Co-selection of Mercury and Multiple Antibiotic Resistances in Bacteria Exposed to Mercury in the Fundulus heteroclitus Gut Microbiome

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Abstract: The emergence and spread of antibiotic resistant pathogenic bacteria is currently one of the most serious challenges to human health. To combat this problem, it is critical to understand the processes and pathways that result in the creation of antibiotic resistance gene pools in the environment. In this study we examined the effects of mercury (Hg) exposure on the co-selection of Hg and antibiotic resistant bacteria that colonize the gastrointestinal tract of the mummichog (*Fundulus heteroclitus*), a small, estuarine fish. We examined this connection in two experimental systems: (i) a short-term laboratory exposure study where fish were fed Hg-laced food for 15 days and (ii) an examination of environmental populations from two sites with very different levels of Hg contamination. In the lab exposure study, fish muscle tissue accumulation of Hg was proportional to food Hg concentration (R^2=0.99; $p<0.0001$). In the environmental study, fish from the contaminated site accumulated 3 fold more Hg compared to fish from the reference site ($p<0.05$). Further, abundance of the Hg resistance gene *merA* was more than 8 fold higher ($p<0.0001$) in DNA extracts of ingesta of fish from the contaminated site, suggesting adaptation to Hg. Finally, resistance to 3 or more antibiotics was more common in Hg resistant as compared to Hg sensitive bacterial colonies that were isolated from fish ingesta ($p<0.001$) demonstrating co-selection of Hg and antibiotic resistances. Together, our results highlight the possibility for the creation of antibiotic resistance gene pools as a result of exposure to Hg in contaminated environments.

Keywords: Co-resistance, mercury resistance, antibiotic resistance, mummichog (*Fundulus heteroclitus*)

Introduction:

Microbial resistance to antibiotics is a pressing public health concern that threatens to reverse the last 70 years of medical advancements. The Centers for Disease Control report that an estimated 2 million infections and 23,000 deaths in the United States are caused by antibiotic resistant bacteria each year [11]. Not only are they more deadly, but infections caused by antibiotic resistant bacteria also take longer and are more costly to treat [31]. The widespread use of antibiotics in industries such as healthcare, agriculture, and aquaculture contributes to the spread of antibiotic resistance by selecting for antibiotic resistant bacteria [44]. This selective pressure eliminates the bacteria that are sensitive, thus stimulating the propagation of resistant members in a population. Moreover, the spread of resistance in microbial communities is accelerated by the carriage of antibiotic resistance genes on mobile genetic elements such as plasmids and transposable elements [19]. Since these elements carry fitness-enhancing genes for other environmental stressors, antibiotic resistance may be co-selected indirectly by exposure to

various toxic substances such as heavy metals [17]. This mode of co-selection has been termed co-resistance by Baker-Austin et al., [2] to distinguish it from cross-selection whereby low specificity efflux pumps render resistance to drugs and toxic metals [44].

The demonstration that metal exposure has resulted in co-resistance to antibiotics requires evidence that (i) exposure to metals has taken place, (ii) this exposure resulted in the selection of metal resistant bacteria, (iii) the metal resistant bacteria are more resistant to antibiotics than metal sensitive strains, and (iv) both metal and antibiotic resistance genes are carried on the same mobile genetic elements. Previous studies have shown increased frequency of antibiotic resistance (requirements [i] and [ii] above) was related to copper and zinc concentrations in pig manure [21] and in agricultural soils [24], to mercury (Hg) concentrations in stream sediments [50], and among gold miners in French Guyana [46]. Mercury and antibiotic resistant strains, naturally present in the oral cavity [41], are highly enriched by exposure to Hg in dental amalgam; moreover, this exposure affects selection of co-resistant strains not only among oral flora but also in the intestinal microbiome [49]. However, only a few studies (e.g., [57]) demonstrated that resistance to antibiotics was more prevalent among metal resistant than sensitive strains (requirement [iii]). The co-carriage of metal and antibiotic resistance genes on conjugative plasmids [17, 32], transposons [30] and integrons [8] is well documented (requirement [iv]) and co-resistance to Hg and several antibiotics in aerobic heterotrophic bacteria from mine tailing ponds was attributed to co-carriage on large conjugative plasmids [3]. Previous research clearly demonstrated co-resistance of Hg and antibiotics among gastrointestinal (GI) tract microbes of feral brook trout *Salvelinus fontinalis* [33], a predatory fish exposed to Hg through the food chain.

Mercury is a highly toxic metal known to biomagnify in the aquatic food web leading to high exposure risks for humans. Despite the toxicity and abundance of anthropogenic and natural sources of Hg in the environment, some microorganisms have adapted to its presence by employing detoxification systems [4]. The most extensively studied Hg detoxification mechanism

is bacterial reduction of Hg (II) to the volatile elemental form mediated by the *mer* system [6]. This system is particularly suited for the study of the role of co-resistance on the dissemination of antibiotic resistance because genes encoding the *mer* system often travel on mobile genetic elements with antibiotic resistance genes [8, 30]. Furthermore, conjugative and mobilized plasmids captured by the exogenous plasmid isolation approach [20] are often Hg resistance plasmids (e.g., [47]). A recent genomic survey of thousands of bacterial genomes and plasmids showed that among biocide/metal resistance genes, resistances to Hg and quaternary ammonium compounds were commonly linked to antibiotic resistance genes [38]. Here we describe a fish exposure study that was designed to test if exposure to Hg selects for Hg resistant bacteria and if this selection results in the co-selection of antibiotic resistances.

In this study we examined the effect of environmental and laboratory exposure to Hg on the selection of Hg and antibiotic resistant bacteria among microbes that colonize the GI tract of the mummichog (*Fundulus heteroclitus*), a small, estuarine fish. Mummichogs forage for benthic invertebrates, which directly exposes them to Hg and other contaminants in sediments [12]. The GI tract of this organism may serve as a model in which to study co-resistance as a pathway for the enrichment of antibiotic resistance because it provides an environment conducive for genetic exchange: a stable temperature, abundance of microbial growth substrates, and a plethora of bacteria that may be directly exposed to Hg. Therefore, relating antibiotic resistance phenotypes of bacteria in the GI tract to Hg exposure may shed light on the extent that Hg contamination may co-select for antibiotic resistance in the environment.

Materials and Methods

An expanded version of the Materials and Methods is available on line in the Supplementary Materials.

Laboratory Hg exposures

To prepare Hg-laced fish food, 9 g of TetraMin Tropical Fish Food Flakes (Tetra®, Blacksburg, VA) were soaked in 10 mL $HgCl_2$ solutions for 24 hours and then dried in an oven (70°C) for 2 days. A control was treated in a similar manner, but prepared in the absence of Hg. Dried food stocks were homogenized using a Teflon mortar and pestle into pieces small enough for fish to eat, approximately 1 mm [28]. Concentrations of total Hg (THg) in the food after treatment were 24.4 ± 4.2 μg g^{-1} THg for the low exposure and 132 ± 4.1 μg g^{-1} THg for the high exposure treatment. The control food with no Hg amendments had a background concentration of 0.08 ± 0.01 μg g^{-1} THg and 0.026 ± 0.004 μg g^{-1} MeHg (Table 1). Selection of Hg food concentrations was based on known Hg levels in Berry's Creek, which is near an EPA Superfund site in the Hackensack Meadowlands, NJ, where THg concentrations of 42 – 1360 ng L^{-1} in surface waters [10] and 20 to 51 mg kg^{-1} in sediments [9] have been recorded.

Mummichogs for the laboratory feeding experiment were trapped without bait in tidal channels among the salt marshes of Great Bay, Tuckerton, New Jersey, in January 2014. Upon arrival in the laboratory, fish were randomly separated into a control, low Hg exposure, or high Hg exposure aquariums. Fish were fed for 15 days during which each aquarium received 0.45 g of fish food per day, in an attempt to provide the recommended feeding level of 0.5 to 1.0% of fish body weight per day [18]. At the completion of the experiment, it was determined that each aquarium received between 1.0 to 1.8% of fish body weight per day.

Environmental Hg exposures

Mummichogs for the examination of environmental Hg exposures were collected from Berry's Creek (BC), part of the Hackensack River Estuary, a legacy Hg contaminated site in the Hackensack Meadowlands [59]. As a reference site with little anthropogenic input, we sampled fish from Great Bay (GB), Tuckerton, NJ. Fish were trap-netted in GB in Jun. 2014 and Aug. 2014 (Figure S1). Fish collected in June were analyzed for Hg concentration, while fish collected in Aug. were used for the Hg resistance and antibiotic resistance experiments. A previous study

has shown no significant increase in fish Hg tissue concentrations over a period of weeks when mummichogs were exposed to Hg contaminated sediments (55). Mummichogs from BC were captured via cast net in July 2014. No bait was used to trap any of the fish collected for this study. During transport to the lab, fish were stored in an aerated bucket containing site water.

Processing of fish

Laboratory and environmentally collected fish were sacrificed at the end of the 15 day aquarium experiment or within 2 hours after collection according to the American Veterinary Medical Association Method for Humane Euthanasia for Finfish [1], including euthanasia in 0.4% tricane methanesulfonate. Prior to dissection, the length and weight of each fish were determined (Tables S1-S5). The intact GI tract was then surgically removed and its contents aseptically transferred to a sterile microfuge tube. Additionally, a muscle tissue sample was taken and freeze dried for Hg analysis. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Analysis of total Hg and methylmercury

Total Hg was quantified in fish tissue, fish food, aquarium water, and site water via cold vapor atomic fluorescence spectroscopy (CVAFS) [51] using a MERX-T Hg Analyzer (Brooks Rand Laboratories). Analysis of methylmerucry (MeHg) was performed by CVAFS following isothermal gas chromatographic separation of ethylated derivates according to Liang et al. [29] using a Tekran 2500. For further details on the procedures, see the Supplementary Materials.

Collection of gut ingesta

Fish GI tracts were washed in sterile 0.85% sodium chloride solution before gut contents were extracted using tweezers into sterile pre-weighed 1.5 mL microfuge tubes. Gut ingesta from 4 to 7 fish were pooled to obtain enough biomass for plating. Samples were placed on ice until processing, which was performed on the same day no longer than 4 h

following fish euthanasia. Gut ingesta were diluted 1:10 in sterile saline. Remaining ingesta was stored in 50% glycerol at -80°C.

Growth media preparation

Tryptic soy agar (TSA) plates were prepared using trypticase soy broth (BD) and 2% agar (ACROS). TSA plates amended with Hg (TSA-Hg plates) were prepared by addition of $HgCl_2$ from a 50 mM stock solution to a final targeted concentration of 25 μM to autoclaved media that was cooled down to 60ºC before plates were poured. Concentrations of Hg in stocks were routinely analyzed to determine actual concentrations of Hg for every batch of prepared plates and actual Hg concentrations are reported throughout this manuscript.

Testing of colonies for antibiotic resistance

To assess resistance to antibiotics and Hg, colonies were randomly chosen from the initial TSA and TSA-Hg plates on which gut ingesta dilutions had been plated. Colonies were spot plated on TSA-Hg plates, on individual plates containing each antibiotic (Table S6), finishing with TSA only plates as a control. Antibiotics were chosen based on their modes of action (Table S7). Colonies that failed to grow on TSA plates were not used in any analysis. A colony was considered resistant to Hg and/or an antibiotic if growth was apparent after 5 days of incubations at 23°C. Because the minimum inhibitory concentrations of antibiotics for environmental bacteria are not well known [26], we based the tested concentrations on those reported in a variety of studies [37, 48, 49, 53, 58].

DNA extractions

DNA was extracted from thawed fish gut ingesta samples using the PowerLyzer PowerSoil DNA Isolation Kit (Mo Bio Labs, Carlsbad, CA). Due to the small sample size, ingesta from several fish were combined prior to extraction to obtain sufficient quantities of DNA for qPCR analysis. Unfortunately, we did not have enough ingesta from fish collected in the aquarium experiment for DNA extraction and gene quantitation by qPCR.

Quantitative PCR (qPCR)

qPCR analysis was performed to quantify *glnA* (glutamine synthetase) and *merA* (mercuric reductase) genes in pooled ingesta samples. The primer set GS2γ and GS1β [23] was used to quantify the number of *glnA* copies, an estimate of the total number of bacteria in the sample, and primers MerA2 F and MerA2 R were used [39] to quantify *merA*, the gene specifying the central function of the *mer* system. This primer set was designed to capture the diversity of *merA* among the proteobacteria (A. Poulain, personal communication). Samples were analyzed in triplicate and standard deviations (1SD) of the triplicate readings are reported in the results. For further details on the procedure, see the Supplementary Materials.

Results

Exposure of fish in the lab – aquarium experiment

Mummichogs exposed to Hg in the laboratory, which were collected in winter, were smaller than those collected in the summer (Tables S1 to S5). At the end of the 15 d exposure period, the average mass of the fish from all treatments (n = 52) was 2.0 ± 2.7 g and the average length was 40 ± 8 mm (Table 1) compared with 6.7 ± 3.2 g and 66 ± 3 mm for the summer fish (n = 36) (Table 2). In all experiments, fish had little gut contents and therefore ingesta of 1 to 5 fish were pooled to obtain 4 to 5 samples for each treatment of 0.03 g to 0.12 g each.

Accumulation of Hg in aquarium exposed fish

After 15 days, accumulated Hg in fish muscle was directly proportional to the amount of Hg in the food for each treatment (Tables 1, S1, S2, and S3; Fig. 1). Muscle tissue from the high Hg exposure fish (132 μg g^{-1}) contained 0.149 ± 0.012 μg g^{-1} THg compared to 0.041 ± 0.010 μg g^{-1} in the low exposure and 0.031 ± 0.015 μg g^{-1} in the control fish. Therefore, the accumulation of Hg in fish muscle was nearly 5 fold higher after 15-days of exposure to high

Hg as compared to the control. The high variability of fish muscle Hg concentrations, as indicated by large standard deviations (8 to 48% of the means) may be attributed to the natural variability of various physiological processes, including ingestion, assimilation, and excretion, among individual fish. The difference in Hg tissue accumulation among the 3 treatments was significant ($p < 0.0001$, one-way ANOVA) clearly showing that fish exposure to Hg resulted in tissue accumulation. Furthermore, the Hg concentration in the fish tissue increased in a linear relationship to the Hg concentration in the food (Fig. 1), connecting consumption of contaminated food to tissue accumulation. There was no significant difference ($p > 0.05$, one-way ANOVA) in tissue MeHg accumulation between fish fed different levels of Hg (Table 1).

At the completion of the experiment, unfiltered water samples from each aquarium were analyzed for Hg concentration (Table 1). Results showed concentrations lower than those we recorded in GB field samples and little consistency between Hg concentration in food and water. The most highly dosed aquarium had a lower Hg concentration, 3.97 ng L^{-1}, than the low exposure aquarium, 16.55 ng L^{-1}, suggesting that release from food added little Hg to the aquarium water. Together, the data suggest that the main and perhaps only exposure route of fish to Hg was via ingestion of Hg-amended food.

Linked antibiotic and Hg resistances in bacterial isolates from the fish GI tract

Co-selection of Hg and antibiotic resistance was tested by randomly picking colonies of aerobic heterotrophic GI bacteria from plates on which diluted ingesta was plated and spot transferred to plates each containing a different antibiotic or 12.5 μM Hg (Table S8). A total of 136 colonies, 53 Hg resistant (Hg^R) and 83 Hg sensitive (Hg^S) were thus tested. The results indicate that colonies resistant to 3 or 4 antibiotics are almost twice as likely to be Hg^R than Hg^S, 32% and 18%, respectively, though this association was not statistically significant ($p > 0.05$, Chi squared test) and therefore the results are not shown. Co-selection could not be related to the

different levels of Hg exposure in the aquaria. However, these results suggest a connection between Hg exposure and multi-drug resistances among ingesta bacteria.

Fish exposed to Hg in contaminated and reference sites in NJ – field samples

To examine if long-term exposure to Hg resulted in tissue accumulation and in selection of Hg and antibiotic resistant GI microbiota, as was observed in the short-term aquarium exposure, mummichogs were collected from two field sites in New Jersey. Total Hg concentrations in the sediment clearly reflected the contamination status of the two sites with 12.3 ± 4.0 mg kg_{dw}^{-1} in BC and 0.39 ± 0.03 mg kg_{dw}^{-1} in GB while water concentrations at the time of sampling were 20.7 ± 1.9 ng L^{-1} and 28.0 ± 6.3 ng L^{-1} for BC and GB, respectively (Table 2). The Hg concentrations in the sediment are more relevant to this study because mummichogs forage in the sediment and are therefore exposed to Hg in their food.

Accumulation of Hg in fish tissue

Fish from both sites were of similar size and weight (Table 2) and about twice as long and 4 to 5 times heavier than the mummichogs that were exposed in the aquarium experiment (Table 1). Muscle tissue accumulation of THg in BC fish, 0.166 ± 0.072 µg g^{-1}, was almost three times higher than fish from GB (0.058 ± 0.017 µg g^{-1}, Tables 2, S4, and S5). Levels of THg in BC fish were greater than the high Hg exposure aquarium treatment while those in GB fish were between the low and high Hg exposure aquarium treatments. The accumulation of THg in fish from the two sites was significantly different (paired t test, $p < 0.05$).

Muscle tissue MeHg concentrations were low in both populations of mummichogs (Table 2) with 0.11 ± 0.01 µg g^{-1} in BC and 0.04 ± 0.01 µg g^{-1} in GB. The proportion of THg as MeHg in mummichog muscle tissue was similar in both sites: 66% in BC and 69% in GB. The relatively high percentage of THg that is not MeHg in mummichog muscle tissue is expected since these are predominantly benthic detritus feeders and are low in the aquatic food chain [54].

Selection of Hg resistant microbes in fish ingesta

Selection for Hg resistance among cultured aerobic heterotrophic bacteria was examined in fish ingesta from both the aquarium exposure and field studies (See Supplementary Materials for methods). As observed in the aquarium exposure study, there was no difference in the number of Hg^R bacteria between the two study sites (Table S9) even though the proportion of resistant organisms in both sites was much higher, about half of all CFU, as compared to fish exposed in aquaria, <2% (Table S8). The difference between the two sites was not significant ($p > 0.05$, two-way ANOVA).

Because only a small proportion of the microbial community from any environment can be cultured aerobically on rich growth medium such as TSA [22], it was possible that adaptation to Hg in the fish ingesta did not occur among cultured aerobic heterotrophs. Therefore, we quantitated the copy number of *merA*, the gene encoding the mercuric reductase [30], in DNA extracts of fish ingesta to measure the abundance of Hg^R genes in the whole community. The abundance of *glnA*, a house-keeping gene encoding for glutamine synthetase in DNA extracts was used as a measure of total microbial biomass [39]. Ingesta from several fish were combined (Table 3) to obtain sufficient amounts of DNA for this analysis and results represent a single sample from each site analyzed in triplicate. qPCR results show that the ratio of *merA*/*glnA* gene copy number was 1/6 in BC and 1/52 in GB, suggesting adaptation to Hg in the GI tract of fish from the contaminated site, BC, as *merA* enrichment in community DNA extracts is a hallmark of adaptation to Hg [7]. Although analysis of more samples is desirable, the highly significant difference in *merA* abundance between fish ingesta from the two sites, ($z=-8.139$, $p < 0.0001$, z-test), strongly supports our conclusion.

Co-occurrence of Hg and multi-drug resistances among ingesta isolates

We examined the co-occurrence between Hg and antibiotic resistances by testing Hg^R and Hg^S colonies for their resistance to 5 (GB) and 6 (BC) antibiotics (Table S6). Compared to the aquarium study, we sought to expand the number of antibiotics tested and include those of public health concern. A total of 181 ingesta colonies (84 Hg^R and 97 Hg^S) were tested from BC and a

total of 106 colonies (80 Hg^R and 26 Hg^S) were tested from GB. As was observed above for the aquarium-exposed mummichogs, a larger proportion of the resistant strains were resistant to at least 3 antibiotics (Figure 2). For GB colonies these proportions accounted for 68% and 23% of the Hg^R and Hg^S colonies, respectively, and for BC these proportions were 84% and 55%. Interestingly, overall a higher percentage of multi-drug resistance was observed among colonies from the contaminated BC site compared to GB. Mercury resistance was significantly associated with resistance to multiple antibiotics in both sites (Chi squared test, BC: $p < 0.0001$; GB: $p < 0.001$).

Discussion

This study examined the effects of Hg exposure on the Hg and antibiotic resistances of the gut microbiota of a small estuarine fish through a short-term laboratory exposure study and examination of environmental populations from two sites with very different levels of Hg contamination. We report that (i) Hg exposure resulted in increased Hg concentrations in fish muscle tissue, (ii) gut microbiomes of exposed fish had an increased abundance of the *merA* gene, suggesting adaptation to Hg, and, (iii) Hg^R bacteria from fish caught in the environment were more likely to be resistant to multiple antibiotics as compared to Hg^S bacteria. Together, our study shows that the creation of antibiotic resistance gene pools, likely by co-selection with Hg resistance, may occur in the environment.

Fish exposure to Hg was evident by the accumulation of Hg in fish tissue. In the aquarium study, Hg concentrations in fish muscle were directly related to the amount of Hg in the food (Fig. 1). Similarly, Hg concentrations in field-collected fish corresponded to the degree of Hg contamination of each site with BC fish containing three-fold more Hg than GB fish consistent with the 32-fold higher sediment Hg concentrations in the former as compared to the later (Table 2). Mummichogs are known to feed on sediment detritus [16] and others have reported concentrations of Hg in BC mummichogs spanning a range of 0.5 to 1.1 μg g^{-1} [14]. A

substantial proportion of the accumulated Hg in fish tissues was in the inorganic form. As mummichogs feed relatively low in the estuarine food chain, the proportion of MeHg of THg in their tissues likely reflects its amount in their food and the dynamics of Hg intake and removal. That Hg accumulated in mummichogs tissue is not related to biomagnification is strongly suggested by the absence of correlations between fish length and weight and tissue Hg content (Fig. S2 and S3); such correlations are commonly observed in predatory fish [33]. For the short-term laboratory exposure study, close to 33% of the Hg in the food was MeHg prior to dosing with inorganic Hg. A majority of the THg in the control and low exposures was in the methylated form, while the fish from the high exposure aquarium contained a much lower proportion of MeHg: <10% (Table 1). In a previous study, mummichogs that were collected from a reference site and incubated in aquaria containing sediments from our polluted site, BC, accumulated as much as 0.11 μg g^{-1} Hg, almost all as MeHg, after 2 weeks of exposure [55]. Thus, a short-term incubation may result in the preferential accumulation of MeHg by fish, possibly due to removal of inorganic Hg and retention of MeHg [52], as well as to different partition patterns into fish fillets [13]. In the highly dosed fish, the MeHg originally present in the food, 0.08 ± 0.01 μg g^{-1}, was diluted more than 5,000 times by inorganic Hg likely leading to the observed lower proportion of MeHg in their tissue. The possibility of methylation by microbes in the fish gut, although rarely observed [42], could also contribute to MeHg accumulation patterns.

In both studies, fish tissue Hg concentration was directly related to their exposure (Fig. 1 and Table 2). We present strong evidence that the fish were exposed through their food. First, the fish aquaria were given the appropriate amount of food to prevent excessive food debris as suggested by the little accumulation of Hg in aquarium water. At the completion of the experiment, water Hg was at most 17 ng L^{-1} (Table 1), less than its concentration in the water at both field sites (Table 2). In the field studies, fish from BC accumulated an average Hg concentration of 0.166 ± 0.072 μg g^{-1}, compared to fish from GB that accumulated 0.058 ± 0.017 μg g^{-1} (Table 2). This three-fold difference in tissue Hg concentration is most likely related to

sediment Hg concentration because (i) mummichogs are sediment feeders [16], and (ii), BC sediment had 32 times more Hg than GB sediment (12.3 ± 4.0 and 0.39 ± 0.03 mg kg^{-1}_{dw}, respectively; Table 2). We therefore conclude that the mummichogs' GI tracts contained Hg at levels that reflected concentrations in their food thus potentially exposing GI microbes to Hg toxicity.

Evidence that Hg toxicity leads to the enrichment of resistant populations in the impacted microbial community may be obtained by observations of increased proportions of Hg^R microbes [4, 34, 40] or of presence [7] and expression [36, 43] of *mer* gene homologs. When these two approaches were applied here, the molecular approach clearly showed an enrichment of *merA*, specifying the major function of the *mer* system, in BC as compared to GB fish (Table 3). On the contrary, the culturing approach clearly showed no relationships between fish exposure and the proportion of Hg^R among the total number of heterotrophic aerobes and this trend was common to the aquarium exposure and the field studies (Tables S7-S8). The discrepancy between the results of the plating experiment (no enrichment for Hg resistance) and the qPCR (almost nine fold enrichment of *merA* in ingesta from BC fish relative to GB fish) may be explained by low culturability of ingesta microbes that do not contain *merA*. Consistent with this suggestion, Hg resistant isolates obtained from ingesta rarely contained *merA* homologs (Lloyd et al., manuscript in preparation). Less than 1% of all microbes in the environment are culturable [56] and plating results may not represent the entire community in the fish gut. The molecular qPCR-based approach scored the presence of *merA* in the microbial microbiome of the gut ingesta, not only those microbes that could be cultured. Thus, prior studies reporting no significant co-selection of antibiotics with metal resistances (e.g., [24, 50]) may have underestimated its occurrence by targeting only cultured strains.

The five most abundant core species, those shared by the gut metagenomes of four individual mummichogs that were collected near Sapelo Island, GA, USA, included *Vibrio* sp., *Photobacterium* sp., *Pseudomonas* sp., *Halomonas* sp., and *Propionibacterium* sp. [15]. Several

cultured isolates from the BC and GB ingesta (Lloyd, unpublished), and numerous *mer*-carrying genomes [5], are affiliated with these genera. It is therefore not surprising that *merA* homologs were detected in the fish ingesta; their enrichment in BC samples, where sediment and fish tissue Hg concentrations were significantly higher than those in GB, is a clear indication for the selection of resistant gut populations, likely as a result of Hg exposure.

Mercury resistant bacterial colonies that were isolated from BC and GB fish ingesta were more likely to be resistant to several antibiotics as compared to Hg^S colonies (Fig. 2); results of the aquarium study suggested a similar trend, although they are not statistically significant. These results clearly suggest that selection for Hg resistance inadvertently enriched for multi-drug resistant strains, consistent with the findings of Wireman et al., [57] who studied co-selection among gram-negative fecal bacteria. While a significant connection between Hg^R and multi-drug resistance in colonies from the Hg contaminated BC site was expected, the GB site may experience other selective pressures for antibiotic and Hg resistances, e.g., pesticides may select for antibiotic resistance [27]. Our sampling sites, even the so-called "cleaner" GB site, are exposed to multitude of contaminants, including industrial and domestic runoffs, from variable sources [25, 45, 35] leaving open the possibility that other effectors may select for antibiotic and Hg resistant bacteria in these fish.

The most plausible explanation for the co-selection of Hg resistance and multi-drug resistance is genetic co-occurrence of resistance genes in the genomes of gut microbes. When such co-occurrences occur on mobile genetic elements, selective pressure for one gene that under the prevailing environmental conditions enhances fitness could lead to enrichment and spread by horizontal gene transfer of all genes, potentially creating an antibiotic resistance gene pool as a result of exposure to unrelated contaminants. This process may be particularly important in environments that are conducive for horizontal gene transfer such as the fish GI tract. This indirect path to the enhancement and spread of antibiotic resistance should be integrated into our thinking of how to best address this looming public health challenge.

Conflict of Interest

The authors declare no conflict of interest.

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Figure Captions

Figure 1 Effect of Hg exposure on Hg tissue accumulation in mummichogs (*F. heteroclitus*) exposed to the indicated Hg concentrations for 15 days in the aquarium study. Values are means ± 1SD of all determinations; see Table 1 for number replicate analyses.

Figure 2 Co-selection of Hg and antibiotic resistances in field studies. The above graphs depict the patterns of Hg and antibiotic resistance in the field sites BC (A) and GB (B). Colonies were scored as either Hg^R or Hg^S, and the number of antibiotic resistances was counted.