Winter 2023

DNA Methylation and the Response to Infection in Introduced House Sparrows

Melanie Gibson

DNA METHYLATION AND THE RESPONSE TO INFECTION IN INTRODUCED HOUSE SPARROWS

by

MELANIE GIBSON

(Under the direction of Aaron Schrey)

ABSTRACT

Epigenetics is the study of molecular modification of a genome without changing its base pairs. The most studied type of epigenetic mechanism is DNA methylation, which is capable of turning a gene “on” or “off.” Epigenetic potential is the capacity to which an individual can have methylation on its genome. The more CpGs available, the greater the epigenetic potential. In invasive species, genetic variation has been observed to be paradoxical: not much of it exists on a genomic level, but epigenetically, phenotypic variation can occur. The focus on shift in gene expression in this study is on Toll-Like Receptor 4 (TLR4). TLR4 is a gene responsible for microbial surveillance measured here in a controlled environment where house sparrows are challenged with *Salmonella enterica*. Utilizing Qiagen DNeasy kits and targeted enzymatic methyl-sequencing on 38 hepatic tissue samples from house sparrows, it was found that sex and body mass do not impact DNA methylation of an individual sparrow, but the acute stressor (*Salmonella*) does impact methylation. This is the first study to prove not only that DNA methylation is impacted by stress and not other biological factors in sparrows, but that epigenetic potential provides the potential for response, DNA methylation states actualize the response, and the response is mediated through changes in gene expression.

INDEX TERMS: Epigenetics, DNA methylation, House sparrow, Invasive species, TLR4

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B.S., Armstrong State University, 2017

A Study in Fulfillment for
Master of Science in Biology
December 2023

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Electronic Version Approved December 2023

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CHAPTER 1

INTRODUCTION

Epigenetics is the study of heritable physical changes to the DNA molecule without modification of DNA sequence (Mohtat et al. 2010). There are three molecular epigenetic mechanisms, DNA methylation, chromatin structure, and histone modification (Mohtat et al. 2010), DNA methylation being the most well-studied (Mohtat et al. 2010). Most commonly, DNA methylation presents as the addition of a methyl group to the cytosines of the DNA strand, typically in locations where a cytosine is immediately followed by a guanine (i.e. CpG site) (Mohtat et al. 2010). DNA methylation occurs throughout the genome, yet interestingly, DNA methylation occurring on part of a gene's promoter region, or distal regulatory sequence, may alter gene expression (Mohtat et al. 2010). Epigenetic mechanisms can fine-tune gene expression by altering the locations of the DNA that cellular machinery can access (Mohtat et al. 2010). Further, individuals may vary in their epigenetic potential (EP), which is the extent by which an organism can display variation in epigenetic states (Kilvitis et al. 2017). In this study, EP is defined by how many CpG sites are present in a promoter. The study of one epigenetic mechanism, DNA methylation, and the differences in epigenetic potential among individuals, in relation to the individual response to stress is the focus of my thesis research.

The genetic paradox of invasion addresses how, although low genetic diversity likely exists in introduced populations, mechanisms such as multiple introductions and epigenetics allow invasive species to exhibit phenotypic plasticity (Kilvitis et al. 2017, Frankham 2005). Bet-hedging is another mechanism by which phenotypes can be altered. Here, phenotypic variants are produced in response to the environment via epigenetic mechanisms that are dictated by vertically transmitted "tools" (e.g. DNA methylation) in the genome. This can lead to rapid

adaptation to novel environments: CpG methylation varies in neurological, immune, behavioral, and endocrine responses to the new stimulus (Caizurgues et al. 2022). Also, stress can impact DNA methylation (Gervasi et al. 2016, Schrey et al. 2012). EP is the propensity for an individual's genome to epigenetically respond to environmental changes through phenotype (Kilvitis et al. 2017). This is measured by the amount of available CpG sites in the target gene's promoter. The axis responsible for triggering of immune pathways, the hypothalamus-pituitary-adrenal (HPA) axis, has been focused on due to its hypothesized association with range expansion capabilities (Kilvitis et al. 2017, 2018). EP concerning CpG sites, influencing genes associated with the HPA axis (such as TLR4), can be evaluated regarding impact on response to infection (Kilvitis et al. 2017).

The house sparrow (*Passer domesticus*) is an excellent model organism for research in epigenetics because it has successfully endured introduction and range expansion across globally diverse habitats (Caizergues et al. 2022, Kilvitis et al. 2019). The genome of the house sparrow has been studied under epigenetic contexts numerous times to give insight into their expansion success (Hanson et al. 2020a). Two geographically separated populations of house sparrows (Nairobi, recently introduced, and Tampa, introduced roughly 150 years ago) exhibited limited genomic variation between populations, but had significant differences in intrapopulation DNA methylation levels (Schrey et al. 2012). These results imply that the shifts in DNA methylation may contribute to the success of introduced species. Also, DNA methylation in the house sparrow is associated with shifts in immune response and microbial surveillance (Caizergues et al. 2022, Kilvitis et al. 2019). Kilvitis et al. (2019) found an association between DNA methylation in the putative promoter of the Toll-Like Receptor 4 (TLR4) and an increase of TLR4 expression: less DNA methylation led to greater expression. Additionally, data from

Hanson et. Al 2020a imply that sparrows with higher epigenetic potential use this mechanism (particularly higher count of CG sites in promoters) for success in range expansion.

My research focuses on DNA methylation of the putative promoter of TLR4. TLRs are key proteins associated with the innate immune response that are specifically involved with inflammation and microbial surveillance (Caizergues et al. 2022, Martin et al. 2013). While TLR4 receptors bind a variety of ligands, TLR4 has been demonstrated to be responsible for inflammatory immune response to bacterial lipopolysaccharide in house sparrows (Martin et al. 2013). TLR4 gene expression varies with the range expansion of house sparrows, which likely affects their ability to thrive via rapid phenotypic adaptations (Kilvitis et al. 2017). Kilvitis et al. 2017 showed that TLR4 expression was greater toward the edge of the range expansion as compared to the core area of the population. My objective was to screen DNA methylation in the putative promoter of TLR4 in house sparrows that have been exposed to the pathogen *Salmonella enterica* (Sheldon et al. 2023). My goal was to determine if: 1) DNA methylation of the TLR4 promoter is altered by *Salmonella* infection, 2) if DNA methylation is correlated to epigenetic potential, 3) if DNA methylation varies with sex or body mass, and 4) if DNA methylation is correlated to TLR4 expression.

MATERIALS AND METHODS

Sample Collection

Colleagues in the Martin lab at the University of South Florida conducted all research with live animals (Sheldon et al. 2023). House sparrows were caught in Tampa, Florida and housed in separate enclosures with measures in place to ensure no transmission of other infection or feces. Individuals were challenged via inoculation with *Salmonella enterica* and their response

to the infections was measured via TLR4 gene expression from multiple tissues. *Salmonella* burden was identified for each individual, and epigenetic potential was estimated for the TLR4 promoter for each individual. DNA was extracted from each sample with the QIAGEN DNeasy kit (Qiagen, Valencia, CA).

Screening DNA Methylation

I screened DNA methylation at the promoter of TLR4 in 38 liver samples from house sparrows from the *Salmonella enterica* exposure experiment using targeted enzymatic methyl-sequencing (New England Biolabs, Ipswich, MA) at the Pritzker Laboratory for Molecular Systematics and Evolution at the Field Museum, Chicago IL.

Data Analysis

I assigned the DNA methylation state for each CpG site, as well as one CHG site (it is the second most commonly methylated motif in the genome) in the TLR4 promoter for each bird. Individual sites were designated 1 for methylated, and 0 for not methylated, and individual house sparrows were characterized by a binary string for the full number of sites resolved (Table 1). I characterized the variability of DNA methylation at each CG site (Table 1) and the average DNA methylation (DNAm) across sites (Table 3). I used Pearson correlations to compare DNAm to EP, sex, body mass, *Salmonella* burden, and TLR4 expression in 4 regions of the intestine of each individual (Table 2, Table 4). I further evaluated the relationship between DNA methylation and EP, and between DNA methylation and individual response to *Salmonella enterica* by testing each site individually against the same suit of phenotypes.

RESULTS

I resolved 7 CpG sites and 1 CHG site in the putative promoter of TLR4 in house sparrows. I found that sites CpG-1, 4-7, and CHG-1 had variable DNA methylation, while CpG-2 and CpG-3 were not variable (Table 3). Across individuals, there were more methylated sites than unmethylated sites (Tables 1, 3). Across sites, there were more methylated sites for CpG-5-7 and CHG-1. Individual 3188 was excluded from further analysis based on the amount of missing data.

DNA methylation was related to *Salmonella* burden, EP, and gene expression in house sparrows. I found that the combined methylation state of CpG-1 and CpG-5 was inversely correlated to *Salmonella* burden ($r = -0.569$, $P = 0.02$) (Figure 4). Further, mean DNA methylation was positively correlated to EP ($r = 0.411$, $P = 0.007$). Also, individuals with high EP had significantly higher mean DNA methylation: high EP mean DNA methylation = 0.55, low EP mean DNA methylation = 0.46, t-test $P = 0.004$ (Figure 1). Mean DNA methylation was negatively correlated to TLR4 expression in the proximal intestine ($r = -0.321$, $P = 0.04$, Figure 2), and medial intestine ($r = -0.321$, $P = 0.04$, Figure 3). The mean DNA methylation was not correlated to, yet showed a similar trend, to distal intestine ($r = -0.257$, $P = 0.09$). This trend did not occur for the cecum ($r = 0.126$, $P = 0.25$). I did not detect differences in mean DNA methylation between sexes (M vs F t-test $P = 0.21$; F vs J t-test $P = 0.34$; M vs J t-test $P = 0$ .40). Also, mean DNA methylation was not correlated to body mass ($r = 0.142$, $P = 0.21$).

I did not detect significant relationships among the DNA methylation of each site and body mass, sex, and TLR4 expression in the proximal and distal intestine. The medial intestine demonstrated higher DNAm and less TLR4 expression.

DISCUSSION

Experimentally infecting house sparrows with a pathenogenic *Salmonella* showed DNA methylation is related to important factors in their response. The key finding of my study is that *Salmonella* burden was inversely correlated to the DNA methylation state of sites CG-1 and -5 (greater Salmonella burden led to a decrease in DNA methylation). Further, DNA methylation was related to TLR4 gene expression and EP. These findings provide the first direct evidence in house sparrows that DNA methylation is related to the response to stress. Further, it supports the hypothesis that EP provides the potential for response, DNA methylation states actualize the response, and the response is mediated through changes in gene expression.

My findings extend what we know about the effect of DNA methylation in the promoter of TLR4 and TLR4 gene expression. Previously, Kilvitis et al (2017) found that methylated state variation at a CpG site in the promoter of TLR4 was correlated to TLR4 gene expression among house sparrows across an introduced range. In the context of response to infection, I show that mean DNA methylation across multiple sites is also correlated to gene expression. My results extend those of Kilvitis et al. (2017) by finding a stronger relationship between DNA methylation and gene expression in response to a more acute stressor, in a more controlled environment. Further, comparing my study with Kilvitis et al. 2017 suggests that those sparrows, which in an introduction are likely responding to multiple stressors at the same time, might use DNA methylation in a complicated manner. In both studies sex and body mass were not predictive of gene expression of TLR4 further supporting DNA methylation is responding to stress rather than basic biological factors.

I found DNA methylation in the TLR4 promoter was related to EP, providing data on the benefit of EP with regard to exposure to novel stressors. These findings are evidence that

regulatory plasticity is partially mediated by environmentally-induced epigenetic variation. I've found direct evidence for environmentally-induced modulation of such regulation by examining *Salmonella* burden introduced to the house sparrow, emphasizing that EP facilitates DNA methylation being responsive to the environment. Simply put, the more CpG sites you have, the greater the opportunity to modify them differently via DNA methylation. Hanson et al. (2021) found that EP predicts gene expression of TLR4, which my data also support. Infection with lipopolysaccharide increased expression of TLR4 in Hanson et al. (2021). My study supports this finding by demonstrating that *Salmonella* burden decreases DNA methylation, thus increasing expression (Figure 4). While my results mirror similar outcomes in related house sparrow studies, my study utilized a greater amount of CpG sites, used a different pathogen, and included the neighboring CHG site.

Previous studies of EP in house sparrows showed that EP is highest at the range edge of an expansion, and lowest at the core of an introduced area (Hanson et al . 2020b, 2022). These findings indicate that house sparrows facing the greatest amount of stress are benefited by having more EP. My study provides direct evidence as to why the individuals facing the most stress need more EP. I show that EP is related to the ability to methylate the genome differently in response to an acute stress. This is the first time an empirical study has found support for the utility of EP through a direct relationship to DNA methylation states in response to stress, thus, it greatly increases the amount of support for the hypothesis that EP is critical for plasticity.

My findings support other house sparrow studies' results regarding variance in DNA methylation across a population and the implication of epigenetic variance existing to mediate the effects of unpredictable stress to a population (Schrey et al. 2012, Liebl et al. 2013). Lauer et al. (in review) demonstrated that invading house sparrows had greater variance in DNA methylation

compared to native and established house sparrows. Liebl et al (2013) found that variation in DNA methylation compensated for decreased genetic diversity associated with introduction. Also, Schrey et al. (2011) and Sheldon et al. (2023) found differences in DNA methylation among introduced populations of house sparrow. My data, addressing how house sparrows from the introduced population in Tampa, Florida respond to an acute *Salmonella* infection provide functional context to these previous findings. Previous studies showed the patterns of variation in DNA methylation supports the hypothesis that variation in DNA methylation is important to the house sparrow individual as the rapidly adapt to new locations. My study shows what this response looks like in a controlled, acute setting.

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TABLES

Table 1. Individual data for the DNA methylation state of each house sparrow infected by *Salmonella* at each CpG or CHG-01 (0 = not methylated, 1 = methylated). NA indicates missing data. Due to a substantial amount of missing data, individual 3188 was excluded from analysis.

Bird	CG-1	CG-2	CG-3	CG-4	CG-5	CG-6	CG-7	CHG-1
3138	0	0	0	0	1	1	1	1
3139	0	0	0	0	1	1	1	1
3142	1	0	0	0	1	1	1	1
3144	0	0	0	0	1	1	1	1
3145	1	0	0	0	1	1	1	1
3150	0	0	0	1	1	1	1	1
3153	1	0	0	0	1	0	1	0
3154	0	0	0	0	1	1	1	0
3155	0	0	0	1	1	1	1	1
3157	NA	0	0	0	1	1	1	0
3158	NA	0	0	0	1	1	1	1
3160	0	0	0	0	1	1	1	1
3162	NA	0	0	0	1	1	1	1
3163	0	0	0	0	1	1	0	0
3166	0	0	0	0	1	1	1	1
3167	NA	0	0	0	1	1	1	0
3168	NA	NA	0	0	1	1	0	0
3170	NA	NA	0	0	1	1	1	0
3171	NA	NA	0	0	1	1	1	0
3173	0	0	0	0	1	1	1	1
3174	0	0	0	0	1	1	1	1
3176	1	0	0	0	1	1	1	1
3178	0	0	0	1	1	1	1	1
3179	NA	0	0	0	1	1	1	0
3180	0	0	0	0	1	1	1	1
3181	0	0	0	0	1	1	1	1
3182	0	0	0	0	0	1	1	1
3184	1	0	0	1	1	1	1	1
3185	1	0	0	0	1	1	1	1
3186	0	0	0	0	1	1	1	0
3187	0	0	0	0	1	1	1	1
3188	NA	0	0	0	0	0	NA	0
3190	0	0	0	0	1	1	1	1
3191	0	0	0	0	1	1	1	1
3192	0	0	0	0	1	1	1	1
3138	0	0	0	0	1	1	1	1

Table 2. Phenotypic data for hosue sparrows, with Sex and Age (S/A), Body Mass (BM), Epigenetic Potential (EP), Epigenetic Potential Category (EPC), Salmonella Burden log genome equivalents (SBL), TLR4 expression relative quantification in the proximal intestine (TLR4 PI), TLR4 in medial intestine (TLR4 MI), TLR4 in distal intestine (TLR4 DI), and TLR4 in cecum (TLR4 C).

Bird	S/A	BM	EP	EPC	SBL	TLR4 PI	TLR4 MI	TLR4 DI	TLR4 C
3138	Female	21.2	8	high	NA	3.01	3.85	4.96	3.04
3139	Male	27.1	8	high	0	1.06	2.07	1.33	2.62
3142	Male	23.2	8	high	NA	1.41	1.28	2	1.20
3144	Juvenile	23	7	low	NA	2.83	0.46	3.08	1.51
3145	Juvenile	19.8	8	high	NA	0.159	0.35	0.45	17.33
3150	Female	22.2	9	high	NA	0.27	0.39	0.15	1.19
3153	Female	24.6	7	low	1.30	6.73	9.58	7.75	9.42
3154	Male	23.7	7	low	3.16	2.07	4.30	4.44	2.53
3155	Male	25.3	10	high	NA	1.69	1.911	1.01	1.01
3157	Male	20.5	9	high	2.28	0.50	0.53	1.95	1.02
3158	Male	21.7	8	high	3.58	2.60	3.25	4.35	9.22
3160	Female	23.5	8	high	1.53	0.37	0.63	0.83	3.58
3162	Male	20.4	7	low	2.79	0.55	0.66	6.41	14.93
3163	Female	19.6	6	low	2.95	0	0	NA	6.15
3166	Female	21.3	6	low	NA	0.72	0.08	NA	NA
3167	Juvenile	21.5	8	high	NA	1.169	1.40	4.067	3.19
3168	Male	22	7	low	NA	2.90	2.13	5.10	4.30
3170	Male	24.3	7	low	NA	1.201	2.83	1.33	2.32
3171	Juvenile	20.2	9	high	NA	NA	NA	NA	NA
3173	Female	21.8	7	low	NA	NA	NA	NA	NA
3174	Male	24.1	8	high	4.26	0.54	1.06	0.64	0.77
3176	Male	NA	7	low	3.15	0.95	0.59	0.46	4.53
3178	Male	23.2	8	high	3.22	NA	0.44	NA	3.49
3179	Male	21.5	8	high	2.20	0.86	1.89	2.23	1.22
3180	Juvenile	22.2	6	low	NA	6.52	5.50	8.17	6.89
3181	Juvenile	23.8	7	low	3.54	3.82	12.00	19.49	3.48
3182	Female	23.6	7	low	5.05	0.88	1.62	1.07	1.10
3184	Female	22.7	8	high	NA	0.51	0.56	0.52	1.33
3185	Male	24.7	8	high	0	NA	NA	NA	NA
3186	Female	21.4	7	low	NA	NA	NA	NA	NA
3187	Male	24.8	7	low	NA	1.02	1.65	1.71	1.95
3188	Juvenile	19.9	7	low	NA	5.62	0.34	3.48	1.19
3190	Male	24	8	high	3.63	1.22	1.71	1.84	2.38
3191	Male	22.1	9	high	2.15	0.60	1.06	1.97	2.37
3192	Female	21.1	7	low	NA	1.38	6.48	0.23	3.04
3138	Female	21.2	8	high	NA	3.01	3.85	4.96	3.04

Table 3. Summary of DNA methylation (DNAm) for each CG site and CHG-1 sites across all screened house sparrows, where N is the total amount of methylated CGs across all individuals.

Site	N	Mutated Sites	Present Sites	Mean DNAm	DNAm Variance
CG-1	26	1	25	0.24	0.19
CG-2	32	1	31	0	0
CG-3	35	0	35	0	0
CG-4	35	1	34	0.12	0.11
CG-5	35	1	34	0.97	0.03
CG-6	35	1	34	0.97	0.03
CG-7	34	0	34	0.94	0.06
CHG-1	35	0	35	0.69	0.22

Table 4. Pearson coefficients (r) for each CG and CHG-1 methylation value in house sparrows infected with *Salmonella* compared to *Salmonella* burden and each TLR4 expression relative quantification in four regions of the intestine. An asterisk indicates statistical significance at α= 0.05.

DNAm vs	**CG-1 r (*P*)**	**CG-2 r (*P*)**	**CG-3 r (*P*)**	**CG-4 r (*P*)**	**CG-5 r (*P*)**	**CG-6 r (*P*)**	**CG-7 r (*P*)**	**CHG-1 r (*P*)**
SB	-0.42*	NA	NA	0.11*	-0.46*	0.25*	-0.06	0.12*
TLR4 PI	0.07	NA	NA	-0.18*	-0.22*	-0.66*	0.03	-0.21*
TLR4 MI	-0.01	NA	NA	-0.20*	0.12*	-0.26*	0.12*	-0.06
TLR4 DI	-0.11*	NA	NA	-0.25*	0.07	-0.17	-0.09	-0.09
TLR4 C	0.42*	NA	NA	-0.25*	0.19*	-0.09	-0.08	0.08

FIGURES

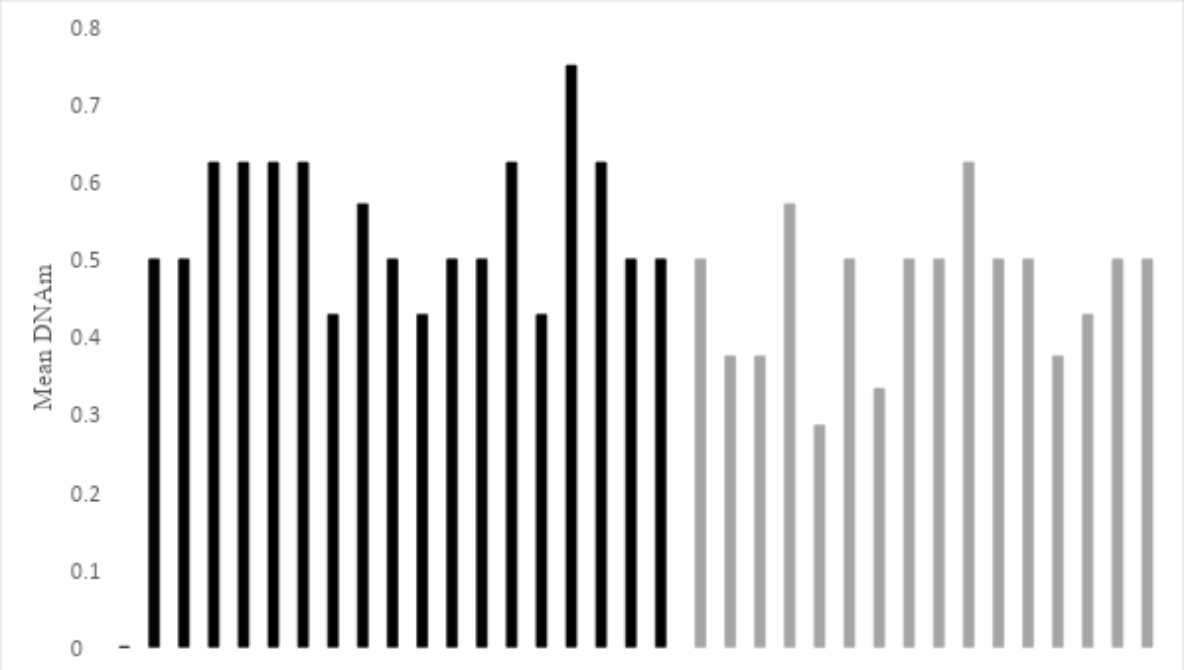

Figure 1. Summary of mean DNA methylation for each house sparrow infected by *Salmonella* (each bar is an individual). Individuals are color coded by epigenetic potential category (high EP black bar, low EP gray bar).

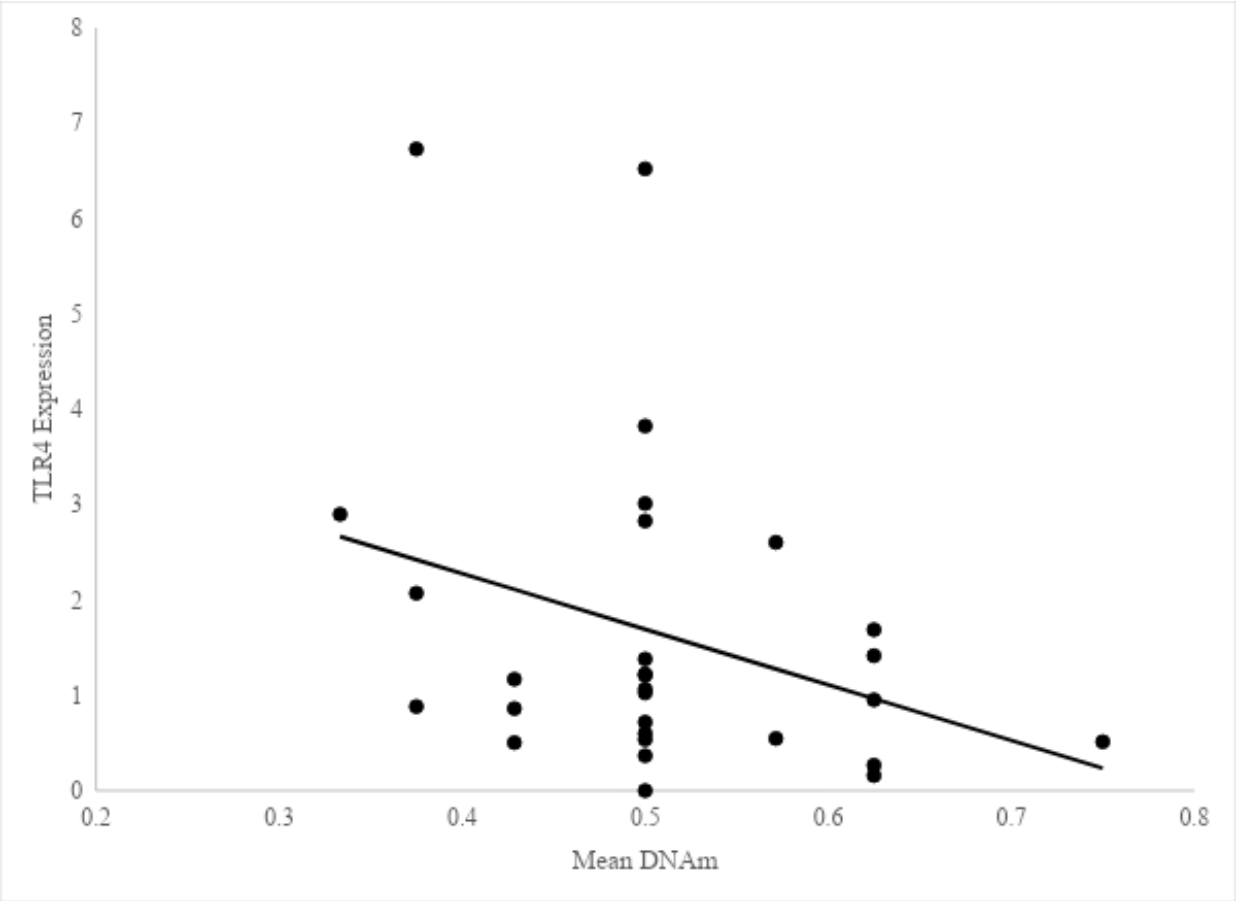

Figure 2. Mean DNA methylation for each house sparrow infected with *Salmonella* plotted against Proximal Intestine TLR4 expression.

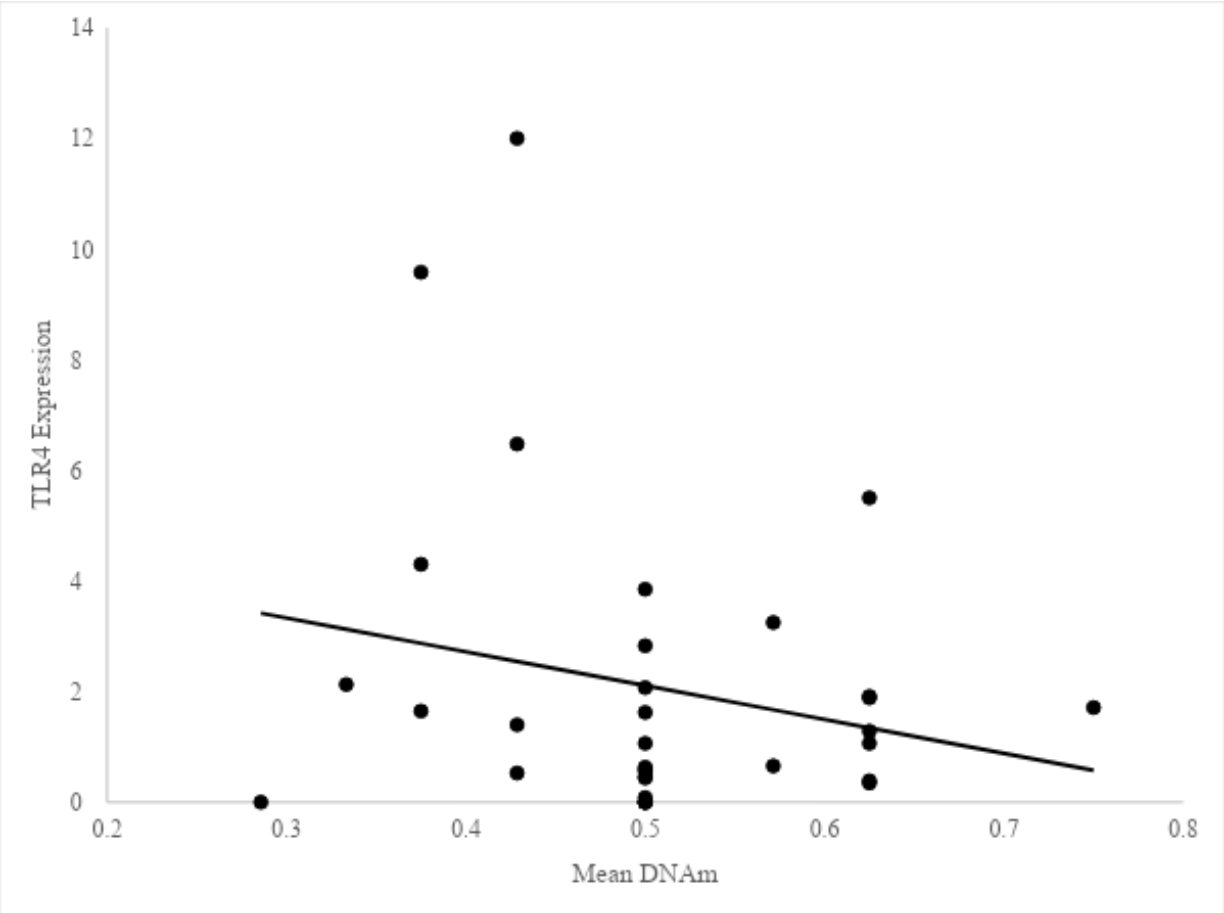

Figure 3. Mean DNA methylation for each house sparrow infected with *Salmonella* plotted against Medial Intestine TLR4 expression.

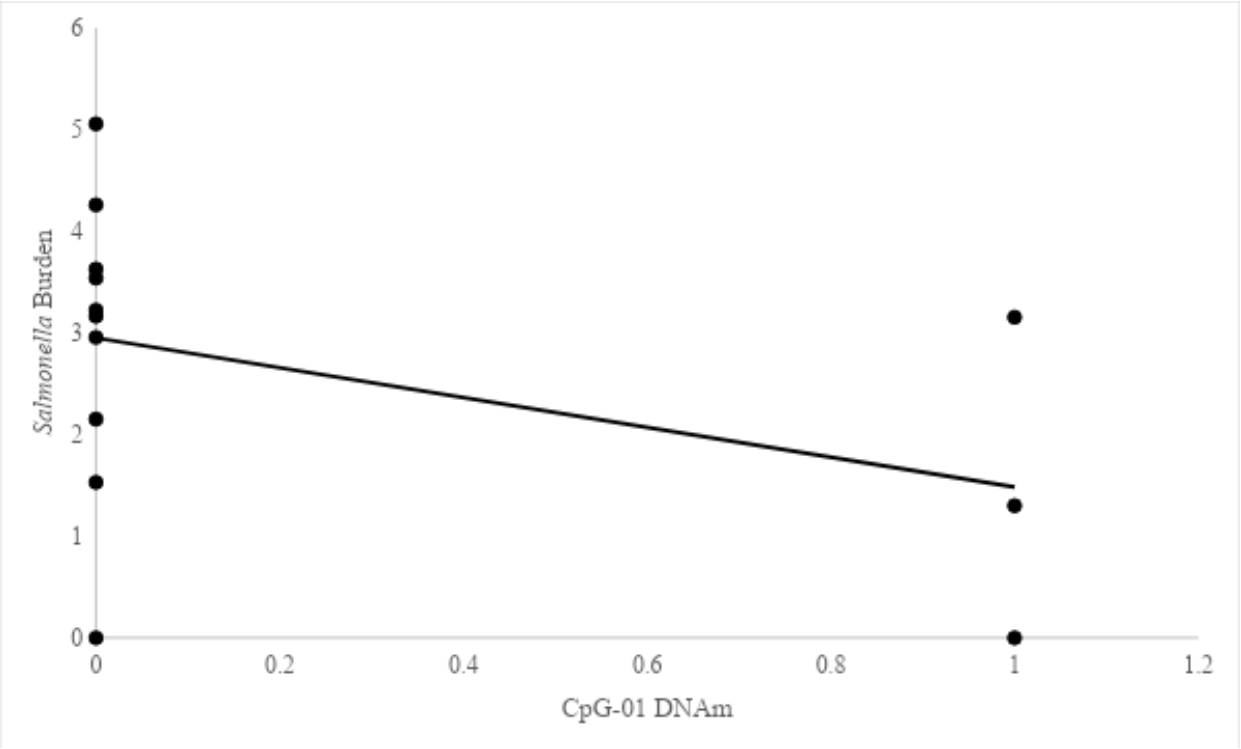

Figure 4. *Salmonella* burden compared to combined DNA methylation state of CG-1 and CG-5 for each house sparrow infected with *Salmonella*.