Detection of *Salmonella* in the intestine of *Hypostomus plecostomus* from the upper San Marcos River, Texas

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ABSTRACT

The prevalence of salmonellae in the intestines of the invasive suckermouth catfish *Hypostomus plecostomus* was assessed in the San Marcos River, just down-stream of its spring-fed headwaters. In 2014, *H. plecostomus*, sediment, and water samples were collected during 15 sampling events. A combination of semi-selective enrichment and quantitative polymerase chain reaction (*q*PCR) revealed the presence of salmonellae in 45% of the fish intestines across the entire year, with a prevalence range of 13–100% per sampling event. Repetitive element sequence-based PCR (rep-PCR) and multi-locus sequence typing (MLST) revealed a high diversity of salmonellae from fish intestine samples at individual sampling times, single or multiple presence of rep-PCR patterns and serotypes within individual fish, and identical rep-PCR patterns and serotypes for different fish within and across sampling events. Overall, 15 serotypes were identified by MLST, with a diversity range between one and seven serotypes per sampling event. Some serotypes were retrieved only once, while others were detected more frequently. A few serotypes were retrieved at several sampling times, nearly evenly distributed over the entire sampling period. Prevalence and diversity were independent of precipitation events, indicating the potential presence of environmental strains that are capable of long-term persistence in the environment.

Key words | diversity, multilocus sequence typing (MLST), rep-PCR, serotype, spring lake, suckermouth catfish

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INTRODUCTION

Members of the genus *Salmonella* are Gram-negative, facultative anaerobic bacteria that are recognized worldwide as major zoonotic pathogens for both humans and animals (Humphrey 2000). The intestinal tract of a taxonomically diverse group of vertebrates is considered to be the principle habitat of salmonellae (Gray 1995; Refsum *et al.* 2002; Briones *et al.* 2004). Salmonellae can be shed into terrestrial and/or aquatic environments through the release of feces (Cherry *et al.* 1972; Polo *et al.* 1998). The presence of salmonellae in the environment is therefore often linked to contamination through, e.g., manure or wastewater discharges (Polo *et al.* 1998; Martinez-Urtaza *et al.* 2004) or the fecal droppings of livestock (Miner *et al.* 1967) and wildlife (Polo *et al.* 1998; Arvanitidou *et al.* 2005). Salmonellae, however, have also been detected in aquatic systems in areas seemingly devoid of fecal pollution (Thomason *et al.* 1975; Patchanee *et al.* 2010; Gaertner *et al.* 2011). Salmonellae have been found to persist in non-enteric habitats (e.g., water, sediment, soil, biofilm) for extended periods of time (e.g., Holley *et al.* 2006; Englebert *et al.* 2008; Budzinska *et al.* 2009; Byappanahalli *et al.* 2009; Cevallos-Cevallos *et al.* 2014; Nyberg *et al.* 2014). While growth might be supported in eutrophic environments such as wastewater, manure-amended soils, or biosolids (Zaleski *et al.* 2005; You *et al.* 2006), oligotrophic environments such as surface waters, sediments, and soils are usually suboptimal for

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growth and induce stress responses that allow salmonellae to survive and persist (Foster & Spector 1995; Spector & Kenyon 2012).

Spring Lake is a small (6 ha) spring-fed headwater pool of the San Marcos River in San Marcos, Texas and considered one of the most pristine waters in Texas (Slattery & Fahlquist 1997). The detection of salmonellae in water and sediments in Spring Lake was generally related to runoff after strong rainfall events (Gaertner *et al.* 2009, 2011). Unrelated to runoff, however, salmonellae were commonly detected in intestines of animals such as fish, turtles, and crayfish residing in these waters (Hahn *et al.* 2007; Gaertner *et al.* 2008a, 2008b, 2011), and in multi-species biofilms on the carapace of turtles and on concrete linings in the water (Hahn *et al.* 2007; Gaertner *et al.* 2011; Sha *et al.* 2011, 2013b). Salmonellae have been isolated from the intestines of several species of fish from the San Marcos River, including the suckermouth catfish *Hypostomus plecostomus* (Gaertner *et al.* 2008a). *H. plecostomus* is an invasive fish species thought to have first been introduced to the San Marcos River in the 1990s through illegal release of aquarium fish (Pound *et al.* 2011). The suckermouth catfish is estimated to now account for 25–50% of the total fish community in the upper San Marcos River, with its food sources consisting primarily of detritus of algal origin (Pound *et al.* 2011). Although salmonellae have been detected in fish, the pathogen does not seem to be part of the natural flora of fish, but its presence might rather be related to food sources and the surrounding water (Cahill 1990). Due to its high population numbers and its food sources that include biofilms, *H. plecostomus* could be an important vector for the dissemination of salmonellae along the San Marcos River.

The aim of this study was to investigate the prevalence of salmonellae in *H. plecostomus* in the San Marcos River at repeated sampling events during an entire year. *H. plecostomus*, sediment, and water samples were collected from the San Marcos River and analyzed for salmonellae using a combination of semi-selective enrichment and quantitative polymerase chain reaction (*q*PCR). Data on prevalence were analyzed as function of rainfall events. Isolates from infected fish were characterized using repetitive element sequence-based PCR (rep-PCR) typing (Foley *et al.* 2006; Albufera *et al.* 2009) followed by multi-locus sequence typing (MLST) (Hughes *et al.* 2010; Shi *et al.* 2015), in order to retrieve information on the potential presence of environmental strains (i.e., those present long term, and thus detected at different times during the year).

MATERIALS AND METHODS

Detection of salmonellae

Sampling

Fish and environmental samples were retrieved from the San Marcos River, San Marcos, Texas between Rio Vista Park and I-35 (29.878633, −97.933024) (Figure 1) beginning

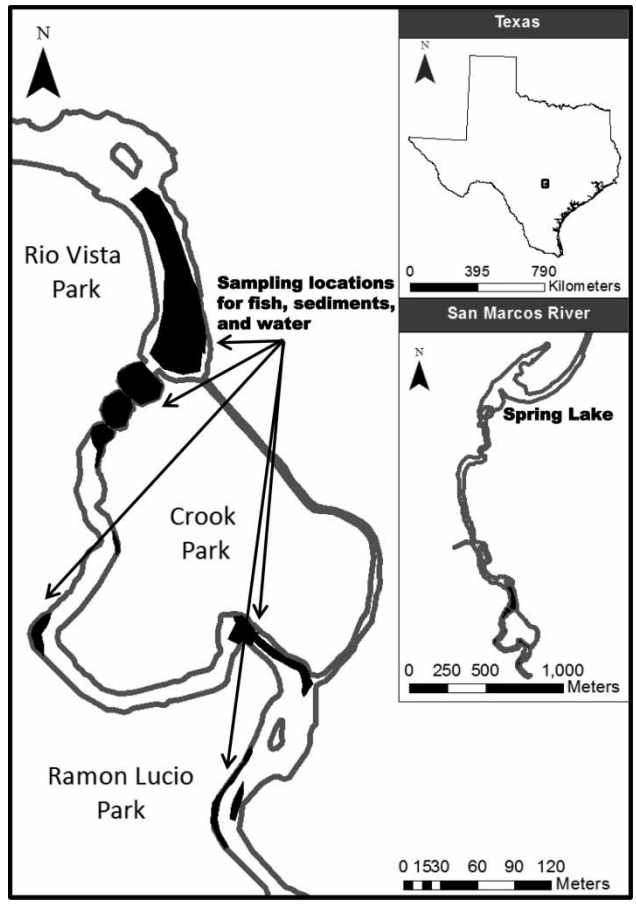

Figure 1 | Sampling sites (black-marked areas) of fish (*Hypostomus plecostomus*) and environmental samples (sediment and water) in the San Marcos River, San Marcos, Texas, between Rio Vista Park and I-35 (approximately 29.878633, −97.933024), starting in February 2014 and ending January 2015, as part of an ongoing study by the City of San Marcos on the effectiveness of spear fishing for the potential removal of the invasive *H. plecostomus*.

in February 2014 and ending January 2015, as part of an ongoing study by the City of San Marcos on the effectiveness of spear fishing for the potential removal of the invasive *H. plecostomus* (Texas Parks and Wildlife Department permit SPR-0601–159). Per sampling event, five to ten dead individuals of *H. plecostomus* and environmental samples (i.e., surface water and river sediment) were provided by the contractor for the City of San Marcos, usually 24 hours after large rainfall events (>12.7 mm) 15 times during 2014 (Figure S1, available with the online version of this paper). Directly after capture, fish were transported to the laboratory, cleaned with 70% ethanol, and dissected. Fish observed to have a spear laceration to the abdominal cavity were not evaluated, but discarded. The entire intestinal tract, i.e., from anus to pyloric stomach, was removed. Samples were obtained by homogenizing a small portion of the upper tract by vigorous shaking and vortexing in sterile distilled water to dislodge intestinal content from the mucosal lining. Homogenized intestinal contents were decanted to separate contents from intestinal mucosa.

Sediment samples (30 mL) were collected in sterile 50 mL centrifuge tubes at the time and site of fish collection, as were 100 mL of surface water. River sediments were pelleted by centrifugation at 3,000 × g for 15 min, and residual water decanted. Remaining sediments were mixed prior to use. Cells from surface water were concentrated by centrifugation at 3,000 × g for 15 min, and resuspended in 1 mL sterile distilled water.

Semi-selective enrichment

For the detection of salmonellae by *q*PCR from each of the three sources, 100 μL of homogenized intestinal contents, 100 μL of concentrated surface water, and 100 mg of sediment samples, respectively, were incubated in 1 mL of buffered peptone water (BPW) (per L: 10 g peptone, 5 g NaCl, 9 g Na_2HPO_4, 1.5 g KH_2PO_4, pH 7.2) at 37 °C for 24 hr (Thomason *et al.* 1977). Subsamples of 100 μL of the BPW incubations were transferred to 1 mL aliquots of Rappaport-Vassiliadis selection (RVS) broth (per L: 4.5 g peptone, 29 g $MgCL_2{\cdot}7H_2O$, 8 g NaCl, 0.4 g K_2HPO_4, 0.6 g KH_2PO_4, 0.036 g Malachite Green) and incubated at 37 °C for 24 hr (Vassiliadis *et al.* 1981). This semi-selective enrichment of salmonellae was repeated with 100 μL from each of these samples (Sha *et al.* 2013b), and 100 μL of these enrichments finally transferred to sterile 1.2 mL microcentrifuge tubes. Cells were pelleted by centrifugation at 14,000 × g for 3 min, washed with sterile nuclease-free water and lyzed in 100 μL of 50 mM NaOH by incubation at 65 °C for 30 min. Lysates were stored at −20 °C until analyses (Sha *et al.* 2013a).

qPCR

Quantitative polymerase chain reaction (*q*PCR) quantification was performed targeting a 284 bp *inv*A gene fragment for the detection of all *Salmonella* spp. using primer set 139 ($^{5'}$GTG AAA TTA TCG CCA CGT TCG GGC AA) and 141 ($^{5'}$TCA TCG CAC CGT CAA AGG AAC A) (Malorny *et al.* 2003). SYBR Green based *q*PCR was performed in a total volume of 10 μL containing 5 μL SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 100 ng each of primers 139 and 141, and 1 μL lysate in an Eco Real-Time PCR System (Illumina, San Diego, CA, USA) using an initial denaturation at 95 °C for 3 min, 40 cycles of 95 °C denaturation, 64 °C annealing, and 72 °C extension for 30 s each, followed by a melting curve analysis (Sha *et al.* 2013c). A standard curve was generated using ten-fold serial dilutions of cells of *S. enterica* serovar Give quantified by *in situ* hybridization (Sha *et al.* 2013a). *q*PCR was performed in triplicate on the intestinal samples, semi-selective enrichments, and environmental samples (Sha *et al.* 2013c).

Characterization of isolates

Isolation

Attempts to obtain isolates used enrichments positive for *Salmonella* that were plated onto Bismuth Sulphite agar (Himedia, Nashik, MH, India) and incubated at 37 °C for 24 hr (Thomason *et al.* 1975). Using sterile toothpicks, individual colonies were transferred to 5 mL sterile Luria Broth (LB) (Difco, Detroit, MI, USA), which were incubated at 37 °C for 24 hr with agitation. After alkaline lysis, cultures were screened for the presence of the *inv*A gene by *q*PCR as described above. Cultures positive for *Salmonella* spp. were recultured in LB and stored in sterile 20% glycerol solution at −80 °C. Culture lysates for *Salmonella* spp. were stored at

−20 °C until analysis by rep-PCR. A minimum of ten isolates per positive sample was attempted for the analysis.

Rep-PCR

Rep-PCR typing of *inv*A positive isolates was performed in an Eppendorf Mastercycler (Eppendorf, Hauppauge, NY, USA) in a total reaction volume of 25 μL containing 5 μL culture lysate, 390 ng BoxA1R primer (5'CTA CGG CAA GGC GAC GCT GAC G), 5 μL of 5 × Gitschier buffer (83 mM $(NH_4)_2SO_4$, 33.5 mM $MgCl_2$, 335 mM Tris/HCl, 33.5 μM EDTA, 150 mM β-mercaptoethanol, pH 8.8), 10% di-methyl-sulfoxide, 3 μg bovine serum albumin (BSA), 5 mM each dNTPs, 2 U *Taq* polymerase (Genscript, Piscataway, NJ, USA) using an initial denaturation of 95 °C for 2 min, 30 cycles of 94 °C for 3 s, 92 °C for 30 s, 50 °C for 1 min, 65 °C for 8 min, followed by a final incubation at 65 °C for 8 min (Versalovic *et al.* 1994; Gaertner *et al.* 2011; Sha *et al.* 2013b). The 2 μL PCR products were visually examined using gel electrophoresis on a 2% agarose gel containing ethidium bromide in TAE buffer followed by subsequent post-electrophoresis staining with ethidium bromide (Versalovic *et al.* 1994). Representative unique isolates were then further analyzed using the Agilent 2100 Bioanalyzer and the DNA 7500 (Agilent, Santa Clara, CA, USA) for a cleaner banding pattern and comparative analyses (Sha *et al.* 2011, 2013b).

Multi-locus sequence typing

Isolates with seemingly unique rep-PCR banding patterns were analyzed using multi-locus sequence typing (MLST) of seven housekeeping genes (*thr*A, *dna*N, *aro*C, *pur*E, *his*D, *hem*D, and *suc*A) for serotype identification and given allele assignments designated by the University of Warwick, UK (Kidgell *et al.* 2002; Achtman *et al.* 2012; Dione *et al.* 2012; Nair *et al.* 2014). PCR products for each gene were amplified using an Eppendorf Mastercycler in a total volume of 25 μL containing 1 μL culture lysate, 2.5 μL 10 × PCR buffer (Genscript), 6.25 pmol of each primer (Table S1, available with the online version of this paper), 0.2 mM dNTPs, 1.25 U Taq polymerase (Genscript), 60 μg BSA using an initial denaturation of 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by a final extension of 72 °C for 10 min (Dione *et al.* 2012). 5 μL PCR products were loaded with GelRed (Biotium, Hayward, CA, USA) in 10 × DNA loading dye and visually examined by electrophoresis on a 2% agarose gel in TAE buffer.

PCR products were then cleaned and concentrated using the UltraClean® 15 DNA Purification Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's guidelines. These products were used as the template for a nested PCR reaction, in the same manner described first using the sequencing primers in place of the primary PCR primers (Table S1).

PCR products were purified by adding 5 μL product to 1.5 μL sterile nuclease-free water, 0.4 U Shrimp Alkaline Phosphatase (Affymetrix, Santa Clara, CA, USA), and 1 U Exonuclease-1 (Affymetrix) (Werle *et al.* 1994). Cycle sequencing was performed on both forward and reverse strands of each gene using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations and ABI 3500XL Genetic Analyzer (Applied Biosystems). Sequences were aligned and edited using Geneious version 8.1 (Biomatters Ltd, Auckland, New Zealand) (Kearse *et al.* 2012). Consensus sequences were submitted to the University of Warwick MLST database for assignment of allele type numbers and strain type identification (Aanensen & Spratt 2005). Sequences of housekeeping genes *thr*A, *dna*N, *aro*C, *pur*E, *his*D, *hem*D, and *suc*A used to identify specific serotypes were deposited at the EMBL Nucleotide Sequence Database under accession numbers LT840127 to LT840166, and LT840241 to LT840340.

Determination of environmental conditions

The presence of salmonellae in intestinal samples of *H. plecostomus* and in environmental samples was compared to precipitation patterns and selected environmental conditions using principle component analysis in SigmaPlot (Systat Software, San Jose, CA). Precipitation patterns throughout the year were obtained from NOAA National Centers for Environmental Information (http://www.ncdc.noaa.gov/oa/ncdc.html for US1TXHYS074 and USC00417983). Data on other environmental conditions affecting the San Marcos River, including temperature and turbidity were obtained at the sampling site as part of the

ongoing studies of the Edwards Aquifer Habitat Conservation Plan, and kindly provided by the PI of that project, Dr. Thomas Hardy. Water quality measurements were taken every 15 min using a 6920 V2-2 Multi-Parameter Water Quality Sonde (Yellow Springs Instruments, Yellow Springs, OH, USA) placed at 29.8800, −97.9333. River discharge and gage height were obtained from USGS National Water Information System (http://waterdata.usgs.gov/tx/nwis/uv?site_no=08170500).

RESULTS

Analyses of intestines of *H. plecostomus*

Intestines from a total of 119 *H. plecostomus* and 30 environmental samples, i.e., 15 water and 15 sediment samples, were analyzed for the presence of salmonellae by *q*PCR. Samples required semi-selective enrichments performed twice for any detection of the *inv*A gene in both fish intestines and environmental samples. Without or with a single enrichment, none of the samples was positive for the *inv*A gene. After double semi-selective enrichments, the *inv*A gene was detected in 53 (45%) intestinal samples, with at least one intestinal sample being positive for each sampling date (Table 1). Only two water samples (13%) and one sediment sample (7%) were positive for the presence of the *inv*A gene, across all sampling events.

Table 1 | Results from the semi-selective enrichment detection of salmonellae in fish (*hypostomus plecostomus*), sediment, and water samples taken from the San Marcos River, San Marcos, TX, USA

	Samples positive for salmonellae	
Date	**Fish (percent of total)**	**Environmental samples**
2014		
February 9	1 of 5 (20%)	nd
February 18	4 of 11 (36%)	nd
April 7	1 of 6 (17%)	Water
April 15	3 of 7 (43%)	nd
June 3	3 of 5 (60%)	nd
July 15	4 of 10 (40%)	nd
July 24	5 of 8 (63%)	nd
September 8	10 of 10 (100%)	nd
October 3	7 of 7 (100%)	Sediment
October 21	7 of 9 (78%)	nd
October 31	6 of 10 (60%)	nd
November 10	1 of 5 (20%)	nd
December 20	1 of 8 (13%)	Water
2015		
January 11	4 of 8 (50%)	nd
January 18	2 of 10 (20%)	nd

nd, not detected.

Characterization of isolates

Of the 53 intestine samples positive for salmonellae, 52 samples provided at least one isolate confirmed as *Salmonella* using the detection of the *inv*A gene as proxy. Of these, 47 samples provided at least ten isolates, for a total of 728 confirmed *Salmonella* isolates that were further characterized by rep-PCR. Visual comparison retrieved a total of 180 unique rep-PCR patterns that were re-analyzed using the Bioanalyzer to reduce subjectivity in the analyses of electropherogram patterns of rep-PCR amplicons. This resulted in the detection of 112 unique rep-PCR patterns. Profiles of rep-PCR patterns from all fish obtained at one sampling could be highly diverse, with an individual fish harboring salmonellae represented by one rep-PCR pattern only, while others displayed several rep-PCR patterns, and identical patterns were observed among different fish (Figures 2 and 3). Multi-locus sequence typing (MLST) of

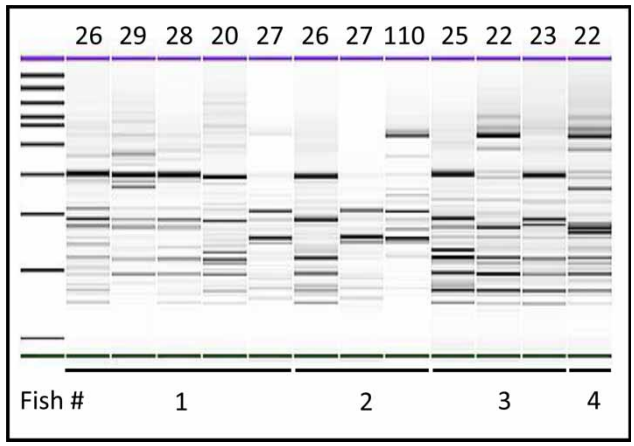

Figure 2 | Rep-PCR characterization of unique isolates of salmonellae from four individuals of Hypostomus plecostomus sampled on July 15, 2014 and analyzed on the Agilent Bioanalyzer. Patterns 22, 26, and 27 are each present in two individuals, i.e., fish 3 and 4 (pattern 22), and fish 1 and 2 (patterns 26 and 27), respectively.

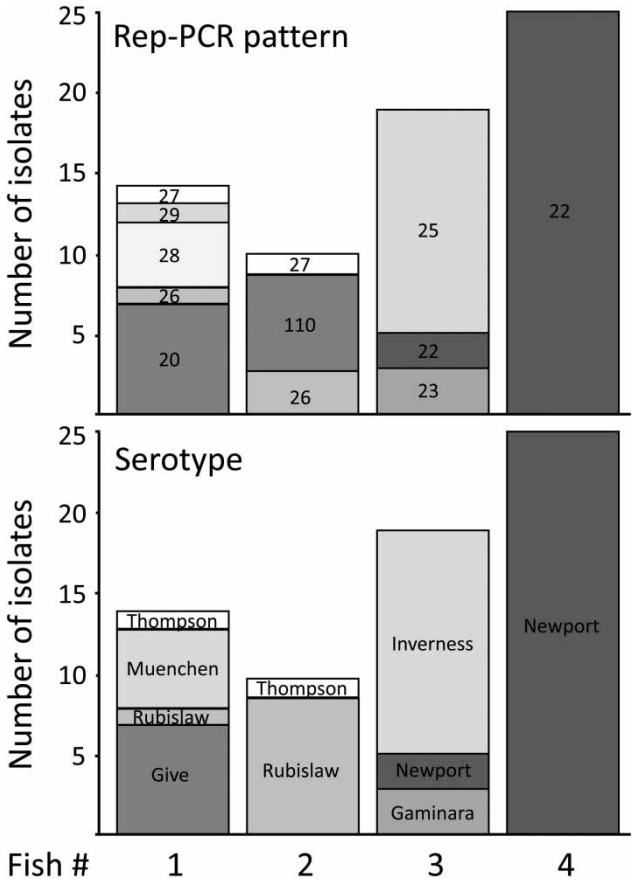

Figure 3 | Characterization of isolates of salmonellae from intestines of four individuals of *Hypostomus plecostomus* sampled on July 15, 2014, using rep-PCR and multi-locus sequence typing (MLST) of seven housekeeping genes (*thr*A, *dna*N, *aro*C, *pur*E, *his*D, *hem*D, and *suc*A) for *Salmonella* serotype identification.

at least two representatives of each unique rep-PCR pattern revealed that identical rep-PCR patterns represented the same serotype, while rep-PCR patterns identified as being different could represent the same serotype (Figure 3). MLST reduced the diversity of salmonellae from 112 unique rep-PCR patterns to 15 serotypes (Figure 3). Nevertheless, detection of serotypes demonstrated the potential for high diversity at one sampling time and the presence of distinctive serotypes for individual fish (Figure 3). For example, four fish from the same sampling event harbored a total of seven serotypes, with one fish harboring only one serotype, while the others carried two, three, and four serotypes. Three serotypes were found in two different fish (Figure 3).

The most prominent serotype detected was Newport retrieved at seven sampling events nearly evenly distributed over the entire sampling period, followed by serotype Give and Gaminara (six sampling times), Inverness (five sampling times), Rubislaw and Typhimurium (three sampling times) and Muenchen and Braenderup (two sampling times). Serotypes Bredeney, Oranienburg, Mbandaka, Saintpaul, Infantis, Thompson, and Paratyphi B var Java were detected at one sampling event only (Figure 4). Serotypes from some samples (e.g., intestines from fish obtained February 9 and 18, June 3, or January 11) could not be determined since MLST did not detect any representatives in the database. Diversity of serotypes differed significantly at specific sampling times, covering a range from one serotype (Newport on January 18) to a maximum of eight serotypes (Give, Rubislaw, Muenchen, Infantis, Saintpaul, Mbandaka, Bredeney, and Orainenburg; September 8) (Figure 4).

Serotypes Newport and Give showed two strains present for each with different alleles for the seven genes. The two Newport strains showed different alleles at all seven of the genes, while the two Give strains were different at six out of the seven genes. The Newport strains occurred in four collection dates each with both occurring in one date, January 18. One of the Give strains occurred in three collection dates, while the other strain occurred in two collection dates. The two Give strains did not occur together in any of the dates as was seen with the Newport strains.

Isolates from both surface water samples were represented by two rep-PCR patterns and identified as serotypes Newport and Inverness. Isolates from the sediment sample were identified as serotype Inverness.

Environmental conditions

Due to the proximity of the sampling site to the spring-fed headwaters of the San Marcos River, major environmental conditions of the water remained relatively constant throughout the year: water temperature was 21–24 °C, conductivity was stable at around 600 $\mu S\,cm^{-1}$, except for a few times correlated to large rainfall events when conductivity briefly declined to a minimum of 500 $\mu S\,cm^{-1}$, pH was between 7.3 and 7.6, dissolved oxygen was between 7 (morning) and 11 (afternoon) mg L^{-1}, gage height was between 120 and 140 cm, and the water discharge was between 3 and 5 $m^3\,s^{-1}$. Turbidity was generally low, i.e., between 0 and 5 NTU, with occasional 100- to

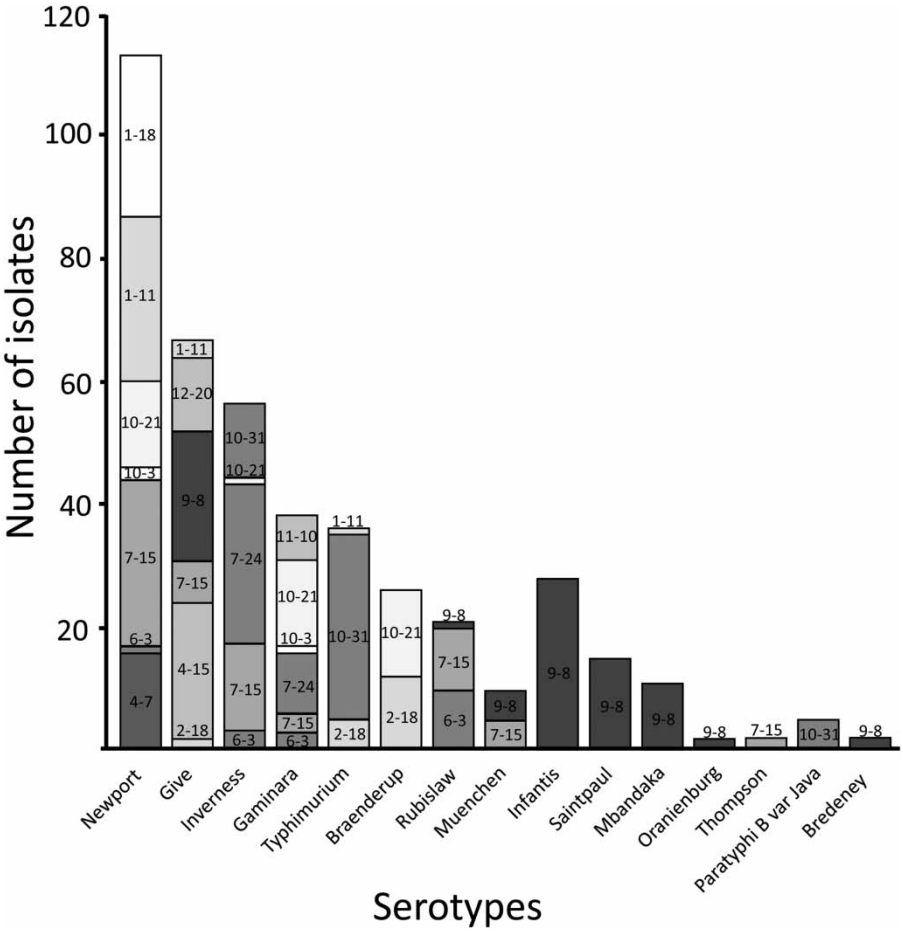

Figure 4 | Frequency of isolation of the most prominent strains of *Salmonella enterica* from *Hypostomus plecostomus* intestinal samples across different sampling events during the period from February 2014 to January 2015, as identified by unique rep-PCR patterns, and identification of the corresponding serotypes.

1,000-fold hourly spikes due to public recreational activity in the fall. Mass developments of organisms such as algal blooms were never detected. A total of 12 days received more than 12.7 mm of precipitation, with eight events in the range of 14 to 21 mm and four events between 51 and 90 mm (Figure S1). Sampling events occurred directly after precipitation (e.g., July 15, September 8, December 20, and January 11), but also without preceding precipitation for weeks (e.g., February 9 and 18, April 7, June 3, and October 21) (Figure S1). For environmental samples positive for salmonellae, only one of the sampling times (December 20, 2014) was preceded by heavy precipitation, while the other two were not. A principle component analysis with chemical and physical factors (pH, conductivity, water temperature, turbidity, river discharge, and river gage height), precipitation, and salmonellae prevalence revealed that prevalence of salmonellae was not correlated to precipitation. This was confirmed using multiple linear regression with the chemical and physical factors listed above and precipitation as independent variables and prevalence of salmonellae as the response variable (data not shown).

DISCUSSION

Several studies have reported on the prevalence of salmonellae in fish intestines, with values between 14% and 24% prevalence in intestine samples (Hatha & Lakshmanaperumalsamy 1997; Budiati *et al.* 2013; Traore *et al.* 2015; Sing *et al.* 2016). Salmonellae have been previously detected in fish intestines from the San Marcos River, with prevalence in samples ranging from 17% to 33%, depending on fish

species and sampling site (Gaertner *et al.* 2008a). That study, however, represented a proof-of-concept analysis of salmonellae in intestine samples from only 32 fish representing six species obtained from three distinct locations during a short sampling period in spring 2007. Salmonellae were detected in intestines of three out of 16 (18%) individuals of *H. plecostomus*, all retrieved from the same location close to the site now used in our current study. Our current study reduced variables by focusing on one fish species, *H. plecostomus*, at one location, and expanded the analyses to include different sampling events during an entire year. The analyses of 119 individuals obtained at 15 sampling events during the year 2014 revealed the presence of salmonellae in 45% of the fish intestines, with a prevalence range of 13–100% in the intestine samples per sampling time. Salmonellae are not expected to be part of the normal intestinal flora of fish (Janssen & Meyers 1968; Pal & Dasgupta 1991), but fish exposed to salmonellae can become asymptomatic carriers of this pathogen (Brunner 1974; Bocek *et al.* 1992). Salmonellae have been detected in the digestive tract of fish with numbers up to one order of magnitude higher than in the water (Pal & Dasgupta 1991; Ampofo & Clerk 2003). They have also have been shown to multiply in the intestine and be shed in the feces (Morse *et al.* 1978a, 1978b; Lesel & Legac 1983). Consequently, fish constitute an important factor potentially affecting the dissemination and persistence of salmonellae in aquatic environments (Lawton & Morse 1980).

Our previous proof-of-concept analyses (Gaertner *et al.* 2008a) required at least one, if not two semi-selective enrichments for the detection of the *inv*A gene in intestine and environmental samples indicating that salmonellae were present and persisting in a viable state in these samples in very low numbers. The study also revealed the presence of salmonellae in sections of the intestine only, e.g., the upper part, but not lower part of the intestine of three *H. plecostomus*. In contrast, salmonellae were only detected in the lower intestine of a largemouth bass, two channel catfishes, and a common carp, while they were only detected in the upper part of the intestine of another carp (Gaertner *et al.* 2008a). These results indicate that salmonellae are not likely permanent members of the intestinal flora of fishes, but transient occupants most likely acquired through ingestion of food sources. The transient nature of salmonellae might have impacted their detection in our current study that focused on the analyses of the upper part of the intestine, and thus we might have failed to detect salmonellae if present in the lower part of the intestine.

Salmonellae were shown to colonize multi-species drinking water biofilms, to survive and grow into microcolonies in these biofilms and to be released from the biofilms into the drinking water (Schaefer *et al.* 2013). Salmonellae have been detected in aquatic biofilms (Hahn *et al.* 2007; Gaertner *et al.* 2011; Sha *et al.* 2011, 2013b) that are a food resource for *H. plecostomus* (Pound *et al.* 2011). Aquatic biofilms consist of highly heterogeneous and active communities of auto- and heterotrophic microorganisms (Geesey *et al.* 1978), that provide protection from toxic compounds, reduce environmental stress (Johnson 2008), and provide rapidly available carbon resources for heterotrophic organisms (Geesey *et al.* 1978; Augspurger *et al.* 2008). Aquatic, multi-species biofilms might therefore not only provide habitats suitable for long-term survival and persistence of salmonellae as seen for other pathogens (Watnick & Kolter 1999; Yildiz & Schoolnik 1999), but also resources considered to support growth (Jones & Bradshaw 1996, 1997). Growth of introduced salmonellae has been detected in feces directly after release for a short time followed by fast reductions in numbers (Sha *et al.* 2013a), however, fast declines of inoculated salmonellae were shown in biofilms with no obvious growth detected (Sha *et al.* 2013a, 2013c). Salmonellae were still found in biofilms, although in small numbers that required semi-selective enrichments, supporting speculations about long-term adaptations of strains to environmental stress and persistence in biofilms in low numbers without considerable growth (Sha *et al.* 2013a). Uptake by *H. plecostomus* and passage through the intestine could therefore activate persisting salmonellae and release them into the environment, with fish feces serving as source of salmonellae, but also as an environment providing nutrient resources for short-term growth of salmonellae.

Clonal persistence of distinct serotypes of *Salmonella* over long periods of time has been discussed as a reason for repeated contaminations of final products from agriculture feed production environments (Prunic *et al.* 2016). This discussion is supported by the detection of isolates with identical rep-PCR patterns, and their identification to

the same serotype, at repeated samplings during the season. In contrast to other suggestions (Gaertner *et al.* 2009, 2011), precipitation and resulting run-off were not correlated to prevalence of salmonellae in fish intestine. Similar results were obtained for prevalence of salmonellae in surface waters where no correlations were found to precipitation, and only weak ones to environmental conditions (McEgan *et al.* 2013). Other studies had identified environmental conditions such as temperature as cause for seasonal variation in the detection of salmonellae, both in the intestine of fish (Al-Harbi & Uddin 2004), as well as in water samples (Haley *et al.* 2009). In our study, environmental conditions were virtually identical throughout the season, and thus environmental conditions were not associated with prevalence of salmonellae in fish intestines.

Our analyses showed a 45% prevalence of salmonellae in fish intestine samples, with at least 15 serotypes detected, many of which were frequently detected during the sampling season. Analyses of serotypes in fish intestines produced highly variable results, covering a range from low prevalence (4%) and low diversity (two serotypes) (Basti *et al.* 2004) to medium prevalence (20%) and low diversity (three serotypes) (Sing *et al.* 2016), medium prevalence (28%) and medium diversity (seven serotypes) (Budiati *et al.* 2013), and medium prevalence (20%) and high diversity (34 serotypes) (Traore *et al.* 2015). Semi-selective enrichment of salmonellae and concomitant growth of background microflora can affect the diversity of *Salmonella* serotypes, or its detection (Gorski 2012), and thus it is possible that some serotypes may have been inhibited or not detected due to competition with other serotypes or other bacteria. Our analyses might therefore underestimate overall diversity of *Salmonella* serotypes.

CONCLUSIONS

In conclusion, our study demonstrated high prevalence of different serotypes of *Salmonella* in intestine samples of the suckermouth catfish *H. plecostomus* in the San Marcos River close to its headwaters across different sampling events during the year 2014. High diversity of salmonellae could be detected in intestine samples at individual sampling times, with single or multiple presence of serotypes in individual fish, and identical serotypes in different fish from the same, but also from different sampling events. Prevalence and diversity was independent of precipitation events, and thus potential runoff, indicating the potential presence of environmental strains able to persist long term in the environment.

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