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DNA METHYLATION DIFFERS IN BLUEGILL AMONG TEMPERATE, ANTHROPOGENICALLY WARMED, AND TROPICAL LAKES

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ABSTRACT

Epigenetic mechanisms can alter gene expression and phenotypes in organisms responding to environmental changes. Within environmental parameters, temperature changes are notably the most pervasive abiotic factor for ectotherms, directly affecting organismal survival and fitness. The goal of this study was to investigate the scale of DNA methylation in populations of a cosmopolitan freshwater species occupying disparate thermal regimes. DNA methylation levels were compared among bluegill sunfish (*Lepomis macrochirus*) populations from an ambient temperature, temperate lake (Lake Mattoon), a nearby power plant-cooling lake (Lake Coffeen), and a tropical lake (Lake Lucchetti). We used epiRADseq to screen levels of DNA methylation at 105,811 loci among fish. We found levels of total DNA methylation increased among specimens as water temperature of lakes increased from the ambient temperature, temperate lake to the tropical lake. We identified loci with statistically significant differences in the frequency of DNA methylation among individuals between lakes: 654 loci between Lake Mattoon and Lake Lucchetti fish and 373 loci between Lake Coffeen and Lake Lucchetti fish. Considering that a previous study on the same power plant-cooling reservoirs showed a shorter lifespan, decrease in growth performance, and the populations skewed towards younger fish, this study observed DNA methylation pattern may be an important mechanism contributing to the observed phenotypic variations in bluegill from a anthropogenically-warmed lake compared to an ambient temperature lake. Our study suggests that epigenetic regulation of phenotypic plasticity in aquatic organisms may be a critical factor in understanding the organismal response to environmental stress.

Keywords: epigenetics, epiRADseq, response to stress, power plant-cooling

INTRODUCTION

Organismal survival and fitness are tightly linked to environmental parameters, and habitat temperature directly affects ecological distribution in ectotherms. Epigenetics is the study of changes in gene expression without changes in DNA sequence, and temperature is known to directly impact DNA methylation status in organisms (Bossdorf et al. 2008; Angers et al. 2010; Dowen et al. 2012; Metzger and Schulte 2017). DNA methylation is the most widely studied molecular mechanism of DNA modification, largely because of the availability of simple and efficient methods to screen variation in DNA methylation (Schrey et al. 2013). DNA methylation can change gene expression and induce phenotypic variation within organisms (Cubas et al. 1999; Darnaudéry and

Maccari 2008), although the relationship between DNA methylation and gene expression is complex (Nätt et al. 2012). DNA methylation may play a role in phenotypic adjustments to environmental stressors in individuals (Angers et al. 2010; Richards et al. 2012). Moreover, epigenetic-mediated variations may allow for phenotypic plasticity to support a population's persistence in the face of environmental changes that is not strictly dependent on inheritance as it is classically thought of (Verhoeven et al. 2010; O'Dea et al. 2016).

Investigating epigenetic responses in nonmammalian vertebrates that are mediated by DNA methylation is an area of great interest (Bossdorf et al. 2008). In fish, the role of epigenetic mechanisms in response to the environment is a growing area of research (Shao et al. 2014; Metzger and Schulte 2017). DNA methylation levels differed among individuals of three-spine stickleback (*Gasterosteus aculeatus*) collected in the wild (Smith et al. 2015). For the same species, changes in temperature during development affected DNA methylation levels (Metzger and Schulte 2017). Furthermore, variation in DNA methylation of rainbow trout (*Oncorhynchus mykiss*) was detected between migratory (smolt) and nonmigratory (resident) juveniles (Baerwald et al. 2016) and DNA methylation patterns changed in response to environmental conditions, such as pH, and increased phenotypic plasticity in the asexual hybrid fish, *Chrosomus eos-neogaeus* (Massicotte and Angers 2012). Massicotte et al. (2011) found that epigenetic variation may account for phenotypic changes observed in the absence of genetic variation in *Chrosomus eos-neogaeus*. Also, Leung et al. (2016) demonstrate that epigenetic variation is greater in *C. eos-neogaeus* among lineages in unpredictable environments when compared to predictable environments.

Several important insights about the importance of DNA methylation have been observed in the model organisms zebrafish (*Danio rerio*) and medaka fish (*Oryzias melastigma*). DNA methylation levels in zebrafish vary in response to environmental stressors, such as acute exposure to xenobiotics. For example, early life exposures of zebrafish to dexamethasone have statistically significant effects on DNA methylation and gene expression patterns, resulting in reproductive dysfunction (Khor et al. 2016). The medaka fish has displayed differential gene expression in spermatogenesis related genes that contribute to reproductive impairments in both the F1 and F2 generations after exposure (Wang et al. 2016).

Changes in environmental temperature thorough space (through species' distribution range) and time (e.g., seasonally or daily) are pervasive and, similarly to xenobiotics, organisms experiencing these temperature changes respond to them by altering gene expression through DNA methylation. Among fish species, an inverse relationship between DNA methylation and body temperature has been observed, indicating that differences in water temperature have precipitated evolutionary stable changes in DNA methylation (Varriale and Bernardi 2006). In small and relatively shallow freshwater systems, such as lakes and reservoirs, the magnitude and direction of the temperature change largely influence the phenotypic response of fishes. Elevated water temperatures in these systems can lead to increased physiological stress and mortality in fish assemblages, unless thermal refuges are available (De Stasio et al. 1996). Fishes able to survive in small lakes experiencing elevated temperatures without thermal refuge are forced to phenotypically adapt to the suboptimal abiotic factor.

The present study evaluates the intrapopulation variation in DNA methylation levels of bluegill sunfish (*Lepomis macrochirus*) among reservoirs with disparate thermal regimes: Lake Mattoon, a small (less than 5 km^2) natural reservoir in the Midwest of the United States; Lake Coffeen, a power plant-cooling reservoir (less than 115 km from Lake Mattoon); and Lake Lucchetti, a tropical reservoir in Puerto Rico. Increased water temperature in the power plant-cooling Lake Coffeen has led to phenotypic changes in

resident species (Martinez et al. 2016; Mulhollem et al. 2016). Specifically, comparisons of bluegill between Lake Coffeen and Lake Mattoon showed a shorter lifespan, decrease in growth performance, and the populations skewed towards younger fish (Martinez et al. 2016). Further, gizzard shad (*Dorosoma cepedianum*) spawn earlier, juvenile bluegill and largemouth bass (*Micropterus salmoides*) are larger, and the growth of adult largemouth bass is greater in anthropogenically warmed lakes (Mulhollem et al 2015).

In warm lentic ecosystems of Puerto Rico, bluegill are a widespread species (Neal et. al., 2009). This dominance is primarily due to their ability to withstand and survive elevated temperatures (Holland et al. 1974; Pierce and Wissing 1974). For example, Holland et al. (1974) investigated the acclimation capacity of bluegill from various cooling reservoirs and found that individuals can rapidly adjust their physiology and acclimate to temperatures ranging from 25 to 35°C. Lake Lucchetti experiences the lowest seasonal habitat variability, which also could affect patterns of DNA methylation (Leung et al. 2016). Since bluegill have rapid phenotypic responses to thermal stress, they represent an excellent focal species to study the physiological responses and adaptations to temperature variation (Martinez et al. 2016), which may be driven partly by DNA methylation.

MATERIALS AND METHODS

Study Sites

We collected bluegill from three man-made impoundments with different temperature characteristics: the ambient temperature, temperate Lake Mattoon (n = 7, replicate sites MA = 4; MB = 3); the anthropogenically warmed, temperate Lake Coffeen (n = 7, replicate sites CA = 3; CB = 4); and the tropical Lake Lucchetti (n = 4). Lake Mattoon is a 4.2 km^2 water reservoir located near Mattoon, Illinois. Annual water temperature ranges from 0.3 to 32.9°C, with an average of 16.8°C (Martinez et al. 2016). Lake Coffeen is a 4.5 km^2 power plant-cooling reservoir, near Coffeen, Illinois. Lake Coffeen supplies cooling water to a power station since 1972, thus forcing a year-round warmer thermal regime of 6.5 to 42.9°C, with an average of 24.7°C (Martinez et al. 2016). Fish in this lake have a population structure shifted towards younger, faster growing fish that do not reach as old an age compared to those in Lake Mattoon and Lake Lucchetti (Martinez et al. 2016). Lake Lucchetti is a 1.9 km^2 impoundment located in the Commonwealth of Puerto Rico. The impoundment was built in 1952 by the Puerto Rico Water Resources Authority (Johnson 1958). The temperature range in Lake Lucchetti is higher still, 23.0–30.0°C, with an average of 26.5 °C. We obtained epaxial white muscle tissue from adult fish, which were collected via 15 min pulse-amplified DC electrofishing surveys. All samples were stored in 95% ethanol or RNAlater until DNA extraction with the Qiagen DNeasy Blood & Tissue Kit (Qiagen).

Next-Generation Sequencing to Screen DNA Methylation

We conducted next-generation sequencing with the Ion Torrent PGM platform (Thermo Fisher Scientific). We used epiRADseq (Schield et al. 2016) following Mascher et al. (2013) substituting the DNA methylation sensitive restriction enzyme *HpaII* for *MspI* (New England Biolabs) to screen variation in DNA methylation. We used Torrent Suite version 4.4.3 for demultiplexing and quality control. We used Geneious v. 11.1.4 to trim sequences (50–300 bp) and for de novo assembly.

Data Analysis

We followed two analysis approaches. First, we mapped individual fragments (BWA; Li and Durbin 2009, 2010) and determined read counts for 50 bp bins, generating 105,811 bins (featureCounts; Liao et al. 2013). We collapsed the read count data into a binary matrix and compare methylated loci (zero) with unmethylated loci (one). We compared total DNA methylation of individuals among sites with *t* tests and *F* tests and correlated total DNA methylation to average water temperature with a Pearson correlation. All statistical tests used $\alpha = 0.05$ and α was corrected for multiple tests when appropriate.

Second, we compared read counts at each locus among groups (edgeR; Robinson et al. 2010; Liu et al. 2015). We tested for statistically significant frequency differences between the following: MA and MB, CA and CA, all M and all C, M and PR, and C and PR, using a false discovery rate of 0.05. We determined the number of statistically significant loci in each comparison using a conservative approach and collapsing statistically significant adjacent bins.

RESULTS

We observed 8,817 to 25,614 reads, post-QC and trimming, among the 18 individuals from the three lakes sampled. These reads formed a pseudoreference sequence of 5,290,456 bases after de novo assembly. Mapping resulted in 7,211 to 21,655 unique mapped reads per individual (Table I). We counted reads at 105,811 bins for all individuals.

Table I. Observed read number and unique mapped reads for each bluegill (*Lepomis macrochirus*) sorted by location: ambient temperature, temperate Lake Mattoon (MA, MB); power plant-cooling, temperate Lake Coffeen (CA, CB); and tropical Lake Lucchetti (PR).

Individual	Reads	Mapped reads	Total DNA methylation
MA-04	18,966	14,687	0.85
MA-06	12,855	9,707	0.88
MA-10	24,297	20,012	0.73
MA-11	15,974	13,080	0.81
MB-37	23,017	20,014	0.73
MB-41	20,274	17,653	0.76
MB-42	25,614	21,655	0.72
Total M			0.78
CA-25	17,922	13,783	0.85
CA-26	22,808	17,736	0.82
CA-35	10,540	9,222	0.85
CB-16	13,789	11,533	0.82
CB-20	8,817	7,211	0.88
CB-23	9,510	7,898	0.87
CB-24	11,546	9,932	0.85
Total C			0.85
PR-08	17,725	12,195	0.87
PR-11	19,354	12,861	0.85
PR-13	12,547	9,522	0.92
PR-17	14,709	11,317	0.92
Total PR			0.89

Total DNA methylation among individuals was statistically correlated to average water temperature (r = 0.696, p = 0.0006; Figure 1). Total DNA methylation statistically differed among fish from the different lakes (all pairwise t tests of proportions, $p < 0.019$; Figure 2). Lake Mattoon bluegill had less DNA methylation than Lake Lucchetti bluegill (0.78 vs. 0.89, p = 0.006), and Lake Coffeen bluegill (0.78 vs. 0.85, p = 0.019); and Lake Coffeen bluegill had lower DNA methylation than Lake Lucchetti bluegill (0.85 vs. 0.89, p = 0.013). We failed to detect statistically significant differences between MA and MB (0.82 vs. 0.74, p = 0.06) or between CA and CB (0.84 vs. 0.86, p = 0.18). Also, Lake Mattoon bluegill had statistically higher variance when compared to Lake Coffeen bluegill (0.003 vs. 0.0005, F test, p = 0.03).

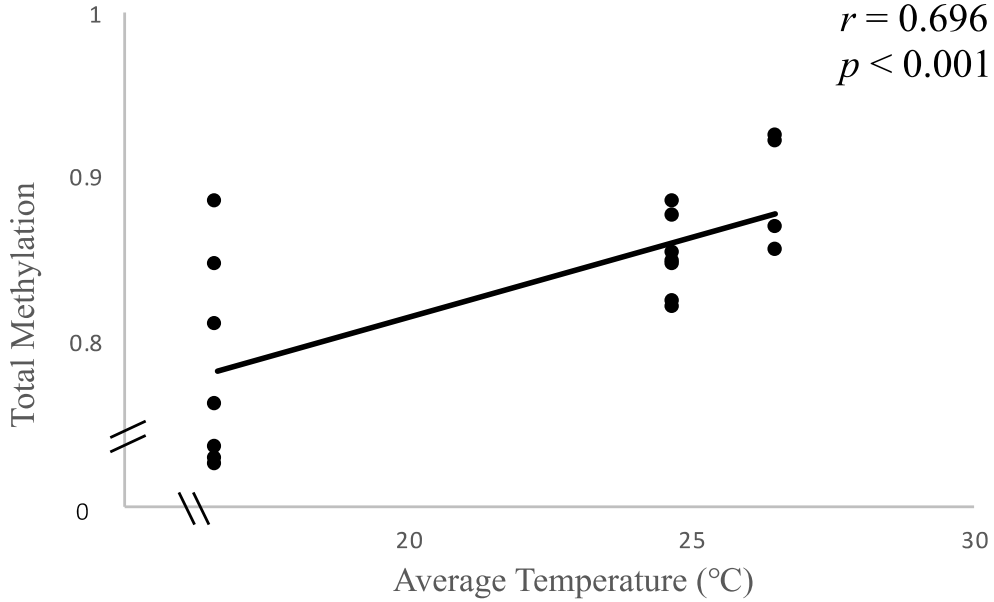

Figure 1. Statistically significant positive correlation (r = 0.696, p = 0.0006) between total DNA methylation for each bluegill (*Lepomis macrochirus*) collected and the average temperature for each site: ambient temperature, temperate Lake Mattoon (16.8 °C); power plant-cooling, temperate Lake Coffeen (24.7 °C); and tropical Lake Lucchetti (26.5 °C).

In the edgeR analysis, we detected statistically significant differences in DNA methylation between Lake Mattoon (the ambient temperature, temperate lake) and Lake Lucchetti (the tropical lake) bluegill, and between Lake Coffeen (the power plant-cooling, temperate lake) and Lake Lucchetti bluegill (Table I). There were 654 loci with statistically significant differences in DNA methylation between Lake Mattoon and Lake Lucchetti bluegill, with more than 99% of the cases indicating higher levels of DNA methylation in Lake Mattoon bluegill. There were 373 loci with statistically significant differences in DNA methylation between Lake Coffeen and Lake Lucchetti bluegill and more than 99% of the cases indicated higher levels of DNA methylation in Lake Coffeen bluegill.

The statistically significant loci detected between Lake Coffeen and Lake Lucchetti bluegill were, for the majority, a subset of those detected between Lake Mattoon and Lake Lucchetti bluegill (Table II). Of the loci that were statistically different in methylation status between Lake Mattoon and Lake Lucchetti bluegill, 51% were also statistically different between Lake Coffeen and Lake Lucchetti bluegill, while 92% of statistically different loci between Lake Coffeen and Lake Lucchetti bluegill were a subset of those statistically different between Lake Mattoon and Lake Lucchetti bluegill. We failed to detect loci with statistically significant differences in all other comparisons (MA with MB, CA with CB, all M with all C).

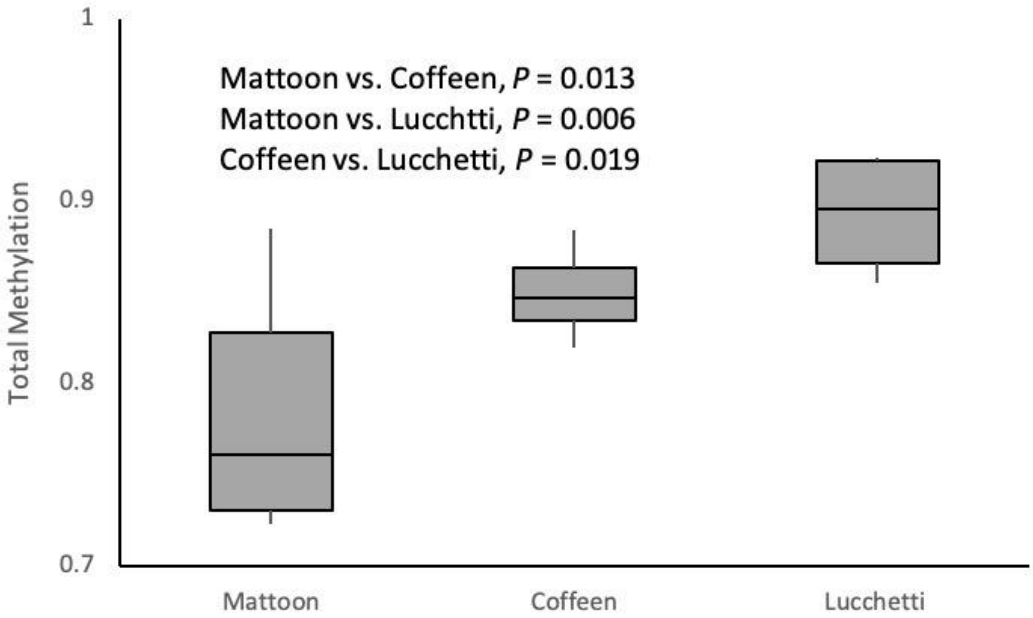

Figure 2. Total DNA methylation for each bluegill (*Lepomis macrochirus*) sampled from the ambient temperature, temperate Lake Mattoon (0.3–32.9°C); the power plant-cooling, temperate Lake Coffeen (6.5–42.9°C); and the tropical Lake Lucchetti (23.0–30.0°C). Statistical significance is indicated for each comparison. Boxes outline the upper and lower quartiles and indicate the median with a horizontal line. Whiskers extend to the upper and lower extreme values.

Table II. Summary of loci with statistically significant differences (false discovery rate = 0.05) in DNA methylation frequency among bluegill (*Lepomis macrochirus)* sampled from the ambient temperature, temperate Lake Mattoon (M); the power plant-cooling, temperate Lake Coffeen (C); and the tropical Lake Lucchetti (PR). Only comparisons between the temperate lakes and the tropical lake returned statistically significant differences. Further, a comparison of the statistically significant loci detected between tests to identify the shared and unique loci is provided.

	M versus PR	**C versus PR**
Statistically significant loci	654	373
DNA methylation up	648	370
DNA methylation down	7	3
Shared loci	335	344
Unique loci	319	29

DISCUSSION

We investigated the impact of elevated habitat temperature on DNA methylation in bluegill. We detected different levels of DNA methylation among bluegill from all sites, which followed a pattern that may be related to the temperature profile of their habitats. Among individuals, total DNA methylation increased with increased temperature. Bluegill from the ambient temperature, temperate lake (0.3–32.9°C) were more different from bluegill from the tropical lake (23.0–30.0°C) than they were from bluegill from the power plant-cooling lake (6.5–42.9°C). Thus, comparing bluegill from the two temperature extremes showed the greatest number of loci with changes in DNA

methylation, the highest magnitude of differences, and the most unique pattern of differences. Also, bluegill from the ambient temperature, temperate lake had greater variance in total DNA methylation compared with bluegill from the power plant-cooling lake. These results suggest that the DNA methylation of bluegill responds to habitat temperature by both increasing amounts of DNA methylation with increasing temperature and by changing patterns of methylation at specific loci.

When looking at individual loci, we detected a pattern in DNA methylation that ran counter to the pattern in an individual's total DNA methylation. The vast majority (99%) of statistically significant individual loci showed decreased DNA methylation in bluegill from the higher temperature Lake Lucchetti. This counter-direction pattern could be detected because of differences in the data analysis methodology. For total DNA methylation, we compared loci that were totally methylated (i.e., zero read count) too all other outcomes. Thus, we did not account for differing levels of DNA methylation at a locus (i.e., read count variation). When we looked at individual loci, we did look at individual read count variation, and subsequently detected the counter pattern. This analysis indicates that bluegill from the two lakes in Illinois were more similar to each other, and that bluegill from the tropical Lake Lucchetti had the most different DNA methylation pattern. We found differences in both increasing and decreasing levels of DNA methylation among bluegill as temperature increases depending on the specific loci in question. This outcome would be consistent with DNA methylation being used to fine-tune gene expression among bluegill based on their habitat, with some genes being upregulated and other genes being downregulated, similar to the patterns detected by Dixon et al. (2014). Future work, to link changes in DNA methylation at specific loci to changes in gene expression in a controlled exposure setting, will be necessary to untangle the consequences of the epigenetic response to temperature.

At the individual locus level, there were a larger number of statistically significant differences in DNA methylation (281 more loci) between bluegill from Lake Mattoon and Lake Lucchetti, than between bluegill from Lake Coffeen and Lake Lucchetti. The loci that were different between bluegill from the power plant-cooling and the tropical lake were largely a subset of those also detected between bluegill from the ambient temperature, temperate and tropical lakes. Thus, DNA methylation patterns in bluegill from the power plant-cooling lake were closer to patterns observed in bluegill inhabiting a tropical lake, yet these populations still harbored 373 loci with statistically different frequencies of DNA methylation (Table I) and statistically different levels of total DNA methylation (Figure 2). These results suggest that the increased temperature may have induced epigenetic changes in bluegill inhabiting an anthropogenically warmed lake and that some loci may show a consistent response in DNA methylation to an increased temperature across sampling sites.

While bluegill in all sampled lakes show differences in epigenetic profiles based on local habitat variation, there was a detectable pattern that followed habitat temperature. Because the ambient temperature, temperate lake likely experiences greater variation during seasons compared to anthropogenically warmed lake or tropical lakes, it is possible that epigenetic markers are responding to variation in habitat. Further, the power plant increases lake temperature on average, but not constantly, which may contribute to the observed differences between fish from the power plant-cooling lake and the tropical lake. This finding supports the hypothesis that unpredictable environments or environments with greater habitat variation would have different epigenetic responses when compared to predictable, less variable environments (Leung et al. 2016). Our experimental design precludes us from identifying any additional environmental factors that affected DNA methylation. It is possible that availability and quality of forage, vegetation, availability of thermal refugia, physiological stress, metabolic differences, and

other factors affect DNA methylation in addition to temperature. Exploring these factors will be a future direction of this research.

In the anthropogenically warmed habitat of Lake Coffeen, bluegill phenotypes respond with reduced lifespan, a decrease in somatic growth performance, and their populations are structured towards younger fish when compared to an ambient temperature lake (Martinez et al. 2016). Our results indicate that some portion of this phenotypic response may be caused by induced changes in DNA methylation. However, our data do not allow us to identify specific functional genomic elements that are differently methylated. Our data support bluegill having a multifaceted response to thermal stress. In one extreme case, highlighting complex phenotypic responses, a nuclear reactor's cooling pond had its surface water temperature raised to 45°C, which caused all plant cover to be lost (Eager 2011). The resident bluegill showed modifications in their morphology to be more fusiform (more hydrodynamically efficient) and they stored lipids differentially compared to fish from an ambient temperature lake. More than 20 years after the nuclear reactor was decommissioned and the pond's water returned to ambient conditions, the bluegill maintained their modified morphology, yet their lipid storage returned to that of nonthermally challenged conditions (Eager 2011). These results indicate that bluegill have a multifaceted response to both direct and indirect effects of changes in environmental temperature. Interestingly, part of the response appears to be governed by nonreversible genetic adaptation, while other aspects of the response seem to rely on phenotypic plasticity. Thus, it is possible that both genetic and epigenetic mechanisms are active underlying this response and that some habitat may have multiple interacting stressors that induce different changes in DNA methylation.

The presence of statistically significant differences in DNA methylation in bluegill among different temperature lakes supports the previous findings in winter skate (*Leucoraja ocellata*), which showed that epigenetic regulation of gene expression was important in the rapid adaptation of a population to a 10°C increase in water temperature (Lighten et al. 2016). Increases and decreases in temperature, ranging from 5 to 25°C during development, also caused long-term changes in DNA methylation levels in the three-spine stickleback (Metzger and Schulte 2017). That temperature range is within that naturally occurring for bluegill from the lakes sampled in this study. Finally, our results support findings from an all-female clonal fish system, *Chrosomus eos-neogaeus*, where changes in the DNA methylation in response to different habitat characteristics increased the phenotypic plasticity in instances that lacked changes in genetic variation (Massicotte et al. 2011; Massicotte and Angers 2012).

In summary, our data suggests that DNA methylation patterns in bluegill are affected by environmental temperature. Thus, epigenetic variation in anthropogenically warmed environments is a candidate for the underlying cause of observed changes in phenotype of bluegill. Future experiments will be necessary to directly link the observed phenotypic changes in the fish to known states of DNA methylation at the identified DNA sequences. The use of next-generation bisulfite sequencing, coupled with RNAseq techniques, and controlled experiments exposing bluegill to different temperature regimes, will be helpful to identify candidate genes that are differentially methylated among fish in different temperature lakes. More specifically, it would be possible to identify what regions of the genome are being epigenetically altered due to the temperature variation and determine the effect of DNA methylation on patterns of gene expression in response to the temperature changes.

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