Methylation and Pigmentation of the Three-Spine Stickleback

by

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The newer methods and techniques which today are so successfully applied in many fields of biology can become more than better tools. In a sense they can create new problems, and allow us to ask more searching questions to which our curiosity demands an answer.

C.H. Waddington
From the conclusion of his book *The Strategy of the Genes*, 1957
Abstract

Recent literature in the field of ecological developmental biology has implicated epigenetic regulation as a factor in development. While genomes are determined by inheritance, the epigenome can be directly affected by the environment. Seven populations of both freshwater and anadromous three-spine sticklebacks, *Gasterosteus aculeatus*, were observed to exhibit different pigmentation patterns from environmentally distinct locations (specifically in water color and opacity). Based on these observations, our study aims to determine how much of this pigmentation difference can be correlated with varying patterns in methylation. Using the gene *dct*, known to play a role in fish pigmentation, primers for the promoter regions were generated. DNA was extracted from 35 fish, a Zymo bisulfite-conversion kit was used, the promoter regions of the samples were amplified by PCR, and finally sequenced. Due to low quality of sequencing, the samples had to be cloned using a Promega vector cloning kit and six colonies from each sample were chosen for sequencing. This protocol allows for visualization of the exact location of methylation of the DNA. We hypothesize that there exists a correlation between environment, pigmentation, and pattern of methylation. This paper is a description of the development of the protocol for this ongoing study as well as a description of its relevance in the literature. This study will provide a novel characterization of variation of methylation patterns for pigmentation in sticklebacks in relation not only to population but also to environment.
1. Introduction

1.1 Epigenetics, History of the Term and Modern Uses

In his 1940 book *Organizers and Genes*, C.H. Waddington coined the term “epigenetics” as a combination of the words epigenesis (development) and genetics. He first used this word in the phrase “epigenetic landscape,” the title of his revolutionary model of development. He developed his landscape model as a three-dimensional alternative to the then-popular two-dimensional model of developmental pathways (Waddington 1957). The two-dimensional model depicted changes in cellular development as branching lines allowing the cell to ultimately meet one of multiple potential fates. The original image that Waddington created for his model is that of water flowing down an inclined plane and winding through multiple valleys (Figure 1.1 a). The single stream of water, representing the undifferentiated cell, bifurcates repeatedly. As it reaches the sea, it is flowing in numerous individual streams which represent the multiple possible developmental fates. The three-dimensionality of this model better represents the complexity of development. Waddington later created a more theoretical image of this model consisting of an unknown surface with hills and valleys down which a ball, representing the cell, rolls (Figure 1.1 b). The variation of the surface creates the multiple possible developmental pathways that the cell could take and therefore the multiple possible fates of the cell (Slack 2002).
Figure 1.1 Waddington’s models of the epigenetic landscape.
a. Waddington’s original image of the epigenetic landscape as painted by John Piper, first appearing in Waddington’s book *Organisers and Genes* (1957).
b. The most well-known image of the epigenetic landscape with a ball representing the cell.
c. An elaboration of b, this image shows the underside of the landscape with the black boxes representing genes pulling strings to shape the landscape.
Waddington further elaborated his model with a drawing depicting the underside of the landscape, where the shape is shown to be controlled by the genes (Figure 1.1 c). It is interesting that Waddington chose to place genes in the role of “controllers” of development even though he created this model before the nature of genes was really understood. His view of genes as driving development was further evidenced by the title of his book *The Strategy of the Genes* (1957). This idea of genes as controllers became popular as more was discovered about the structure and mechanism surrounding DNA. Books such as Richard Dawkins’ *The Selfish Gene* (1976) popularized the anthropomorphization of genes as developmental biology switched its focus from the whole organism to individual genes (Gilbert 2001). In his essay *Metaphors and the Role of Genes in Development* (1990), H. F. Nijhout explains why these images of controller genes are no longer useful in our view of development. Genes are necessary but not sufficient for the process of development. During development, gene expression is largely regulated by stimuli external to the gene, not by the gene itself. Additionally, the sequential nature of development is a product of a large number of components working together at the right time, not any one “program” of the genes (Nijhout 1990). Waddington did manage to capture some of this complexity of development in his epigenetic landscape but he overemphasized the role of genes in the control of development.

This complex image of development becomes more complete with the addition of not only the environment surrounding the gene but also the environment surrounding the organism. The ecological developmental biology movement (often referred to as ecodevo) serves to make this connection. The importance of the
environment in development, however, is not a new idea. In an essay on the subject, Gilbert (2001) describes the emphasis placed on the role of the environment by many of Waddingon’s predecessors—the embryologists of the mid- to late-19th century and arguably the “founding fathers” of developmental biology. These scientists were primarily interested in environmentally dictated developmental processes such as the effects of temperature on polyphenisms, environmental sex-determination, and numerous others. The emphasis on the role of the environment was subsequently lost as the embryologists of the 20th century began to limit their studies to what was occurring inside the embryo (Gilbert 2002). If we were to modify Waddington’s model of the epigenetic landscape to include the effects of both the external and internal environments, it would consist of the ball rolling down a surface representing the developmental pressures inside the organism (e.g., transcription factors, enzymes, chemicals), which are in turn shaped by both the genes and the environment external to the organism (Figure 1.1 b).

Modern uses of the term “epigenetics” involve explanations for any observed difference between the genotype and the predicted phenotype (Wu and Morris 2001). Previously, this discrepancy was vaguely referred to as “intangible variation” or “developmental noise” because the mechanisms were not known (Rakyan 2001). These terms were used to describe any method of alteration of the expression of the genotype that creates a phenotype that does not necessarily directly correspond to the nucleotide sequence. This terminology has decidedly negative connotations, implying that any difference between the genotype and phenotype is due to
inefficiency and interference. In *The Strategy of the Genes* (1957), Waddington gives the following description of developmental noise:

> It can hardly be expected that any epigenetic mechanism can operate with complete precision. Quite apart from any disturbances due to the external environment of the embryo there are likely to be slight irregularities in the interactions between different parts of the germ, which, in a sense, provide an environment for each other (Waddington 1957).

Here, Waddington uses the term epigenetic mechanism to mean a mechanism of development and distinguishes it from developmental noise. With our increased understanding of the process of development, however, we can see that rather than interfering with epigenetic mechanisms, environmental effects both inside and outside of the cell are an integral part of epigenetic mechanisms and therefore development, creating both variation and regulation. Current uses of the term “epigenetic mechanism” refer to mechanisms that alter the expression of DNA, and therefore development, without changing the actual genotype (Rakyan 2001). The use of the term “epigenetics” has, therefore, evolved in recent years from a theoretical term to one describing concrete mechanisms.

If we insert these mechanisms into the modified model of Waddington’s epigenetic landscape proposed above by first imagining the ball at the top of the hill to have a fixed genotype and a predicted phenotype while at the bottom, it maintains its fixed genotype but has a number of possible phenotypes (Figure 1.1 b). The epigenetic mechanisms are part of the surface that contributes to differentiation. It is still beneficial, therefore, to look back to Waddington and other earlier embryologists
for an understanding of the complete developmental picture, that is, the complexity of the journey from genotype to phenotype.

The differentiation of cell types that Waddington (1940) explains through his epigenetic landscape is the most obvious example of the implementation of epigenetic mechanisms. Multi-cellular organisms are made up of numerous cells with very different phenotypes. This difference cannot be explained by the genetic code because within an organism every cell has an identical genetic background. In order for these cells to differentiate beyond their constant genotype, therefore, there must exist a molecular mechanism of regulation above the genome, creating differences in gene expression between cells. Recently, much of this regulation above the genome has been attributed to mechanisms that have been labeled “epigenetic” (Gilbert 2009).

From the perspective of evolutionary theory, we can imagine how epigenetic mechanisms could have been involved in the step from single-cellular to multi-cellular organisms (Jablonka and Lamb 2002). It is intuitive that a mechanism that controls the genome without actually modifying it would be selected for. With the advent of epigenetic regulation, organisms were able to “use” their genomes in different areas with very diverse results.
1.2 Epigenetic Mechanisms

Many different molecular mechanisms are considered epigenetic. These mechanisms work on different levels of DNA organization but all ultimately alter DNA transcription in some way. At the most general level, sections of the chromosome can interact with each other over a long distance to change transcription, the exact mechanisms of which are not well understood (Grimaud et al. 2006). More specifically, epigenetic regulation can occur at the level of chromatin (DNA wrapped around histone proteins) organization. The chromatin can be packed tightly (restricting transcription) or loosely (enabling transcription) (Kiefer 2007). On an even smaller level, the histone itself can then be modified via methylation, ubiquitinylation, phosphorylation or ADP-ribosylation, all of which alter the amount of transcription (Kiefer 2007). Acetylation and methylation are the most common modifications. Acetylation lowers the affinity that histone protein H4 has for DNA, thereby loosening the binding of the DNA to the histone and increasing transcription (Hong et al. 1993). Methylation does the opposite by blocking transcription (Gilbert 2009).

On the most specific level, the DNA itself can be modified by a number of mechanisms. DNA Methylation is the best-understood and most researched mechanism of epigenetic regulation. Methylation of the DNA occurs when proteins called DNA methyltransferases (Dnmts) transfer a methyl group to the 5-position of the pyrimidine ring of a cytosine, (Figure 1.2) referred to as 5-methyl cytosine (Rakyan et al 2001). Methylation almost always occurs on cytosines that are bound by a phosphate to a guanine, referred to as a CpG site (Cedar et al. 1979).
Approximately 70% of CpGs are methylated (Cooper and Krawczak 1989). These methylated cytosines are usually found on promoter regions, where they can affect expression of a specific gene (Bird 1986). The methylation creates a physical barrier directly on the DNA, thereby stopping transcription of the selected DNA sequences by impeding the binding of transcription factors to recognition sites (Kiefer 2007). The DNA is therefore inaccessible because of a physical obstruction of transcription by the methyl group. Additionally, methylation inhibits transcription when methyl-CpG binding domain proteins (MBPs) recruit other proteins such as histone deacetylases (HDACs) or histone methyltransferases (HMTs) that affect the transcription at a histone/chromatin level (Kiefer 2007).

Figure 1.2. Methylation of cytosine by DNA methyl-transferases.

While there are a number of differences in the molecular mechanisms of these methods of epigenetic modification, they frequently occur at the same time and seem to largely accomplish the same ultimate epigenetic results. Evidence for the redundancy of these mechanisms comes from the fact that drosophila have no methylation of their DNA in the adult stage (unlike most other eukaryotes). Lyko et al. (2000) showed this by performing high-performance liquid chromatography for 5-methylcytosine on DNA taken from drosophila embryos. This showed that there is
low level of methylation in early drosophila development, but that the level of methylation in embryos decreases with time, disappearing completely in the fully developed adult (Lyko et al. 2000). This finding is interesting because adult drosophila, while lacking methylation, are as equally differentiated as other eukaryotes. The findings of this study therefore, indicate that there must be regulation of the genome using mechanisms other than methylation in drosophila that accomplish the same goals as methylation in other organisms.

Methylation was chosen as the form of epigenetic regulation investigated in this study for several reasons. First, the fact that the mechanism and its function are well known and understood by the scientific community as well as the mechanism’s relative simplicity (shown above) made it attractive. Second, there is a large body of work concerning certain developmental processes in which the specific role of methylation is well understood. Finally, and most importantly, there exists a developed method of detection of the position of 5-methyl-cytosines: bisulfite conversion of DNA. When DNA is bisulfite converted, the cytosines are converted to thymines by the removal of an amine (NH2) (Figure 1.3). When a methyl group is attached to the cytosine, however, this mechanism, called deamination, cannot occur, and the cytosine thereby remains a cytosine (Fernandez and Esteller 2004). When the modified DNA is sequenced, therefore, one can observe the pattern of methylation by the location of any remaining cytosines.

1 In this paper, we will use the term “pattern” both in relation to epigenetic patterning or more specifically methylation patterns to refer to the actual positioning of epigenetic markers on the DNA in relation to each other.
Figure 1.3 mechanism of bisulfite conversion.
a. unmethylated cytosine becomes thymine
b. methylated cytosine remains cytosine
1.3 Importance of Methylation

Methylation has been shown to be important in many fundamental developmental processes. One such process is parental imprinting, the silencing of one parental allele of many genes in mammals and plants. This limitation of expression is caused by methylation of either the DNA or the histone (Jullien and Berger 2009). The importance of parental imprinting and its epigenetic role can be seen in Angelman and Prader-Willi syndromes. Both syndromes occur when there is a mutation at a certain locus. If the paternal allele is mutated, the mutation will manifest itself as Prader-Willi syndrome, a syndrome characterized by low muscle tone, short stature, incomplete sexual development, and a chronic feeling of hunger that, coupled with a low metabolism, causes life-threatening obesity. Angelman syndrome, which occurs when the same mutation is on the maternal chromosome, is characterized by a youthful appearance, difficulty communicating, very happy demeanor and difficulty developing motor skills (Nicholls et al 1998). A genetically identical mutation can lead to extremely diverse phenotypes (Figure 1.4) due to differences in the epigenome\(^2\). In this instance, methylation has a greater phenotypic effect than the genes themselves.

\(^2\) The term “epigenome” is defined as the combination of all epigenetic markers located above a given genome.
Methylation has gained a lot of attention in recent years because of the discovery that an excess of methylation is strongly linked to the progression of certain cancers (Issa 2007). Much of the current research concerning methylation, therefore, is in relation to oncogenesis. While overall, cancer is caused by a lack of regulation and, therefore, carcinogenic tissues tend to exhibit hypomethylation, recent studies have shown that this is coupled with hypermethylation on specific promoter regions (Herman and Baylin 2003). The hypermethylation is located so that it represses antitumoral genes and thereby allows tumorigenesis. With this recent revelation, researchers have been performing clinical trials of epigenetic therapies to treat a specific methylation-caused cancer: Myelodysplastic Syndrome (Oki and Issa 2007). They have had significant success in treating this cancer by administering chemotherapy with histone deacetylase inhibitors and hypomethylating agents that serve to undo the anti-tumoral gene silencing. A number of oral hypomethylating agents have recently been approved by the FDA (Issa et al. 2005). The advantage of
epigenetic chemotherapy is that only a small dosage is necessary in order to undo the methylation as opposed to near-lethal levels of traditional chemotherapy, which aim to kill all cancer cells. Hypomethylation therapy appears to be one of the fastest growing and most successful new fields of cancer research. Our continually improving understanding of methylation is helping us to develop therapies to stop cancer growth.
1.4 Epigenetic Heritability

Richards (2006) defines epigenetics as:

an alternative inheritance system, operating at the interface of the familiar stable genetic system that is encoded in primary nucleotide sequence and the transient protein-DNA interactions that mediate gene-expression changes in response to developmental signals and environmental stimuli.

The image that Richards (2006) conjures up is that of another “genome” located above our traditional genome. This epigenome is more flexible in its response to the environment and its regulation of proteins. He also suggests that these regulatory mechanisms have a degree of heritability, a relatively controversial idea. While it is easy to become preoccupied with the debate surrounding this alternative inheritance system, it should not detract from the important roles that epigenetics play in development. Instead, we can look at inheritance as a mechanism by which epigenetic regulation could have evolved and continued to persist. The possibility of mechanisms of epigenetic inheritance indicates the possibility for purely developmental information to be directly passed on to future generations.

Heritability of epigenetic marks\(^3\) poses something of a paradox since it is understood that the epigenetic marks must be erased at some point of gametogenesis in order to allow the cells of the early embryo to retain totipotency (Rakyan et al 2001). We therefore have to ask the question: how can embryonic cells be totipotent when there is an epigenetic “memory?” Numerous studies have recently been

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\(^3\) For the purpose of this study, epigenetic marks are defined as factors that are added to the DNA on any level (nucleotides, histones etc.). For example, a methyl group (attached to a cytosine) would be an epigenetic mark since it is a physical addition to the DNA.
performed in order to determine if and how an epigenetic memory is transmitted. In one such study, gestating rats were exposed to endocrine disrupters during the period of gonadal sex determination. Their male offspring exhibited disrupted fertility, defects that were shown to persist in the following generations. This inheritance was attributed to epigenetic inheritance because the observed fertility defects correlated with DNA methylation patterns found in the germ line (Anway et al. 2005). Another study showed evidence for epigenetic inheritance by observing the records of a small town in Sweden. The researchers found transgenerational trends in the nutritional state of the grandparent linked to the risk of diabetes and heart disease. While they are unsure of the epigenetic mechanisms that are implicated by their results, they have tried to eliminate all other possibilities for this phenomenon and believe that the results point strongly towards epigenetic inheritance (Kaati et al. 2007). Both of these examples show correlations that indicate an epigenetic method of passing on information that cannot be directly tied to genomic heredity.

There is definitely a possibility, however, that the inherited aspect of epigenetics is linked to classical genetic inheritance. Richards (2006) describes three kinds of possible relatedness between the epigenome and the genome: Obligatory (complete), facilitated (partial) and pure (autonomous) variation (Figure n). These different ways in which the epigenome may be tied to the genome illustrate that there are varying degrees with which the genome could regulate the epigenome. While there are definite differences in the mechanisms of the epigenome and the genome, there is a possibility for the inheritance of the epigenome to be derived from the
genome in some way. If the hereditary information for the epigenetic marking comes from the genome, however, can we really call it epigenetic inheritance?
1.5 Epigenetics and the Environment

Multiple studies have shown a relationship between environment and epigenetic patterning. One of the most striking areas where this relationship has been shown is in monozygotic twins. Fraga et al. (2005) performed a large analysis wherein they looked at a number of epigenetic differences including X-inactivation, Histone H3 and H4 Acetylation, total DNA methylation, sequence-specific methylation and RT-PCR levels between 80 twins. They found that while early in life there are no epigenetic differences between twins, as they age the epigenetic differences increase (Fraga et al. 2005). This study shows the importance of environmental effects on separating the epigenome from the genome.

Studies by Cooney et al. have shown that a change in diet can change gene expression in mice with a mutation that causes an over-expression of the Agouti gene (the gene that determines the wild type coat color). An increase in the folic acid and vitamin B 12 in the diet increases the abundance of the central methyl donor metabolite S-adenosylmethionine that promotes methylation. This increase in the methyl donor elevates DNA methylation of the upstream IAP element that is responsible for the Agouti overexpression. The Agouti overexpression is suppressed by the excess methylation and the phenotype is changed (Cooney et al. 2003). A similar phenomenon whereby the epigenetic patterns are changed by the environment has been shown in rats by Weaver et al. (2007). The offspring of mothers that are bad nurturers exhibit an increased methylation of their glucocorticoid receptor. This methylation leads to an increase in stress when in traumatic situations. The social environment of the offspring’s rearing affects their methylation patterns and
phenotype (Weaver et al. 2007). Interestingly, both of these environmental effects have also been shown to affect future generations, giving more evidence for an epigenetic system of heredity (Richards 2006).

The environmental effect on epigenetic patterning is a very attractive aspect of epigenetics, placing it firmly in the center of ecological developmental biology. Because of its already established importance at the intersection of evolution and development, the exploration of epigenetics brings together three aspects of biology: evolution, ecology and development. With the combination of these classically distinct terms, we as biologists are realizing that many of the barriers that have been created in the last century or so must now be broken down. The more we discover, the more interactions we unearth between what had been considered discrete disciplines. While the human and especially the scientific mind has a tendency to compartmentalize information in order to better understand it, ultimately, our comprehension of this information is greatly furthered by expanding our view to the “big picture.”

An important characteristic of ecodevo is that it necessitates a shift in the study of development from the lab into the field (Gilbert 2001). Most of the aforementioned environmental epigenetic studies were conducted inside the laboratory. Since epigenetics is at the center of ecology and development, if we are truly to understand the effect that environment has on epigenetics, we must look at the epigenetics of organisms in their natural environment. We will only be able to truly understand the effect of the environment on development if we study the development of animals not raised in a laboratory setting.
There have been a number of studies that have looked at methylation outside of the clinical context. (Cai and Chinnappa 1999; Cubas et al. 1999; Bernardi and Varriale 2006). The species *Linaria vulgaris*, or Common Toadflax, has a naturally occurring mutant form with a symmetry pattern so radically different from the non-mutant form (Figure 1.5) that when in 1742 Linnaeus was trying to catalogue the two forms into species, he referred to this mutant as *Peloria* (monstrosity) (Gilbert 2009). Cubas et al. (1999) discovered that this mutant form is actually not due to a genetic mutation, but to an excess of methylation. The hypermethylation is located on the gene *Lcyc*, which is involved in formation of symmetry (Cubas et al. 1999). Cai and Chinnappa (1999) have shown a relationship between environmental temperature and methylation in plants. Using high performance liquid chromatography, they observed that changing the growing conditions of Long-stalk Starwort, *Stellaria longipes* from colder to warmer climates causes a decrease in methylation (Cai and Chinnappa 1998).

In fishes, extensive research has been performed on the effects of environment on methylation in terms of temperature (Jabbari et al. 1997; Varriale and Bernardi 2006; Bucciarelli et al. 2009). They have shown through whole-genome analyses of
methylation levels that fishes and amphibians show an increase in methylation when compared to mammals, a difference believed to be caused by the higher body temperatures of mammals (Jabbari et al. 1997). Bernardi et al. (2006) have also shown through reverse-phase high-performance liquid chromatography that body temperature (measured through environmental temperature due to the ectothermic nature of fish body temperature) is correlated both with the percent of the genome that consists of guanines and cytosines as well as the percent of 5-methyl cytosine. They divided up the samples into polar/subantarctic fishes (living at around 0 °C) and temperate/tropical fishes (living at around 10-30 °C) and observed a significant decrease in both average 5mC % and GC % in the fish from warmer climates (Bernardi and Varriale 2006).
1.5 Model Species

The 3-spine stickleback, *Gasterosteus aculeatus* (Figure 1.6), was selected as the model system for this study because of its presence in the wild, as well as because of the near-complete sequencing of its genome (Ensembl.org). The combination of these two attributes allows for both an environmental and a molecular study.

![G. aculeatus](image)

**Figure 1.6 G.aculeatus**
Catherine Bursch

*G. aculeatus* are small (~5.1 cm) fishes characterized by three separate spines (commonly between two and four) in front of their dorsal fin. It is found in freshwater, marine, brackish, and anadromous (migrating from a marine to a freshwater environment to spawn) populations. Its environmental range has made this species especially appealing, as populations in different salinity levels have different morphologies. For example, the anadromous populations have been shown to be nearly five times more massive than freshwater populations (Bell Pers. Comm.). Additionally, all forms are protected by lateral and dorsal bony plates (Wootton 1976). Their segregation from marine populations into distinct freshwater populations encourages population genetics studies (Bell and Orti 1994; Bell et al. 2004). Additionally, *G. aculeatus*’ relative hardiness makes it an ideal species to be raised in a laboratory setting (Wootton 1976). *G. aculeatus* are widely distributed
among the temperate and sub-polar regions of the northern hemisphere and are found along the coasts of North America, Western Europe and Eastern Asia (Figure 1.7). It is usually found in slow moving streams and lakes (Wootton 1976). It is predominately carnivorous, feeding mainly on annelids, crustaceans, and the larvae and pupae of chironomids in addition to some zooplankton and algae (Wootton 1976).

Figure 1.7 Worldwide native distribution of *G. aculeatus*

1.6 Samples and Site Descriptions

All of the samples used in this study were collected by Dr. Michael A. Bell, professor of ecology and evolution at SUNY Stony Brook. The samples come from six sites in the Matanuska-Susitna Borough and the Kenai Peninsula Borough, Alaska. Both of these boroughs are located on the edge of Cook Inlet. Cook Inlet was almost entirely filled with glacial ice until 20,000 years ago (Bell and Orti 1994). There were a few cold deserts and ice lakes on the Kenai Peninsula that most likely did not support stickleback populations ancestral to the existing ones. It is possible, however, that some of the Kenai Peninsula populations may predate the last glacial advance (Bell Pers. Comm.). During this time, the Matanuska-Susitna Valley was covered entirely with ice. All of the freshwater samples listed below, therefore, can be assumed to have been derived from anadromous (sea run) stickleback within the last 15,000 to 20,000 years (Bell Pers. Com.).

Brief descriptions of the six sites are below, provided by Dr. Bell (Pers. Comm.):

Bear Paw Lake – This lake is located in the Matanuska-Susitna Borough. The population from which samples were taken was also used to sequence the first G. aculeatus genome. This is a small lake with numerous sphagnum swamps surrounding it and no inlet or outlet streams. The water residence time is long, conductivity is low, and the water is relatively opaque in the red.
Meadow Creek – This creek is located in the Matanuska-Susitna Borough and is the only stream sample. There are not a lot of stream populations because *G. aculeatus* winter kill below a certain size, and rivers are generally too fast-moving for freshwater populations to inhabit.

Rabbit Slough - Matanuska-Susitna Borough. This is the only anadromous population.

Mud Lake – Located in Matanuska-Susitna Borough, t is a shallow lake and its resident freshwater population specializes in eating benthos. Although the lake has inlet and outlet streams and high conductivity water, it is fairly turbid and probably slightly stained in the red end of the spectrum.

Tern Lake - Kenai Peninsula population. This is an ecological replicate of the Mud Lake population. However, it is about 100 km away and there is good reason to believe that it evolved from anadromous stickleback independently of the Matanuska-Susitna Borough populations. This population and Mud Lake, therefore, are phylogenetically independent populations of benthic-feeding freshwater stickleback.

Stormy Lake - Kenai Peninsula population. This is a relatively large lake with a tributary and outlet streams. The mineral content is relatively high and the water is relatively clear (not stained in the red). Although it is on the Kenai Peninsula, like
Tern Lake, they are far apart and were almost certainly founded by separate invasions of anadromous stickleback.

Seymour Lake – This lake is located in the Matanuska-Susitna Borough. This is also a relatively large lake with tributary and outlet streams. It is comparable to Stormy Lake with respect to water quality but geographically distant and almost certainly independently derived from anadromous stickleback. This and Stormy Lake populations are phylogenetically independent samples of largely planktivorous, clear-water populations.
1.7 Study Purpose

The purpose of this study is to characterize the variation of epigenetic patterning and its correlation with morphology, population, and environment. In order to achieve this, we must ask the questions (1) how much variation in methylation is there between and among distinct populations of sticklebacks, and (2) is this variation correlated with pigmentation differences and/or environmental differences? First, we hypothesize that the pigmentation is more similar within a population of sticklebacks than between populations. Second, we hypothesize that by sequencing bisulfite-converted DNA from these populations, we will find that the pattern of methylation is more similar within a population than between populations. Third, we hypothesize that by comparing sequences from freshwater and anadromous populations, we will see that the patterning of methylation is more similar within the group of freshwater fish than between this group and the anadromous fish. Lastly, by testing the top three hypotheses, we hypothesize that we will determine that there exists a correlation between pigmentation and methylation, there exists a correlation between pigmentation and environment, and, finally, there exists a correlation between methylation and the environment.
2. Materials and Methods

2.1 Animals

This study is being conducted using 5 whole fish samples from each of 5 freshwater populations of *G. aculeatus*. Additionally, we have 5 caudal fin clips from the stream population and anadromous population. Upon receipt of samples, each individual fish (or fin clip) was assigned a number 1 to 35. When we refer to sample number n, therefore, we are referring to DNA extracted from one individual fish.

All samples were collected by Dr. Michael A. Bell, Professor of ecology and evolution at the State University of New York at Stony Brook.

Table 2.1 Collection sites (Matanuska-Susitna Borough and Kenai Peninsula Borough, AK) and corresponding dates of collection.

<table>
<thead>
<tr>
<th>Collection Site</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meadow Creek</td>
<td>June 6, 2008</td>
</tr>
<tr>
<td>Rabbit Slough</td>
<td>May 30, 2009</td>
</tr>
<tr>
<td>Tern Lake</td>
<td>June 3, 2008</td>
</tr>
<tr>
<td>Stormy Lake</td>
<td>June 3, 2008</td>
</tr>
<tr>
<td>Bear Paw Lake</td>
<td>May 26, 2007</td>
</tr>
<tr>
<td>Mud Lake</td>
<td>N/A</td>
</tr>
<tr>
<td>Seymour Lake</td>
<td>May 31, 2007</td>
</tr>
</tbody>
</table>

Individual *G. aculeatus* were captured with minnow traps (chamber 44.45 cm long, 22.86 cm diameter; openings 2 cm; mesh 0.32 cm) baited with sharp cheddar cheese and set over night (about 20 h). Traps were always set 0-5 m from shore in less than 2 m of water and usually adjacent to rocks, aquatic plants, or fallen trees, which were almost always present. Trapped *G. aculeatus* and a few (usually <5)
specimens of other fishes were anesthetized in MS-222, washed to remove the anesthetic, and fixed in 10% formalin. Specimens were soaked in water and transferred to 100% ethanol (Bell and Orti 1994). Samples were shipped to us by Dr. Bell wrapped in 100% ethanol-soaked cheesecloth. They are stored in 100% ethanol.
2.2 Gene Studied

We decided to examine the methylation of the promoter region of genes associated with pigmentation for multiple reasons. First, when examining the samples, it was the most striking morphological difference. Second, it seems a likely candidate for environmental effects because it is important for fish to blend into their environment in order to avoid predation. Third, Braasch et al. (2007) listed genes associated with pigmentation in *G. aculeatus*. This study begins with the examination of the gene *dct* from Braasch et al 2007; also called tyrosinase-related protein 2, it is a member of the Tyrosinase family and is involved in melanin synthesis in most vertebrates. In the stickleback genome it is located on chromosome XVI (Braasch et al. 2007).
2.3 Protocol

DNA was extracted from caudal fins using the QIAquick DNA extraction kit (QIAGEN). The provided protocol for extraction from whole animal tissues using spin columns was followed to isolate and purify DNA. The last step of the provided protocol was changed to 30 µl of buffer added to the membrane and incubated at room temperature for 10 minutes before centrifugation to increase DNA concentration.

DNA was bisulfite converted using the EZ DNA Methylation Kit (Zymo). Prior to conversion, DNA concentrations were taken with a Nanodrop so that the amount of DNA converted was between 300 and 400 nanograms. After problems with incomplete C to T conversion, the protocol was modified so that 7.5 µl of M-Dilution Buffer was added instead of 5 µl, the volume of M-Dilution Buffer added to the CT-Conversion Reagent was changed from 210 µl to 185 µl and 97.5 µl of CT-Conversion Reagent was added to sample instead of 100 µl. Additionally, the denaturing was extended to 20 minutes at 37°C. The DNA was denatured at 37°C with a dilution buffer, converted during an overnight incubation at 50°C with the CT-conversion reagent, cleaned up, desulfonated, and finally eluted. The bisulfite-modified DNA was kept at 4°C for immediate use or at -20°C for long-term storage.

The section of the bisulfite converted DNA to be amplified by PCR was found on Ensembl (Ensembl.org) by searching for the dct gene in G. aculeatus and choosing a non-coding region 548 base pairs long directly upstream of the start codon. Primers which flanked this region were designed manually according to specifications by Fernandez and Esteller (2004). They suggest using primers without
CG dinucleotides to avoid methylation-biased amplification, but with non-CpG cytosines to optimize the amplification of bisulfite-converted DNA (Fernandez and Esteller 2004). Additionally, the primers were chosen to be between 15 and 25 base pairs and with as close as possible annealing temperatures and as few repeated bases as possible to avoid non-specific binding.

The sequences of the primers from Ensembl are:

Forward—GAGGTGTCAGGGTGAAAG
Reverse—CATCTGTGGAAACAGTTAAAGG

These were then modified to be specific for bisulfite-converted DNA by changing all Cytosines to Thymines to produce this primer set:

Forward—GAGGTGTAGGGTGAAAG
Reverse—TATTTGTGGAAATAGTTAAAGG

All primers were supplied by IDTDNA

Amplification by PCR was run using GoTaq Hotstart Green Master Mix (Promega). The following amounts were used for each sample:

25 µl of master mix,
1.25 µl of each (forward and reverse) primer,
2.5 µl of DNA,
19.5µl of dd H2O

for a final volume of 47.25 µl

The following PCR program was designed in order to maximize specificity of annealing for both primers:
1) 94°C 2:00
2) 94°C :30
3) 44°C 1:00
4) 72°C 1:30
5) 4°C ∞

The entirety of the PCR product (~50 µl) was then run with 1µl 6x gel loading dye (NEB) on a 1% agarose gel for 30 minutes at 100 volts to verify amplification of desired band. The band of interest was cut out of the gel on an open UV-Transilluminator using a sterile scalpel. The DNA was then extracted from the gel and purified using the QIAquick Gel Extraction Kit (QIAGEN). The protocol was modified to increase DNA concentration by eluting with 30 µl of Buffer EB and letting the spin column stand for 10 minutes prior to centrifugation.

PCR products were cloned into vectors using the Pgem-T Easy Cloning Kit (Promega). Concentrations of DNA were taken with a Nanodrop prior to ligation in order to determine the volume of insert that would create an approximately 3:1 ratio of insert to vector. The following formula was used to determine the nanograms of DNA that would be required:

\[
\text{ng of vector} \times \frac{\text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}
\]

Where ng of vector = 50, kb size of insert = 589, kb size of vector = 3000 and insert : vector molar ratio = 3:1. We therefore used as close to 29.45 ng of insert as possible (concentrations permitting).

All materials were sterilized prior to ligation by exposure for 15 minutes to UV light. Ligation reactions were set up in a 1.0 ml microcentrifuge tube as indicated in the protocol for the pGEM-T Easy Vector System (Technical Manual 042 available
on their website, Promega.com hereafter referred to as TM 042). Ligation reactions were then incubated overnight at 4°C.

Transformation was performed according to TM 042 in a 1.5 ml microcentrifuge tube using SOC medium and the JM109 Competent Cells (stored at -80°C) provided with the pGEM-T Easy Vector System II. Recipes for LB/Amp/IPTG/X-Gal plates, SOC medium, and IPTG are included at the end of the methods section.

100 µl of each transformation culture was added to one LB/amp/IPTG/X-Gal plate and spread using sterile glass beads. Plates were then incubated overnight at 37°C. After overnight incubation, Plates were transferred to 4°C for 12-16 hours. Six (large) white colonies from each transformation culture were then chosen for further analysis.

Colonies were removed from plates using a metal loop, which was heated to glowing red over a Bunsen burner before each use. The loop holding the colony was then shaken in a 15 ml glass tube (Fisher) containing 5 ml of LB/amp to release the colony. Samples were grown overnight at 37°C while shaking at ~200 rpm.

Following overnight growth, a plasmid miniprep was performed using the PureYield Plasmid Miniprep System (Promega). The centrifugation protocol was followed (Technical Bulletin 374) and the alternative protocol for larger culture volumes where 3 ml of culture medium is processed was used in order to maximize DNA yield.

In order to verify that the colony contained the correct length insert, a PCR was performed using pUC/m13 primers.
Forward (17mer): 5´-d(GTTTTCCCAGTCACGAC)-3´
Reverse (17mer): 5´-d(CAGGAAACAGCTATGAC)-3´

Amounts of reagents listed in Table 2.2

The following PCR program was used:

1) 94°C 2:00
2) 94°C :30
3) 44°C :45 40x
4) 72°C 1:40
5) 72°C 5:00
6) 4°C ∞

Table 2.2 Reagents and amounts for PCR

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH2O</td>
<td>35.75</td>
</tr>
<tr>
<td>Standard Taq Buffer (NEB)</td>
<td>5.00</td>
</tr>
<tr>
<td>2.5 mM dNTPs</td>
<td>1.00</td>
</tr>
<tr>
<td>25 mM MgCl2</td>
<td>3.00</td>
</tr>
<tr>
<td>20 M Primer (Forward)</td>
<td>1.25</td>
</tr>
<tr>
<td>20 M Primer (Reverse)</td>
<td>1.25</td>
</tr>
<tr>
<td>DNA</td>
<td>2.50</td>
</tr>
<tr>
<td>Taq DNA Polymerase (NEB)</td>
<td>0.25</td>
</tr>
</tbody>
</table>

The PCR products were then run with 1µl 6x gel loading dye (NEB) on a 1%
agarose gel for 30 minutes at 100 volts to verify amplification of desired band. The
samples that were successful were sent to the Yale DNA analysis facility for sanger
sequencing. Sequences were then curated and aligned using Clustal W multiple
alignment in Bioedit.
2.4 Buffers and Solutions

**IPTG Stock Solution, 0.1 M**

1.2g IPTG (Promega Cat. # V3955)

H2O to 50 ml, filter sterilized through 0.2 m filter unit, stored at -20°C

**SOC Medium**

2.0g Baco-tryptone

0.5g yeast extract

1ml 1M NaCl

0.25ml 1M KCl

1ml 2M Mg2+ stock solution (101.5g MgCl2, 123.3g MgSO4, H2O to 500 ml filter sterilized through 0.2m filter unit)

1ml 2M glucose, filter-sterilized
ddH2O to 100ml everything added except Mg2+ and glucose stirred, autoclaved, and cooled before addition of remaining ingredients and filter-sterilized through a 0.2 m filter unit.

**Amp/X-Gal/IPTG LB Plates**

10g tryptone

5g yeast extract

5g NaCl

1LH2O

15g Agar
Autoclaved and cooled to 50°C before addition of 0.1g ampicillin (TEKNOVA)

Pour plates

Plates were made up to 1 month before use

When ready for use, 100 μl IPTG and 20 μl of X-gal (Promega) were added to each plate, spread with a glass spreader sterilized with alcohol and flamed between every plate. Plates sat for 30 minutes before use to absorb liquids.
3. Results and Discussion

This paper constitutes an ongoing study. Because of the difficult nature of the experiment, this “results” section consists of a discussion of the development of our protocol as well as an explanation of the decisions made.

This study was originally designed to be performed using the blacknose dace, *Rhinichthys atratulus*. Another study in our lab being conducted currently is a phylogeography of the blacknose dace, looking at different gene haplotypes in relation to geography. We thought it would be interesting to expand this study by looking at the epigenome and therefore characterizing methylation patterns in a similar manner. When we examined fish from two different tributaries of the Connecticut river, we noticed a difference in pigmentation. The study therefore changed slightly as we wondered whether this difference was due to methylation in relation to differences in environment or in population.

After extracting DNA from the caudal fin clips of dace, we designed primers for PCR amplification using the genome of zebrafish, *Danio rerio*, which is in the same family, *Cyprinidae*. In order to improve our chances of successfully amplifying the promoter region, we chose genes that multiple Cyprinids possess *dct, silvb, spr, pcbd2, pam,* and *clot* (Braasch et al. 2007). Use of these primers lead to unspecific binding on the DNA (Figure 3.1). By raising the annealing temperature to increase specificity, we found that no DNA was amplified resulting in no bands on the gel. We concluded that the nucleotide sequences must have been too dissimilar to be amplified by PCR. Interestingly, another study in our lab has shown that primers designed using the *R. atratulus* genome are able to amplify another species in the
Rhinicthys genus, *R. Obtusus*. These findings pose the question: how related do two species have to be in order to have successful annealing of primers to DNA of both species? This is a question that, interestingly, has not been sufficiently addressed in the literature.

The decision was therefore made to attempt the same study with *G. aculeatus* because of the availability of nearly the entire genome (Ensembl.org).

![Figure 3.1. Gel of PCR of R. atratulus DNA with Danio rerio primers. Note the multiple bands indicating non-specific binding of primers. 100 bp ladder in lane 1.](image-url)

From observation of the samples we received from Dr. Michael A. Bell, we expected that *G. aculeatus* from different sites would differ with respect to pigmentation (Figure 3.2). In order to verify this, we placed fish into individual Petri dishes assigned random numbers. We then asked 7 individuals to group the fish based solely on pigmentation. This experiment was based upon Fraser et al. (2005) in which they aimed to verify morphological differences between *R. atratalus* and *R.*
obtusus. We found that the trials grouped the 25 samples into between 3 and 8 groups. Generally, they were able to separate the fish according to pigmentation with a few outliers. Bear Paw Lake and Seymour Lake were the easiest groups to separate. One trial had 100% success in separating the 25 fish into their respective 5 groups of 5 samples. Graphs of this data are located in appendix B. Further statistical analyses will be performed to better characterize the extent of this pigmentation difference. We expect to establish a correlation between water opacity and pigmentation level because of the advantages in escaping predation of blending in with the environment.
Figure 3.2 Pictures showing pigmentation differences between populations.

- a. Meadow Creek
- b. Rabbit Slough
- c. Tern Lake
- d. Stormy Lake
- e. Bear Paw Lake
- f. Mud Lake
- g. Seymour Lake

Note: pigmentation of only the caudal fin can be shown for Meadow Creek and Rabbit Slough populations due to limited availability of these samples. (other pictures can be found in Appendix A)
The initial protocol involved DNA extraction, bisulfite conversion, PCR, purification of the PCR product using the QIAquick PCR Purification Kit (QIAGEN) and direct sequencing of the PCR product. Unfortunately, when this protocol was attempted, the sequencing was of a very poor quality and therefore completely illegible (Figure 3.3).

In consultation with both the technicians at the Yale DNA Sequencing Facility and the scientists at Zymo Research Corporation, we made the decision to clone the PCR products into vectors thereby separating any other DNA sequences that may be amplified by the PCR and improve quality of the sequencing.

Cloning of PCR products with the pGEM-T Easy Cloning kit (Promega) was therefore added to the protocol. White colonies were selected and scraped off the plate using an autoclaved toothpick and put directly into a PCR tube with reagents listed in methods. Examination of the gel from this PCR (Figure 3.4), however, showed that the DNA needed to be extracted from the bacteria and purified before this PCR could be performed.
In order to maximize the volume of DNA before purification, the overnight growth step was added allowing each colony to multiply in growth medium (and therefore create more DNA). The Pure Yield Plasmid Miniprep System (Promega) was then used to isolate and purify the plasmid DNA. This protocol change lead to more successful gels after PCR (Figure 3.5).
Figure 3.5 Gel of PCR products after purifying plasmid DNA with miniprep.
Because the control insert with the cloning kit is ~500 bp and our insert is ~550 bp, we are able to use the positive control band to determine which clones contained the insert. Lanes 2-7 (sample 22) do not have the same insert since they are at the same level as the negative control in lane 15. Lanes 8-13 (sample 27) do contain the insert since they are at the same level as the positive control in lane 14. 100 bp ladder in lane 1.

Using this protocol, we successfully sequenced one sample (27), obtaining a good quality sequence that showed our initial primers (Figure 3.6). Unfortunately, however, there seemed to be a discrepancy in cytosine and thymine throughout the sample. This indicated an incomplete C to T conversion during the bisulfite conversion. The second step of the protocol was therefore modified in an effort to improve the chances of achieving complete conversion.
Figure 3.6 Curated and aligned sequences of sample 27. Blue boxes indicate forward primers for insert.

a. aligned sequences. Purple arrows indicate discrepancies between the two sequences between cytosine and thymine

b. and c. show the base calls supplied by the Yale DNA Analysis Facility. Note that sequences show equal clarity of sequencing.

Figure 3.7 Most recent curated and aligned sequences, note consistency between forward and reverse. The presence of Cs not bound to a G, however, indicates incomplete bisulfite-conversion.
Subsequent attempts to sequence the bisulfite converted DNA, however, proved unsuccessful. We repeatedly received sequences from the Yale facility that did not contain our primers, initially making us think that there had been a mix-up with our samples. This was both frustrating and baffling. The gels appeared to contain one clear band with the right base pair length (Figure 3.8). After months of repeating the experiment with small modifications of protocol, we hypothesized that there may be non-specific binding of our initial primers that for whatever reason was not visualized on the gel. In fact, when we looked at the gel on another UV transilluminator it became clear that there were additional smaller and less present products that could be interfering with the final sequencing. We therefore decided to use the QIAquick gel extraction kit (QIAGEN) to extract the DNA only from the band with the correct base pair length. This purification step therefore replaced the original purification of PCR product using the QIAquick PCR Purification Kit (QIAGEN).
Figure 3.8 Gel of bisulfite converted PCR product prior to Ligation. Note existence of only one band at the correct location on the gel (~500 bps). 100 bp ladder in lane 1.

After altering our protocol to include gel purification, we were finally able to successfully sequence samples, confirming our previous hypothesis that the incorrect sequences were due to non-specific amplification during PCR (Figure 3.7). Unfortunately, while these sequences do contain our primers and, unlike our first attempt, show consistency between forward and reverse sequences, there appear to be ongoing problems with incomplete conversion. This is most likely due to a problem of which we were initially unaware where a buffer in the EZ DNA methylation kit (Zymo) denatures with prolonged exposure to air. Now that the protocol has been proved successful otherwise, we expect to receive fully converted sequences when this protocol is repeated with new reagents.
4. Conclusion and Future Directions

While we have no conclusive results at this time because this paper is a discussion of an ongoing project, it is nonetheless valuable as an introduction to this study, an explanation of the design of this experiment, and a description of a working protocol for sequencing of bisulfite-converted DNA. The development of this protocol has unearthed some technical issues of which we were previously unaware. Bisulfite conversion is a notoriously difficult procedure and the development of an “easy” kit by Zymo is a huge step in making this procedure more accessible. Zymo’s protocol, however, is lacking in its disregard of the fact that it seems to be necessary to clone bisulfite-converted DNA prior to sequencing. Additionally, Zymo’s failure to advertise the denaturation of M-dilution buffer upon limited exposure to air seems a huge oversight that should be corrected with immediate publication of this issue.

This project will be continued with repetition of the protocol described in this paper on all samples. Additionally, we hope to repeat this study with the promoter regions of other genes associated with pigmentation. Ultimately, we will further study the link between methylation, pigmentation, and environment. This study will serve as a characterization of variation of methylation on the level of individual cytosines in both a population and environmental context.

As our understanding of the importance of epigenetic regulation continues to grow, studies such as this one will become increasingly valuable. While clinical research has, up until now, dominated the field, it is time to begin to characterize and understand the epigenome as we have the genome. This study takes methylation out of not only the clinic but also the laboratory by using samples from the wild allowing for the exploration
of “real-world” environmental effects. The scientific community’s discovery of the molecular mechanisms surrounding epigenetics as well as the development of methods for visualization of this regulation has made it possible for us to now expand investigation of epigenetics outside of the cell. The discovery of epigenetic regulation has increased our understanding of the possible influences the environment has upon the process of creation of a fully differentiated organism. Upon completion, this study will add information to our collective understanding of the nature of epigenetics as the link between ecology and development.
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References


Appendix A. Pictures of Samples

Samples 2-5 from Meadow Creek

Samples 7-10 from Rabbit Slough
Samples 12-15 from Tern Lake

Samples 17-20 from Stormy Lake

Samples 22-25 from Bear Paw Lake
Samples 27-30 from Mud Lake

Samples 32-35 from Seymour Lake
Appendix B. Graphs of Pigmentation Trials

Trial 1

Trial 2

Trial 3

Red: BL  Orange: TL
Green: ML  Blue: SL
Purple: SeL
Red: BL   Orange: TL
Green: ML   Blue: SL
Purple: SeL