Incipient resistance to an effective pesticide results from genetic adaptation and the canalization of gene expression

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Abstract

The resistance of bacteria, disease vectors, and pests to chemical controls has vast ecological, economic, and human-health costs. In most cases, resistance is only detected after non-susceptible phenotypes have spread throughout the entire population. Detecting resistance in its incipient stages, by comparison, provides time to implement preventative strategies. Incipient resistance (IR) can be detected by coupling standard toxicology assays with large-scale gene expression experiments. We apply this approach to a system where an invasive parasite, sea lamprey (*Petromyzon marinus*), has been treated with the highly-effective pesticide 3-trifluoromethyl-4-nitrophenol (TFM) for 60 years. Toxicological experiments revealed that lamprey from treated populations did not have higher survival to TFM exposure than lamprey from their native range, demonstrating that full-fledged resistance has not yet evolved. In stark contrast, we find hundreds of genes differentially expressed in response to TFM in the population with the longest history of exposure, many of which relate to TFM's primary mode of action, the uncoupling of oxidative phosphorylation. One gene critical to oxidative phosphorylation, *ATP5PB*, which encodes subunit b of ATP synthase, was nearly fixed for alternative alleles in comparisons between native and treated populations ($F_{ST} > 9$ SD from the mean). A gene encoding an additional subunit of ATP synthase, *ATP5F1B*, was canalized for high expression in treated populations, but remained plastic in response to treatment in sea lamprey from the native range. These combined genomic and transcriptomic results illustrate that an adaptive, genetic response to TFM is driving incipient resistance in a damaging pest species.

Introduction

The evolution of resistance often occurs in response to the continued and large-scale application of chemical compounds such as antibiotics, herbicides, and pesticides (1-4). The costs associated with the evolution of resistance measure in the billions of dollars per year and cut across diverse fields ranging from healthcare to agriculture (5-7). Increased rates of human mortality, decreased food security, and large-scale environmental costs represent only some of the negative effects when microbes, pathogens, and pests can no longer be effectively controlled (4, 8). In light of these costs, there is a considerable need to prevent or delay the evolution of resistance. Unfortunately, resistance is often detected too late, after most individuals in a population have acquired a resistant phenotype (9). Once this stage is reached, there is little to be done except to implement alternative control measures, which are often less effective (10, 11), or stop employing control measures until resistant genotypes are eliminated from the population, which can take tens to hundreds of generations (12-14).

One solution to mitigating the costs of resistance is early detection; identifying the evolution of resistance in its incipient stages can allow time for developing alternative control measures or implementing management actions that can prevent the onset of population-wide resistance (e.g., adaptive and integrative management strategies (15-17)). One approach to detecting incipient resistance is to screen a large fraction of the population for resistant genotypes or phenotypes. However, such measures can be costly and impractical because of the large sample sizes required to identify small numbers of fully resistant individuals. Detectability issues (e.g., false positives and negatives) compound this problem. Alternatively, the coupling of experimental, eco-toxicological assays with the identification of genomic and transcriptomic responses in populations with varied treatment histories can provide key insights into how and

44 whether resistance is beginning to evolve. We applied this design to an invasive vertebrate pest
45 species that has been controlled with a chemical pesticide for 60 years.

46 Throughout the Laurentian Great Lakes, invasive sea lamprey (*Petromyzon marinus*)
47 have negatively impacted native fish communities by feeding parasitically on adult fishes (18).
48 With the construction and improvement of shipping canals in the early 1900s, sea lamprey
49 expanded their range from the northwestern Atlantic Ocean to the Great Lakes (19) (Fig. 1).
50 Adult sea lamprey are hematophagous parasites of other fishes and are not host specific (Fig.
51 1D). The lack of host specialization, coupled with ample spawning habitat found throughout the
52 Great Lakes region, spurred the growth of parasitic sea lamprey populations into the millions of
53 individuals (19). High abundance of sea lamprey in the Great Lakes contributed to rapid and
54 substantial declines in native lake trout (*Salvelinus namaycush*) and other ecologically and
55 commercially important fishes (20, 21). In response to the effects of sea lamprey on native fish
56 and fisheries, there was an immediate and concerted effort to develop efficient means for
57 controlling sea lamprey populations in the Great Lakes.

58 One effort, initiated in the 1950s, involved testing over 4000 chemical compounds on sea
59 lamprey and other fish species (22). The organic compound 3-trifluoromethyl-4-nitrophenol
60 (hereafter, TFM) was found to effectively kill larval sea lamprey, but had no detectable effects
61 on other fishes at low concentrations (23, 24). The larval stage of sea lamprey (Fig. 1C) are
62 targeted because they are small (typically < 120 mm) and aggregate at high densities. The
63 primary mode of action for TFM is to uncouple oxidative phosphorylation (25, 26), and because
64 sea lamprey have high sensitivity to this mechanism, TFM is a very effective pesticide (Fig. 1B).
65 Since the application of TFM to locations with abundant larval lamprey, invasive sea lamprey
66 populations have declined by up to 90% (19, 27). However, TFM does not kill all larval sea

67 lamprey (28) and it is possible that resistant individuals could survive and reproduce. If this
68 process is repeated over enough generations, then resistant individuals could increase in
69 frequency – a scenario documented in many systems where pests have been controlled by
70 chemical means (29). The evolution of resistance would greatly reduce the effectiveness of TFM,
71 which is currently the primary sea lamprey control method (28), and decreased pesticide
72 effectiveness would likely hamper the restoration of native and commercially-important fish
73 populations throughout the Great Lakes.

74 To test for the evolution of resistance, we first collected larval sea lamprey from three
75 populations with varied histories of TFM treatment (Fig. 1A): Lake Michigan (56 years of TFM
76 treatment), Lake Champlain (26 years of TFM treatment), and Connecticut River (native range; 0
77 years of TFM treatment). Larvae were acclimated in a common environment for 4 months before
78 being experimentally exposed to TFM at lethal, sublethal, and control (no TFM) levels. Detailed
79 survival and toxicological analyses were coupled with tissue-specific RNA-seq. This procedure
80 was followed by the identification of differentially expressed genes, network analysis, and
81 identification of genomic regions under selection. Survival analyses revealed that the outright
82 resistance of sea lamprey to lethal concentrations of TFM could not be detected, but genomic and
83 transcriptomic analyses suggest that the early, incipient stages of resistance are well under way
84 in populations with a history of pesticide exposure.

85 **Results**

86 We performed multiple independent toxicological assays over a two-year period using larvae
87 collected from historically untreated (native range) and treated tributaries (Fig. 1A; Table S1).
88 The larvae collected from treated tributaries were not previously exposed to TFM prior to the
89 toxicological assays (i.e., any resistance, if detected, could not be due to inducible tolerance

90 (30)). If outright resistance has evolved, we would expect to observe higher survivorship in sea
91 lamprey collected from populations with a history of treatment (i.e., Lake Michigan or Lake
92 Champlain) in comparison to sea lamprey collected from areas that have never been treated (i.e.,
93 the native range, Connecticut River). We found no evidence to support this prediction. In 2016,
94 the mortality for treated individuals was highly similar across all populations over a 12-hour
95 exposure period (LM 96.4%; LC 100%; CT 91.1%; Fig. 2A, *SI Text*). In 2017, survivorship
96 through time again did not vary substantially among populations (Fig. S1) and estimates of lethal
97 concentrations required to kill 50% of the population (LC50) varied only slightly among
98 populations and trials (Fig. 2B, *SI Text*). Furthermore, no individuals from Lake Michigan
99 survived TFM concentrations greater than 2 mg/L (Fig. S1). When TFM is applied in the field,
100 stream concentrations of TFM range from 5.5 to 9 mg/L for the pH and alkalinity values used in
101 our experimental treatments (Fig. 2B, *SI Text*). In total, we performed 5 independent tests with 6
102 tested concentrations and never detected any meaningful differences in survival across
103 populations (Table S2, *SI Text*). From these analyses, we conclude that outright resistance to
104 TFM has not yet evolved, as no treated individuals survived TFM exposure at concentrations
105 they would be exposed to under ordinary stream treatment conditions.

106 By contrast, we observed remarkably large differences in patterns of gene expression
107 among sea lamprey from different populations exposed to the same, sublethal concentration of
108 TFM. Briefly, we exposed 168 larvae to either 0.2 or 0.3 mg/L of TFM for 6 hours (Table S2).
109 An equal number of control individuals per population were included (i.e., individuals not
110 exposed to TFM), individuals from all populations had the exact same acclimation history (4
111 months), and all populations were simultaneously included in each of two large experiments to
112 avoid confounding circadian (31) or environmental effects (see Methods, *SI Text,* and Tables S2-

113 S3). Remarkably, when comparing samples treated with 0.3 mg/L TFM to control samples, we
114 found 336 genes that were differentially expressed (275 upregulated, 61 downregulated in
115 comparison to control individuals) in muscle tissue in the Lake Michigan population, dwarfing
116 the number of differentially expressed genes (DEGs) found in both the Lake Champlain (n = 21)
117 and the Connecticut River (n = 68) populations (Fig. 3A-C; Table S4). No DEGs were shared
118 among all three populations and different population responses were observed when lower
119 concentrations (i.e., 0.2 mg/L) of TFM were applied and when different tissues were examined
120 (Figures S2-S5).

121 The primary mode of action for TFM is to uncouple oxidative phosphorylation in the
122 mitochondria resulting in severe ATP depletion and eventual death (25, 26, 32). Of the 336
123 DEGs identified in Lake Michigan, several genes were directly related to this mode of action
124 including *CRCM1* (LogFC = 2.60), a calcium release-activated channel protein that controls the
125 influx of calcium into cells when depleted, and *PLCD4* (LogFC = 2.41), an enzyme responsible
126 for hydrolyzing phosphatidylinositol 4,5-bisphosphate into two secondary messenger molecules,
127 one of which (inositol 1,4,5-trisphosphate or 'IP3') is known to release cellular calcium stores
128 and increase calcium concentrations within the cell (33). Upregulated expression of genes that
129 increase intracellular concentrations of calcium could enhance oxidative phosphorylation in Lake
130 Michigan larvae and partially compensate for the uncoupling effects of TFM (Fig. 4A). In
131 addition, calcium plays an integral role in activating and driving the oxidative phosphorylation
132 cascade in mitochondria, and studies have demonstrated a positive relationship between
133 nanomolar free calcium and the rate of phosphorylation (34, 35). In gene ontology (GO)
134 hierarchy networks, we also found regulation of calcium ion transport, regulation of calcium ion
135 transmembrane transport, regulation of calcium ion transmembrane transporter activity, and

136 regulation of voltage-gated calcium channel activity as significant biological processes (Fig. S6).
137 In Lake Champlain, a gene encoding ADP/ATP translocase3, *SLC25A6*, was significantly
138 downregulated in treated individuals (LogFC = -3.50). The downregulation of *SLC25A6* may
139 disrupt the exchange of ADP and ATP across mitochondrial membranes and inhibit apoptosis,
140 suggesting that individuals from Lake Champlain are retaining ATP within their mitochondria in
141 response to TFM (36, 37).

142 We next aligned our reads to the sea lamprey genome (38, 39), called single nucleotide
143 polymorphisms (SNPs), and identified outliers based on Z-scores of genetic divergence (Weir
144 and Cockerham's F_{ST}) (40, 41). Out of 28,315 SNPs, the single highest outlier, *ATP5PB*, was
145 found in comparisons between Lake Michigan and Connecticut River and had an F_{ST} value
146 greater than 9 standard deviations from the mean (Fig. 3D). *ATP5PB* encodes a subunit of ATP
147 synthase (subunit b) and, similar to many of the genes identified by expression analyses, appears
148 to be responding to strong selection in order to restore normal oxidative phosphorylation in
149 response to TFM's primary mode of action (Fig. 4A). Closer examination of the genotypes at the
150 tri-allelic SNP found in this gene illustrates that a single allele is nearly fixed in both Lake
151 Michigan and Lake Champlain, but only occurs rarely in the native range (Fig. 4B). This outlier
152 was consistently identified across genotype calling pipelines and tissue types (muscle and liver;
153 *Methods,* Figures S7-S9) and was also observed in comparisons between Lake Champlain and
154 Connecticut River (Figures S10-S11). Although the native-range, Connecticut River lamprey are
155 not fixed at the genetic level for this variant, they were able to respond plastically with respect to
156 TFM at ATP synthase. The gene *ATP5F1B*, which codes for subunit beta of ATP synthase, was
157 significantly upregulated in Connecticut River lamprey in response to TFM treatment (LogFC =
158 4.68, FDR-corrected p-value < 0. 0072, Fig. 4C). Interestingly, this gene was not upregulated in

159 control individuals from Connecticut River, suggesting a plastic response, but was upregulated in
160 both control and treatment individuals from Lake Michigan and Lake Champlain in comparison
161 to Connecticut River control individuals, suggesting a genetic response to constitutively increase
162 expression of this gene. Thus, here we show both genomic and transcriptomic evidence of
163 among-population variation in response to TFM; critically, the two populations with a lengthy
164 history of TFM treatment show adaptive responses in ATP synthase at the genomic level, while
165 the naïve, native population shows plastic responses in transcription of the same enzyme in
166 response to TFM.

167 **Discussion**

168 Here we provide evidence for the evolution of incipient resistance in invasive sea lamprey
169 treated with a pesticide. We found 336 genes differentially expressed in response to TFM in
170 larvae from Lake Michigan, the population with the longest history of using TFM (56 years).
171 This transcriptional response represents a nearly 5-fold greater number of differentially
172 expressed genes in comparison to larvae collected from the native range. The pesticide TFM's
173 primary mode of action is to serve as a protonophore, translocating protons across the inner
174 mitochondrial membrane, thus uncoupling oxidative phosphorylation and depleting ATP. Many
175 of the DEGs identified in Lake Michigan represent direct or indirect responses to ATP depletion,
176 TFM's primary mode of action. We also identified genomic evidence of a response to selection
177 imposed by TFM. The gene coding for ATP synthase subunit b, *ATP5PB*, had an F_{ST} value
178 greater than 9 standard deviations from the mean in comparisons between Lake Michigan and
179 Connecticut River (Fig. 3D) and greater than 7 standard deviations from the mean in
180 comparisons between Lake Champlain and Connecticut River (Fig. S10). These large shifts in
181 allele frequencies indicate a response to selection in a gene that is critical to successful oxidative

182 phosphorylation. We also identified one gene, *ATP5F1B*, that was differentially expressed in the
183 native Connecticut River population, but not the other, treated populations. Interestingly, this
184 gene is constitutively expressed at high levels in treated populations, suggesting that an
185 additional component of ATP synthase has responded to pesticide-induced selection.

186 Lake Michigan and Lake Champlain have been treated with TFM for 56 and 26 years,
187 respectively. Both show evidence of selection at *ATP5PB* and *ATP5F1B*, however, both
188 responded to TFM differently at the gene expression level with 336 and 21 genes differentially
189 expressed in Lake Michigan and Lake Champlain, respectively. These large differences in the
190 number of genes differentially expressed may reflect different evolutionary responses to TFM,
191 one of which is mediated exclusively at the genetic level and one that is mediated at both genetic
192 and transcriptomic levels. Alternatively, these differences may reflect the deeper evolutionary
193 histories between these two populations or the different duration and intensities of pesticide
194 usage (42). We also observed large differences in gene expression depending on the
195 concentration of TFM used and the tissue examined (Figures S2-S5). These similarities in
196 adaptive, genetic responses and differences in broad patterns of gene expression suggest that the
197 TFM-mediated selection results in a convergent, genetic response while the different
198 evolutionary and treatment histories of these populations affect overall patterns of gene
199 expression.

200 We identified large genetic and transcriptomic differences among populations but did not
201 detect any individuals able to survive concentrations of TFM applied to streams in practice (Fig.
202 2). Thus, we may be documenting the earliest stages of resistance evolving in treated populations
203 (i.e., incipient resistance). Survival and reproductive advantages by larvae exposed to sublethal
204 concentrations of TFM, for example individuals at the peripheries of TFM treatment or

individuals buried deeper in the sediment, are inherited by their offspring and have spread throughout the treated populations. It may only be a matter of time before further mutations allow individuals to survive higher concentrations of TFM. In fact, recent modelling efforts have revealed that given the estimated strength of selection imposed by lamprey control, full-fledged resistance to TFM is predicted to occur soon (12). Because of the high gene flow and lack of population structure found throughout the Great Lakes (i.e., Lake Michigan is one, large panmictic population, see *SI Text*), fully-resistant individuals may already be spreading throughout the population even though it may take many more years for successful detection (12). Alternatively, it may be that our toxicological assays were simply not sensitive enough to detect resistance to lethal concentrations of TFM. From a pragmatic standpoint, the detection of incipient resistance (IR) means that the development of alternative control measures and the implementation of adaptive management strategies focusing on delaying resistance should be a priority for the continued control of invasive sea lamprey. The detection of resistance in its earliest stages, as documented here, allows time for such actions to assist with the continued restoration of native and commercially-important fishes. Regardless of the system, the detection of incipient resistance can greatly assist in efforts to mitigate and delay the costly consequences of resistance.

Methods

Sample Collection and Experimental Design

We collected a total of 2,035 larval sea lamprey (ammocoetes) from five locations during the summers of 2016 and 2017, focusing our sampling efforts on populations with different durations of exposure to TFM (Fig. 1; Table S1). In 2016, we obtained 565 ammocoetes from the Manistee River in Lake Michigan, 517 ammocoetes from Corbeau Creek and the LaPlatte River

in Lake Champlain, and 404 ammocoetes from the Connecticut River (Fig. 1). Lake Michigan ammocoetes have been treated with TFM since 1960 (43), Lake Champlain ammocoetes have been treated with TFM since 1990 (44), and the Connecticut River is part of the sea lamprey's native range and has never been treated with TFM. All ammocoetes were collected using pulsed-DC backpack electrofishers. To examine lamprey of similar ages and developmental stages, we only collected ammocoetes from a narrow size range (80-120 mm). All ammocoetes were collected within five calendar days (July 11-July 15, 2016) of each other and were transported to Purdue's Aquaculture Research Lab (ARL) immediately after collection (see *SI Text* for housing and acclimation details). In 2017, we collected an additional 284 larval lamprey from the Platte River in Lake Michigan and 265 larval lamprey from higher up the Connecticut River (Turner Falls, Massachusetts) to independently validate our 2016 toxicological analyses (Table S1-S2; *SI Text*).

Lethal Exposure Trials

After a 4-month acclimation period, we constructed an experimental array to conduct exposure trials in a temperature-controlled environmental chamber (12.8°C) consisting of 36, 2.5-gallon glass aquaria (see *SI Text* for details). In 2016, we conducted two toxicological exposure trials to directly assess whether sea lamprey populations exhibited differential mortality in response to TFM exposure (see *SI Text* for details). To analyze survivorship data, we conducted a Kaplan-Meier survival analysis that employed the log-rank test to quantify time to death for ammocoetes in different populations (45). The Kaplan-Meier method generates an estimate of survival probability (S) at time t_i and is calculated as:

$$S(t_i) = S(t_i - 1)(1 - \frac{d_i}{n_i})$$

250 where $S(t_i - 1)$ is the probability of being alive at time $t_i - 1$, n_i is the number of individuals
251 alive just prior to t_i, and d_i is the number of deaths occurring at t_i (45). We performed the survival
252 analysis in R 3.5.1, using both the SURVIVAL and OIsurv packages to create survival curves and
253 assess statistical differences between the survival curves of the three populations (46, 47). *P*-
254 values associated with pairwise tests used to determine which populations differ in their
255 survivorship were FDR adjusted using the Benjamini-Hochberg method (48).

256 To confirm these results, we conducted a second set of three toxicological assays in 2017,
257 using a modified design. These trials were organized to measure lethal concentration (or dose), a
258 different measure of phenotypic response to TFM. The 20-hour trials were conducted with a
259 single population at five different dosage levels (0, 0.5, 1.2, 1.5, and 2.0 mg/L). Trials were
260 limited to a single population to increase power for the detection of lethal concentrations in a
261 given population (see *SI Text* and Table S2 for details). To compare differences in survival,
262 lethal concentrations for each trial were computed in R using the DRC package (v 3.0-1) (49),
263 and comparisons were made among trials at different dosage levels using Kaplan-Meier survival
264 analyses in the SURVIVAL package (v 2.43-3) (47) with visualization using the SURVMINER
265 package (v 0.4.3) (50).

266 *Sublethal Exposure Trials*

267 To assess whether resistance was evolving at a sublethal level, we conducted an exposure trial to
268 assess *(i)* how gene expression profiles changed in response to TFM, and *(ii)* whether TFM
269 exposure altered patterns of gene expression differently among the three populations with
270 varying histories of exposure. The experimental design for sublethal exposure was similar to the
271 lethal exposure trials (outlined above) with two key differences: 1.) we exposed ammocoetes to
272 sublethal levels of TFM (Table S2) and 2.) we preserved tissue from ammocoetes at a

273 predetermined timepoint (t = 6 hr) during the 12-hour gene expression exposure trial for RNA-
274 seq. Sampling consisted of removing a single ammocoete per replicate tank and immediately
275 euthanizing it in a lethal dose of MS-222 before weighing, measuring, and flash-freezing in
276 liquid nitrogen. At the completion of the experiment, cryovials were directly transferred to a -
277 80°C freezer (see *SI Text* for details).

278 *RNA-seq and Differential Gene Expression Analysis*

279 We used tissue-specific RNA-seq to compare patterns of gene expression in ammocoetes from
280 our three populations with varying histories of exposure to TFM. Comparing tissue-specific
281 patterns of expression required that we first dissect specific tissue samples from the body
282 segments collected during the gene expression trial. Given TFM's established mode of action,
283 we targeted ammocoete muscle, liver, and brain tissues for downstream analyses of gene
284 expression profiles (see *SI Text* for details). RNA extractions were performed using a Qiagen
285 RNeasy Mini Kit. In total, we performed 71 RNA extractions across three populations (Lake
286 Michigan, Lake Champlain, and Connecticut River), and three tissue types (muscle, liver, and
287 brain) (Table S3). Library preparation and RNA sequencing (RNA-seq) were performed by the
288 Purdue Genomics Core Facility. We obtained a total of 3.25 billion 150bp paired-end reads that
289 we used to create a high-quality, annotated transcriptomes (see *SI Text* for details).

290 We aligned all reads to our assembled sea lamprey transcriptome and estimated transcript
291 abundances using RSEM (51). Differentially expressed genes (hereafter, DEGs) were identified
292 in R using edgeR's GLM functionality (46, 52, 53). We compared gene expression profiles of
293 treated versus untreated ammocoetes within each population to identify genes that were
294 significantly up- and down-regulated in response to TFM treatment and were specific to each
295 population. For genes to be classified as differentially expressed, we set minimum thresholds for

296 significance (FDR correction via the Benjamini-Hochberg method; *p*-value < 0.05). While we
297 did not filter DEGs by log fold change, the minimum log2-fold changes we observed for DEGs
298 were > 2 across all three populations (2.04, 3.50, and 2.74 for Lake Michigan, Lake Champlain,
299 and Connecticut River, respectively). Finally, GOseq was used to determine which gene
300 ontology functional categories were overrepresented among differentially expressed genes in
301 TFM treated individuals for each population-tissue combination (54). Only functionally enriched
302 categories significant below an FDR-adjusted *p*-value threshold of 0.05 were retained. We
303 constructed gene ontology (GO) hierarchy networks of the top 100 significant GO terms
304 identified with differentially expressed genes (FDR-corrected *p*-value = 0.01) using the
305 metacoder package in R (55).

306 *Genomic Analysis*

307 We called SNPs from our RNA-seq data (Table S3) following the methods and algorithms
308 provided by the Genome Analysis Toolkit (GATK – Calling Variants in RNA-seq, (56)) for
309 muscle (n = 44 samples) and liver (n = 19 samples) tissues separately. Variant calling details are
310 reported in *SI Text*. Since the GATK pipeline called variants for each sample separately, we
311 removed indels, and combined variants for all samples into a single variant-call-format file. We
312 filtered out loci genotyped at fewer than 80% of samples followed by samples genotyped at
313 fewer than 80% of loci. After filtering, we used the remaining variants as high-quality SNPs and
314 we kept 42 and 19 samples for muscle and liver tissues, respectively. In each population, we
315 further excluded loci out of Hardy-Weinberg Equilibrium (HWE), which we identified using the
316 R package HWxtest (likelihood ratio *p*-value < 0.05, (57)). A locus fixed for a single allele
317 within a population could represent a genotyping error that would not be removed with HWE
318 tests (e.g., misalignment, insufficient read depth). Thus, we next employed two genotype-calling

319 pipelines to create 4 SNP data sets, based on whether loci fixed for a single homozygote within a
320 population ("fixed homozygotes") were included or not: 1.) muscle – without fixed
321 homozygotes, 2.) muscle – with fixed homozygotes, 3.) liver – without fixed homozygotes, and
322 4.) liver – with fixed homozygotes. We calculated pairwise F_{ST} for each locus among all three
323 populations (i.e., LM vs. LC, LM vs. CT, and LC vs. CT populations) (40). To detect significant
324 outliers, we z-transformed F_{ST} in each comparison and defined loci with Z(F_{ST}) greater than five
325 standard deviations from the mean as outlier loci. We then identified the corresponding genes on
326 which outlier loci were located by mapping outlier loci to the sea lamprey genome (38, 39) and
327 calculated the frequency of all alleles at each outlier locus.
328

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340 **Author Contributions**

341 MRC, ASM, and MSS designed the project, all authors provided logistic support, ASM
342 performed molecular analyses, XY, AMH, and ASM performed gene expression and genomic
343 analyses, ASM and MMS performed survival analyses, and XY, ASM, and MRC wrote the
344 manuscript with input from all authors.

345

346 **Competing Financial Interests**

347 The authors declare no competing financial interests.

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476 **Figure Legends**

477 **Figure 1:** Sample collection sites, sample sizes, and pesticide (TFM) treatment histories for
478 larval sea lamprey used in this study (A). Connecticut River sea lamprey were collected from
479 their native range whereas sea lamprey from Lake Michigan and Lake Champlain are considered
480 invasive. Sea lamprey abundance and metric tons of TFM applied to Lake Michigan from 1987
481 onwards (B); notice that TFM is an effective pesticide with decrease in lamprey abundance
482 following increases in TFM application. The larval stage of sea lamprey remain buried in the
483 sediment (C), aggregate in high densities, and are targeted with the pesticide TFM. After
484 transformation, the juveniles are generalist parasites, using their circularly-arrayed teeth to attach
485 to hosts and their rasping tongues to feed (D).

486 **Figure 2:** Results of toxicology assays illustrating survival through time and lethal
487 concentrations. In 2016, survival analyses revealed that previously unexposed larvae collected
488 from treated populations did not survive at higher rates than larvae collected from the native
489 population during exposure to lethal concentrations of TFM (A). In 2017, independent
490 toxicology assays from additional larvae collected from Lake Michigan (MI) and Connecticut
491 River (native range; CT) again revealed that larvae collected from populations with a history of
492 exposure did not survive lethal doses of TFM at higher rates than larvae collected from the native
493 range (B). Lethal concentrations for 25% and 50% mortality were calculated and two separate
494 assays were performed for Connecticut River individuals (CT1 and CT2, see *SI Text* for
495 additional analyses). Dashed green lines border the range of TFM values larvae would be

496 exposed to under typical stream application conditions. Collectively, these results illustrate that
497 resistance to lethal concentrations of TFM has not yet evolved.

498 **Figure 3:** Transcriptomic and genomic evidence of incipient resistance (IR) in sea lamprey. A
499 total of 336 genes were differentially expressed in response to sublethal concentrations of TFM
500 in larval sea lamprey collected from Lake Michigan, the population with the longest history of
501 TFM treatment (A). By contrast, only 21 and 68 genes were identified as differentially expressed
502 in lamprey from Lake Champlain (B) and Connecticut River (C), respectively. After aligning
503 reads back to the sea lamprey reference genome (sea lamprey have 99 chromosomes, 90 of
504 which are assembled), calling SNPs and calculating F_{ST}, the largest outlier SNP (9.1 standard
505 deviations greater than the mean) was found to belong to *ATP5PB*, a gene encoding subunit b of
506 ATP synthase (D), a putatively adaptive response to TFM's primary mode of action.

507 **Figure 4:** The pesticide TFM's primary mode of action is to act as a protonophore, translocating
508 protons from the inter-membrane space back into the inner mitochondrial matrix, thus disrupting
509 oxidative phosphorylation (A). Several genes upregulated in Lake Michigan, the population with
510 the longest history of exposure, may play a role in responding to TFM's mode of action. For
511 example, the upregulation of *CRCM1* and *PLCD4* increases intracellular concentrations of
512 calcium and could enhance oxidative phosphorylation in Lake Michigan ammocoetes (A). The
513 gene *ATP5PB*, which encodes subunit b of ATP synthase, was identified as an outlier (Fig. 3D),
514 is nearly fixed at a tri-allelic SNP in the treated Lake Michigan and Lake Champlain populations,
515 but segregates for largely different alleles in the native-range sea lamprey (B). A gene encoding
516 an additional subunit of ATP synthase, *ATP5F1B*, is canalized for high expression in treated
517 populations (i.e., the gene is expressed at high levels in both treatment (TFM+) and control
518 (TFM-) individuals from the treated Lake Michigan (LM) and Lake Champlain (LC)

519 populations) (C). By contrast, this gene is not constitutively upregulated in native-range,
520 Connecticut River (CT) lamprey, but responds plastically to treatment. Error bars depict standard
521 deviation, *p*-value calculated from FDR-corrected tests of differential gene expression.

Figure 1

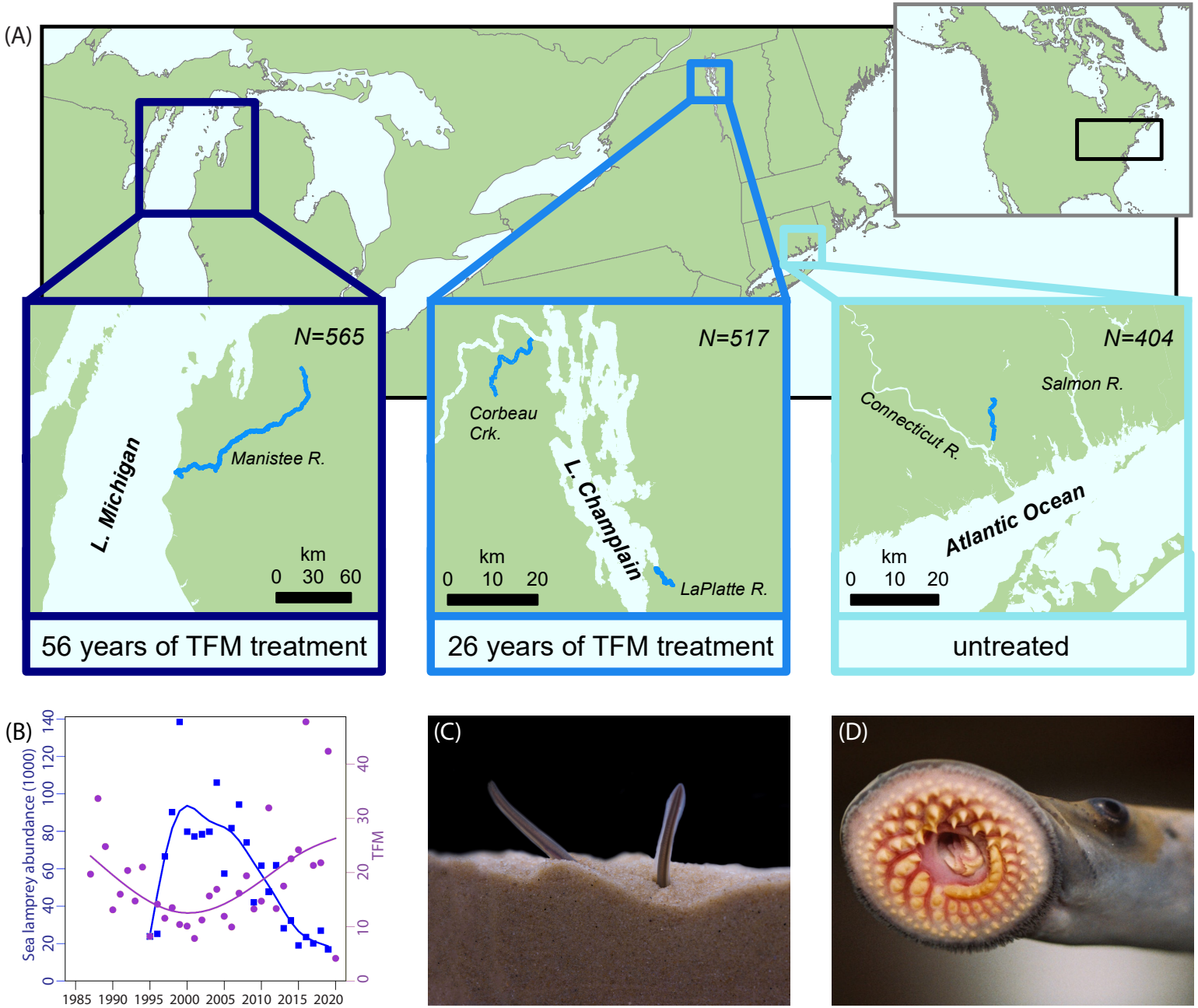

(A)
N=565
L. Michigan
Manistee R.
km
0 30 60
56 years of TFM treatment
N=517
Corbeau Crk.
L. Champlain
LaPlatte R.
km
0 10 20
26 years of TFM treatment
N=404
Salmon R.
Connecticut R.
Atlantic Ocean
km
0 10 20
untreated
(B)
Sea lamprey abundance (1000)
TFM
1985 1990 1995 2000 2005 2010 2015 2020
(C)
(D)

Figure 2

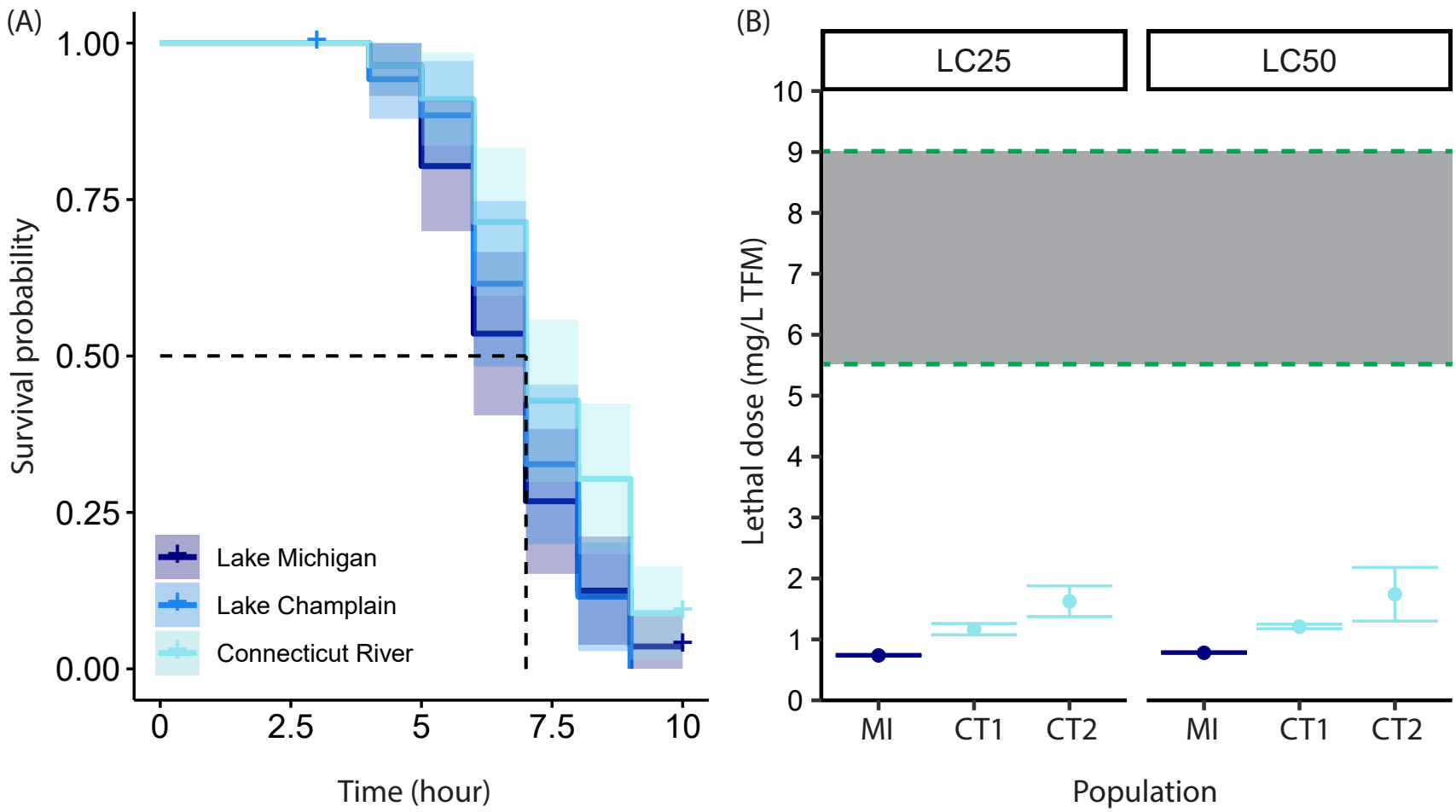

(A)
1.00
0.75
0.50
0.25
0.00
Survival probability
Lake Michigan
Lake Champlain
Connecticut River
0
2.5
5
7.5
10
Time (hour)
(B)
LC25
LC50
10
9
8
7
6
5
4
3
2
1
0
Lethal dose (mg/L TFM)
MI
CT1
CT2
MI
CT1
CT2
Population

Figure 3

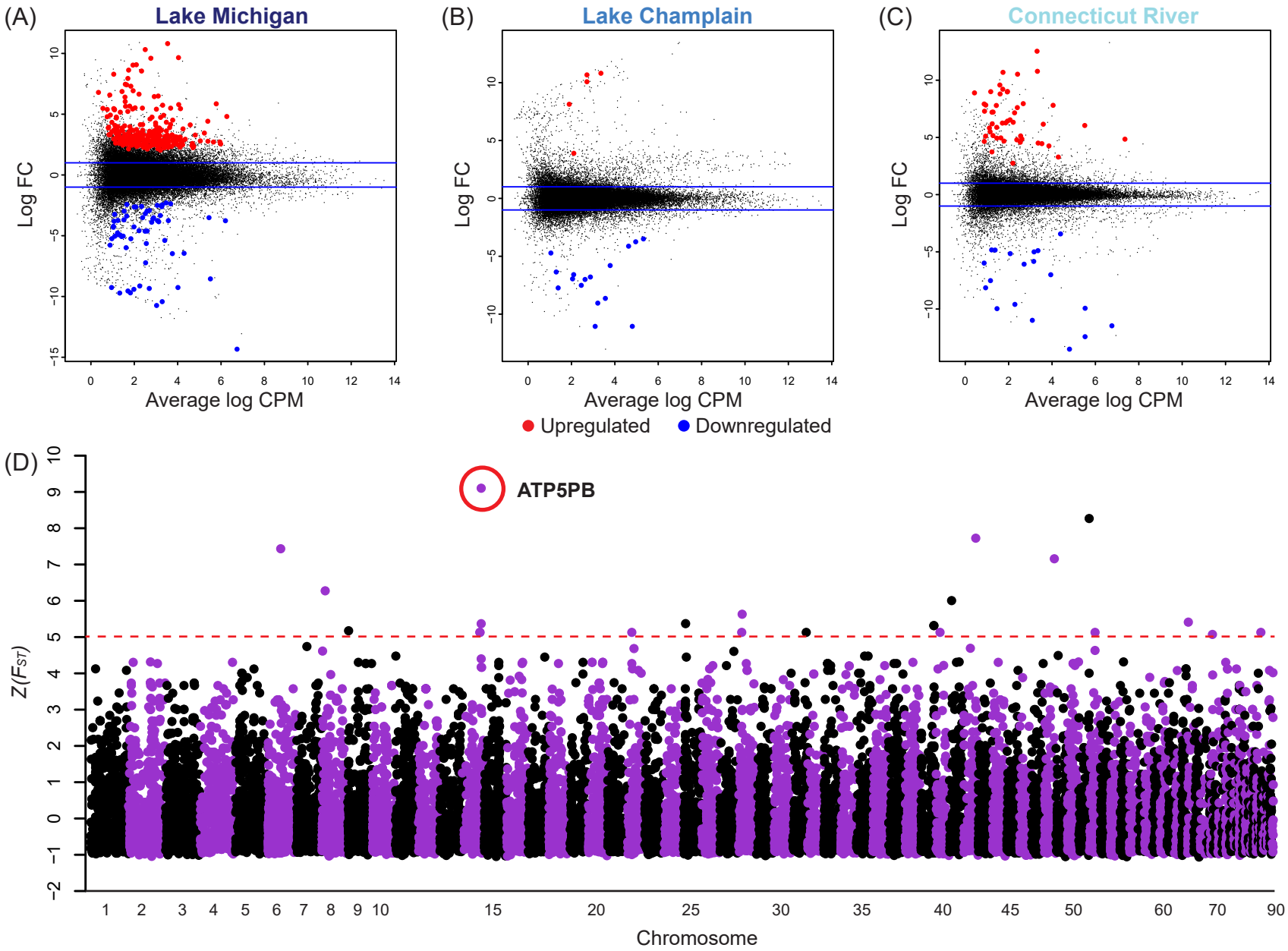
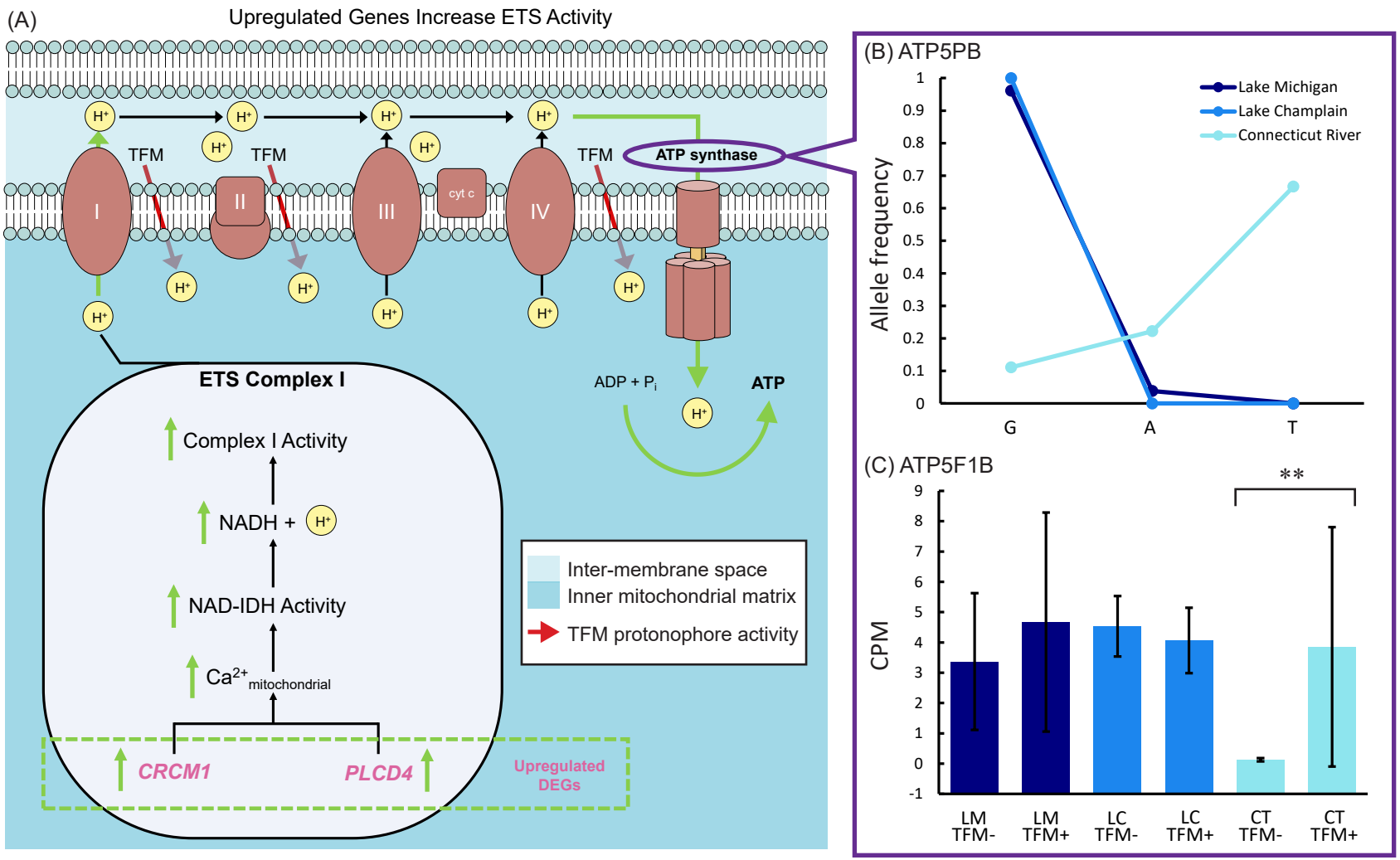

(A)
Lake Michigan
Log FC
Average log CPM
(B)
Lake Champlain
Log FC
Average log CPM
(C)
Connecticut River
Log FC
Average log CPM
Upregulated
Downregulated
(D)
ATP5PB
Z(FST)
Chromosome

Figure 4


(A)
Upregulated Genes Increase ETS Activity
TFM
TFM
TFM
I
II
III
IV
cyt c
ATP synthase
ADP + Pi
ATP
ETS Complex I
Complex I Activity
NADH +
NAD-IDH Activity
Ca2+ mitochondrial
CRCM1
PLCD4
Upregulated DEGs
Inter-membrane space
Inner mitochondrial matrix
TFM protonophore activity
(B) ATP5PB
Allele frequency
Lake Michigan
Lake Champlain
Connecticut River
G
A
T
(C) ATP5F1B
CPM
**
LM TFM-
LM TFM+
LC TFM-
LC TFM+
CT TFM-
CT TFM+