of intestinal tumours than uninfected fish exposed to DMBA (Spitsbergen et al., 2000). While this nematode was implicated in the original diagnosis of several affected fish, it was not prevalent amongst the affected laboratory zebrafish in our retrospective study (Paquette et al., 2013).

Within mammals, gastrointestinal neoplasms are often linked to bacterial agents (Sears & Garrett, 2014), particularly *Helicobacter pylori*, which is the most common cause of gastric cancer (Peek, 2016). Other bacteria that have been suggested to contribute to gastorintestinal neoplasms include *Fusobacterium nucleatum*, enterotoxigenic *Bacteroides fragilis* and colibactin-producing *Escherichia coli* (Brennan & Garrett, 2016). While bacteria have not previously been associated with gastrointestinal neoplasms in fishes specifically, we previously showed that medaka, *Oryzias latipes*, exposed to benzo-a-pyrene have an increased incidence of liver tumours when co-infected with *Mycobacterium marinum* (Broussard et al., 2009). Other evidence points to pathologic shifts in the resident gut bacteria, referred to as dysbiosis, as an instigator or driver of gastrointestinal cancers. Multiple studies have reported altered microbiota in colon cancer patients versus healthy controls and even in tumour versus adjacent, healthy tissue biopsies (Louis, Hold, & Flint, 2014). Experimentally, mice deficient for the NOD-like receptor family pyrin domain containing 6 (NLRP6) gene develop inflammation-associated colorectal cancer that is transmissible to cohouse wild-type mice (Hu et al., 2013). We demonstrated in gnotobiotic zebrafish that excessive intestinal epithelial cell proliferation in zebrafish larvae with an oncogenic mutation in *axon1* was reduced in the absence of microbiota and enhanced by the presence of particular bacteria (Cheesman, Neal, Mittge, Seredick, & Guillemin, 2011). Therefore, a bacterium or perhaps a consortium of bacterial species are reasonable candidates as the cause for the zebrafish intestinal neoplasia. Although experimental evidence has not linked specific bacteria to carcinogenesis in zebrafish to date, the chronic inflammation elicited by certain pathogenic strains of bacteria, and even the natural microbiota, of zebrafish could potentially serve as promoters of intestinal carcinogenesis.

The possibility of an infectious aetiology combined with the evidence against diet, water-borne carcinogenic chemicals, or genetics as likely causes suggests these tumours could be spread amongst zebrafish populations through exposure to a shared environment. We performed a series of experiments with the primary goal of testing whether this intestinal neoplasm could be transferred from afflicted to healthy zebrafish populations through cohabitation. In addition, we also assessed the composition and distribution of intestinal bacteria in zebrafish in the study to see if changes in the intestinal microbiota were linked to disease transmission. This combined approach allows us to test the transmissibility of the disease and to generate hypotheses and tools to further investigate the aetiology of the disease. We also observed liver lesions in some populations exposed to donor fish, and these data are presented in the Supporting information.

2 | METHODS

2.1 | Ethics statement

All zebrafish experiments were done in accordance with protocols approved by the Oregon State University and the University of Oregon Institutional Animal Care and Use Committees and conducted following standard protocols as described (Westerfield, 2007).

2.2 | Experimental transmission

Approximately 200 1-year-old zebrafish from a laboratory with a high prevalence of the intestinal tumours, previously designated as "primary facility" (Paquette et al., 2013), were transferred to our laboratory in Nash Hall, Oregon State University, and used to establish a series of transmission experiments to assess the transmissibility of the intestinal tumours across zebrafish populations. These donor zebrafish, and all other experimental animals, were maintained on a single-pass flow-through system in which the incoming water is city source, dechlorinated with activated carbon and heated to 28 C. A total of five transmission experiments were performed in which healthy recipient zebrafish were either cohoused in a shared tank with primary (fish from the primary facility) or secondary (originally healthy recipients exposed to primary donors) donor fish (exposures A, B and E) or exposed to unfiltered effluent from tanks housing primary or secondary donor fish (exposures C and D) for between four and twelve months each (Figure 1a). The primary donor zebrafish represented a mixture of three populations of wild-type AB background fish and ranged in age between 315 and

441 days post-fertilization (dpf), while healthy recipient fish alternated between similarly aged Casper zebrafish lacking pigmentation (exposures A, B and E) and wild-type outbred 5D zebrafish (exposures C and D) to allow for simple visual differentiation of donor and recipient individuals. In addition, for each transmission experiment, zebrafish from the same population as the recipients were maintained under similar conditions but not exposed to donor fish to act as controls. Details about each individual transmission experiment are as follows:

Exposure A: A total of 175 donor fish were place in a 100-L circular tank. Then 3 months later, 58 recipient fish (3-week-old Casper line obtained from Children's Hospital) were exposed to effluent from the donor fish tank for 2 weeks due to their small size and then transferred to the tank with the donor fish and cohabitated with the donor fish for an additional seven and a half months.

After a total of 8 months exposure, the recipient fish were removed and maintained for an additional 4 months in a separate 16-L tank. Sixteen control fish from the same population were maintained on the same water system and fed the same diet, but not cohabitated with the donor fish. Six of these fish were examined at 6 months post-exposure, and the remaining ten were examined at twelve months post-exposure (Figure 1b).

Exposure B: Fifty adult Casper zebrafish from the Sinnhuber Aquatic Research Laboratory (SARL) were exposed to seventy of the donor fish in a 16-L tank for 5 months, and then the donor fish were removed. Ten control fish from the same population were maintained on the same water system and fed the same diet, but not cohabitated with the donor fish. These fish were examined at eleven months post-exposure.

Exposure C: A total of 50 5D fish were exposed to the effluent of a tank holding Exposure B (as donor fish) for 3 months. The effluent flowed at approximately 1L per 5 min. The recipient tank water was supplemented with fresh, unexposed water at a rate of 1L per 2.5 min to maintain water quality. Recipient fish (Exposure C) were examined at 9, 12 and 15 months post-exposure, and moribund fish were examined at 6 and 9 months post-exposure (Figure 1b). Ten control fish (unexposed) from the same population were examined at 12 months post-exposure.

Exposure D: As with Exposure C, 5D fish were exposed to effluent from Exposure B for 4 months. In this exposure trial, the 300 recipient fish were divided into three 16-L tanks, receiving the effluent from Exposure B in the same manner as Exposure C. Recipient fish were examined at 8, 10, 12, 15 and 18 months post-exposure. In addition, several emaciated fish were collected and examined at 10 and 15 months post-exposure (Figure 1b). Control fish were examined as follows at 10 (n = 10), 12 (n = 8), 15 (n = 10) and 18 (n = 15) months post-exposure.

Exposure E: This group represented fish exposed to Exposure C. Here, a total of 37 Casper fish (starting age of 6 months) were cohabitated with Exposure C (as donor fish) for 12 months. Sixteen control Casper fish from the same population were also examined at 13 months post-exposure.

2.3 | Histology and analysis of disease prevalence

Following each transmission experiment, recipient zebrafish were removed from the exposure treatment and maintained in separate tanks. The intestines of individual fish were then sampled for histopathology, while some collections involved histological examination of the entire fish. Fish were killed by icing (Matthews & Varga, 2012), the abdomen was then opened with a longitudinal cut, and whole fish were preserved in Dietrich's solution. Fish were processed for histology with sagittal cuts prepared from whole fish, and slides were then stained with haematoxylin and eosin.

The pathological changes in the intestine of each fish was designated as inflamed, preneoplastic (including fish with hyperplasia or dysplasia of the intestinal epithelium), neoplastic (either small cell tumours or adenocarcinomas) or normal as described by Paquette et al. (2013). All fish with neoplasms exhibited preneoplastic tissue regions in other regions of the intestine. A Cochran–Mantel–Haenszel chi-squared test of independence (CMH test) was used to determine whether exposed fish were statistically enriched for the development of neoplasia relative to control fish while controlling for the exposure group covariate.

At multiple time points post-exposure, a subset of fish from the donor population as well as exposures D and E were selected for bacterial DNA analysis in addition to histopathology. For these fish, the anterior half of each intestine was processed for histology to assess disease state while the posterior half was retained for DNA analysis (see below). Here, fish were dissected to expose the coelomic cavity and the anterior half of the intestine was preserved in Dietrich's fixative and processed for histology with multiple slides prepared for each piece of intestine. To verify that histopathology of the anterior portion was sufficient to accurately diagnose fish, we re-examined the slides and the raw data reports from our previous retrospective study (Paquette et al., 2013). Of the 194 fish in this previous study with either tumour or preneoplastic lesions, none showed lesions confined to the posterior intestine.

2.4 | Intestinal microbiome profiling

To assess a possible relationship between disease occurrence and bacterial communities, we sampled and characterized the intestinal bacterial microbiota of zebrafish from both the beginning of the transmission experiment, the donors and from the end, Exposure D, using high-throughput sequencing of the 16S rRNA gene. Donor fish were sampled immediately following Exposure B, while Exposure D fish were sampled 15 months post-exposure. The posterior intestines of 42 donor fish, 30 Exposure D fish and 19 control fish (five controls for donors and 14 for Exposure D) were removed aseptically, placed in a 2-ml screw-cap tube and stored at −80°C prior to subsequent processing, while anterior portions were preserved for histology (described above). Each transmission trial lasted over a year, and hence, the overall study spanned several years. Due to changes in the technology and methodology available over the course of the entire transmission experiment, donor and Exposure D

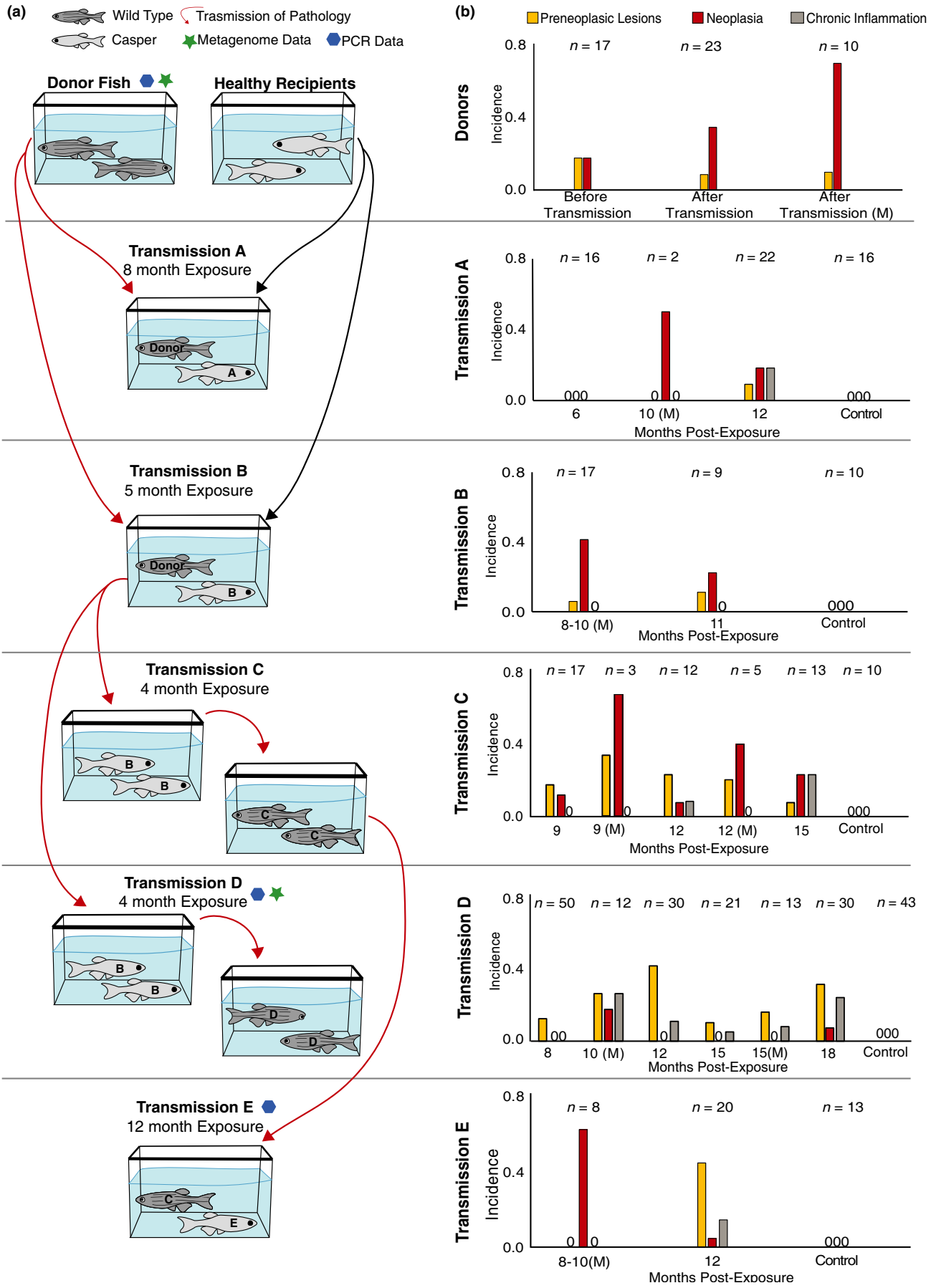
(a)
Wild Type
Trasmission of Pathology
Casper
Metagenome Data
PCR Data
Donor Fish
Healthy Recipients
Transmission A
8 month Exposure
Donor
A
Transmission B
5 month Exposure
Donor
B
Transmission C
4 month Exposure
B
B
C
C
Transmission D
4 month Exposure
B
B
D
D
Transmission E
12 month Exposure
C
E
(b)
Preneoplasic Lesions
Neoplasia
Chronic Inflammation
Donors
Incidence
0.8
0.4
0.0
n = 17
n = 23
n = 10
Before Transmission
After Transmission
After Transmission (M)
Transmission A
n = 16
n = 2
n = 22
n = 16
000
0 0
000
6
10 (M)
12
Control
Months Post-Exposure
Transmission B
n = 17
n = 9
n = 10
0
0
000
8-10 (M)
11
Control
Months Post-Exposure
Transmission C
n = 17
n = 3
n = 12
n = 5
n = 13
n = 10
0
0
000
9
9 (M)
12
12 (M)
15
Control
Months Post-Exposure
Transmission D
n = 50
n = 12
n = 30
n = 21
n = 13
n = 30
n = 43
00
0
0 0
000
8
10 (M)
12
15
15(M)
18
Control
Months Post-Exposure
Transmission E
n = 8
n = 20
n = 13
0 0
000
8-10(M)
12
Control
Months Post-Exposure

FIGURE 1 (a) Transmission paradigm. Donor fish with high levels of intestinal tumours were cohoused with healthy casper fish (exposures A and B). After exposure to donors, transmission of disease was propagated by exposing healthy 5D fish to effluent from tanks of Exposure B fish (exposures C and D). After Exposure C was exposed to Exposure B, Exposure C fish were cohoused with healthy Casper fish (Exposure E). Red arrows indicate transmission of pathology from one group to another. (b) Incidence of intestinal lesions in donor and recipient zebrafish. Preneoplastic designates hyperplasia or dysplasia of epithelium. All fish with neoplasia had preneoplastic lesions in other regions of the intestine, but these fish were not included in the "preneoplastic" data. M = moribund fish.

samples were processed using different methods from one another. DNA was extracted from donor samples using a combination of bead beating and the Qiagen DNeasy Blood and Tissue Kit as described by Stephens et al. (Stephens et al., 2016), while DNA from Exposure D samples was extracted using MoBio PowerMag RNA/DNA Isolation Kit. The V4 region of the 16S rRNA gene was amplified using the 515F and 806R primers (Caporaso et al., 2012). Amplicons were then sequenced using the Illumina NextSeq platform for donor samples and the Illumina HiSeq 2500 platform for Exposure D samples generating 150 base pair paired-end sequences from both. Sequences were then assembled using FLASH (Magoc & Salzberg, 2011) and quality-filtered using the FASTX Toolkit (Hannon Lab, 2010). Host sequences were filtered from the data set by aligning reads to the zebrafish genome using Bowtie (Langmead & Salzberg, 2012). Sequences from both runs were then combined to generate operational taxonomic units (OTUs) *de novo* at 97% sequence similarity using USEARCH (Edgar, 2010). The taxonomy of these OTUs was then assigned using the RDP classifier (Wang, Garrity, Tiedje, & Cole, 2007). Read assembly, quality filtering and OTU clustering were done on the University of Oregon ACISS cluster. The resulting OTU table was rarefied to 40,000 sequences per sample, and downstream community analyses were then performed in R (R Core Team, 2016) using the vegan (Oksanen et al., 2016) and DESeq2 (Love, Huber, & Anders, 2014) packages. Illumina 16S amplicon sequence reads have been deposited under the National Center for Biotechnology Information BioProject accession number PRJNA412387.

2.5 | Mycoplasma PCR

Following results suggesting that a *Mycoplasma* OTU was predominant in fish that had lesions (described in the Results section below), we adapted a genus-specific PCR test (van Kuppeveld et al., 1994) to determine the presence of *Mycoplasma* in faecal samples from tanks during the experiment and to correlate the presence with 16S rRNA gene profiling data. Microbial DNA was isolated from original donor fish intestines, and faecal samples from tanks of exposed and unexposed control tanks were analysed. Faecal samples were collected from tanks of fish and centrifuged, and DNA was extracted using PowerLyzer PowerSoil DNA Isolation Kit (Mo Bio) following manufacturer protocol. 2 μl of DNA isolated from faecal samples and intestinal samples were used as a template in a 25 μl reaction containing 0.25 μl Phusion HF polymerase (Thermo Scientific), 5 μl 5× Phusion GC Buffer (Thermo Scientific), 0.4 mM dATP, dTTP, dCTP and dGTP and 400 nM of each primer, in 14.74 μl nuclease-free water with the following cycle conditions: 98°C 30 s, 35 cycles of 98°C for 10 s, 55.3°C for 15 s and 72°C for 30 s. 70°C for 5 min, reaction held at 4°C. 5 μl PCR product was mixed with 1 μl 6× DNA loading dye and run on 1% agarose gel stained with ethidium bromide. Gel was visualized with UV light. Presence or absence of band at 280 bp indicated presence or absence of *Mycoplasma* species in the sample. *Mycoplasma* group-specific primer set amplifies 280-bp fragment. Forward primer GPO-3 (5′-GGGAGCAAACAGGATRAGATACC CT-3′) and the reverse primer MGSO (5′-TGCACCATCTG TCACTCTGTTAACCTC-3′) (van Kuppeveld et al., 1994). Amplicons were extracted from agarose gel, purified using Zymo Gel DNA Recovery kit and sequenced to verify that sequences from *Mycoplasma* were amplified.

To validate the sensitivity of the PCR assay, the 280-bp fragment amplified by PCR from Exposure D faecal DNA was gel purified and subcloned into an ampicillin-resistant *E. coli* plasmid with synthetic multiple cloning site pGEN-MCS (Lane, Alteri, Smith, & Mobley, 2007) via smaI site and transformed into DH5α cells. The plasmid was amplified by growing overnight in a 5 ml culture in LB containing ampicillin and purified using Zymo Plasmid Miniprep Kit. The number of plasmid copies present in sample was then calculated. PCR was performed as described earlier testing a series of plasmid dilutions. The mean copies of plasmid present to generate a positive result by PCR are 517.23 (geometric mean, ranging from 93.7 to 8,700 copies).

3 | RESULTS

Approximately two hundred zebrafish from a laboratory with a high prevalence of intestinal tumours were used to establish a series of transmission experiments to assess the transmissibility of the intestinal tumours across zebrafish populations (Figure 1a). The main goals of the series of experiments was to (1) assess the transmissibility of the intestinal tumours, (2) assess whether the disease could be transmitted over multiple "generations" of exposure and (3) determine whether exposure to effluent tank water was sufficient to transmit the disease or if direct contact between populations was required.

Preneoplastic lesions and neoplasms were observed in all six groups of recipient fish as well as the donor fish (Figure 1b; $n = 350$). Chronic inflammation was also observed in most of the exposed fish populations. The lesions were consistent with those seen in our retrospective study of affected zebrafish (Paquette et al., 2013) and are described in detail in Figures 2 and 3. These intestinal phenotypes were not observed in any of the control fish for any transmission experiment ($n = 102$). Indeed, a test of independence

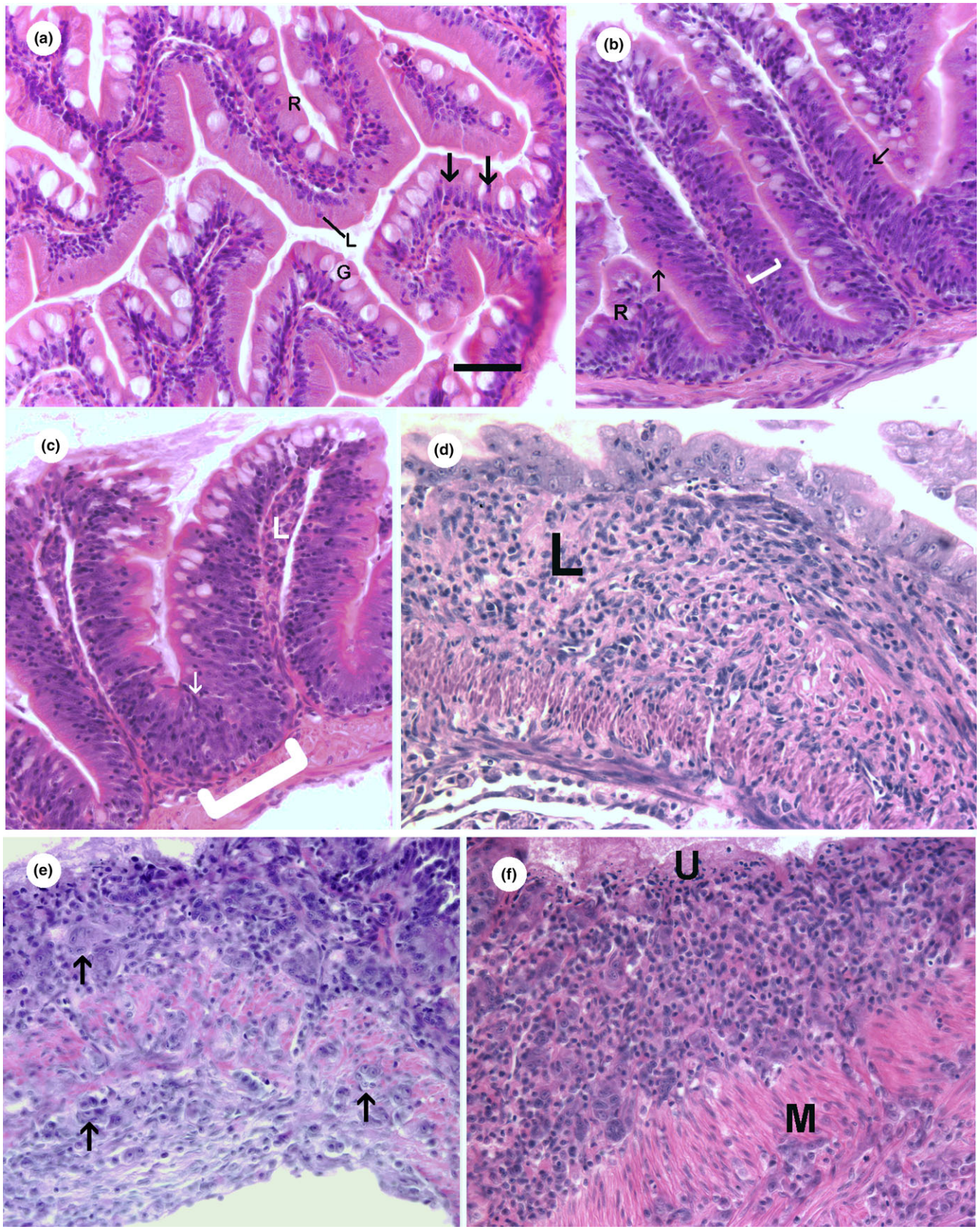
(a)
R
L
G
(b)
R
(c)
L
(d)
L
(e)
(f)
U
M

FIGURE 2 Histological sections of zebrafish intestines. Haematoxylin and eosin. Bar = 25 μm. (a) Normal intestine. Nuclei are basal, lymphocytes (L) are uncommon within the epithelium, and goblet cells (G) were frequently observed. (b) Moderate epithelial hyperplasia in an exposed fish from group A. The layer of basal located nuclei within the mucosal epithelium is diffusely thickened (bracket) and there are numerous intra-epithelial leucocytes composed primarily of lymphocytes (arrows) (c) Severe epithelial hyperplasia from an exposed fish from group A. The layer of basilar nuclei is severely thickened and extends to near the brush border in some places (arrow). At the bases of intestinal folds, the mucosal epithelium is markedly thickened and bulges into the muscularis (bracket). (d) Chronic severe lymphoplasmacytic and fibrosing enteritis in an exposed fish from group D. The lamina propria (L) is severely thickened by loosely organized fibrous connective tissue containing an inflammatory infiltrate. Intestinal folds have been blunted or effaced, and the luminal aspect is lined by a layer of severely dysplastic epithelial cells. (e) Small cell intestinal carcinoma from in an exposed fish from group B. Expanding the lamina propria is a poorly demarcated, unencapsulated neoplasm composed of nests and clusters of polygonal cells (arrow) embedded in a scant fibrovascular stroma. Neoplastic cells have variably distinct cell borders with large amounts of infrequently granular, lightly basophilic cytoplasm. The neoplastic cells invade the tunica muscularis (M) and are present on the serosal surface of the intestine. (f) Small cell carcinoma in an exposed fish from group B. The lamina propria contains large numbers of neoplastic epithelial cells similar to those described in image E. The overlying epithelium is ulcerated (U). Neoplastic cells invade through the tunica muscularis (M)

(Cochran–Mantel–Haenszel, or CMH, chi-squared test) found that there was a significant increase in the frequency of fish that developed neoplasia in the exposed groups relative to the control groups (p = .00049). Likewise, all exposure groups had a greater frequency of preneoplastic lesions, and a CMH test similarly identified a significant enrichment in total intestinal lesions amongst exposed fish relative to controls (p = 7.9e-10).

Multiple moribund fish were observed over the course of the transmission experiments and examined at the time of clinical presentation (Figure 1b). These moribund fish in both the donor group and the recipient groups had a higher prevalence of lesions, and thus, we analysed the results further to determine whether the morbidity was associated with the disease. Grouping all donor and recipient fish together, 32% (34/106) of the moribund fish had intestinal lesions (either preneoplastic, enteritis or neoplasia) and 57% (25/44) exhibited neoplasia. In contrast, only 17% (36/218) or 16% (45/280) apparently healthy fish had intestinal lesions or evidence of neoplasia, respectively. We then used a Fisher's exact test of independence to determine if the frequency of moribund or dead fish that exhibit neoplasia is significantly greater than the corresponding frequency amongst apparently healthy fish. Doing so, we found very significant associations between morbidity and intestinal lesions in general (p = .0014) and even more so with neoplasia (p = 2.95e-08). Moribund fish not exhibiting intestinal lesions or neoplasms frequently had systemic mycobacteriosis, including three in Exposure B, one in Exposure C, one in Exposure D and one in Exposure E.

3.1 | Intestinal microbiotas of donor and exposed zebrafish are enriched for Mycoplasma

Given the evidence that this disease is transmissible across zebrafish populations, we sought to investigate whether there was a relationship between the intestinal microbiota and either disease incidence or exposure, which would implicate a bacterial agent being involved in the aetiology of this disease. We obtained 16S rRNA gene profiles of gut microbiota from affected and normal zebrafish to identify candidate bacterial taxa associated with the disease, using similar approaches to those we have employed to characterize the intestinal microbiota of healthy zebrafish (Roeselers et al., 2011; Stephens et al., 2016). Of the 42 donor fish sampled for microbiota analysis, 12 were diagnosed with preneoplasia and four with neoplasia, while of the 30 Exposure D fish sampled, eight were preneoplastic and only two had developed full neoplasia. This is in contrast to both groups of controls, none of which showed signs of the disease. Across the entire data set, we observed a small but significant difference in bacterial composition between healthy and diseased (both preneoplastic and neoplastic) samples (PERMANOVA: pseudo-F = 1.492, p < .05), as well as a much stronger difference between exposed populations (i.e., donor and Exposure D recipient fish) and their respective controls (PERMANOVA: pseudo-F = 3.24, p < .001 for donors compared to controls and pseudo-F = 7.62, p < .001 for Exposure D recipients compared to controls). These overall differences in composition were largely driven by a single OTU belonging to the *Mycoplasma* genus: one of only two OTUs (the other belonging to the *Vibrio* genus) that was significantly differentially abundant in diseased compared to healthy individuals (Figure 4a; $\log_2$-fold change = 3.46, p < .001), and which most strongly differentiated exposed samples (i.e., donor and Exposure D fish) from controls (Figure 4b; $\log_2$-fold change = 11.37, p < .0001). The average abundance of *Mycoplasma* was similar

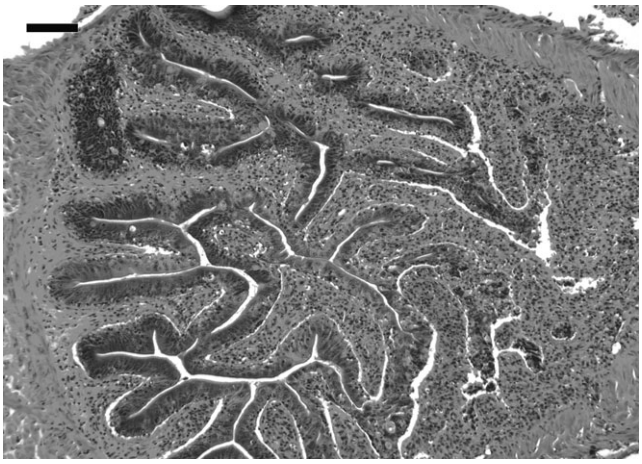

FIGURE 3 Prominent, chronic enteritis in anterior region of intestine, with diffuse inflammatory infiltrate in the lamina propria. Intestine also exhibits moderate epithelial hyperplasia. H&E. Bar = 50 μm

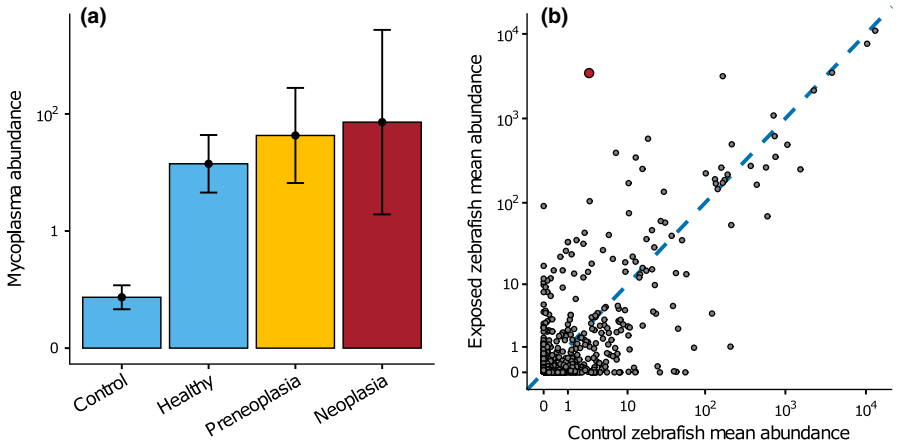

FIGURE 4 Distribution of bacterial taxa in healthy and diseased fish. (a) Average abundance of a *Mycoplasma* OTU in healthy and diseased fish across the data set. (b) The mean abundance of each bacterial OTU in control zebrafish (*x*-axis) and exposed (both donor and Group D) zebrafish (*y*-axis) across the data set. The red point denotes the OTU belonging to the *Mycoplasma* genus and most strongly differentiates exposed and control populations

amongst the exposed fish, regardless of whether they had lesions or were normal (Figure 4a). The relatively low number of fish sampled with neoplasia made it difficult to identify significant correlations between bacterial abundance and disease severity (i.e., preneoplasia vs. neoplasia), such that there was no significant relationship between abundance and disease severity for any OTU in the data set.

The observation of a single OTU that strongly differentiated both exposed populations as well as the occurrence of the disease motivated a more focused investigation of this *Mycoplasma* strain using targeted PCR (see methods). *Mycoplasma* was present in intestinal samples from the donor group and faecal samples from Exposure D and E by PCR (Figure 1). *Mycoplasma* was not detected in the unexposed control groups that were tested (Exposure D and E). Furthermore, in donor samples which indicated a high prevalence of *Mycoplasma* by 16S rRNA gene profiling, brighter bands were visible on the agarose gel compared to individuals with lower levels of *Mycoplasma*.

Whereas most of the other dominate phyla and classes of bacteria in the data set were comprised of multiple lower level taxa (e.g., we detected over 70 genera belonging to the class Gammaproteobacteria), this single *Mycoplasma* OTU was the only member of the class Mollicutes that was detected. Furthermore, despite being relatively dominant numerically, the entire *Mycoplasma* OTU was comprised of only a single unique sequence, suggesting that it represents a single unique strain, or at least species, of bacteria. This sequence most closely matched a 16S sequence belonging to a *Mycoplasma penetrans* strain isolated from salmon intestines (Holben et al., 2002).

4 | DISCUSSION

These exposure trails, comprised of five populations of zebrafish, three independent "generations" of transmission, and both cohousing and effluent exposure transmission modes, provide compelling evidence that a common intestinal neoplasm of zebrafish is caused by a transmissible agent. Tumours were observed in recipient fish from all trials when examined starting at 9 months post-exposure, following exposure by cohabitation or effluent water. Neither preneoplastic lesions nor the tumours were observed in control fish. Moreover, these lesions have never been documented in hundreds of other zebrafish used in other experiments in our laboratory at Oregon State University where the experiments were conducted, including those of the Casper and 5D lines held in the same OSU laboratory and fed the same diet. The pathologic changes observed in the zebrafish in this transmission study were similar to that observed in a retrospective study of some 10,000 zebrafish examined over a 12-year period from several other facilities and in several fish lines, particularly in fish that were older than 1 year (Paquette et al., 2013). We have observed morphologically similar intestinal neoplasms in transgenic zebrafish that overexpressed the *Helicobacter pylori* virulence factor CagA and were homozygous for a loss-of-function allele of *p53* (Neal, Peterson, Kent, & Guillemin, 2013), suggesting that these neoplasms may arise from common carcinogenic processes.

The lesions, particularly neoplasms, were associated with increased morbidity. The association of lesions with disease was more profound than observed previously (Paquette et al., 2013). This previous study actually found a higher prevalence of the preneoplastic or neoplasms in apparently healthy, sentinel fish than those submitted as clinical. One explanation for this difference is that the fish examined by Paquette et al. (2013) included many diagnostic cases with moribund fish that had succumbed to a variety of infectious diseases that are common in zebrafish, such as mycobacteriosis (Whipps, Lieggi, & Wagner, 2012) or microsporidiosis caused by *Pseudoloma neurophilia* (Sanders, Watral, & Kent, 2012). A few moribund fish from Exposure B and one healthy fish from Exposure D exhibited granulomas consistent with mycobacteriosis in the coelom or kidney. *Pseudoloma neurophilia* was observed in on moribund fish from Exposure G. This probably represented a background infection from the original donor fish or the recipient fish from Children's Hospital in group A, as all of the other the recipient fish were specific pathogen free (SPF) for this parasite (Kent et al., 2011). Beyond this single case, we did not detect this, or any other pathogenic nematode, in either donor or recipient fish, suggesting they are not the primary cause of these neoplasms.

In our study, donor and recipient fish were comingled in the same tanks or exposed to effluent that was not filtered, and thus, we cannot exclude the possibility of actual transfer of neoplastic

cells, as seen with in transmissible venereal tumours in dogs and facial tumours in Tasmanian devils (Murchison, 2008) and haemic neoplasms of bivalve molluscs (Metzger, Reinisch, Sherry, & Goff, 2015). However, the high prevalence of concurrent diffuse epithelial hyperplasia in the affected populations without neoplasms suggests that the tumour arise from preneoplastic changes in host cells rather than from direct transfer of foreign neoplastic cells.

The most compelling evidence from our study implicates a strain of *Mycoplasma* as a candidate aetiological agent of the lesions. A single strain of *Mycoplasma* sp. was enriched in populations exposed to diseased individuals compared to controls, and PCR analyses with the *Mycoplasma* genus-specific test consistently yielded positive results in exposed fish, but not controls. While control versus exposed fish showed distinct differences in *Mycoplasma* abundance, the abundance was similar within exposed fish regardless of their lesion status. This is not surprising as gut bacteria could be readily shared amongst fish within a tank due to oral–faecal transmission. This is a plausible candidate to pursue as *Mycoplasma* spp., frequently employ intracellular lifestyles (Rottem, 2003), cause a variety of pulmonary and urogenital infections (McGowin & Anderson-Smits, 2011; Waites & Talkington, 2004) and have been linked to cancers (Rogers, 2011), including associations with intestinal cancers (Barykova et al., 2011; Mariotti et al., 2010; Yang et al., 2010) and induction of malignancy in cell cultures (Feng, Tsai, Rodriguez, & Lo, 1999; Namiki et al., 2009). *Mycoplasma* spp. are frequently dominant in the intestines of adult, but not juvenile or larval, salmon in the wild (Llewellyn et al., 2016), but they have only rarely been associated with disease in fish. A novel species of *Mycoplasma*, *Mycoplasma mobile*, was previously isolated from the gills of tench (*Tinca tinca*) fish with "red disease" and subsequently characterized (Kirchhoff & Rosengarten, 1984; Kirchhoff et al., 1987). This bacterium was later shown to be able to infect host cells and experimental exposure of *M. mobile* to tench resulted in gill epithelial necrosis (Stadtl ander, Lotz, Körting, & Kirchhoff, 1995; Stadtländer & Kirchhoff, 1990).

We have previously detected *Mycoplasma* 16S rDNA gene sequences in a survey of healthy zebrafish in the University of Oregon facility, especially in elderly fish at 300 dfp (Stephens et al., 2016), but in these cases we observed a diversity of mostly rare sequences; in contrast to the single abundant sequence, we detected within the affected donor and recipient fish populations. The apparent clonal nature of the *Mycoplasma* combined with its frequent dominance compared to other bacterial taxa in the transmission studies supports the hypothesis that it is an aetiological agent of the lesions, rather than a background group of bacteria that opportunistically proliferates in zebrafish with intestinal lesions. We have seen the same phenomenon with *Mycobacterium marinum* outbreaks in hybrid striped bass, where one genotype based on RFLP analysis persisted at a farm for several years (Ostland et al., 2007), and one genotype of *Mycobacterium chelonae* predominated the infections at a large zebrafish facility (Whipps et al., 2012).

Whereas the agent has yet to be confirmed, we recommend that veterinarians and technicians manage zebrafish with these intestinal lesions (Paquette et al., 2013) as a communicable disease. Though far from conclusive, preliminary evidence highlights a strain of *Mycoplasma* as a potential agent warranting further investigation. We are not, however, excluding the possibility of a different agent, such as oncogenic viruses, as the primarily cause. This should be considered as cancers often require more than one factor to develop. Furthermore, bacterial profiles associated with gastrointestinal lesions may be the result of the pathological change, rather than the underlying cause (Garrett, 2015; Wroblewski, Peek, & Coburn, 2016). Nonetheless, the association of *Mycoplasma* with exposed populations of zebrafish opens up additional avenues to study the transmission and aetiology of this disease. By utilizing faecal samples, this PCR assay can be employed to track the presence of disease without killing the animals, allowing transmission experiments to continue. Additionally, this PCR assay can be used retrospectively to investigate DNA samples from previous experiments or archives. Our studies highlight the value in taking a combined approach to studying the aetiology of a disease. The research here provides the first step to understanding the cause and perhaps developing a controlled zebrafish model for intestinal cancer.

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SUPPORTING INFORMATION

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