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3 Epigenetic Inheritance of DNA Methylation Changes in Fish Living in 4 Hydrogen Sulfide-rich Springs

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26 **Short title:** Hydrogen sulfide effects on DNA methylation in *Poecilia mexicana*
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Abstract

Environmental factors can promote phenotypic variation through alterations in the epigenome and facilitate adaptation of an organism to the environment. Although hydrogen sulfide (H_2S) is toxic to most organisms, the fish *Poecilia mexicana* has adapted to survive in environments with high levels that exceed toxicity thresholds by orders of magnitude. Epigenetic changes in response to this environmental stressor were examined by assessing DNA methylation alterations in red blood cells, which are nucleated in fish. Males and females were sampled from sulfidic and nonsulfidic natural environments; individuals were also propagated for two generations in a nonsulfidic laboratory environment. We compared epimutations between the sexes as well as field and laboratory populations. For both the wild-caught (F0) and the laboratory-reared (F2) fish, comparing the sulfidic and nonsulfidic populations revealed evidence for significant differential DNA methylation regions (DMRs). More importantly, there was over 80% overlap in DMRs across generations, suggesting that the DMRs have stable generational inheritance in the absence of the sulfidic environment. This is one of the first examples of epigenetic generational stability after the removal of an environmental stressor. The DMR associated genes were related to sulfur toxicity and metabolic processes. These findings suggest that adaptation of *P. mexicana* to sulfidic environments in southern Mexico may, in part, be promoted through epigenetic DNA methylation alterations that become stable and are inherited by subsequent generations independent of the environment.

Keywords**:** Epigenetic, Inheritance, Sulfidic Environment, Adaptation

64 **Significance Statement**

65

66 Environmental factors can promote phenotypic variation through alterations in the
67 epigenome and mediate adaptation of an organism to the environment. Observations suggest the
68 adaptation of *Poecilia mexicana* fish to toxic, hydrogen-sulfide-rich environments in southern
69 Mexico may, in part, be promoted through epigenetic DNA methylation alterations that became
70 generationally stable and are inherited to subsequent generations independent of the environment.
71 Environmental epigenetics may provide an important mechanism mediating adaptation in this
72 species. This is one of the first observations that the epigenome is stably inherited generationally
73 through the germline after the removal of an environmental stressor (i.e. hydrogen sulfide) from a
74 wild population.

75

Introduction

Toxicants are present in a wide variety of ecological contexts, including natural and man-made, and environmental toxicants are becoming increasingly abundant due to anthropogenic activities. While many chemicals are categorized by their mutagenic effects, it has become increasingly clear that indirect modes of action are as important for short-term (e.g. phenotypic plasticity) and long-term (e.g. adaptive) effects [1-4]. Responses to the toxicity of environmental chemicals has been shown, in part, to be mediated by epigenetics [4-6]. For example, exposure to heavy metals, including cadmium and arsenic, has been associated with changes in DNA methylation in mammals [7-10]. However, the findings are often complex and contradictory. *In vitro* cadmium exposure experiments in rat liver cells suggested initial inductions of DNA hypomethylation occurred, while prolonged exposure led to DNA hypermethylation [7]. A wide variety of environmental compounds, such as the agricultural fungicide vinclozolin and the herbicide glyphosate, have been shown to modify the location and abundance of DNA methylation in rats [11-13]. Moreover, vinclozolin and glyphosate promote the epigenetic transgenerational inheritance of DNA methylation states and disease susceptibility in the third generation following transient exposures [6, 11-13]. The ability of these environmental toxicants to shape phenotypic variation through epigenetic changes that are stable across generations has potential evolutionary consequences that remain largely unexplored.

Natural systems with environmentally derived toxicants provide a framework in which it is possible to study the evolutionary effects of toxicants. By comparing populations adapted to a toxicant to non-adapted ancestral populations, we can determine the effect of the toxicant on the evolution of the populations. To make predictions about the outcomes from environmental exposure, it is necessary to have a model stressor with clearly defined and predictable effects.

99 Hydrogen sulfide (H_2S) is an ideal toxicant to study the role of epigenetics in responding to
100 environmental toxicants, because H_2S exposure has clear effects on sulfide processing and energy
101 metabolism [14].

102 H_2S is one of the most toxic inorganic gases for metazoan organisms. It occurs both
103 naturally (e.g. in deep-sea hydrothermal vents, marine sediments, and sulfide springs) and as a by-
104 product of pollution and industrial processes (e.g. in habitats impacted by farming, tanning, paper
105 manufacturing, sewage treatment, oil refining, and gas exploration and refining [15, 16]). For
106 humans with a recommended industrial daily exposure of 10 ppm (293 μM) (National Institute for
107 Occupational Safety and Health, 2020), H_2S can have detrimental health effects, including
108 headache and eye irritation, are seen even at low concentrations of 2.5 to 5 ppm (73 to 146 μM)
109 [16] and exposure to high concentrations (>1000 ppm / 29,343 μM) can lead to instantaneous death
110 [17]. The primary reason for H_2S's high toxicity is that it directly inhibits cytochrome c oxidase,
111 which is Complex IV of the mitochondrial electron transport chain [16, 18, 19]. The inhibition of
112 cytochrome c oxidase by H_2S results in a shutdown of the electron transport chain and cellular
113 ATP generation [16, 18, 19].

114 While H_2S is toxic at high concentrations, H_2S is produced at low concentrations
115 endogenously as a product of cysteine catabolism and by intestinal bacteria [20, 21], and
116 physiologically relevant concentrations are likely in the nanomolar range [22, 23]. Due to the
117 endogenous production of H_2S, most organisms are able to detoxify low concentrations of H_2S,
118 and there are known sulfide detoxification enzymes present in most metazoans [24]. Sulfide
119 oxidation to thiosulfate is mediated by sulfide quinone oxidoreductase (SQR), a dioxygenase, and
120 sulfur transferase [25]. H_2S is highly membrane permeable [26] and plays an important role as a
121 cell signaling molecule in the cardiovascular and nervous systems [27]. For example, H_2S is

122 involved in the regulation of vasodilation and inflammation [28]. The effect of H_2S on epigenetic
123 changes has not been investigated.

124 Very few organisms are able to tolerate exposure to high H_2S concentrations [16, 29].
125 However, some fish in the livebearing family Poeciliidae live in habitats with naturally occurring
126 sustained and high concentrations of H_2S [30-35]. The current study focuses on the species
127 *Poecilia mexicana,* which inhabits H_2S-rich springs in southern Mexico (Figure 1) [36]. H_2S in
128 these springs ranges from 30 μM to over 1000 μM, depending on the site [36]. The concentrations
129 fluctuate little across seasons and years [31, 37]. There are also closely related populations of *P.*
130 *mexicana* residing in nonsulfidic habitats adjacent to sulfidic springs. The populations are
131 genetically differentiated but considered the same species [38-40]. Wild-caught fish from sulfide
132 spring populations exhibit significantly higher sulfide tolerance than fish from adjacent nonsulfidic
133 populations [36]. We have previously shown that *P. mexicana* from sulfidic populations can
134 survive in high levels of H_2S by constitutively expressing high levels of important H_2S
135 detoxification genes [41-43], and some sulfidic populations have evolved a resistant cytochrome
136 c oxidase [44]. Moreover, there is heritable variation in gene expression, especially in key genes
137 related to H_2S toxicity and detoxification [45], some of which may be driven by expression
138 differences of relevant microRNAs [46]. Despite these observations, a key gap in our knowledge
139 is the effect of H_2S exposure on epigenetics and whether expression changes due to H_2S exposure
140 and local adaptation are potentially mediated by DNA methylation changes. Fish are known to
141 have an increased number of DNA methylation enzymes DNMTs, and respond to environmental
142 insults through DNA methylation.

143 The current study was designed to understand the long-term epigenetic changes that can
144 occur in response to H_2S adaptation. This study also sets the stage for future studies examining

145 how short- and long-term environmental exposure may shape the epigenome and the role of
146 epigenetic transgenerational inheritance in adaptation to H_2S. The primary research objectives
147 were to test whether: 1) the abundance and distribution of differential DNA methylated regions
148 (DMRs) differ between sulfidic and nonsulfidic populations for both sexes, when samples were
149 derived from wild versus laboratory-reared individuals; and 2) the methylation differences
150 between populations are consistent and related to living in H_2S. We hypothesized that long-term
151 H_2S exposure in *P. mexicana* involved alterations in epigenetics (DNA methylation) and that those
152 changes are stable over generations.

153 **Results**

154 Red blood cells (RBC) from male and female *P. mexicana* from H_2S-rich habitats and
155 nonsulfidic habitats in southern Mexico were collected to assess the environmental impacts of the
156 sulfidic habitats on the fish epigenome (Figure 1). The RBC in fish species contain nuclei that
157 allows the DNA extraction from an easily purified single cell type. Another set of *P. mexicana*
158 from sulfidic or nonsulfidic habitats were collected as adults (pregnant females) in May 2013 and
159 transported to a nonsulfidic laboratory environment and propagated for two generations in the
160 nonsulfidic environment (Figure 1C). Only adult F2 generation laboratory fish harvested in August
161 2015 were used for analysis and comparisons (Figure 1C). Genomic DNA from the nucleated RBC
162 was isolated and used to identify differential DNA methylation regions (DMRs) between the
163 sulfidic and nonsulfidic populations for males and females, separately. This experimental design
164 allowed us to determine population differences in DMRs of wild fish populations and the stability
165 of the epigenetic alterations in the laboratory propagated fish.

166 The RBC DNA was fragmented and the methylated DNA immunoprecipitated (MeDIP)
167 with a methyl cytosine antibody to identify DMRs between the sulfidic and nonsulfidic
168 populations. On average, similar numbers of DMRs were observed for the various comparisons
169 between sulfidic and nonsulfidic populations (Table 1). Two-thirds of DMRs had one significant
170 100-bp window, the other third of DMRs exhibited multiple, significant 100-bp regions with the
171 DMR (Table 2). The DMRs for each comparison are presented in SI Appendix, Tables S1-S4. For
172 both wild male and female comparisons between sulfidic and nonsulfidic populations,
173 approximately 50% of the DMRs had an increase in DNA methylation in the sulfidic population,
174 and the remainder of the DMRs had a decrease in methylation (SI Appendix, Figure S1). Although

175 a limitation of the study is the small number of fish analyzed, the robust and significant epigenetic
176 data obtained supports the observations presented.

177 The genomic features associated with DMRs for each comparison were similar. The
178 primary cytosine nucleotide followed by a guanine nucleotide (CpG) density was 1 to 6 CpG per
179 100 bp (Figure 2). The average size of a DMR was around 1 kb, with a range from 1 to 6 kb (Figure
180 2). Therefore, the CpG density was low in 1-2 kb regions, similar to CpG deserts previously
181 observed [47].

182 A principal component analysis (PCA) was performed to assess the genomic components
183 of the DMRs and comparisons between the data sets (Figure 3). All identified DMRs were included
184 in the PCA. For samples collected in the wild, the sulfidic males and females clustered and were
185 distinct from the nonsulfidic males and females (Figure 3A). Therefore, the sulfidic habitat
186 influences the PCA clustering for both sexes. For the laboratory samples from fish that were
187 propagated in a nonsulfidic environment for multiple generations, the PCA clustering again
188 showed that the individuals derived from the sulfidic population clustered distinctly from the
189 nonsulfidic one (Figure 3B). Hence, the samples were separated by habitat type in the DMR PCA
190 analysis for both the wild and laboratory populations. The PCA analysis with all the DMR data
191 sets from all comparisons further demonstrated that the wild DMR data sets clustered separately
192 from the laboratory DMR data sets (Figure 3C). Therefore, both the population of origin and the
193 rearing environment (wild vs. laboratory) impact DMRs.

194 The majority of the DMRs were unique to a specific dataset at $p<1e-07$, with the wild or
195 laboratory male and female comparisons having the highest level of overlap (Figure 4).
196 Interestingly, 94 DMR overlapped between all four comparisons (see SI Appendix, Table S5) and
197 nearly 20% of DMRs were shared between laboratory and field conditions at $p<1e-07$. We also

198 performed an extended DMR overlap that compared the DMRs with $p<1e-07$ for each comparison
199 with the genomic windows in a second comparison at $p<0.05$ (Table 3). DMRs in one analysis
200 were considered present in the second analysis if any overlapping genomic window in the second
201 analysis had a p-value of 0.05 or less. A comparison of the male and female data sets within the
202 wild or laboratory comparisons demonstrated greater than 90% overlap. Therefore, as indicated by
203 the PCA (Figure 3) and Venn diagram (Figure 4), the overlaps are very high between the sexes for
204 each laboratory or wild DMRs. Interestingly, the extended overlaps for the comparisons between
205 laboratory and wild samples for both males and females were greater than 80% (Table 3).
206 Therefore, even after two generations of maintaining fish under nonsulfidic laboratory conditions,
207 the majority of DMRs documented in the laboratory overlapped with those documented in the wild
208 with the extended overlap comparison. Although this extended overlap with multiple p-values is
209 not standard, it does reveal a high degree of overlap not observed in the high statistical threshold
210 comparison.

211 The DMR gene associations were investigated using DMRs identified within 10 kb of a
212 gene, so the promoter regions could be considered (SI Appendix, Tables S1-S4). The signaling,
213 transcription, cytoskeleton, metabolism, and receptor functional categories were the most
214 predominant the DMR associated genes in all comparisons (Figure 5). There were consistent
215 similarities in the DMR associated gene numbers for all the categories. Analysis of the DMR
216 associated gene functions using a Pathway Studio™ program analysis identified a large number
217 of genes related to sulfur toxicity, either directly or indirectly (Figure 6A). A variety of related
218 protein ligands, transcription factors, receptors, kinases, and phosphatases were present for the
219 comparison data sets. Another relevant DMR associated gene functional category involved sulfur
220 metabolism (Figure 6B). A number of different components of sulfur metabolism processes are

221 listed, and all have connections to DMR associated genes. The most predominant were chondroitin
222 sulfate associated genes that are a critical component of the extracellular matrix and proteoglycans.
223 The final set of DMR associated genes analyzed was the 94 overlapping DMR between all the
224 comparisons (Figure 4) with the DMR list and DMR associated genes (SI Appendix, Table S5).
225 These DMR associated genes and correlated gene processes are presented in Figure 7. Although
226 this does not demonstrate direct links with sulfidic environments or toxicity, the pathways and
227 gene families influenced are anticipated to be indirectly linked to the sulfur environment exposure
228 and potential adaptation.
229

230 ## Discussion

231 There is evidence that epigenetic modifications change in response to exposure to environmental
232 toxicants [4, 7, 11] and that modified placement of epigenetic modifications are associated with
233 phenotypic variation and disease [3]. The analysis of methylation changes in populations adapted
234 to a physiological stressor allows for the assessment of short-term responses to environmental
235 stress as well as potential forecasting of long-term responses. Here, we used H_2S-sensitive and
236 H_2S-tolerant fish, *P. mexicana*, living in naturally nonsulfidic and sulfidic environments to
237 elucidate the epigenetic changes in response to living in H_2S-rich springs.

238 The current study investigates the epigenetic inheritance stability of putatively adaptive
239 epigenetic alterations following the removal of the abiotic selective pressure. Although the *P.*
240 *mexicana* genome [48] is not a chromosome-level assembly, sufficient information was available
241 to assess the environmental influences on the DMRs, associated genomic features, and potential
242 gene associations. The lack of a well characterized genome will underestimate the DMR-gene
243 associations and future improved genomic information will likely improve and expand on the gene
244 association information. The DMR size and locations in regions of low density CpG (termed CpG
245 deserts) found in this study are similar to environmentally responsive DMRs previously identified
246 in other species [47, 49]. Low density CpG regions generally constitute over 90% of the genome
247 for most species, while the high density CpG islands generally constitute less than 5% of the
248 genome [47]. As shown in most species from plants to mammals, CpG DNA methylation is
249 predominant, with non-CpG methylation being negligible with no known functional significance
250 currently identified [50]. Although the methylated DNA immunoprecipitation (MeDIP) procedure
251 used is biased to low density CpG regions, it is the most efficient to examine the majority of the
252 genome compared to other procedures, such as bisulfite sequencing, that can be biased to higher

253 density CpG analyses [47, 51]. High density CpG regions are predominantly restricted to 50% of
254 promoters in mammals, so less than 5% of the genome, these regions are not detected efficiently
255 with MeDIP-Seq, but greater than 90% of the genome is detected with MeDIP-Seq [52].

256 The differential DNA methylation regions (DMRs) associated genes were investigated to
257 provide insights into the potential impacts of the epigenetic alterations on the genome and
258 physiology. Although a gene association is identified, this does not confirm a functional causal
259 link of the epigenetic alteration and gene regulation. This is a limitation that will require future
260 studies at the gene expression level to confirm. Due to the sulfidic versus nonsulfidic focus of the
261 study, the initial analysis of the DMR associated genes involved sulfur toxicity and metabolism.
262 A large number of toxicity-associated genes were either directly or indirectly correlated with sulfur
263 that were associated with the DMRs identified including genes such as *MTOR* or *TPMT* have been
264 shown to have direct impacts on sulfur toxicity [53-56]. Sulfur metabolism genes were also
265 associated with the DMRs identified. The chondroitin sulfate processes, which has been associated
266 with sulfur exposures [57, 58], had the most represented associated genes. A number of cellular
267 processes (e.g. apoptosis and angiogenesis) were also associated with the DMRs. Although these
268 DMR-associated genes have links with sulfur toxicity and metabolism, further molecular work is
269 needed to correlate these molecular processes and genes to adapttaion of *P. mexicana* to H_2S, and
270 the observed epigenetic inheritance.

271 An overlap of the DMRs between the comparisons and data sets demonstrated the majority
272 of DMRs were distinct for the comparison with DMRs at $p<1e-07$. The primary principal
273 component in the DMR clustering separated laboratory and wild samples, suggesting that there are
274 largescale changes in DMRs when populations are brought into the laboratory for multiple
275 generations. Approximately a 20% overlap was observed between the male and female wild or

276 laboratory comparisons. To expand this analysis, an extended overlap was performed that
277 compared the p<1e-07 DMR with the p<0.05 DMRs for the other data sets. Although the extended
278 overlap with a high and low statistical threshold is not standard, this allows for the identification
279 of overlapping DMRs that may be marginally significant at any given p-value threshold. The
280 comparison showed that a greater than 90% overlap between the male or female DMR
281 comparisons for the wild or laboratory DMRs. Greater than 80% overlap was observed between
282 the wild and laboratory comparisons; this level of overlap demonstrates the majority of the sulfidic
283 versus nonsulfidic DMRs identified were in common between the F0 generation wild populations
284 and the F2 generation laboratory populations. Therefore, the majority of the methylation changes
285 identified comparing wild individuals from sulfidic and nonsulfidic environments were found to
286 be propagated for two generations in the laboratory in the absence of the sulfidic environmental
287 exposure. These observations demonstrate an epigenetic inheritance of DMRs in the absence of
288 the continued sulfidic environment. This is one of the first observations of methylation inherited
289 through the germline when an environmental stressor (i.e., H_2S) is removed from a wild population
290 and suggests future studies on transgenerational inheritance beyond the F2 generation would be
291 useful. This is consistent with previously described environmentally induced epigenetic
292 transgenerational inheritance phenomena [4, 6].

293 Environmentally induced epigenetic inheritance of phenotypic variation and disease was
294 first described in rats exposed to an agricultural fungicide vinclozolin [11], but now has been
295 shown to occur in all species investigated from plants to humans [4, 6]. Previous studies have
296 primarily used the transient exposure of an individual to induce epigenetic alterations in the
297 germline (sperm or egg) to promote the epigenetic transgenerational inheritance phenomenon [4,
298 6]. The transmission of epigenetics between generations requires the involvement of the germline,

299 as they are the only cells that can transmit molecular information between generations [4, 6]. Direct
300 exposure to an environmental factor can promote epigenetic alterations, and this can occur at any
301 time during development. For example, the exposure of a gestating female mammal exposes the
302 F0 generation mother, the F1 generation fetus, and germline that will generate the F2 generation
303 individual [4, 59]. *Poecilia mexicana* is a livebearing fish that requires the same consideration.
304 Therefore, the F3 generation is the first generation that has no direct exposure toxicity [59]. For a
305 non-livebearing fish model, exposure of the F0 generation male or female will directly impact the
306 F0 generation and the sperm or egg that will form the F1 generation, therefore the F2 generation
307 is the first generation (i.e. transgenerational) not having direct exposure toxicity [59]. An example
308 involves the exposure of zebrafish to mercury that promoted neurodevelopmental abnormalities in
309 zebrafish in the F2 generation offspring, which was the first epigenetic transgenerational
310 inheritance observation in fish [60]. However, livebearing fish are more like mammals in the
311 exposure of a gestating female will expose the germline that will generate the F2 generation. The
312 current study observations suggest an important future study in these livebearing fish is the
313 investigation of DMRs in the F3 generation, the first with no direct exposure to H_2S, which would
314 be considered epigenetic transgenerational inheritance. Although the current study did not analyze
315 the F3 generation in the nonsulfidic environment, propagation of the F2 generation in the
316 laboratory environment demonstrates survival of the fish to the F3 generation and beyond. Future
317 studies will need to compare the F1 generation of laboratory fish to determine whether there is a
318 steady decrease in DMR shared with each subsequent generation or whether the changes are due
319 to the initial transition to an aquarium environment and are stable and maintained at similar levels
320 thereafter. It is also unknown whether fish from sulfidic habitats that have been raised in a
321 nonsulfidic laboratory environment for multiple generations may have a higher survival than fish

322 with nonsulfidic ancestry when transmitted back into a sulfidic environment. The question is if an
323 ancestral sulfidic environment adapted epigenetics is maintained, which may facilitate
324 reintroduction into the sulfidic environment. This will be an important future experiment to
325 consider for understanding how epimutations altered in an aquarium environment play a role in
326 phenotypic variation and survival.

327 Although the ability of environmental stressors to alter the epigenome of the individual
328 exposed and impact the physiology of the individual is critical, the ability to alter the germline
329 epigenome and propagate this transgenerationally to subsequent generations allows for an adaptive
330 mechanism that can impact evolution [61]. The phenotypic plasticity and molecular alterations in
331 the epigenome and transcriptome to survive the sulfidic environment is essential to allow an
332 organism to respond to their environment. The epimutations documented here may contribute to
333 heritable variation in gene expression, which we have documented in detail in the field, the lab,
334 and across multiple species [41, 42, 45, 62]. The sulfidic and nonsulfidic populations of *P.*
335 *mexicana* studied here are genetically differentiated, with a divergence time of ~10,000 years ago
336 and an average empirical F_{ST} of 0.11 [63], but considered the same species [38-40], and studying
337 how these epigenetic changes play into adaptive changes will be a fruitful avenue of research. The
338 population studied here is one of four pairs of sulfidic and nonsulfidic *P. mexicana* populations in
339 tributaries of the Río Grijalva, and the replicated nature of the independently derived sulfidic
340 populations provides an opportunity to test whether the findings in this drainage are consistent
341 across multiple drainages. Future research will need to explore how genetic variation interacts with
342 epimutations to shape phenotypic variation in this system. This project is a first step to evaluate
343 epigenetic effects of toxicant exposure and sets the stage for future studies examining how long-
344 term environmental exposure may shape the epigenome.

345 **Materials and Methods**

346 *Samples*

347 Male and female *P. mexicana* were collected from H_2S-rich habitats and nonsulfidic habitats in
348 southern Mexico (Figure 1) for the preparation of purified red blood cells. Wild-caught samples
349 were obtained from Southern Mexico in proximate H_2S-rich (sulfidic, N=4 female, N=4 male)
350 (PSO, Lat: 17.43843, Long: -92.77476) and nonsulfidic springs (N=4 female, N=3 male) (Bonita,
351 Lat: 17.42685, Long: -92.75213) in the Tacotalpa Drainage in Southern Mexico. Another set of *P.*
352 *mexicana* males and females from sulfidic or nonsulfidic habitats were collected and transported
353 into a breeding population at Kansas State University and propagated for two generations (F2) in
354 the nonsulfidic laboratory environment (Figure 1C) (sulfidic, N=4 female, N=4 male) and
355 (nonsulfidic, N=4 female, N=4 male). All laboratory animals were reared in nonsulfidic water and
356 N=4 individuals were sampled per population. Adult fish for meDIP sequencing were collected in
357 the wild in May 2015. For laboratory experiments, pregnant females were collected in May 2013,
358 and adult F2 offspring were harvested in August 2015 for blood extractions. Of note, fish from
359 sulfidic habitats are smaller than fish from the nonsulfidic habitats, and males are smaller than
360 females.

361
362
363 *DNA extraction*

364 Fish were sacrificed by cervical transection, and blood was immediately extracted by using
365 microhematocrit capillary tubes (Fisher). Tubes were then spun down at 12,000 rpms for 3 minutes
366 using a ZipCombo Centrifuge (LW Scientific) to separate blood cells from serum. After
367 centrifugation, capillaries were broken to retain only red blood cells. The portion of the capillary
368 containing the red blood cells was placed in a 1.5 ml Eppendorf tube for DNA extraction. DNA

369 was extracted using the Qiagen DNeasy Blood & Tissue kit according to manufacturer's
370 instructions.
371
372 *Methylated DNA Immunoprecipitation (MeDIP)*
373 Extracted genomic DNA was used to isolate methylated DNA with a methyl-cytosine
374 antibody precipitation procedure (MeDIP). The protocol is described in detail in reference [64].
375 Five micrograms of total genomic DNA were sonicated using a Covaris sonicator. Sonicated DNA
376 was diluted with TE buffer to 400 µl, heat denatured for 10 minutes at 95˚C, and cooled on ice for
377 10 minutes to create single-stranded DNA fragments. A total of 100 µl of 5X IP buffer and 5 µg
378 of antibody (monoclonal mouse anti 5-methyl cytidine; Diagenode #C15200006) were added to
379 the fragmented single-stranded DNA. The mixture was incubated on a rotator overnight at 4˚C.
380 Pre-washed magnetic beads (50 µl, Dynabeads M-280 Sheep anti-Mouse IgG; Life Technologies
381 11201D) were added to the 500 µl of DNA-antibody mixture. The mixture was incubated for 2
382 hours on a rotator at 4˚C. After which the samples were washed three times with 1X IP buffer.
383 Washed samples were resuspended in 250 µl digestion buffer (5mM Tris PH 8, 10.mM EDTA,
384 0.5% SDS) with 3.5µl Proteinase K (20mg/ml) and incubated at 55˚ for three hours. A Phenol-
385 Chloroform-Isoamyalcohol extraction was used to clean-up the DNA and the DNA was
386 resuspended in 20µl of H2O. DNA concentration was measured in Qubit (Life Technologies) with
387 the ssDNA kit (Molecular Probes Q10212).
388
389 *MeDIP-sequencing*
390 For library preparation, we used the NEBNext Ultra RNA Library Prep Kit for Illumina
391 starting at step 1.4 of the manufacturer's protocol to generate double stranded DNA. After this step

392 the manufacturer's protocol was followed. Each individual received a unique barcode. Enriched
393 methylated DNA libraries were sequenced at the WSU Genomics Core in Spokane, WA, using an
394 Illumina HiSeq 2500 with paired-end 50 bp reads. All sequencing data has been deposited to NCBI
395 GEO (GEO # GSE157730).
396
397 *Identifying and analyzing differentially methylated regions*
398 Differentially methylated region (DMR) identification and annotation followed previously
399 published approaches [65, 66]. The FastQC program
400 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to assess data quality.
401 Reads were trimmed to remove adapters and low-quality bases using Trimmomatic [67]. The reads
402 for each MeDIP sample were mapped to the *P. mexciana* [48] genome using Bowtie2 [68] with
403 default parameter options. The mapped read files were then converted to sorted BAM files using
404 SAMtools [69].
405 Differential coverage between sulfidic and nonsulfidic populations was calculated using
406 the MEDIPS R package [70]. P-value from edgeR [71] was used to determine the significance of
407 the difference between the two groups for each 100 bp genomic window. Windows with an edgeR
408 p-value less than a specified threshold ($p<1e-07$) were considered the initial start of the DMR.
409 DMR edges were extended until no genomic window with an p-value less than 0.1 remained within
410 1000 bp of the DMR. The extended DMR overlap compared the DMRs, with at least one 100 bp
411 window with $p<1e-07$ from one comparison, with the genomic windows (100 bp regions) in a
412 second comparison. Windows that had a p-value <0.05 in the second comparison were considered
413 overlapping. The Ensembl database [72], accessed with the biomaRt R package [73] was used to
414 annotate DMRs. Genes that were overlapping a DMR, including 10 kb on either side of the DMR,

415 were input into a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway search [74, 75] to
416 identify relevant associated pathways. The DMR associated genes were sorted into functional
417 groups using information provided by the DAVID [76] and Panther [77] databases incorporated
418 into an internal curated database (www.skinner.wsu.edu/). DMR-associated gene correlations
419 present in published literature were further analyzed using Pathway Studio software (version
420 12.2.1.2: Database of functional relationships and pathways of mammalian proteins; Elsevier). All
421 sequencing data has been deposited into the public database at NCBI (GEO # GSE157730) and R
422 code computational tools are available at GitHub (https://github.com/skinnerlab/MeDIP-seq) and
423 www.skinner.wsu.edu.

424 **Data Accessibility**

425 GEO Accessions: GSE157730

426 Code and internal databases can be found at https://github.com/skinnerlab/MeDIP-seq and

427 www.skinner.wsu.edu.

428

429 **Competing Interests**

430 We declare we have no competing interests.

431

432 **Ethical Statement**

433 Procedures for all experiments were approved by the Institutional Animal Care and Use

434 Committee at Kansas State University (Protocol #3418). Field work was approved by the

435 Mexican government (Fieldwork Permit DGOPA.00093.120110.-0018).

436

437 **Author Contributions**

438 MKS, JLK, MT conceived the study. MT, LAR conducted fieldwork. CQ, ISR performed

439 sequencing work. DB conducted analyses. MKS, JLK wrote the manuscript. All authors edited

440 and gave final approval.

441

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654

655

Table and Figure Legends

Table 1. DMR identification. The number of DMRs found using p-value threshold of p<1e-07. The All Window (100 bp) column shows all DMRs. The Multiple Window column shows the number of DMRs with at least one additional adjacent significant window.

Table 2. The number of DMRs with specific numbers of significant windows at a p-value threshold of p<1e-07 are presented. Male and female sulfidic wild versus nonsulfidic wild comparison DMRs. Male and female sulfidic laboratory versus nonsulfidic laboratory comparison DMRs.

Table 3. Extended DMR Overlap. The p<1e-07 left axis and p<0.05 top axis with number of DMR overlap and percent of total for the specific comparison. Comparisons sulfidic female wild (SFW), nonsulfidic female wild (NFW), sulfidic male wild (SMW), nonsulfidic male wild (NMW), sulfidic female laboratory (SFL), nonsulfidic female laboratory (NFL), sulfidic male laboratory (SML), and nonsulfidic male laboratory (NML).

Figure 1. Collection site, *Poecilia mexicana* fish morphology, experimental design. **(A)** Collection sites in southern Mexico. **(B)** Images of *Poecilia mexicana* adult male and female fish from sulfidic and nonsulfidic environments. **(C)** Experimental design.

677 **Figure 2.** DMR genomic features for DMRs with a p-value < 1e-07. **(A)** The number of DMRs
678 at different CpG densities for each of the pairwise comparisons. **(B)** The DMR lengths in
679 kilobase (kb) for each of the pairwise comparisons.
680
681 **Figure 3.** Principal component analysis (PCA). **(A)** Wild sulfidic versus nonsulfidic. **(B)**
682 Laboratory sulfidic versus nonsulfidic. **(C)** Combination of all comparisons. Comparisons
683 sulfidic female wild (SFW), nonsulfidic female wild (NFW), sulfidic male wild (SMW),
684 nonsulfidic male wild (NMW), sulfidic female laboratory (SFL), nonsulfidic female laboratory
685 (NFL), sulfidic male laboratory (SML), and nonsulfidic male laboratory (NML).
686
687 **Figure 4.** DMR overlap for DMRs with a p-value threshold of $p<1e\text{-}7$. Comparisons sulfidic
688 female wild (SFW), nonsulfidic female wild (NFW), sulfidic male wild (SMW), nonsulfidic
689 male wild (NMW), sulfidic female laboratory (SFL), nonsulfidic female laboratory (NFL),
690 sulfidic male laboratory (SML), and nonsulfidic male laboratory (NML).
691
692 **Figure 5.** DMR associated gene categories. The number of DMRs for each comparison
693 presented for each category. Comparisons are sulfidic female wild (SFW), nonsulfidic female
694 wild (NFW), sulfidic male wild (SMW), nonsulfidic male wild (NMW), sulfidic female
695 laboratory (SFL), nonsulfidic female laboratory (NFL), sulfidic male laboratory (SML), and
696 nonsulfidic male laboratory (NML).
697

698 **Figure 6.** DMR gene associations from Pathway Studio™. **(A)** DMR gene associations with
699 sulfur toxicity. **(B)** DMR gene associations with sulfur metabolism. Sulfur metabolism pathway
700 components are highlighted in blue. Gene symbols presented and the functional categories listed.
701
702 **Figure 7.** Overlapping 94 DMR $p<1e-07$ gene associations with cellular processes and
703 pathologies from Pathway Studio™. Gene symbols presented and the functional categories
704 listed.
705

706 **Table 1**

707 Number of sulfidic versus nonsulfidic DMRs with a p-value < 1e-07

Treatment	Sex	All Window	Multiple Window
wild	male	1049	440
wild	female	1461	606
laboratory	male	1619	641
laboratory	female	1451	544

708

709 **Table 2**

710 Number of sulfidic versus nonsulfidic DMRs containing different numbers of genomic windows
711 with a p-value < 1e-07

Number of significant windows	1	2	3	4	5	6	7	8	9	10	11	≥12
Wild, male	609	240	76	45	23	17	10	4	8	5	3	9
Wild, female	855	331	119	61	37	17	12	3	9	2	4	10
Laboratory, male	978	377	120	57	33	17	12	3	6	4	1	9
Laboratory, female	907	329	110	44	18	12	6	6	3	2	2	12

712

713 **Table 3**

714

p<0.05 / p<1e-07	SFWvsNFW	SMWvsNMW	SFLvsNFL	SMLvsNML
SFWvsNFW		1372 (94%)	1207 (83%)	1205 (82%)
SMWvsNMW	984 (94%)		871 (83%)	860 (82%)
SFLvsNFL	1192 (82%)	1155 (80%)		1324 (91%)
SMLvsNML	1356 (84%)	1291 (80%)	1496 (92%)	

715