DOCTOR OF SCIENCES

Adaptation and evolution with low genetic diversity

a combined field and laboratory study on DNA methylation variation in the mangrove rivulus Kryptolebias marmoratus

Chapelle, Valentine

Award date:
2023

Awarding institution:
University of Namur

University of Namur
Faculty of Sciences
Department of biology
Laboratory of evolutionary and adaptive physiology

Adaptation and evolution with low genetic diversity: a combined field and laboratory study on DNA methylation variation in the mangrove rivulus *Kryptolebias marmoratus*

A dissertation submitted by
Valentine CHAPELLE
In partial fulfillment of the requirements
for the degree of PhD in Biological Sciences
Mars 2023

Members of the jury:
Pr. Alice Dennis (President, UNamur, Belgium)
Pr. Frédéric Silvestre (Supervisor, UNamur, Belgium)
Pr. Caroline Nieberding (Co-supervisor, UCL, Belgium)
Pr. Ryan L. Earley (UAlabama, USA)
Pr. Sofia Consuegra (Swansea University, UK)
Dr. Victoria Suarez-Ulloa (Panacea Cooperative Research, Léon, Spain)

SUMMARY

Many studies emphasized epigenetic mechanisms as a third source of phenotypic variation on a range of fitness-related phenotypic traits including animal behaviors. Investigating the origins and patterns of epigenetic variation (e.g. epimutations) with an evolutionary point of view is challenging as it requires natural (non-artificial) control of genetic variation among individuals to avoid genetic interference. An emerging and valuable vertebrate model, the mangrove rivulus *Kryptolebias marmoratus*, possesses a mix-mating reproductive strategy that allows us: (1) to naturally produce isogenic lineages through self-fertilization and therefore to focus on epigenetic origins independently from underlying genetic variation and (2) to generate a genetic diversity gradient by controlling the balance between selfing and outcrossing rates and therefore to investigate how epigenetic and genetic sources interact. By using this model species, this thesis aimed to **determine the role of DNA methylation in the adaptation and evolution of mangrove rivulus *Kryptolebias marmoratus* by investigating its variation and sources within and among mangrove rivulus populations from the wild or reared under standardized laboratory conditions.** We choose individual and consistent behavioral variation (i.e. personality traits) as an endpoint to represent the capacity of DNA methylation to generate phenotypic variability

Ecological epigenetics have mostly progressed with studies that investigate DNA methylation variation under laboratory conditions, and later with population epigenetics studies on natural plant populations. Now that this research has established a solid basis on the extent of epigenetic variation and its dynamics over time and context, scientists are reaching the next level with wild animal population epigenetics. **The first article of this thesis is a review** describing epigenetic variation in wild animal populations encountering natural levels of genetic and environmental heterogeneity. This field requires a review because it is fast moving research area, and there is a set of recent advances that need to be brought together to provide new insights in this domain. As insights from this review, DNA methylation diversity has been found to be an important parameter to characterize natural animal populations. There was as much obligatory (genome-dependent) as pure (genome-independent) epigenetic variation in wild populations depending on the studies and the species of interest. These results contrast with similar studies in plants that mainly show a strong correlation between patterns of epigenetic variation and underlying genetic variants. Otherwise, as genetic variation can blur

the role of epigenetic variation, studies in which the confounding effects of genetic variation have been controlled or reduced may be useful for isolating the contributions of epigenetic mechanisms in evolutionary processes.

Research focused on populations with a lack of genetic variation showed substantial epigenetic diversity and habitat-specific methylome created by pure epimutations. The mix-mating reproduction system of the mangrove rivulus *Kryptolebias marmoratus* can be used to go even further into the analysis of epigenetic-genetic variations interaction. For the first time, this question can be addressed in a species naturally found under genetically-diverse or isogenic and homozygous populations, allowing us to cover a vast spectrum of genetic diversity configurations of a single species. **The second article of this thesis is a field experiment** comparing epigenetic and behavioral variation in four wild rivulus populations possessing a gradient of genetic diversity, including a quasi-clonal population at Emerson Point Preserve (EPP). We found similar level of epigenetic variation within all four populations, regardless of genetic heterogeneity. Moreover, the functional enrichment analysis of genes where differentially methylated cytosines occur within a population showed shared pathways between the four populations, while a smaller proportion were population-specific. Another intriguing result is that individuality (consistent among-individual behavioral variation) emerged in EPP and another Floridan population, but not in the most genetically-diverse population from Belize.

Emerson Point Preserve is a very special population as we found almost no genetic diversity, similar level of epigenetic diversity to other genetically-diverse populations, and significant variation in personality traits i.e. individuality. We hypothesized that this significant phenotypic variation could come from pure epimutations, including environmentally-induced and random epimutations, but we cannot evaluate their contribution to the generation of individuality as environmental variation in this field study is difficult to describe. It is therefore not possible to link an epigenetic pattern to a given environment. **The third article of this thesis is a laboratory work** investigating the question of random and environmentally induced epimutations in an isogenic lineage of rivulus from EPP under standardized environment. We ran an ecotoxicological experiment, with an exposition to methylmercury (MeHg) as it can create permanent transgenerational effects on DNA methylation and behaviors in the zebrafish. Thus, we aim to distinguish potential environmentally (MeHg)-induced epimutations in the exposed groups, and natural rate of random epimutations in the control group of mangrove

rivulus, and to link them to gene expression and behavioral variation. We exposed rivulus larvae to MeHg from 0 to 7 days post-hatching (dph), and evaluated immediate effects on DNA methylation, gene expression and behaviors at the end of the exposure, but also delayed effects in adults rivulus (90 dph). As results, MeHg exposures are associated with behavioral alterations and gene expression changes in 7dph larvae, but none of those genes underwent methylation changes in targeted CpGs. None of the significant behavioral and molecular impairments observed in 7-dph larvae were found in 90-dph adults, which highlight a distinction between immediate and delayed effects of developmental MeHg exposure. Interestingly, we showed that behavioral variation arises in isogenic lineage reared in standardized environments, but not individuality as shown in the previous field study with their wild parents.

In our discussion, we compared results from the three articles to reach our main objective to investigate the role of DNA methylation in the adaptation and evolution of mangrove rivulus *Kryptolebias marmoratus* by studying its variation and sources of within and among populations from the wild or reared under standardized laboratory conditions. The comparison of our laboratory and field studies generated new insights to achieve this goal by helping us to disentangle epimutations sources, to understand how behavioral individuality arises and what could be the underlying adaptive and evolutionary strategies. It turns out that there is a discontinuity between epigenetic and genetic distance among rivulus populations, and between epigenetic and genetic diversity within populations, highlighting that pure epimutations (environmentally-induced and/or random) do occur in wild rivulus populations. There is less epigenetic variation under standardized experimental conditions than observed into the wild for the same genotype, supporting the role of environmental variation, random (epigenetic drift) and non-random (epigenetic clock) events in the generation of epimutations. However, obligatory and facilitated epimutations are not excluded as genetic and epigenetic distances match for some populations. Our results on individuality suggest that natural conditions (environmental heterogeneity, selection pressure, epigenetic drift,…) may maintain individuality in some rivulus population, while high intra-individual variation (plasticity and predictability) could prevent the emergence of individuality in others. Mangrove rivulus can be useful to investigate adaptive and evolutionary processes mediated by epigenetic mechanisms such epigenetic buffering, phenotypic plasticity, bet-hedging strategies, the Baldwin effect, phenotypic convergence, and genetic assimilation.

THESIS CONTEXT AND STRUCTURE

The current evolutionary biology theory primarily involves genetic alterations and random DNA sequence mutations to generate the phenotypic variation required for natural selection to act. This is the Modern Evolution Synthesis (MS) and has been the primary evolutionary theory for nearly 100 years. Nowadays, many biologists feel that the foundations of the MS are crumbling, and that the construction of a new evolutionary paradigm is underway. The winds of change in evolutionary biology are blowing from many directions (from developmental biology, behavior, microbial biology, ecology, and cultural studies) and highlight non-genetic processes and non-genetic inheritance that can underwrite the development of organisms and the inheritance of characters. Among them, epigenetics is an interesting target as it has been demonstrated to generate heritable phenotypic variation independent of genetic sequence alterations, in response to environmental changes or randomly, and hence can contribute to evolutionary changes. Epigenetics mechanisms are included in the 'extended evolutionary synthesis' (EES) which is characterized by the view that the direction of evolution does not depend on selection alone, and need not start with mutation. This synthesis aims to extend, rather than refute, the Modern Synthesis (Gilbert et al., 1996; Pigliucci et al., 2010; Skinner et al., 2015; Skinner & Nilsson, 2021)

Recent studies emphasized the effect of epigenetic mechanisms as a "third source of phenotypic variation" on a range of fitness-related phenotypic traits including animal behaviors, as significant behavioral variation among individuals (i.e. personalities) arises in the absence of genetic diversity and environmental variation (Bierbach et al., 2017; Laskowski et al., 2022). Behavior constitutes the central core of the interactions between an organism and its environment, and personality traits (individual behavioral differences that are consistent across time and context) are phenotypes of particular interest for behavioral ecologists due to their high influence on organisms' fitness and therefore on ecological and evolutionary processes. Investigating the role of epigenetic variation in the generation of behavioral variation under an evolutionary point of view is challenging as it requires the natural (non-artificial) control of genetic variation among individuals. An emerging vertebrate model, the mangrove rivulus *Kryptolebias marmoratus*, presents a mix-mating reproductive strategy that allows us (1) to naturally produce isogenic and homozygous lineages through self-fertilization and therefore to focus on epigenetic sources of behavioral variability and (2) to generate a genetic diversity

gradient by controlling the balance between selfing and outcrossing rates and therefore to investigate how epigenetic and genetic sources interact in the generation of behavioral variability. Beyond answering fundamental questions of the molecular bases of personality traits, investigating how random and environmentally-induced (i.e. genetic-independent) epigenetic variation generate phenotypic variation and how it balances with genetic variation in this process will join the accumulating evidences supporting the construction of a new evolutionary paradigm including non-genetic processes.

In this context, this thesis research project aimed to **determine the role of DNA methylation in the adaptation and evolution of mangrove rivulus *Kryptolebias marmoratus* by investigating its variation and sources within and among populations from the wild or reared under standardized laboratory conditions**. This manuscript is separated in 6 chapters:

- **Chapter 1: State of the art.** A bibliographic synthesis presenting the general concepts involved in this thesis. First, the place of epigenetic mechanisms in evolutionary mechanisms is developed, with a description of epigenetic mechanisms and more precisely, DNA methylation marks, their causes, consequences, and inheritance. Then, several concepts of animal behavior as well as personality traits in the animal kingdom are described. In this section, particular attention is dedicated to behavioral individuality (among-individual variation) and to plasticity and predictability (within-individual variation). Finally, the ecology, reproductive features, behavior and the genetic and epigenetic characteristics of the model organism are presented.

- **Chapter 2: Objectives**. Based on the state of the art, this section develops the general and specific objectives of this thesis, as well as our hypotheses and how we will test them.

- **Chapter 3: Epigenetic variability in wild animal populations**. This chapter aims to describe epigenetic variation in wild animal populations encountering natural levels of genetic and environmental heterogeneity. It is divided in two sections. Section 1 includes a review on population epigenetics and the extent of DNA methylation in wild animal populations, as no review has been written on this subject before (**ARTICLE 1**). Section 2 includes a field study of epigenetic, genetic and behavioral variation within and among four

wild populations of mangroves rivulus showing a selfing rate gradient, which generate genetic diversity gradient (**ARTICLE 2**).

- **Chapter 4: Epigenetic variability in isogenic lineage reared under laboratory conditions**. This chapter aims to characterize the sources of DNA methylation variation (named epimutations) in mangrove rivulus reared under controlled conditions and exposed to an environmental stressor. It includes an ecotoxicological study on the effects of early-life exposure to methylmercury on personality traits and the underlying mechanisms (gene expression changes and environmentally-induced epimutations) in one isogenic lineage of mangrove rivulus fish *Kryptolebias marmoratus,* which are offsprings of wild rivulus involved in the previously described field study (**ARTICLE 3**).

- **Chapter 5: General discussion and perspectives**. This chapter includes a synthesis of the main results obtained during this thesis, new results arising from the comparison of our different studies data, as well as a general discussion and proposes research perspectives to this work**.**

- **Chapter 6: Conclusions.**

TABLE OF CONTENTS

CHAPTER 1: STATE OF THE ART

1. Epigenetics: an underappreciated component of evolution

1.1 Embedding epigenetics in the Extended Evolutionary Synthesis

Understanding the processes that lead to between-individual phenotypic variation, on which natural selection can act, is a central issue in biology. According to the Modern Evolution Synthesis, evolutionarily significant phenotypic variation arises from genetic mutations that occur at a low rate independently of the strength and direction of natural selection. This current evolutionary biology theory has been challenged by the advances in developmental biology, genomics and ecology. For example, the slow spread of genetic mutations does not explain all of micro- and macroevolutions observed in natural populations, and they cannot keep pace with the rapidly changing environment (Eva & Lamb, 2008; Huneman & Walsh, 2017; Laland et al., 2015; Skinner & Nilsson, 2021). Quantitative genetic studies also pointed out some inconsistency of this theory. By manipulating environmental factors and genotypic variation, they confirmed that genotype, environment and their interaction contribute to phenotypic variation, illustrated by the following equation: $V_P = V_G + V_E + V_{GxE}$, where V_G is the genetic variability (differences in alleles), V_E the environmental variability (differences in environmental cues that modify gene expression) and V_{GxE} the variability of genetic x environment interaction. Previous research tested this paradigm by using highly-inbred, isogenic, monozygotic or clonal organisms reared in highly standardized environmental conditions, and showed substantial amount of between-individual variation in morphological, physiological and behavioral traits (Bierbach et al., 2017; James et al., 2018; Vogt, 2017; Vogt et al., 2008). Arguments from evolutionary developmental biology ('evo-devo'), developmental plasticity, inclusive inheritance (beyond the transmission of traits by passing on of genes) and epigenetics are particularly instructive, and points toward an alternative framework labeled the 'extended evolutionary synthesis' (EES) as it will extend, rather than refute, the Modern Synthesis (Gilbert et al., 1996; Pigliucci et al., 2010; Skinner et al., 2015; Skinner & Nilsson, 2021). The EES is characterized by the view that the direction of evolution does not depend on selection alone and need not start with mutation. The EES included additional processes that generate novel variation (e.g. epigenetic effects, regulation of gene expression, construction of internal and external developmental environments), processes that

bias the outcome of natural selection (developmental bias and niche construction) and also processes that contribute to inheritance (epigenetic, cultural and ecological inheritance) (Table 1), and thus provides a considerably more complex account of evolutionary mechanisms than traditionally recognized by including non-genetic processes (Figure 1).

Table 1: Glossary of processes included in the extended evolutionary synthesis

Term	Definition
Phenotypic accommodation	Adaptive adjustment, without genetic change, of variable aspects of the phenotype following a novel input during development.
Niche construction	The process whereby organisms transform environmental states, and thereby modify the selection pressures to which they, and other organisms, in current and subsequent generations, are exposed.
Developmental bias	The non-random generation of phenotypes by developmental systems, with variants sometimes being channeled or directed by the processes of development towards functional goals.
Inclusive inheritance	Inheritance extends beyond genes to encompass (transgenerational) epigenetic inheritance, ecological inheritance, social (behavioral) transmission and cultural inheritance.
Ecological inheritance	The accumulated environmental changes, and associated selection pressures, that previous generations have brought about through their niche-constructing activity, and which descendant organisms inherit.
Cultural inheritance	Transmission of information by communication, imitation, teaching and learning from members of a generation to the next generation, e.g. from parents and other members of the population to which they belong.
Epigenetic inheritance	Transmission of epigenetic markers from cell to cell, or to one organism to the next (transgenerational epigenetic inheritance) that affects the traits of offspring without altering the DNA sequence.

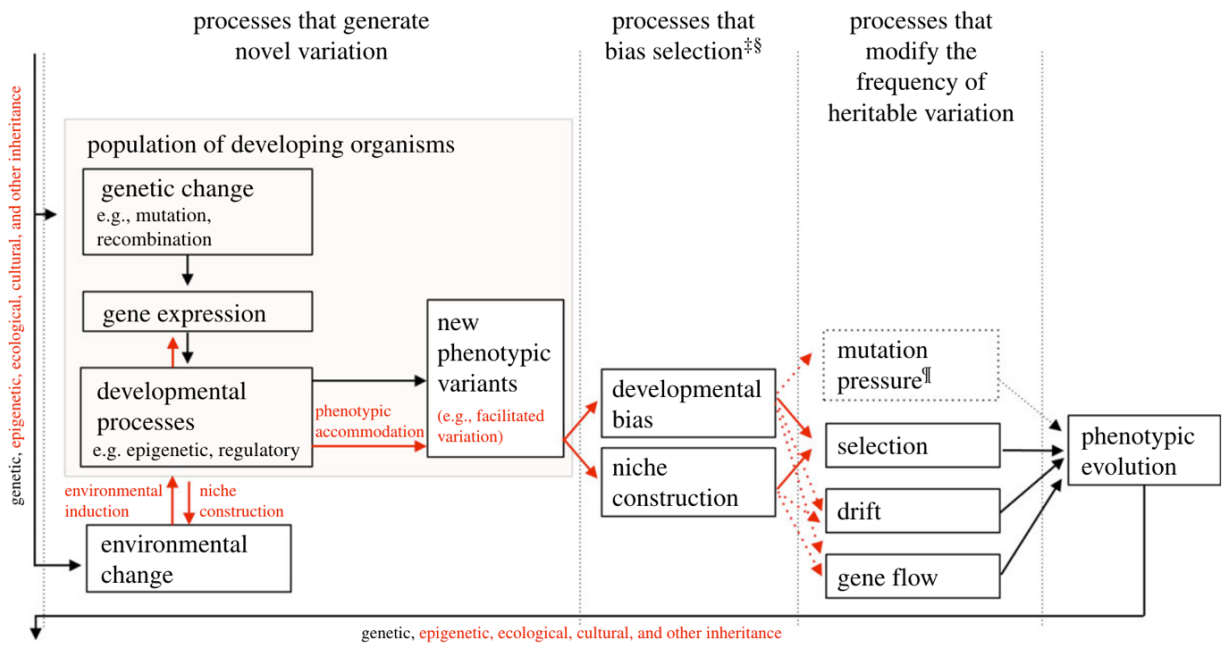


Figure 1 *The structure of the EES. The EES includes as evolutionary causes processes that generate novel variants, bias selection, modify the frequency of heritable variation (including, but not restricted to, genes) and contribute to inheritance. A variety of developmental processes (e.g. epigenetic effects, regulation of gene expression, construction of internal and external developmental environments) contribute to the origin of novel phenotypic variation, which may be viable and adaptive (i.e. 'facilitated variation'). In addition to accepted evolutionary processes that directly change gene frequencies, the EES recognizes processes that bias the outcome of natural selection, specifically developmental bias and niche construction. All processes that generate phenotypic variation, including developmental plasticity and some forms of inclusive inheritance, are potential sources of bias. A broadened conception of inheritance encompasses genetic, epigenetic and ecological (including cultural) inheritance. Arrows represent causal influences. Processes shown in red are those emphasized by the EES, but not a more traditional perspective. ¶ Mutation pressure refers to the population-level consequences of repeated mutation, here depicted as dashed because mutation is also represented in 'processes that generate novel variation'. ‡In the EES, this category of processes will often need to be broadened to encompass processes that modify the frequencies of other heritable resources. §Developmental bias and niche construction can also affect other evolutionary processes, such as mutation, drift and gene flow. Figure from figure 2 Laland et al., (2015).*

Epigenetics, one of the emerging non-genetic areas in the EES, is the focus of this thesis. Epigenetics refers to the study of heritable changes in gene expression and function without alterations in the DNA sequence (Richards, 2006). The three main epigenetic mechanisms that have been well studied so far are (1) chromatin remodeling mainly by chemical modification of histones, (2) RNA interference by non-coding RNAs, and (3) DNA methylation (Russo et al., 1996; Stedman & Stedman, 1950) (Figure 2). The importance of these epigenetic mechanisms has long been valued at the molecular level (e.g., its role in cellular differentiation, metabolism and self-recognition), but their role in evolution and ecology is a more recent focus. It turns out that epigenetic mechanisms interact with genetic, physiological, and morphological

systems and may play critical roles in phenotypic plasticity (Kilvitis et al., 2017; Schlichting & Wund, 2014), soft inheritance (Burggren, 2016; Richards, 2006) and, more generally, in organism-environment interactions (Angers et al., 2010; Richards et al., 2010).

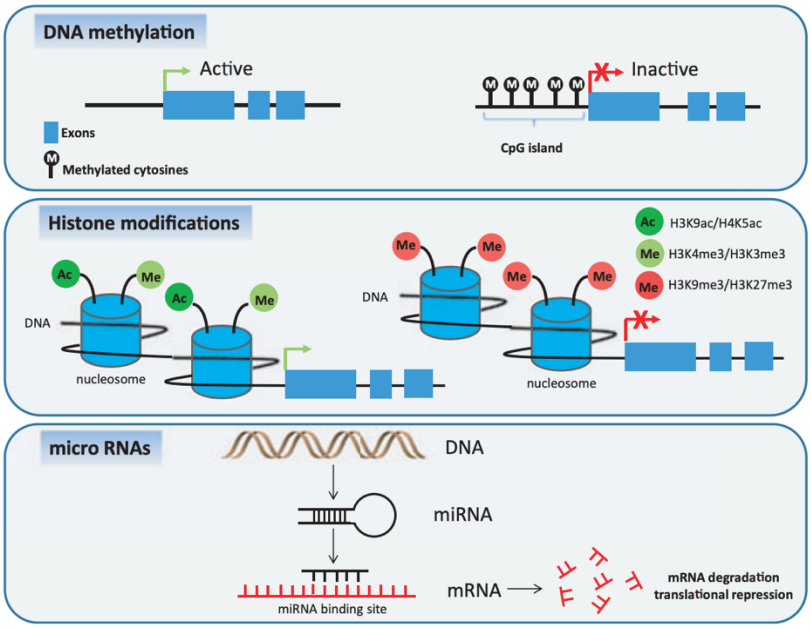


Figure 2: Three epigenetic mechanisms. (1) DNA methylation is the covalent modification of cytosine residues within gene sequences, typically in CpG dinucleotides in animals. It leads most of the time to transcriptional silencing, with some exception of transcriptional activating cases. (2) The N-terminal tails of histones can undergo a variety of post-translational covalent modifications, including (de)methylation and (de)acetylation. Histone modifications can lead to either activation or repression of gene transcription, depending upon which residues are modified and which modifications take place. (3) Noncoding RNAs include microRNAs (miRNAs) that regulate gene expression through post-transcriptional silencing of target genes. Sequence-specific base pairing of miRNAs with 3' UTRs of messenger RNAs results in target degradation or inhibition of translation. Figure from (D'Addario et al., 2013).

1.2 DNA methylation: mechanisms and distribution

This thesis focuses on DNA methylation, the most extensively characterized epigenetic mechanism in both plants and animals (de Mendoza et al., 2020). It plays important roles in diverse cellular processes such as DNA imprinting (Ferguson-Smith, 2011), X-inactivation (Lyon, 1961), silencing transposable elements (Slotkin & Martienssen, 2007), and in response to environmental stressors (Ardura et al., 2018). It is found across all taxa of life and refers to the transfer of a methyl group ($-CH_3$) from S-adenosyl-L-methionine (SAM) on the 5-methylcytosine bases (m5C, the 5^{th} carbon of the pyrimidine ring) in eukaryotes and prokaryotes. The transfer of a methyl group predominantly happens on cytosine followed by

guanine residues (CpG) in animals. Methylation of high-density CpG regions are called CpG islands and has been extensively described as a mechanism associated with the regulation of gene expression, mostly, with gene expression silencing. The methyl groups cause either a modification of the DNA surface which disrupt its recognition by various proteins including transcription factors, or allow the binding of methylated DNA binding proteins (MDBPs), which bind to certain DNA sequences only when they contain m5C residues at specific positions. This can create a chain reaction ending in recruitment of histone desacetylase (HDAC) enzymes leading to chromatin compaction. MBPs can also lead to steric congestion that inhibits the binding of transcription factors to DNA (Wu et al., 2011). The DNA methylation of regulatory regions is thus generally associated with gene down-regulation or silencing, but that is not always the case. Recent studies have showed that gene body methylation is positively correlated with transcriptional activity in most animal species (Rauluseviciute et al., 2020; Spainhour et al., 2019).

Three conserved DNA methyltransferases (DNMTs) responsible of DNA methylation have been described in vertebrates: DNMT1, DNMT3a and DNMT3b (Figure 3) (Goll & Bestor, 2005). DNMT1 is responsible of restoring DNA methylation after DNA replication, which ensures the fidelity of this DNA methylation patterns across cell divisions. In line with this role, it has a strong preference towards methylation of hemimethylated DNA. DNMT3a and DNMT3b catalyze *de novo* methylation by targeting hemimethylated and unmethylated CpG at the same rate. It is primarily responsible for the establishment of genomic DNA methylation patterns and play an important role in processes such as DNA imprinting and X-inactivation. Active and passive demethylation processes can also take place in the organism. This reversibility of DNA methylation is essential as the erasure of epigenetic markers is required for some processes including the proper development of embryo (He et al., 2017). Active demethylation occurs through the oxidation of the 5-methyl group of cytosine by the TET proteins (TET1, TET2 and TET3). The 5-hydroxymethylcytosine (5hmC) generated is further oxidized into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) followed by base excision repair resulting in unmethylated cytosine (Ito et al., 2011). The active process can also occur through deamination of 5mC and 5hmC resulting in unmethylated cytosine (Jang et al., 2017). Passive demethylation takes place in the absence of methylation of newly synthesized DNA strands by DNMT1 during replication. Due to this impairment, 5mC is progressively diluted after DNA replication. Passive DNA demethylation has been shown to promote reprogramming

(He et al., 2017). This mechanism occurs both in the germline and in the zygote immediately after fertilization in animals and corresponds to an extensive erasing and remodeling of DNA methylation during gametogenesis and embryo development. Its purposes include the erasure and reestablishment of parental genomic imprints in germ cells, and the correct development of the embryo through the generation of totipotent or multipotent cells. The reprogramming process has been described in a species such as mice (Reik et al., 2001), zebrafish (Mhanni & McGowan, 2004), killifish (Fellous et al., 2018), and medaka (Wang & Bhandari, 2019), and show species-specific characteristics.

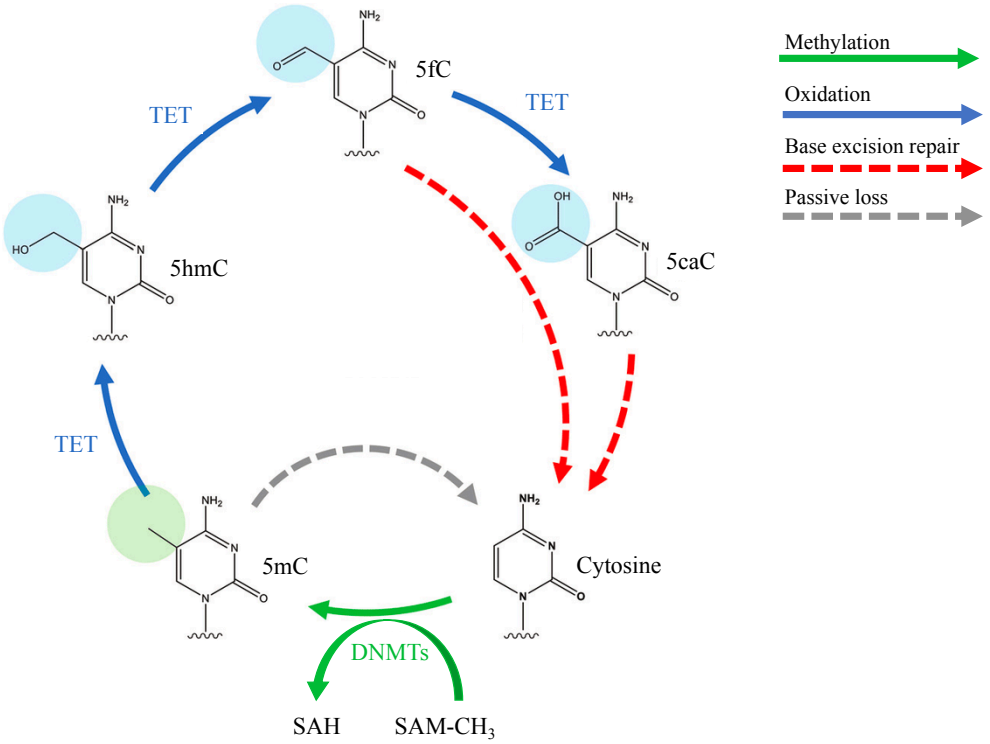


Figure 3: DNA methylation and demethylation. DNA methyltransferases (DNMTs) deposit and maintain methyl-groups on selected cytosines. TET enzymes successively oxidize 5mC to 5hmC, 5fC and 5caC. 5fC and 5caC are recognized, excised and replaced via base excision repair. All modifications can be by a passive mechanism via dilution during DNA replication in the absence of maintenance activity. Adapted from Ravichandran et al., 2017.

The distribution of DNA methylation across genome has been described in many clades of animals, but there are some differences in how and where it occurs. In vertebrates, the pattern of DNA methylation is well conserved across species; DNA methylation occurs nearly throughout the entire genome, with 70–80% of cytosines in the CpG dinucleotides being methylated (Feng et al., 2010). Gene bodies, including exons and introns, are typically methylated, while CpGs in the gene promoter regions are often lowly methylated (Hon et al., 2013; Suzuki & Bird, 2008). The idea that only vertebrates have a highly methylated genome

has recently been challenged as this phenomenon has also been found in the sponge *Amphimedon queenslandica* and an unicellular green alga from the genus *Chlorella* (de Mendoza et al., 2019; Zemach et al., 2010). Regarding invertebrates, DNA methylation patterns are extremely variable across taxa. Some invertebrate genomes lack cytosine methylation such as the nematode *Caenorhabditis elegans*, the platyhelminth *Schmidtea mediterranea*, the fruit fly *Drosophila melanogaster,* and the rotifer *Adineta vaga* (Glastad et al., 2014; Rae & Steele, 1979; Simpson et al., 1986), while others are similar to plants as they have a mosaic of heavily methylated DNA domains (predominantly in exons) that are interspersed with domains that are methylation-free, such as the sea anemone, honey bee, and sea squirt (Xu et al., 2019; Zemach et al., 2010).

1.3 DNA methylation variation: evolutionary relevance

To determine the implications of DNA methylation in evolution, a major concern is to identify the degree of autonomy between epigenetic and genetic variation and ultimately, the degree of phenotypic variation that can be explained only by genotype-independent epigenetic variation. This is important because the effects of epigenetic variation on phenotypic plasticity and evolution can be subsumed into the effects of genetic variation if epimutation is guided by underlying genetic variation (Hu & Barrett, 2017). Another concern is to determine if epigenetic marks variation (named epimutations) can be transmitted to the offspring through transgenerational epigenetic inheritance (TEI). Laboratory studies on plants and animals have shed light on some of the general features of epigenetics, with important evolutionary implications related to these concerns.

Firstly, epimutations can be independent from the underlying genotype. Based on their degree of autonomy from genome, epimutations are categorized into three types: obligatory, which is completely dependent on the genetic variation; facilitated, which is directed or loosely potentiated by the genotype; pure, which is independent of the genetic variation and is generated by stochastic events or environmental changes (Richards, 2006). Random epimutations can arise at any time in the lifespan of the organism, and they are not induced by environmental factors. They were assessed to be up to five orders of magnitude more frequent than genetic mutations were (10^{-4} versus 10^{-9} per base pair and generation) in the model plant *Arabidopsis thaliana* (Schmitz et al., 2011; van der Graaf et al., 2015). Random epimutations have been

proposed to be among mechanisms underlying the diversified bet-hedging strategy, which is based on the production of phenotypically variable offspring, irrespective of environmental conditions. According to this evolutionary strategy, a single genotype produces a range of phenotypes across offspring with the aim to increase the likelihood that, at least, some individuals are well-adapted to the selection pressure of unpredictable environments. On the other hand, environmentally-induced epimutations can create habitat-specific methylome and has been proposed to mediate phenotypic plasticity, as they can shape phenotypic responses to environmental variation, facilitating adaptation, speciation, and adaptive radiation (Angers et al., 2010). The responsiveness of environmentally-induced epimutation can help populations facing rapid, fluctuating environmental changes. This phenomenon is named epigenetic buffering, and could facilitate evolutionary rescue through heritability of environmentally induced phenotypes, reducing genetic loss and increasing the probability of genetic mutation, all reviewed in (O'Dea et al., 2016).

Although the transfer of environmentally-induced epigenetic modifications through mitotic and meiotic processes is relatively well established, research on multigenerational epigenetic inheritance is considerably more challenging. It is important to distinguish between intergenerational and transgenerational epigenetic inheritance (Figure 4) (Tuscher & Day, 2019). Intergenerational inheritance involves exposure of the parent generation (F0, male or non-pregnant female) to an environmental factor that leads to epimutations in the parent and likely the parental germline cells (F1; sperm or oocytes). Observation of similar epimutations or phenotypic traits in offspring through this type of F0 to F1 transmission would be considered an intergenerational effect (Figure 4). Unlike intergenerational inheritance, transgenerational inheritance relies strictly on germline transfer of epimutations in the absence of any direct environmental exposure. As such, for an effect to be considered transgenerational, an inherited epigenetic mark and corresponding trait would need to persist in the F2 generation. For pregnant females that experience environmentally-induced epimutations, the pregnant female (F0), fetus (F1), and fetal germline cells (F2) would all be exposed to the external stimulus. In this case, intergenerational effects encompass transmission from F0 to F2, and only persistence to the F3 generation would be considered a transgenerational effect. This TEI has been reported in many plant and animal taxa such as mammals (Manikkam et al., 2012), birds (Guerrero-Bosagna et al., 2018), fish (Bhandari et al., 2015), and invertebrates (Liew et al., 2020; Seong et al., 2011). Moreover, pure epimutations can cause heritable environmentally-induced phenotypic

variation through transgenerational epigenetic inheritance (Blanc et al., 2021; Casier et al., 2019; Guerrero-Bosagna et al., 2018).

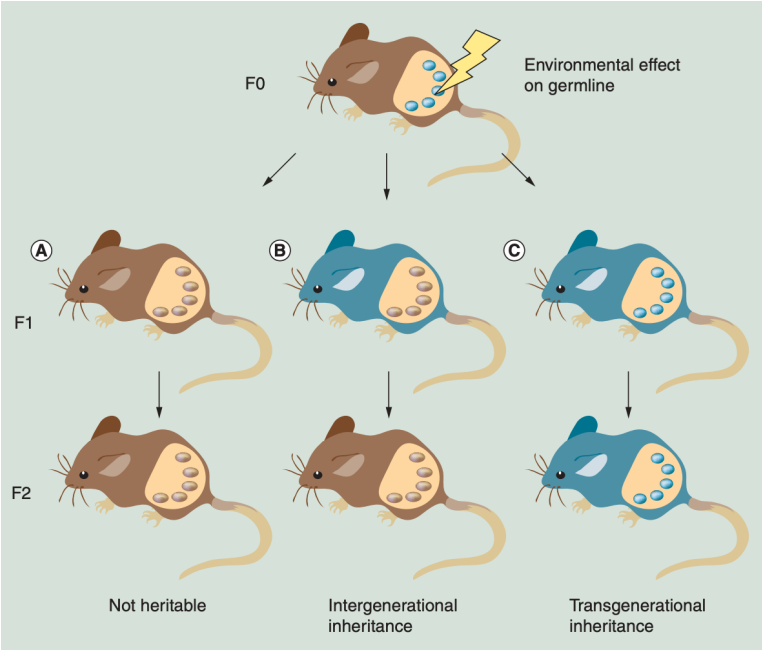


Figure 4: Modes of epigenetic inheritance. Exposure of the F0 germline to an environmental change can cause epimutations that may or may not be inherited. (A) No inheritance: epimutations in the parental F0 germline do not affect the offspring (F1). Such epimutations are presumably corrected during F1 reprogramming. (B) Intergenerational inheritance: epimutations transmitted through the F0 germline escape reprogramming and alter development in the F1 generation. However, these epimutations are corrected in the germline of F1 animals and are not transmitted to the F2 generation. (C) Transgenerational inheritance: epimutations escape reprogramming in F1 and subsequent generations and affect development over multiple generations. From Prokopuk et al., 2015.

From these relevant characterizations of epimutations under different laboratory settings, an important step is now to examine the extent of epigenetic variation and the way that this variation changes over time and across generations in wild populations that encounter natural levels of environmental complexity, genetic structure and dynamics, and natural ecological processes. This endeavor represents part of the field of population epigenetics. This topic is not detailed here, as Chapter 3 includes a review on "Population epigenetics: The Extent of DNA Methylation Variation in Wild Animal Populations". This review brings together natural animal population epigenetic studies to generate new insights into ecological epigenetics and its evolutionary implications. We first provide an overview of the extent of DNA methylation variation and its autonomy from genetic variation in wild animal population. Second, we discuss DNA methylation dynamics which create observed epigenetic population structures by

including basic population genetics processes. Then, we highlight the relevance of DNA methylation variation as an evolutionary mechanism in the extended evolutionary synthesis.

2. Animal behavior: a relevant downstream phenotype

2.1 General concept of behavior

Ethology is the study of animal behavior and focuses on how animals interact socially, how they move in their environment, how they learn about their environment, and how an animal might achieve cognitive understanding of its environment (Breed & Moore, 2021). With this definition, we understand how wide the panel of behaviors can be. Expanding our knowledge on animals' behaviors is crucial as behavioral change is one of the major mechanisms used by animals to acclimate and adapt to their environment. It constitutes the interactive link between the organism and the environment in which it lives and evolves (Sih et al., 2010). Moreover, behavior is considered as the most plastic phenotype, preceding morphological and physiological adaption. For a long time, change in behavior has been proposed as the first stage in the process of speciation (West-Eberhard, 1986). By moving to another area or by expanding their range, animals may need to change their behaviors to rapidly adapt to the new environment. In doing so, animals are subject to new selection pressures that facilitate divergent evolution and speciation processes.

The study of animal behaviors addresses four main questions: what are the mechanisms that produced a behavior? How does a behavior develop? What is the survival value (utility) of a behavior? How did the behavior evolve from an ancestral state (Tinbergen, 1963) ? Advances in areas such as genomics, endocrinology, neurobiology and ecology provided countless insights to these questions. At the same time, ethology has undergone a new shift from considering individual behavioral differences as background noise to a key target for animal behavior research (Réale et al., 2007; Wolf & Weissing, 2012). It is through model species such as the nematode *Caenorhabditis* and the fruitfly *Drosophila* that the existence of individual behaviors within a species as well as the effect of genetics and environment on behavior could be highlighted (Carere & Maestripieri, 2013). Thus, ethology, which is used to target the study of the behavior of entire populations or an entire species, is now focusing on consistent individual behavioral differences, also called animal personality.

2.2 Personality, plasticity, predictability: among and within individual behavioral variation

The study of animal personalities aims to fill a gap in our understanding of animal behavior, as it studies the adaptive significance of behavioral differences among individuals. Understanding why and how behavior differs among individuals, how personalities evolve over time, how selection acts on it are important questions. Personalities (also called behavioral traits, or temperaments) are individual behavioral differences that are consistent across time and context (Stamps & Groothuis, 2010). There are five major personality categories: shyness-boldness, exploration-avoidance, activity, sociability and aggressiveness. Shyness-boldness corresponds to an individual's reaction to any risky situation, but not new situations. Exploration-avoidance corresponds to an individual's reaction to a new situation. This includes behavior towards a new habitat, new food, or novel objects. Activity refers to the general level of locomotor activity of an individual. It is often quantified while measuring other personality traits, such as boldness or exploration (Conrad et al., 2011; Réale et al., 2007). This activity variation is strongly related to the resting metabolic rate of individuals (Nespolo & Franco, 2007). Aggressiveness corresponds to an individual's agonistic reaction towards conspecifics. Finally, sociability corresponds to an individual's reaction to the presence or absence of conspecifics (excluding aggressive behavior) (Réale et al., 2007). Personalities strongly influence social relationships, predator avoidance, food access and reproduction, and therefore organisms' fitness (Figure 5). For example, boldness of bighorn ewes *Ovis canadensis* was related to survival during years of high predation but not during years of low predation by cougars *Puma concolor* (Réale & Festa-Bianchet, 2003). In great tits *Parus major*, slow-exploring females had the lowest probability of nest failure (Both et al., 2005). Studies on personality traits may help to understand if selection favors plasticity or canalization of these traits, and also to find adaptive explanations for the maintenance of variance in personality among organisms. Moreover, many species live in temporally fluctuating environments, and it is an open question as to why some individuals are relatively inflexible in their behavior instead of showing higher plasticity, which correspond to intra-individual variation.

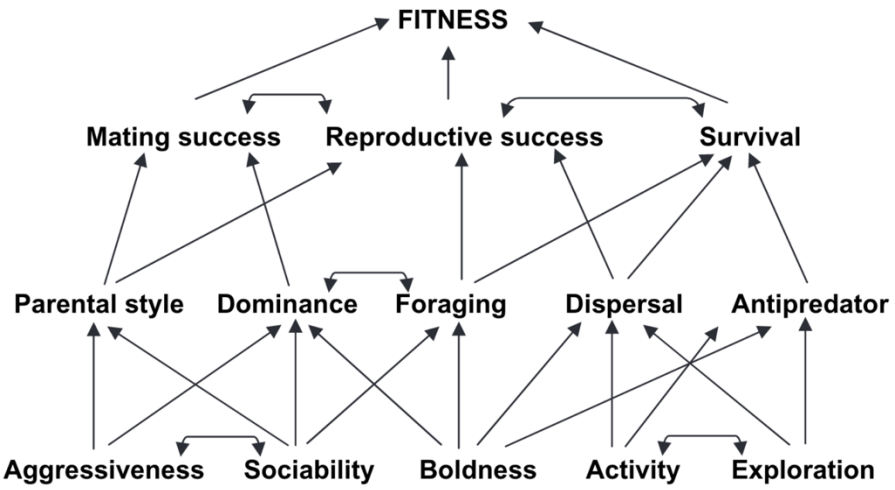


Figure 5 : Flow diagram illustrating the links between the five main personality traits and organism's fitness. Note that the arrows do not represent all possible links between variables. Adapted from Réale et al. (2007).

A meta-analysis across 114 studies and 98 species (both in the laboratory and the wild) showed that approximately 40% of the total behavioral variation observed can be attributed to consistent differences between individuals (Bell et al., 2009). Thus, personality is an interesting trait to investigate among but also within individuals, as most behavioral variation is due to intra-individual variation. This remaining variation is often attributed to intra-individual variation linked to behavioral plasticity (Figure 6). Behavioral plasticity corresponds to reversible changes in behavior in response to biotic and abiotic environmental conditions within the same individual (Dingemanse et al., 2010, reviewed in Stamps, 2016), which allow organisms to adjust their behavior along environmental gradients or over time. For example, individuals have been shown to differ consistently in their behavioral responses to food deprivation, predation risk and temperature fluctuations (Biro et al., 2010; Mitchell & Biro, 2017). Previous studies have indicated that behavioral plasticity is complex and likely to be dependent on the species under investigation. Behavioral plasticity can depend upon an individual's behavior, as bold dumpling squid *Euprymna tasmanica* become increasingly bold whereas shy individuals maintained a shy phenotype (Sinn et al., 2008). In contrast, bold three-spined sticklebacks *Gasterosteus* aculeatus are less plastic than shy fish (Jolles et al., 2019). Another study on this species showed that behavioral plasticity was dependent upon the population of origin (high and low predation) (Bell & Stamps, 2004).

Personality and individual plasticity can be jointly quantified by adopting a reaction norm framework. A reaction norm is defined as the range of behavioral phenotypes that a single

individual produces along a given environmental gradient (Dingemanse et al., 2010). The individual's personality is the Y-intercept of the reaction norm while the slope is the individual plasticity. Even after accounting for consistent among-individual variation (i.e. differences in personality) and behavioral plasticity (i.e. responsiveness to environmental/temporal change), there may still remain unexplained behavioral variability. This individual variation in residual within-individual variance is the deviation from this reaction norm, corresponding to behavioral predictability (Figure 6) (Westneat et al., 2015). Unpredictable individuals are characterized by high variability around their average behavioral type and reaction norm slope. Predictable individuals on the other hand have little residual variance around their behavioral type and reaction norm slope. Among-individual variation in predictability may be evolutionarily adaptive, e.g. diversification in bed-hedging (see review of Westneat et al., 2015 on the biology of residual phenotypic variance).

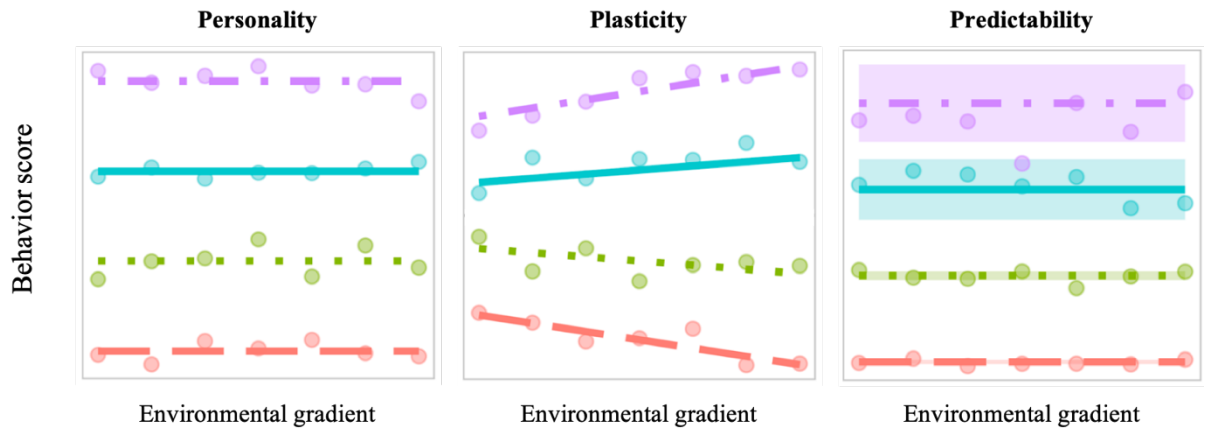


Figure 6: Partition of behavioral variation in among and within individual variation: ***a*** *Personality: Among-individual differences in mean behavioral expression over repeated measures.* ***b*** *Linear reaction norm plot: individuals differ in their behavioral plasticity (slope) along an environmental gradient and there is a positive correlation between an individual's behavioral type (intercept) and its plasticity (slope).* ***c*** *Predictability: individuals differ in within individual behavioral variability from more predictable individuals (red ribbon) to less predictable individuals (purple ribbon). Adapted from Hertel et al., (2020).*

Some studies revealed correlations between behavior variation. Behavioral syndrome, i.e. correlation of two or more personality traits at the individual level in repeated measures data, is well described (Conrad et al., 2011; Dochtermann & Dingemanse, 2013). For example, correlation between aggressiveness and boldness (the most studied behavioral syndrome) is positive in many species such as in the European grayling (*Thymallus- thymallus*) or the stickleback (*Gasterosteus- aculeatus*), but the syndrome can vary and/or disappear due to

diverse factors such as predation pressure (Bell & Stamps, 2004). In the mangrove rivulus, correlation between boldness and aggression was only expressed by secondary males (hermaphrodites that turn into males, see the next section on the mangrove rivulus), indicating potential differences between sexes (Edenbrow & Croft, 2012). The correlation between personality traits can be adaptive but also maladaptive. On the one hand, the association of behavioral traits can be linked to an adaptive process involving life history traits. In fact, bolder and more aggressive individuals could experience a better growth and reproduction rate. This concept is referred as the "pace of life" syndrome. On the other hand, a bold and aggressive individual is more easily exposed to dangerous situations (predators, parasites) which greatly increases the mortality risk. Moreover, behavioral syndrome enforces trade-offs that limit the plasticity of each trait independently. Consequently, the ability of an individual to behave optimally in a specific situation and/or to an environmental factor is limited, which impacts individual fitness, species distribution and speciation rate and therefore ecology and evolution of the concerned population (Conrad et al., 2011; Sih et al., 2010; Wolf & Weissing, 2012). These examples picture how behavioral syndrome can be adaptive or maladaptive according to the context and time.

Besides correlation between behavioral traits, studies highlighted evidence of correlations between all three variance components, meaning that individual differences in personality, plasticity and predictability can be linked. Research using the behavioral reaction norm approach has revealed that personality and plasticity are correlated for a range of traits and across different species (see Mathot et al. (2012)). For example, fast moving Caribou *Rangifer tarandus* decreased movement speed more strongly with increasing resource aggregation than individuals that moved more slowly (Webber et al., 2020). Bold individuals of different species tend to show lower plasticity in their responses to risk (Cole & Quinn, 2014; Jones & Godin, 2010) and to changing social environment (Jolles et al., 2014; Magnhagen & Bunnefeld, 2009). In the same manner, both personality and behavioral plasticity can be correlated with behavioral predictability, as shown in wild-caught three-spined sticklebacks *Gasterosteus aculeatus* where bold fish are less plastic and more predictable than shy fish (Jolles et al., 2019). Correlated behaviors or traits can restrict a population's capacity to adapt to changing conditions, as it can enforce trade-offs that limit the plasticity of each trait independently (Dochtermann & Dingemanse, 2013; Wolf & Weissing, 2012). Consequently, the ability of an individual to behave optimally in a specific situation and/or to an environmental factor is limited, which can

impact individual fitness. The occurrence of behavioral syndrome could be explained by underlying mechanism shared by behavioral trait. For example, pleiotropy can facilitate behavioral syndrome as the same genes influence different behaviors (Bell & Aubin-Horth, 2010).

2.3 Repeatability: Quantifying intrinsic behavioral variation

To quantify the extent of individual variation in a population, researchers commonly calculated repeatability (R), where among-individual variance is standardized by the total phenotypic variance, ranging from 0 to 1. Repeatability indicates the proportion of phenotypic behavioral variance in a population that can be attributed to individual differences in behavioral expression, also named individuality (Figure 7) (Bell et al., 2009). A significant repeatability corresponds to individual intrinsic (non-reversible) variation, that is not driven by external factors or internal state (i.e. reversible variation), creating individuality within a population.

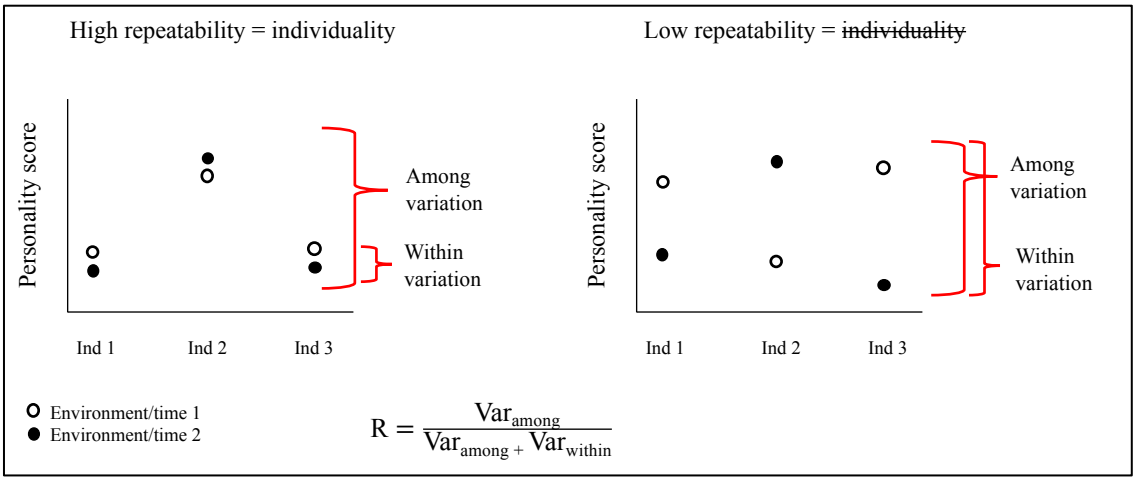


Figure 7: Repeatability R as a measure of individuality. Three individuals ("Ind") each have personality measurements made 2 times (black and white dots). In the left panel, there is low within-individual variation and a high among-individual variation, resulting in a high repeatability value and a significant individuality. In the right panel, there is a higher within-individual variation, resulting in a lower repeatability value and no individuality.

This intrinsic variation is typically associated to differences in genetic sequences. Previous research challenged this paradigm by investigating genetically identical individuals reared in highly standardized environmental conditions, and showed substantial between-individual variation in several behavioral traits (Bierbach et al., 2017; James et al., 2018; Vogt, 2017; Vogt et al., 2008). These studies suggested that high repeatability and individual consistency can also come from several other non-genetic sources: epigenetic mechanisms, maternal effects,

experience and learning (Hertel et al., 2020). Thus, repeatability provides initial evidence that among-individual variation is caused by factors intrinsic to the individual, but does not allow for separation of the genetic and non-genetic components of the variance in personality, that is individuality. Taking into consideration the previous section on epigenetics, DNA methylation variation could be another source of behavioral individuality and could be consider as another intrinsic variation among individual, as some DNA methylation marks can be irreversible and can even be transferred to the next generation (Bhandari et al., 2015; Guerrero-Bosagna et al., 2018; Liew et al., 2020).

3. Model organisms: the mangrove rivulus *Kryptolebias marmoratus*

3.1 Ecology

The mangrove rivulus, *Kryptolebias marmoratus* (Poey, 1880), is an oviparous teleost fish belonging to the rivulidae family (order: Cyprinodontiformes) that lives in mangrove ecosystems of the Caribbean, Florida, Central and South America. There are no data on the longevity of fish in the wild, but *K. marmoratus* can be long-lived in the laboratory, with one specimen noted to survive 8.2 years (D. S. Taylor, unpublished data). It has the widest distribution of any inshore-dwelling coastal fish species in all of North, Central, and South America (Taylor, 2012), as it colonizes the Florida peninsula and keys, almost the entire Central American and northern South American coastline, and likely south to the mouth of the Amazon River (Avise & Tatarenkov, 2015; Tatarenkov et al., 2012; Taylor, 2012). This species spends its entire life cycle within mangroves, and more precisely in red mangrove forests *Rhizophora mangle*. It occupies a wide range of microhabitats such as stagnant pools, crab burrows (specifically, those of *Cardisoma guanhumi*, great land crab, and *Ucides cordatus*, mangrove land crab), temporally flooded swales, inside/under logs and mangrove leaf litter and in coconuts (Taylor, 2000) (Figure 8a). A common feature among these micro-habitats includes their being either fossorial, intermittently dry, or having adverse water quality conditions that preclude the establishment of other species of fish.

Physico-chemical conditions of mangrove ecosystems are highly variable due to the alternation of tidal/rainfall flushing and drier periods through the day. Parameters such as dissolved oxygen, ammonia, salinity, temperature and water level vary drastically on a seasonal and daily basis (Taylor, 2000, 2012). A relevant example is salinity. In natural habitats, *K. marmoratus*

is euryhaline as it has been collected at salinities of 0–70 ppt (average salinity of seawater is about 35 ppt) (Davis et al., 1995; Turko et al., 2011, 2018). Extremely high salinities are a common feature of mangroves during drought or periods of reduced tidal inundation, while salinities drop drastically following heavy rainfall. It seems that the mangrove rivulus has a great tolerance to environmental variations as it has been collected over a wide thermal range (7–38°C) and survives hypoxic conditions (<1.0 ppm) (Ellison et al., 2012). When environmental conditions become extreme such as elevated concentration of hydrogen sulfide (H_2S) and oxygen depletion, the rivulus jump out of the water, behavior referred as emersion[1] (Figure 8b). Their survival in terrestrial environment up to 2 months is possible through gill and skin remodeling ensuring osmo- and iono-regulation (Costa et al., 2010; Taylor, 2012; Wright, 2012).

Relatively little information exists on rivulus interactions (diet, predation, competition) with other species living in the red mangrove. There are limited studies of the diet of *K. marmoratus* in the wild, but this fish is clearly a predator, with various terrestrial and aquatic invertebrates such as insects, arachnids, copepods, gastropods, polychaetes, and fish scales, but can also be cannibalistic and commonly eats its eggs in captivity (LeBlanc et al., 2010; Taylor, 1990). Regarding predation, given the extremely fossorial nature of rivulus, it is hard to envision intense avian predation. During periods of heavy tidal flooding, estuarine predators (e.g., snapper, barracuda, and blue crab) do move into *K. marmoratus* habitat and there may be incidental predation on *K. marmoratus* then. A more significant predator may be the mangrove water snake, *Nerodia fasciata.* Regarding competition, other species of fish are not commonly sympatric with *K. marmoratus*, presumably due to their preferred microhabitats. Only a few other species (e.g., *Gambusia spp., Poecilia spp., Fundulus spp., Cyprinodon spp., Adenia xenica*, and *D. maculatus*) have been collected in the intermittently flooded swales/potholes/ditches where rivulus may be found (Taylor, 2012).

[1] See https://www.youtube.com/watch?v=DSRT-RPgU48 from Frédéric Silvestre.

a b

Figure 8: ***a*** *Habitat of the mangrove rivulus in red mangrove forest. Small pound formed with rain and/or tide.* ***b*** *Rivulus Jump and emersion behavior. Photo credits:* ***a*** *Frédéric Silvestre,* ***b*** *Benjamin Perlman (top) and Andy Turko (down).*

3.3 Reproduction

Rivulus populations are exclusively composed of hermaphrodites (Figure 9a) and a small but variable proportion of males (Figure 9b). This reproductive strategy deprived of females is called androdioecy (Taylor, 1990). The mangrove rivulus expresses a mix-mating system. On the one hand, hermaphrodites produce eggs and sperm by meiosis and reproduces by self-fertilization, meaning that each hermaphrodite fertilizes itself inside its body and lay fertilized eggs (Figure 9c). It reproduces sexually contrary to Amazon molly or *Chrosomus eosneogaeus* which are asexual species. Along with its close relative species, *K. hermaphroditus*, mangrove rivulus are the only known self-fertilizing hermaphroditic vertebrates (Tatarenkov et al., 2010). Consistent self-fertilization is an extreme form of inbreeding (J. C. Avise & Tatarenkov, 2015) and it consequently naturally produces isogenic lineages. It has been suggested that selfing would have possibly evolved to face low probability to meet a male in their complex environment (Sakakura et al., 2006; Soto et al., 1992). On the other hand, and more rarely, external cross-fertilization events occur when males drop sperm near unfertilized eggs produced by hermaphrodites. This hermaphrodite-male outcrossing has only been observed in laboratory, but population genetics studies of *K. marmoratus* have confirmed that this type of reproduction exists in rivulus natural environment (Ellison et al., 2015). Genetic tests highlighted that cross-fertilization between two hermaphrodites does not exist (Turner et al., 2006).

a b c

Figure 9: The mangrove rivulus Kryptolebias marmoratus. ***a*** *Hermaphrodite adult rivulus.* ***b*** *Male adult rivulus.* ***c*** *Embryo of rivulus at stage 32 according to Mourabit et al. (2011). Photo credits* ***(a-b)*** *Frédéric Silvestre,* ***(c)*** *Valentine Chapelle.*

Regarding rivulus gonads, the anterior part of the bilobed structure of gonads is made of ovarian tissues that develop during the first days post hatching (dph). Then, testicular tissues develop in the posterior part of gonads and constitute less than 10% of the gonads (Figure 10) (Sakakura et al., 2006; Soto et al., 1992). Sexual maturity is generally achieved between 2 to 3 months (80 to 100 dph). The androdioecy strategy of rivulus presents two types of males. Primary males exclusively present testicular tissues in their gonads and produce sperm throughout their entire life. Depending on environmental conditions, about 60% of hermaphrodites can lose their ovarian tissues and therefore become a secondary male (Harrington, 1971; Soto et al., 1992). With time, the testicular zone of ovotestis increases progressively to the detriment of the ovarian zone. When the ratio of testicular tissue to ovarian tissue exceeds a certain threshold, the testis tissue proliferates and causes involution of the ovarian tissue to give a secondary male. Once sexually mature, males and hermaphrodites are easily distinguishable according to external characteristics. Males exhibit orange color, faded ocellus, and black margins on anal/caudal fins, while hermaphrodites express silver to brown skin with a black ocellus on their caudal fins (Harrington, 1971) (Figure 9a-b). Recently, cryptic males were discovered that subtly vary from hermaphrodite phenotypes with absent orange colors (Marson et al., 2019).

Information about oviposition and hatching are still scarce due to difficulties of observing rivulus in its natural environment. However, by combining natural and laboratory observations, it appears that embryos will fully develop without standing water, provided they are kept damp (Taylor, 1990). They can be laid in the terrestrial environment or at the water-air interface, enter diapause when they are fully developed and hatch as autonomous larvae after flooding event (Scarsella et al., 2018).

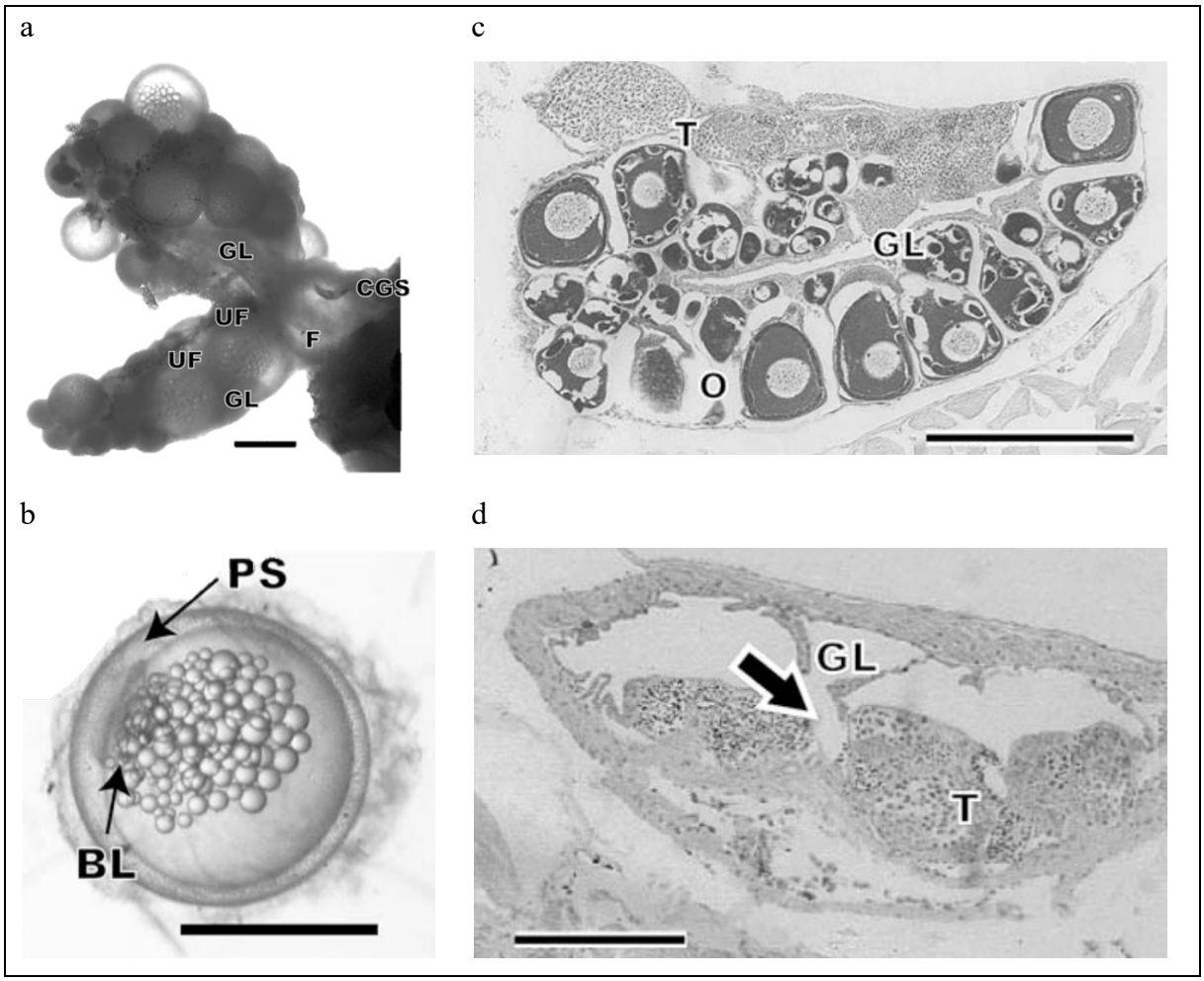


Figure 10: ***a*** *Dorsal view of hermaphrodite mangrove rivulus gonads. It is a bilobed structure, with the posterior tips of the lobes fused and a common genital sinus (CGS). There are unfertilized eggs (UF) in the anterior part of the gonadal lumen (GL), and fertilized egg (F) in the posterior part of the GL.* ***b*** *Fertilized egg with blastodisc (BL) and perivitelline space (PS).* ***c*** *Cross section in the anterior part of gonad with a large ovarian region (O) and a smaller testicular region (T).* ***d*** *Cross section in the posterior part of gonad with only testicular region (T). Arrow in* ***d*** *indicates the region where discharge of spermatozoon into GL was observed. Bar* ***a-b*** *1 mm;* ***c*** *200 µm;* ***d*** *10 µm. Adapted from (Sakakura et al., 2006).*

3.4 Behavior and personality variation

Living in fossorial microhabitat makes the mangrove rivulus behavior difficult to observe in its natural environment (Taylor, 2012) although some field studies have described rivulus behavior, such as emersion, habitat preference, and escape behavior (Taylor, 2000). Variation in personality traits has been the focus of several laboratory studies on mangrove rivulus, which showed considerable plasticity in boldness (Edenbrow & Croft, 2011, 2013; James et al., 2018), aggressiveness (Edenbrow & Croft, 2012, 2013) and exploration (Edenbrow & Croft, 2011, 2013) in response to abiotic and biotic environmental changes. For example, adult fish decrease

the intensity of aggression behavior in contact with kin and familiar individuals compared to non-kin and unfamiliar individuals (Edenbrow & Croft, 2012). Rivulus aggressiveness is also modulated by winning and losing experience, and by hormone levels, as aggressiveness positively correlated with cortisol and testosterone level and individuals that received a winning experience were quicker to display aggressive behaviors (Chang et al., 2012). Regarding boldness and exploration traits in *K. marmoratus*, Edenbrow and Croft (2011) showed that these behaviors scores correlated with genotype. These correlations emerged at 61 dph and were maintained through 151 dph. Furthermore, they showed that these behaviors were not repeatable. They suggested that developmental flexibility may be characteristic of this species, as they showed a general age effect upon these behavioral traits and low repeatability estimates. This highlights the importance of maturation in the covariance of behavioral traits. In another study of Edenbrow and Croft (2013), the presence of conspecifics as well as low food intake significantly decreased the exploration of *K. marmoratus*, whereas simulated predation risk created no significant difference with controls. Like exploration, boldness of individuals also decreases in the presence of conspecifics, with an interaction with age. Indeed, it is the presence of conspecifics during ontogeny that most affected the boldness of rivulus. The presence of conspecifics during ontogeny increased the aggressiveness of the individuals. A behavioral syndrome between boldness and aggressiveness was described in adult rivulus with these traits positively correlated to each other and to testosterone and cortisol levels (Chang et al., 2012). Another study on boldness and exploration revealed that the intensity of these traits increased during ontogeny and then stabilized at sexual maturity suggesting a developmental plasticity of these behavioral traits (Chang et al., 2012). This plasticity could allow individuals to adapt to the highly variable local environmental conditions in which rivulus evolves, and therefore would be directly linked to temporal and spatial heterogeneity of the mangrove forests (Edenbrow & Croft, 2011). Moreover, some studies suggest that behaviors forming a behavioral syndrome could maintain flexibility at different degrees to respond to environmental variations (Edenbrow & Croft, 2011).

By comparing personality traits among isogenic lineages, some studies highlighted genetic influences upon behavioral traits with genotypes differing in their average behavior and varying in the extent of environment-specific behavior plasticity. A relevant example is the study of Edenbrow and Croft (2013) were they compared boldness, exploration and aggressiveness of 5 different isogenic lineages exposed to different environmental changes including conspecific

presence, simulated predation risk and low food. Their findings highlighted genetic influences upon behavioral expression, with genotypes differing in their average behavior and varying in the extent of environment-specific boldness plasticity (Figure 11).

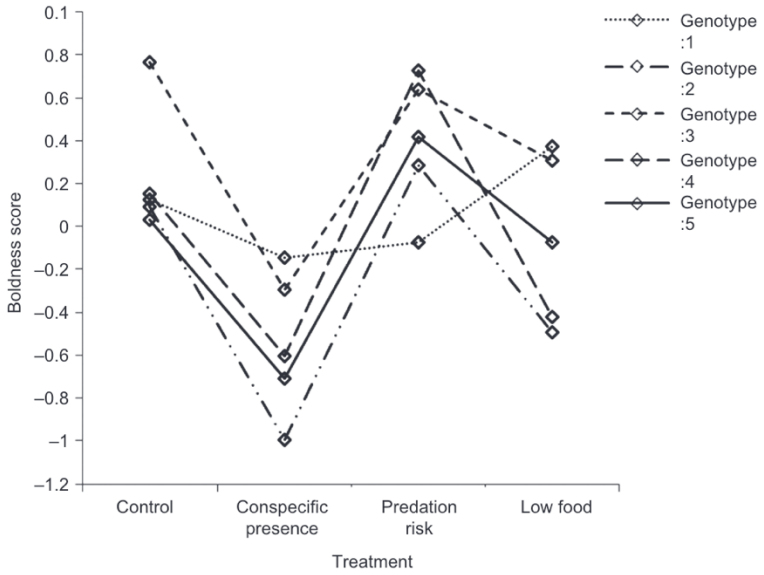


Figure 11: Mean boldness reaction norms for each of genotype (1–5) in response to each of the experimental rearing environments. From Edenbrow & Croft (2013).

3.5 Genetics: diversity in laboratory and wild populations

When conducting research on laboratory animals, researchers often try to maximize replicability (low variation between measurements in a given test), repeatability (low variation among replicate tests within a laboratory), and reproducibility (similar outcomes in comparable experiments from different laboratories). To do so, one possibility is to use animals with uniform genetic backgrounds. However, natural clonal reproduction in vertebrates is relatively rare, and this fact has led researchers to develop artificial techniques and breeding schemes that allow the clonal production of genetically uniform animals in several vertebrate taxa (Avise, 2008).

The unique selfing capacity of *K. marmoratus* to generate natural highly homozygous isogenic lineage have made this species a model system for a variety of studies such as population genetics (Avise & Tatarenkov, 2015; Tatarenkov et al., 2007, 2015), behavior (Edenbrow & Croft, 2011, 2013; James et al., 2018), developmental biology (Mourabit et al., 2011) and ecotoxicology (Carion et al., 2018, 2020; Voisin et al., 2021). Homozygosity level in rivulus

lineages was first assessed with microsatellite analysis, but now has evolved to whole genome sequencing coupled with single nucleotide polymorphisms (SNPs) analysis (Chang et al., 2012; Kelley et al., 2016; Lins et al., 2018). This last method highlighted genetic variability within inbred isogenic lineages initially described as 100% homozygous via microsatellite markers revealing that genetic variability can occur within inbred isogenic lineages through *de novo* mutations, but also residual heterozygosity (genetic variation retained and/or segregated from variation in the ancestral wild progenitor), intermittent outcrossing within or between strains in the laboratory, and any inadvertent mislabeling or misidentification of the genetic stocks (Lins et al., 2018; Tatarenkov et al., 2010). Lins et al. sequenced whole genomes from 15 lineages that were completely homozygous at microsatellite loci and used single nucleotide polymorphisms (SNPs) to determine heterozygosity levels. They found missense polymorphisms most often in genes associated with immune function and reproduction. Luckily for researchers that justified the use of rivulus on its natural production of isogenic lineages, this variation is rare, as most SNPs were found as singletons and were found mainly in intergenic regions of the genome, with only 3.5% of the SNPs found in coding regions even though 9.8% of the genome is made up of coding sequences (Lins et al., 2018).

The mix-mating system of *K. marmoratus* allows researchers to study populations with different levels of selfing, supporting different levels of genetic diversity. Previous field works established that each natural population of rivulus has its own genetic diversity generated by its own selfing rate, and a variety of lineages with different levels of homozygosity, which contrasts with the almost exclusive use of isogenic lines in the laboratory (Table 2). See Tatarenkov et al. (2017) for a large screen of rivulus populations genetics from the northern and southern distribution limits including 35 populations and 4 anterior studies (Tatarenkov et al., 2007, 2010, 2012, 2015). The highest gene diversity (i.e. expected heterozygosity, H_E) is in the Belizean populations, followed by the Florida Keys and Panama. The lowest H_E values were observed in central Florida and Exuma Island in the Bahamas. All populations showed significant deficiencies of heterozygotes, expressed as positive values of inbreeding coefficient F_{IS}. Rates of selfing are high in most of the populations (>90%), with the Belizean populations having noticeably lower rates of selfing, ranging from 77% in Northern Caye (NC) to only 39% in Twin Cayes (TC). A neighbour-joining tree based on the microsatellite data set of 33 populations shows that populations are split into two major lineages; one lineage (Northern clade) includes populations from Florida, Bahamas, Belize and Honduras, while the other

lineage encompasses populations from the rest of the Caribbean (southern Cuba, Puerto Rico, Panama, Aruba, Bonaire) and Brazil. The second lineage is further divided into a Central clade (Caribbean populations) and a Southern clade (Brazilian populations) (Figure 12)(Tatarenkov et al., 2017).

Table 2: Examples of descriptive statistics of genetic variation at microsatellite loci in Kryptolebias marmoratus (data from Tatarenkov et al., 2007, 2012, 2015, 2017, summarized in Tatarenkov et al. 2017).

Major area	Population	N	P_{99}	N_A	A_R	H_E	H_O	F_{IS}	s
Belize	Twin Cayes (TC)	59	0.97	9.28	7.24	0.69	0.52	0.25	0.39
	Long Caye (LC, BW)	40	0.94	5.78	5.17	0.61	0.24	0.62	0.76
Florida Keys,	Long Key (LK)	31	0.84	4.2	3.48	0.49	0.06	0.88	0.93
USA	Dove Creek (DC)	26	0.91	4.38	3.54	0.45	0.08	0.84	0.91
Florida, USA	Tampa bay (TPB)	24	0.41	1.56	1.52	0.17	0.00	1.00	1.00
Honduras	Utila Island (HONU)	20	0.81	3.97	3.55	0.50	0,01	0.99	1.00
Bahamas	Exuma Island (EI)	12	0.34	1.38	1.36	0.14	0.00	1.00	1.00
Brazil	Maracaípe River, Ipojuca (MPE)	17	0.66	3.28	2.89	0.37	0.00	0.99	1.00
Note: N, sample size; P_{99}, proportion of polymorphic loci (99% criterion); N_A, average number of alleles; A_R, allelic richness; H_E, expected heterozygosity; H_O, observed heterozygosity; F_{IS}, coefficient of inbreeding; S, selfing rate.									

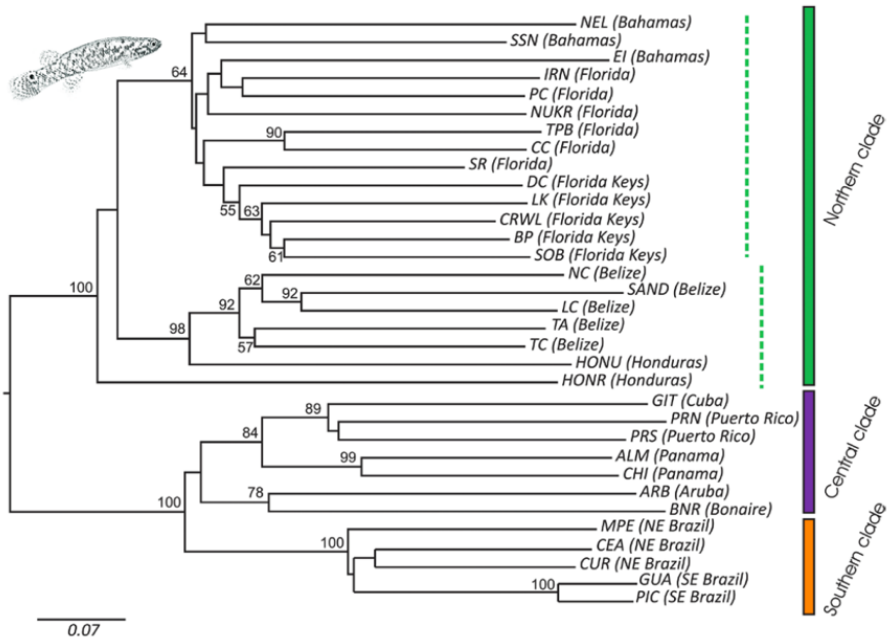


Figure 12: A neighbour-joining tree showing the genetic relationships of 33 populations of Kryptolebias marmoratus species complex. The tree is based on allele frequencies of 33 microsatellite loci. Bootstrap support, shown at the nodes, is based on

1000 replicates; only bootstrap values above 50% are shown. Colored thick lines outline populations forming three main clades. Broken green lines delineate subclades of the Northern clade. From (Tatarenkov et al., 2017).

These population genetics studies provide us precious information on rivulus population composition and dynamics. Based on these data, further studies can select specific populations to investigate ecological and evolutionary questions such as sexual determinism, evolution of mating system, inbreeding and inbreeding depression, effect of genetic diversity on a multitude of traits by comparing populations with contrasted genetic diversity, phenotypic plasticity and bet-hedging strategies, etc. Nowadays, the feasibility of advanced molecular analyses in the mangrove rivulus is supported by the public availability of genome assembly and to current efforts to improve the genome annotation. In this thesis, we used the ASM164957v2 genome assembly which is it is the latest version (Dec 2020) (Table 3).

Table 3: Genome Assembly ASM164957v2 of Kryptolebias marmoratus

Assembly statistics	
Genome size	680.6 Mb
Number of chromosomes	24
GC percent	40
Assembly level	Chromosome
Annotation details	
Genes	25 206
Protein-coding	22 200
Non-coding	2 733
mRNAs	42,520
non-coding RNAs	4,079
Sample details	
Dev stage	Adult
Sex	Hermaphrodite
Tissue	Liver

3.6 Epigenetics

Characterization of mangrove rivulus DNMTs and TETs highlight conserved features with vertebrates and mammals. In the *K. marmoratus* genome, one DNMT1, two DNMT3a (DNMT3a1 and DNMT3a2), three DNMT3b (DNMT3b1, DNMT3b2, DNMT3b3), and three TETs (Tet1, Tet2, and Tet3) genes were identified. Compared with vertebrates DNMTs and TETs, they contained all domains that are essential for their function of methylation and

demethylation (Kim et al., 2016). Moreover, their expression is specific to tissue, sex and developmental stages (Fellous et al., 2018). Regarding global CpG DNA methylation level, Fellous et al., (2018) found consistency with other vertebrates as adult tissues were highly methylated at CpG sites in males and hermaphrodites. A significant difference in CpG global methylation of genomic DNA was observed among tissues of adult rivulus. The male testis was hypermethylated compared to the hermaphrodite ovotestes (Table 4), which is in line with *Danio rerio* where global CpG methylation is higher in sperm than in oocytes (Potok et al., 2013).

Table 4: CpG global DNA methylation levels in different organs of Kryptolebias marmoratus using the LUminometric Methylation Assay (LUMA). Adapted from Fellous et al. 2018.

Tissue	DNA methylation % (Mean ± SEM)		Sidak's multiple comparisons test	
	Males	Hermaphrodite	Male *vs* hermaphrodite	Between tissues
Gonad	87.22 ± 1.14	79.55 ± 1.78	**0.0058**	A
Brain	78.22 ± 0.84	75.88 ± 3.68	0.8349	B
Liver	80.93 ± 1.53	81.67 ± 2.35	0.9995	C
Gills	76.05 ± 0.61	77.36 ± 0.57	>0.9999	B
Muscle	73.71 ± 1.28	73.73 ± 7.32	0.9307	B
Skin	73.71 ± 1.28	74.15 ± 1.96	>0.9999	B

Due to its unique mode of reproduction and the natural occurrence of isogenic lineages, this new model species is of great interest for understanding epigenetic contributions to the regulation of development and reproduction. Several studies investigated the role of epigenetic mechanisms as regulatory system of embryonic development and sex-ratio modulation. Histone modifications such as lysine acetylation/deacetylation (mediated by lysine acetyltransferases (KAT) and histone deacetylases (HDAC)) and lysine methylation/demethylation (mediated by lysine demethylases (Kdm) and methyltransferases (Kmt)) play an important role in embryonic development. Twenty-seven KAT, seventeen HDAC, twenty-five Kdm orthologues and forty-eight Kmt orthologues genes have been identified. Their conserved domains, expression profiles and their phylogenetic analysis suggest that they might have important biological functions in early and late development as well as in male/hermaphrodite gametogenesis and adult neurogenesis, which raises questions about epigenetic regulation of these processes by histone lysine modifications in *K. marmoratus* (Fellous et al., 2019a, 2019b). Besides histone modifications, DNA methylation seems to have a central role in the regulation of rivulus development and reproduction. Characterization of rivulus DNA methylation reprogramming

highlights consistency with that of *Mus musculus* (Kim et al., 2004), *Danio rerio* (Mhanni & McGowan, 2004) and medaka *Oryzias latipes* (Wang & Bhandari, 2019) reprogramming where DNA undergoes global demethylation promptly after fertilization and then becomes remethylated at specific loci and stages. However, reprogramming seems species specific as rivulus reprogramming is later, deeper, and longer than zebrafish reprogramming at the same embryonic stages (Figure 13). It has been suggested that this long and deep DNA methylation reprogramming period could permit environmental signals to be assimilated at the epigenetic level during embryogenesis and could consequently increase phenotypic diversity (Fellous et al., 2018).Temperature seems to be an important environmental factor as Ellison et al. (2015) found a significant interaction between sexual identity (male or hermaphrodite), temperature and methylation patterns when two selfing lines were exposed to different temperatures during development. They identified several genes differentially methylated in males and hermaphrodites that represent candidates for the temperature-mediated sex regulation in *K. marmoratus* including cyp19a and sox9a. Thus, DNA methylation can be a mechanism regulated by temperature that modulates sexual identity in the mangrove rivulus.

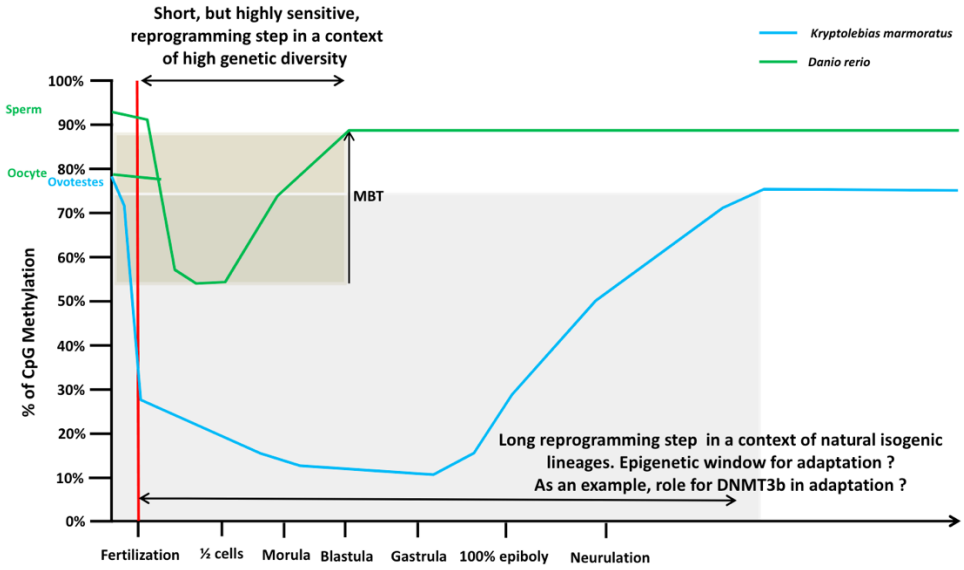


Figure 13: Dynamic of DNA methylation during early zebrafish and mangrove rivulus development. (MBT, Mid Blastula Transition). From Fellous et al. 2018.

Using the mangrove rivulus as a model species help us to disentangle the source of epimutations (obligatory, facilitated or pure), and ultimately, to investigate the transmission of environmentally-induced epimutations and their possible role in generating phenotypic variation. Berbel-Filho et al. (2019) analyzed DNA methylation patterns of two highly inbred strains of rivulus reared in two rearing environments (with or without physical enrichment) to

determine epimutations sources. They found higher methylation differentiation between genotypes than between environments, with a genotype x environment interaction as most methylation differences between environments common to both strains followed a pattern where the two genotypes responded to the same rearing environment with contrasting DNA methylation levels (facilitated epialleles). Later, they investigated the effects of environmental enrichment on the behavior, metabolic rate and brain DNA methylation patterns of parents and offspring of one isogenic lineage of mangrove rivulus. They identified significant physiological (basal metabolic rate and cortisol levels), behavioral (neophobia, activity) and epigenetic differences among parents reared under two different levels of environmental enrichment, some of which influenced the offspring behaviors as offspring activity and neophobia were influenced by the parental rearing environment. Regarding DNA methylation, most of their results indicated a stronger effect of offspring rearing environment than that of their parents on DNA methylation patterns. However, three differentially methylated cytosines between the two rearing conditions maintained the same methylation patterns in both parents and offspring independently to the offspring rearing conditions (Berbel-Filho et al., 2020). These results bring new insights to the reversibility and the memory/erasure balance influencing the transmission of environmentally-induced epimutations in the mangrove rivulus, as environmentally-induced epimutations can be transmitted to the offspring to some extent.

3.7 Ecotoxicological studies and methylmercury as our environmental stressor

The mating system of the mangrove rivulus provides an ideal model for the identification of true cause-effect relationships between the environment, the epigenome and the phenotype, including the role of epigenetic mechanisms in toxicity and ecotoxicity. A growing body of work supports its value as a new model species for studying the potential lasting effects of an early life exposure to an environmental stressor through epigenetic modifications. Voisin et al. showed that early life exposure of rivulus to an endocrine disrupting compound, 17-α-ethinylestradiol (EE2), can induce delayed effects on the adult phenotype, proteome and epigenome (Voisin et al., 2016, 2021). This study highlights some relevant genes to investigate in long-term effects studies such as nipped-B-like protein B (NIPBL) where a methylation change of + 21.9% was shown in NIPBL promoter region in rivulus exposed to 4 ng/L EE2. Carion et al. highlighted immediate effects of the neurotoxin β-N-Methylamino-L-alanine (BMAA) on rivulus larvae behaviors, and delayed effects of BMAA on expression of genes

involved in glutamate turnover, intracellular dopamine levels and astrocyte protective mechanisms (Carion et al., 2018, 2020). Another interesting characteristics of the mangrove rivulus is its inter-individual variability in a large range of phenotypic traits. Although the rivulus used in these studies are from the same isogenic lineage, they still display high individual differences within each experimental condition. This inter-individual variation in behaviors within an isogenic lineage reared in standardized environment could be due to non-genetic factors such as epigenetic variation, as a recent study showed a methylation difference of 40% between aggressive and non-aggressive fish in the Toll-interacting protein (Tollip) coding gene controlling proinflammatory reaction in response to injury (Carion et al., unpublished data). As reviewed in Nikinmaa and Anttila (2019), variability should always be included as an endpoint in data analysis as it would bring new information about the responses of organisms to environmental contamination.

Although these studies show immediate effects of environmental stressor on rivulus behaviors on one side, and delayed effects on epigenome on the other side, no studies show delayed behavioral effects of an environment stressor through environmentally-induced epimutations. However, it has been suggested that delayed and also transgenerational pollutant-altered behaviors (including neurotoxicants) can result from epigenetic changes (Carvan et al., 2017; Skinner, 2011). By using mangrove rivulus from one isogenic lineage, we can investigate if and how their epigenome is modified by an environmental stressor, creating environmentally-induced epimutations underlying immediate but also delayed behavioral alterations as they can be permanent (Carvan et al., 2017; Skinner et al., 2015). To get all the odds on our side, we chose one well-known neurotoxicant, the methylmercury. Methylmercury (MeHg) is a neurotoxic contaminant generated through methylation of heavy metal mercury by anaerobic bacteria in aquatic environments. It enters the aquatic food web through the consumption of these bacteria by zooplankton, and undergoes bioaccumulation and biomagnification resulting in high concentrations in large predatory fish and other top predators including humans (Baeyens et al., 2003). It is established that MeHg crosses the blood-brain barrier and can induce brain damages, impaired neurological development and behaviors. In fish, MeHg exposure causes altered swimming activity and prey capture success (Mora-Zamorano et al., 2017; Samson, 2001; Xiaojuan Xu, 2012; Zhu et al., 2020), visual deficit (Carvan et al., 2017; Weber et al., 2008), learning and memory impairments (Smith et al., 2010; Xu et al., 2016). Several cellular mechanisms have been proposed for MeHg-induced neurotoxicity including oxidative

stress (Carvan et al., 2017; Farina & Aschner, 2019), alterations of neuronal differentiation (Tamm et al., 2008), and disruption of glutamatergic and dopaminergic neurotransmitter systems (Faro et al., 2002).

Most recent studies of MeHg neurotoxicity have focused on adverse outcome pathways and behavioral effects of developmental and early-life stages exposure. MeHg exposure during these stages can cause delayed effects that can be observed later in life, but also transgenerational effects (Carvan et al., 2017; Xu et al., 2016). It has been suggested that delayed and transgenerational MeHg-altered phenotypes result from gene expression modification without changes to the underlying nucleotide sequence of DNA (i.e., epigenetic mechanism) (Carvan et al., 2017; Skinner, 2011). Generally, MeHg alterations of epigenome include downregulation of microRNA expression, reduced histone acetylation and increased histone methylation, and global DNA hypomethylation in brain (reviewed in Culbreth and Aschner, 2019). Even with this extensive compilation of cellular and molecular data, it is still challenging to link MeHg-induced behavioral impairments to specific molecular alterations, and to understand the underlying mechanisms of delayed behavioral effects of early-life stages exposure. As such, MeHg has become an attractive neurotoxicant in the investigation of reversible/permanent environmentally-induced epimutations underlying behavioral alterations in one isogenic lineage of mangrove rivulus, reducing the genetic noise.

Furthermore, rivulus is subject to MeHg exposure in its natural environment since mangroves are considered as potential MeHg hotspots due to their large amount of organic matter and their physico-chemical characteristics (Figure 14) (detailed in Lei et al., 2019). Their location facilitates pollutant accumulation, as showed in Florida where Hg levels were elevated in mangrove transition zone compared to both the upstream canals and the open waters of Florida Bay (Rumbold et al., 2011). Total mercury (THg) concentrations in freshwater ecosystems range from 0.3 ng/L to 450 µg/L, with higher levels found downstream of pollution sources including mines and industrial discharges (Kidd & Batchelar, 2011). There are fewer data available on the distribution of mercury in mangrove waters, and even less on MeHg. THg concentrations in mangroves water ranged from 0.04 to 110 ng/L, with large temporal and spatial variations. MeHg transfer from water into the base of the food web (bioconcentration) and subsequent biomagnification in the aquatic food web leads to most of the MeHg in higher trophic levels (Wu et al., 2019). The mangrove rivulus is a predator and can accumulate MeHg

through contaminated prey consumption including bivalves that showed 100 to 940 ng/g dw Hg concentration (Saha et al., 2006) and polychaetes containing 50 to 280 ng/g dw Hg (Alam et al., 2010) in mangrove ecosystems. To our knowledge, there is no available data on THg and MeHg concentrations in mangroves rivulus.

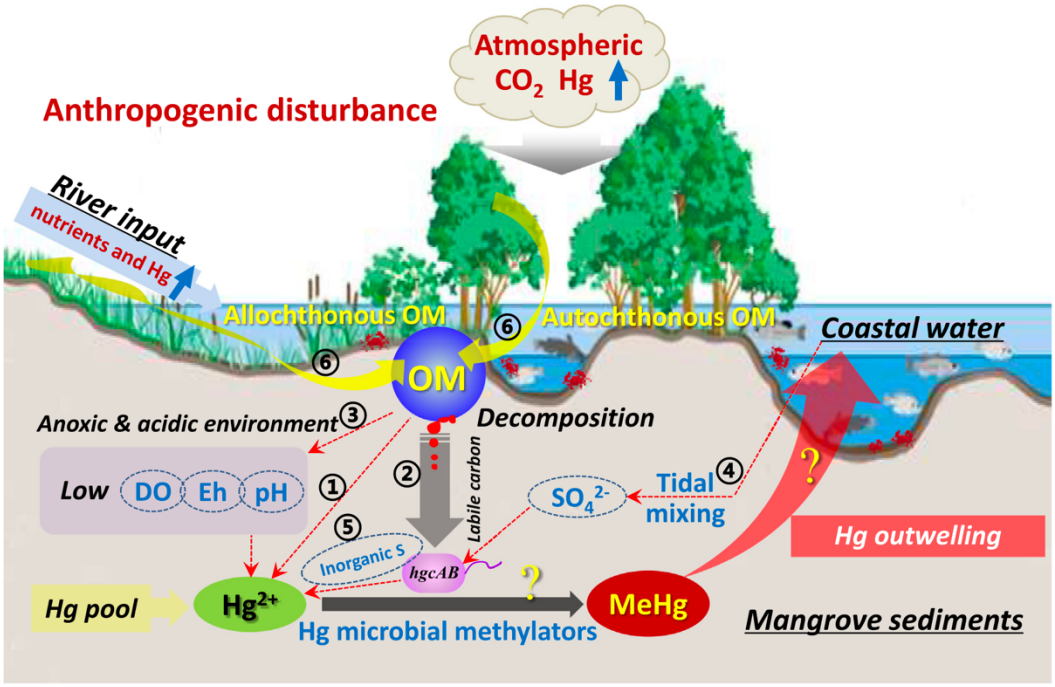


Figure 14: Factors controlling MeHg production in mangrove sediments. ① Organic matters (OM) binds with inorganic mercury (Hg^{2+}) and changes its bioavailability; ② OM acts as labile carbon source of Hg methylators, such as sulfate-reducing bacteria (SRB), iron-reducing bacteria (FeRB), and methanogens; ③ OM decomposition often results in a low pH, Eh, and DO environment, indirectly changing the Hg methylation rate; ④ tidal mixing changes the sulfate concentrations in the sediments, altering SRB activity; ⑤ abundant inorganic sulfur species have significant effects on MeHg production; ⑥ mangrove OM has different origins that differ in their roles in sedimentary MeHg formation. From Lei et al., (2019)

3.7 Summary of relevant features as model species

In its natural habitat, the mangrove rivulus shows exceptional plasticity to face the highly variable physico-chemical conditions of mangrove ecosystems such as emersion behavior and survival in terrestrial environment through gill and skin remodeling (see 3.2 Ecology). The wide reaction norm of the mangrove rivulus is paradoxical considering its reproduction system. This system alternates between the cross-fertilization of a male and a hermaphrodite on the one hand, and self-fertilization (or selfing) of hermaphrodites on the other hand, which is a unique feature among vertebrates (Figure 9a-b). Consistent selfing of wild-caught rivulus that are highly

heterozygous produces isogenic lineages with offspring that are homozygous at all microsatellite loci after approximately 5–10 generations, and heterozygosity decreased by approximately 50% after one generation (Mackiewicz et al., 2006). This allows us to work with natural highly homozygous and isogenic lineages and reduces genetic noise within lineages (see 3.3 Reproduction). In opposition to artificial clonal lineages of other model species, the mangrove rivulus and its unique reproductive system allow us to investigate adaptive and evolutionary processes in a relevant way, including origins of phenotypic variation. Variation in personality traits has been the focus of several studies on mangrove rivulus, which showed considerable plasticity in boldness (Edenbrow & Croft, 2011, 2013; James et al., 2018), aggressiveness (Edenbrow & Croft, 2012, 2013) and exploration (Edenbrow & Croft, 2011, 2013) in response to abiotic and biotic environmental changes (see 3.4 Behavior and personality variation). In the last decade, there have been advances in genomic studies of mangrove rivulus leading to an annotated genome assembly. Moreover, colossal efforts in populations genetic studies showed that each natural rivulus population has its own genetic diversity generated by its own selfing rate, and a variety of lineages with different levels of homozygosity, which contrasts with the almost exclusive use of isogenic lines in the laboratory (see 3.5 Genetics: diversity in laboratory and wild populations). More recently, the mangrove rivulus has become the target of epigenetic studies. After investigating the basis of mangrove rivulus epigenetic machinery, recent studies suggested that regulation of gene expression through DNA methylation could play a role in its plastic response to environmental variation (see 3.6 Epigenetics). Many other advantages related to the use of this new valuable model vertebrate species exist and the following list is probably not exhaustive: its short generation time (≈ 80 to 100 days), its simple rearing environment, the transparency of its embryos, its small size, and its robustness (Figure 9c).

CHAPTER 2: OBJECTIVES

The present study aims to determine the role of DNA methylation in the adaptation and evolution of mangrove rivulus *Kryptolebias marmoratus* by investigating its variation and sources within and among mangrove rivulus populations from the wild or reared under standardized laboratory conditions. We choose individual and consistent behavioral variation (i.e. personality traits) as an endpoint to represent the capacity of DNA methylation to generate phenotypic variability.

The state of the art highlights crucial points that need investigation: (1) In opposition to wild plant populations, there is no review about population epigenetics in wild animal populations. Our first step is to produce a review which will be used as a starting point for our field study on mangrove rivulus. (2) Epigenetic variation as a source of phenotypic variation has been explored in genetically diverse populations, or in clonal populations. For the first time, this question can be addressed in a species naturally found under both configurations, allowing us to compare populations covering a vast spectrum of genetic diversity. (3) Behavioral variation has never been chosen as a phenotypic end point in these previous studies on epigenetic and genetic diversity in wild populations. However, animal behavior mediates the interaction between an organism and its environment, and constitutes the ultimate phenotype influencing ecological and evolutionary processes. Thus, field work comparing epigenetic and behavioral variation in populations with a gradient of genetic diversity, including an isogenic population (Figure 15), would allow us to answer to new evolutionary relevant questions described below:

How epigenetic variation balances with genetic variation in wild populations of mangrove rivulus encountering genetic diversity gradient?

- What are the origins of epimutations? Is there higher epigenetic variation in populations with higher genetic variation (i.e. obligatory epimutations)? Is there epigenetic variation in isogenic populations (i.e. pure epimutations)?
- Is there significant individuality in wild populations of mangrove rivulus? Are there intrinsic parameters that maintain rivulus personality through the time (genetic of non-genetic)?

- What are the origins of individuality? Is there higher behavioral variation in population with higher genetic variation? Is there no significant individuality in isogenic population?

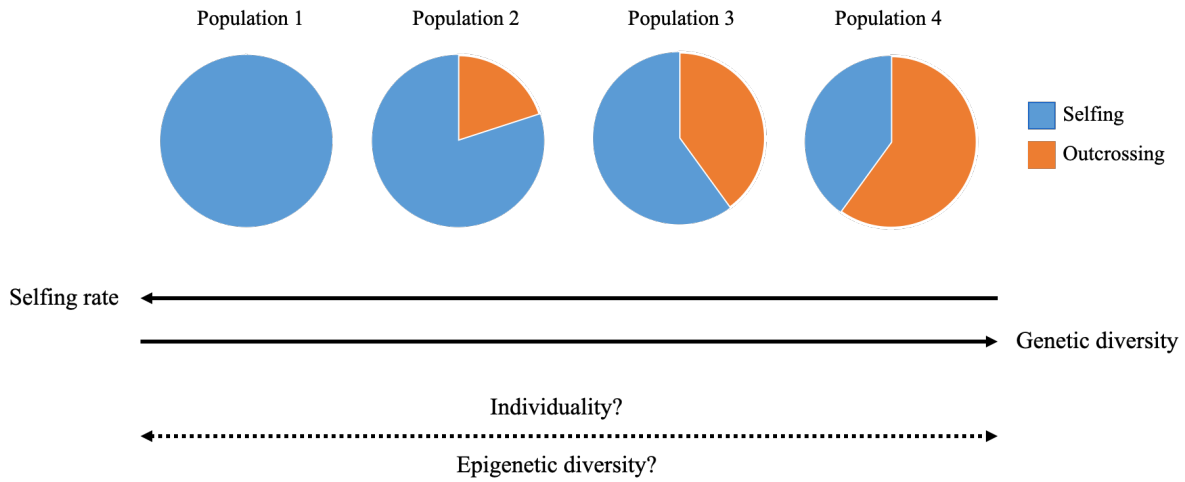


Figure 15: Schematic design of field work investigating epigenetic diversity and consistent individual behavioral variation (i.e. individuality) in wild mangrove rivulus populations with a gradient of genetic diversity. When selfing rate increases, genetic diversity is expected to decrease within a population as it can create isogenic lineages.

As shown in our review (see Chapter 3), the relationship between genetic, epigenetic and phenotypic variation can be species- and even population-specific. Depending on the different levels of genetic, epigenetic and behavioral variation observed within and among population of mangrove rivulus, several scenarios may emerge with different implications for the role of DNA methylation in the adaptation and evolution of mangrove rivulus. (1) Populations with high genetic diversity show high epigenetic variation (obligatory epimutations) and genetic variation is the main cause of individuality. Populations with lower genetic diversity show lower epigenetic variation and no significant individuality. This scenario implies that genetic diversity is the main driver of epigenetic and behavioral diversity generation in rivulus populations (Figure 16a). (2) Population with low genetic diversity show epigenetic variation due to random or environmentally-induced epimutations, which corresponds to an epigenetic buffering as detailed in the introduction. Briefly, this phenomenon can facilitate evolutionary rescue of populations showing low genetic diversity by helping them facing rapid and fluctuating environmental changes thanks to the responsiveness of environmentally-induced epimutations. Population with higher genetic diversity show higher epigenetic variation due to the addition of obligatory, facilitated and pure epimutations effects. Individuality occurs in all populations from different sources and with different intensity (from pure epimutations in

isogenic populations, and from genetic, obligatory + facilitated + pure epimutations in genetically-diverse populations). This scenario implies that epigenetic diversity is crucial for the adaptation and survival of some mangrove rivulus populations showing low genetic diversity, while genetic diversity maintain phenotypic variation within genetically-diverse rivulus populations (Figure 16b). (3) There is a phenotypic convergence between epigenetic and genetic factors in the generation of individuality. In population with low genetic diversity, environmentally-induced and random epimutations underlies individuality. In population with high genetic diversity, obligatory epimutations and genetic variation underlie individuality. All populations show similar level of individuality, which come from different sources (Figure 16c). By comparing epigenetic diversity and individuality in mangrove rivulus populations with different level of genetic diversity, we can investigate if DNA methylation is involved in adaptation and evolution of mangrove rivulus populations, but also if this involvement varies depending on the population.

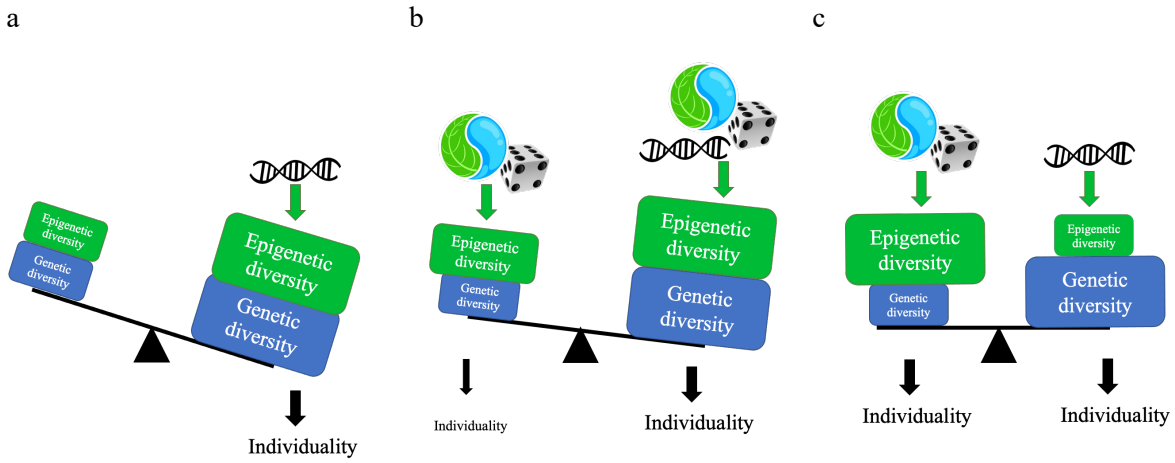


Figure 16: Possible relationship between individuality, epigenetic and genetic variation among wild mangrove rivulus. ***a*** *Genetic diversity is the main driver of epigenetic and behavioral diversity generation in rivulus populations.* ***b*** *Epigenetic buffering helps populations with low genetic diversity to face environmental changes thanks to the responsiveness of environmentally-induced epimutations.* ***c*** *Phenotypic convergence between epigenetic and genetic factors in the generation of individuality.*

The main inconvenience of field studies is that environmental variation within and among a population is difficult to describe and to include in the sources of phenotypic variation. Environmentally-induced and random epimutations (pure epimutations) within population cannot be discriminated. Thus, following this field work which investigate the balance between epigenetic variation and genetic variation underlying behavioral variation, we explored the

effects of a well-known pollutant, the methylmercury (MeHg), on the offspring of the previously studied wild isogenic population. We aim to distinguish potential environmentally (MeHg)-induced epimutations at targeted CpGs in the exposed groups, and natural rate of random epimutations in the control group of mangrove rivulus, and to link them to gene expression and behavioral variation (Figure 17). We exposed rivulus larvae to MeHg from 0 to 7 days post-hatching (dph), and evaluated immediate effects on DNA methylation, gene expression and behaviors at the end of the exposure, but also delayed effects in adult rivulus (90 dph). Early-life is recognized as a sensitive window during which the environment can have long-lasting effects on the organism phenotype later in life. Discovering out how environmental stressors influence epigenetic and possibly phenotypic variance is crucial to understand animals' ability to acclimate to new environmental conditions. This ecotoxicological study would highlight the level of random epimutations and the potential alternations of environmental changes on the methylation level of targeted genes related to personality in the mangrove rivulus by using a methylation gene-specific approach. We do not necessarily intend to study the effects of MeHg on the mangrove rivulus, but rather to study the effects of environmental variation on epigenetic variation in one isogenic lineage of mangrove rivulus, if these effects vary among rivulus, if they are reversible or permanent, which brings us to these experimental questions:

Whether and how an environmental stressor (MeHg exposure) induces epimutations in an isogenic lineage, and how behaviors and related genes expression are modified?

- Does an early-life exposure to MeHg (from 0 to 7 days post-hatching) create environmentally-induced epimutations affecting the expression of genes related to behaviors in rivulus larvae?
- Are the immediate effects on DNA methylation, gene expression and/or behaviors maintained later in life in adults rivulus (90 dph)?
- Do random epimutations occur? What is the rate in control group and in exposed groups?
- Is there significant individuality in exposed and/or control groups? How is it affected by an environmental stressor?

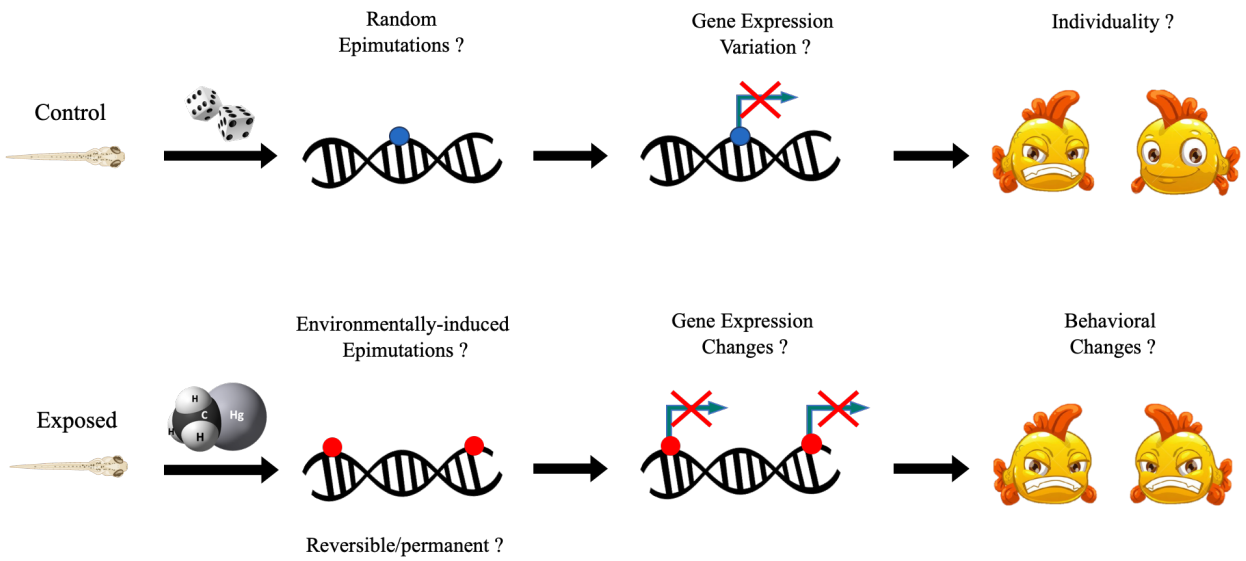


Figure 17: Schematic design of ecotoxicological study investigating immediate and delayed effects of methylmercury exposure (represented as CH_3Hg molecule) on DNA methylation and behaviors in mangrove rivulus Kryptolebias marmoratus. In control group, DNA methylation variation (i.e. epimutations) can arise from random processes (represented as dices), randomly modifying gene expression and resulting behaviors, creating consistent individual behavioral variation (i.e. individuality). In methylmercury-exposed group, environmentally-induced epimutations can arise due to the exposure, modifying gene expression and altering behaviors.

Different results may arise from this experiment, as the effects of methylmercury vary according to the species (detailed in the introduction). (1) **Immediate and reversible effects of MeHg exposure:** MeHg exposure can create DNA methylation changes in rivulus larvae after 7 days of exposure. These environmentally-induced epimutations modify expression of genes including behavior-related genes, which cause behavioral alterations. These epimutations are reversible and disappear when the exposition ends. No effects of MeHg are observed in adults rivulus. (2) **Immediate and irreversible effects of MeHg exposure**: Immediate effects of MeHg on DNA methylation, gene expression and/or behaviors are maintained after the end of the exposure, creating permanent effects in adults rivulus. (3) **Delayed effects of MeHg exposure**: Early-life MeHg exposure does not create immediate effects on larvae, but delayed effects are observed in adults rivulus. (4) **No significant effects of MeHg exposure on our targeted endpoints**.

In addition to comparing experimental groups (control and exposed rivulus), the characterization of epigenetic and behavioral variation within groups can provide us with

crucial information on the basal variability of these parameters among rivulus from the same isogenic lineage reared in standardized environment. These data will be useful in the interpretation of results obtained in the field study, where environmental variation is not controlled. By studying epigenetic and behavioral variation in control group, we can highlight basal levels of random epimutation and intrinsic individuality among rivulus that do not come from environmental or genetic variation. By studying epigenetic and behavioral variation within exposed groups, we can also highlight differences in rivulus sensibility to an environmental variation, and how an environmental stressor can induce higher phenotypic variation within exposed group than control group.

CHAPTER 3: EPIGENETIC VARIABILITY IN WILD ANIMAL POPULATIONS

Ecological epigenetics mostly progressed with studies that investigated DNA methylation variation under laboratory conditions, and later with population epigenetics studies on natural plant populations. Now that this research established a solid data basis for the extent of epigenetic variation and its dynamics over time and context, scientists are reaching the next level with wild animal population epigenetics. Since the first wild animal populations epigenetics study in 2010, more than thirty similar articles have been published on a wide variety of animal species, from invertebrates to vertebrates. The field of population epigenetics requires a review with a focus on wild animal population because it is fast moving research area, and there is a set of recent advances that need to be brought together to provide new insights in this domain. For example, most articles discuss the lack of field study focusing on the transgenerational inheritance of epigenetic marks. Since 2021, there are several publications on this topic. From a broader view, this review contributes to the construction of the extended evolutionary synthesis, a theory which is still under debate. This review will form the basis for our field study on mangrove rivulus.

epigenomes

MDPI

Article

POPULATION EPIGENETICS: THE EXTENT OF DNA METHYLATION VARIATION IN WILD ANIMAL POPULATIONS

Valentine Chapelle [1]* and Frédéric Silvestre[1]

[1]Laboratory of Evolutionary and Adaptive Physiology, Institute of Life, Earth, and Environment, University of Namur, 61 Rue de Bruxelles, 5000 Namur, Belgium

* Correspondence: valentine.chapelle@unamur.be

Abstract:

Population epigenetics explores the extent of epigenetic variation and its dynamics in natural populations encountering changing environmental conditions. In contrast to population genetics, the basic concepts of this field are still in their early stages, especially in animal populations. Epigenetic variation may play a crucial role in phenotypic plasticity and local adaptation as it can be affected by the environment, it is likely to have higher spontaneous mutation rate than nucleotide sequences do, and it may be inherited via non-mendelian processes. In this review, we aim to bring together natural animal population epigenetic studies to generate new insights into ecological epigenetics and its evolutionary implications. We first provide an overview of the extent of DNA methylation variation and its autonomy from genetic variation in wild animal population. Second, we discuss DNA methylation dynamics which create observed epigenetic population structures by including basic population genetics processes. Then, we highlight the relevance of DNA methylation variation as an evolutionary mechanism in the extended evolutionary synthesis. Finally, we suggest new research directions by highlighting gaps in the knowledge of the population epigenetics field. As for our results, DNA methylation diversity was found to reveal parameters that can be used to characterize natural animal populations. Some concepts of population genetics dynamics can be applied to explain the observed epigenetic structure in natural animal populations. The set of recent advancements in ecological epigenetics, especially in transgenerational epigenetic inheritance

in wild animal population, might reshape the way ecologists generate predictive models of the capacity of organisms to adapt to changing environments.

Keywords: population epigenetics; DNA methylation variation; epimutation; natural animal populations; evolution

Citation: Chapelle, V.; Silvestre, F. Population Epigenetics: The Extent of DNA Methylation Variation in Wild Animal Populations. Epigenomes 2022, 6, 31. https://doi.org/10.3390/ epigenomes6040031

Academic Editor: Che-Kun James Shen

Received: 15 August 2022
Accepted: 19 September 2022
Published: 28 September 2022

1. Introduction

Our understanding of an organism's capacity to respond to environmental changes has advanced, in a large way, through studies focusing on genetic variation and the manipulation of environmental conditions. These studies confirm that genotype, environment, and their interaction contribute to phenotypic variability, a fundamental prerequisite for evolution by natural selection. The tremendous development of genetic knowledge during the 20th century has led to the merge of Darwinism and the field of genetics into a modern synthesis. However, we now admit that genetic variation is not the only source of phenotypic variation that can be inherited across generations because only a small proportion of variance in complex traits can actually be explained by genetic variance [1]. The concept of inclusive heritability has been proposed to unify genetic and non-genetic mechanisms of heritability, which encompasses all dimensions of inheritance such as the transmitted parental effect, ecological variation, social variation, and transgenerational epigenetic inheritance (TEI) [2]. There is a growing consensus that epigenetics and, in particular, TEI could be one of the missing factors for understanding phenomena that cannot be explained by the DNA sequence alone, such as incomplete penetrance (i.e., individuals of a given genotype expressing different phenotypes) and the variance in expressivity (i.e., the degree/intensity to which complex trait expression differs among individuals) [3,4]. These two phenomena result in an incomplete correlation between genotype and phenotype, and these may be partly explained by epigenetic mechanisms.

Epigenetics has been narrowly defined as mitotically and/or meiotically heritable changes in gene expression that cannot be explained by changes in the gene sequence [5] (see Table A1 for glossary). These changes include histone modification, DNA methylation, and small RNA regulation, and these are involved in processes such as cellular differentiation, development, diseases, behaviors, and metabolism [6]. Studies exploring phenotypic plasticity have showed that epigenetic variation can play a significant role in the response that an organism has to environmental variation, as epigenetic marks can be directly affected by the environment. In other words, environmentally induced epigenetic variations have been proposed to mediate phenotypic plasticity as they allow the organisms to adapt to the environmental conditions by increasing the phenotypic options of a genotype with no genetic sequence modification [7–9]. Moreover, fitness-related phenotypes that are initially environmentally induced can be selected to become genetically determined, and hence, heritable, a process that is named genetic accommodation [10,11]. Genetic assimilation (i.e., the loss of or decreased plasticity [12]) and

genetic compensation (i.e., the selection for similar phenotypes in different environments [13]) are different types of genetic accommodation. In other words, in addition to being another source of phenotypic variation, epigenetic variation can precede genetic adaptation through genetic accommodation, thus reversing the standard model of evolution from a genotype-to-phenotype to a phenotype-to-genotype information flow [11,14,15].

Understanding the evolutionary implications of epigenetics and how epigenetic mechanisms contribute to phenotypic variability is one of the current greatest challenges in evolutionary biology. The importance of epigenetic variation in environmental adaptation and evolution has been investigated much more in plants than it has been in animals [16,17]. Moreover, most epigenetic data that are available on animals have been collected under laboratory conditions in model organisms such as mice and insects and these studies have mainly focused on epigenetic mechanisms and their responses to environmental stressors [17–20]. Laboratory studies on plants and animals have shed light on some of the general features of epigenetics, with important evolutionary implications. First, epimutations were assessed to be up to five orders of magnitude more frequent than genetic mutations were (10^{-4} versus 10^{-9} per base pair and generation) in the model plant *Arabidopsis thaliana* [21,22]. Second, some epigenetic marks may be stably inherited across generations through transgenerational epigenetic inheritance, as reported in many plant and animal taxa such as mammals [23], birds [24], fish [25], and invertebrates [26,27]. Third, epigenetic variation that is associated with changes in gene expression can be environmentally induced in plants and animals [28,29].

An important step is now to examine the extent of epigenetic variation and the way that this variation changes over time in wild populations that encounter natural levels of environmental complexity, genetic structure and dynamics, and natural ecological processes. This endeavor represents part of the field of population epigenetics. Though the basic concepts of population genetics from the 1930s are well described, these have been extended with the introduction of the modern synthesis (MS), however, the body of knowledge concerning population epigenetics remains largely scarce as it is a new research interest. As for laboratory studies, most of the natural population, epigenetic research projects have been carried out in plant populations, which have been reviewed elsewhere [16,30,31]. The first experimental work investigating epigenetics in natural animal populations was published in 2010 on DNA methylation in rainbow trout *Oncorhynchus mykiss* [32]. Since this study, there has been a growing number of

studies focusing on natural population epigenetic variation, especially DNA methylation variation in animals in terms of their phenotypic diversity generation and local adaptation.

As is the case for most of these articles, the present review focuses on DNA methylation, which is the most extensively characterized epigenetic mechanism in both plants and animals [33]. DNA methylation is found across all taxa of life, and primarily occurs at the 5-methylcytosine bases in eukaryotes and prokaryotes [34]. It involves the addition of a methyl group to cytosine within the CpG dinucleotides in animals. The DNA methylation of regulatory regions is generally associated with gene down-regulation or silencing, but that is not always the case [35,36]. Recent studies have showed that gene body methylation is positively correlated with transcriptional activity in most animal species [33]. The genomic distribution of DNA methylation has been described in many clades of animals, but there are some differences in how and where it occurs. In vertebrates, the pattern and extent of DNA methylation is well conserved across species; DNA methylation occurs nearly throughout the entire genome, with 70–80% of cytosines in the CpG dinucleotides being methylated [37]. Gene bodies, including exons and introns, are typically methylated, while CpGs in the gene promoter regions are often lowly methylated [38,39]. The idea that only vertebrates have a highly methylated genome has recently been challenged as this phenomena has also been found in the sponge *Amphimedon queenslandica* and a unicellular green algae from the genus *Chlorella* [34,40]. Despite this rather consistent DNA methylation pattern across vertebrate species, differences occur in terms of the pattern establishment during early embryogenesis. Taking DNA methylation reprogramming as an example, the demethylation of both parental genomes occurs in the mouse embryo, whereas the paternal pattern of methylation is maintained in zebrafish, with a reprogramming of the maternal DNA to correspond to the paternal template [41]. Regarding invertebrates, DNA methylation patterns are extremely variable across taxa. Some invertebrate genomes lack cytosine methylation such as the nematode *Caenorhabditis elegans*, the platyhelminth Schmidtea mediterranea, the fruit fly *Drosophila melanogaster,* and the rotifer *Adineta vaga* [42,43], while others are similar to plants as they have a mosaic of heavily methylated DNA domains (predominantly in exons) that are interspersed with domains that are methylation-free, such as the sea anemone, honey bee, and sea squirt [34,44]. Although the DNA methylation pattern and its genomic distribution vary widely across animal taxa, it is possible to draw general lines on its diversity and its responsiveness when it is facing natural environmental conditions.

Our discussion starts with a presentation of existing literature on DNA methylation variation and genetic variation within and among natural animal populations. It then focuses on the relationship between epigenetic and genetic variation, which illustrates a degree of autonomy of DNA methylation variation from genetics, and ultimately, its additional value in evolutionary mechanisms. The following section describes the epigenetic dynamics in natural animal populations. Some ecological processes act on epigenetic variation and patterns, and others act on both epigenetic and genetic structures. It is crucial to consider these processes to understand the current patterns of genetic and epigenetic variation, but also the past and the future populations' epigenetic dynamics. Then, we discuss the extended theory of evolution, including epigenetic variation as an evolutionary mechanism in natural populations. Epigenetic variation may be involved in population microevolution (rapid evolutionary events that are adaptations to a new environment during introduction and invasive events in fast-changing habitats and when stressors are occurring), but also in population macroevolution, including radiation and speciation. The review closes with a discussion on the directions of future studies on the epigenetics of wild animal populations. Addressing these topics is essential to achieve a more comprehensive understanding of the relevance and the roles of epigenetic mechanisms, especially DNA methylation, in regulating phenotypic plasticity and facilitating evolution in wild animal populations.

2. Epigenetic Diversity in Natural Animal Populations

DNA sequences are a succession of four different bases (A, C, T, and G), and each mutation switches a base for another one. An allele is a variant of the same gene that is located at the same genetic locus and is characterized by a specific sequence. Each diploid organism owns two alleles at each locus, and it is qualified as heterozygous if the alleles are different, or homozygous if they are the same. The situation is quite different for DNA methylation marks, since a cytosine can only be methylated (M) or unmethylated (U), thus restricting to two the number of possibilities that there can be at each cytosine. For the same allele, each CpG (and at a lower level, each CHG and CHH-H for any base, except for G) can either be M or U, thus producing a succession of single methylation polymorphisms (SMPs). SMPs can accumulate in the DNA sequence, and they produce a specific methylation pattern, or epiallele. While genetic variation refers to the different allele frequencies that there are among individuals or populations, epigenetic variation corresponds to the presence or absence of epigenetic markers

at specific loci that are studied among and/or within populations [45]. The amount of epigenetic variation within a population is called epigenetic diversity, and it refers to SMP diversity [46]. SMP diversity is generated by epimutations, i.e., epigenetic modifications at a given position or region, and its origins can be genetic, environmental, or stochastic [45,47]. Focusing on cytosine methylation (5mC), epimutations are heritable changes in the methylation status of a single cytosine or of a region or cluster of cytosines [48]. To determine the DNA methylation diversity in wild animal populations, most field studies have used methylation-sensitive amplified polymorphism (MSAP or MS-AFLP) [49–51]. Next-generation sequencing is, more rarely and most recently, the method that has been used among other such as reduced representation bisulfite sequencing (RRBS) [52–55], MeDIP-Seq analysis [56,57], and whole-genome bisulfite sequencing (WGBS) [58] (Table A2).

By comparing the epigenetic and genetic diversity in wild animal populations, it is possible to estimate the relative importance of genetic and epigenetic variation in populations phenotypic diversity, and to test the hypothesis that epigenetic divergence acts as the first step in speciation, allowing for the expression of alternative phenotypes in response to environmental changes, which are ultimately fixed by genetic accommodation or assimilation [11,15,59,60]. Many studies have identified extensive epigenetic diversity that exceeds the genetic diversity between natural populations of plants [61,62]. It was suggested that the epigenetic variation in natural plant populations plays a major role for their transient and/or heritable adjustment to the changing environments, as it may be stable and related to environmental variation [63]. This implies that these environmentally induced epimutations may lead to the convergence of individuals that are living in similar habitat conditions; this is a situation that may be exacerbated by TEI. Even though studies on epigenetic variation in natural animal populations are scarce when they are compared to plant studies, some discernible patterns have emerged after we have reviewed them. As result of our review, regardless of whether we focused on crustaceans, mollusks, fish, reptiles, birds, or mammals, the DNA methylation variation was larger than the genetic variation was among and/or within wild animal populations [53,56,64–68]. For example, Smith et al. studied the DNA methylation variation in fish (*Etheostoma olmstedi)* using the MSAP technique. They investigated two North American river drainages, wherein, each of them includes several closely related populations, to characterize the epigenetic variation within and among the populations. They obtained results that demonstrated that there is a significantly greater epigenetic diversity than there is genetic diversity within all

of the populations in both the Patuxent and Potomac rivers. Regarding the diversity among the populations, their analysis demonstrated that there is a substantial epigenetic structure, but no genetic structure, meaning that *E. olmstedi* populations are significantly different from each other in terms of their DNA methylation patterns, but not in terms of their genomes [60]. They assumed that the methylome is changing faster than the genome is in this species, which is in accordance with the general hypothesis that epigenetic divergence can precede genetic divergence in evolution due to its dynamics.

A larger amount of epigenetic diversity in comparison to the amount of genetic diversity can also arise between populations of sister species. Skinner et al. measured the genetic mutations (via copy number variation—CNV) and epimutations (via differential DNA methylation regions—DMRs) across five species of Darwin's finches (*Geospiza fuliginosa, G. fortis, G. scandens, Camarhynchus parvulus,* and *Platyspiza crassirostris*). As a result of these measurements, they found that there were fewer genetic mutations than there were epimutations among the five species, showing that the differences in the methylome are more related to evolutionary relationships than they are differences in the genome. Moreover, they reported that the differentially methylated genes were related to evolutionarily important pathways in birds [65]. Vernaz et al. found a substantial methylome divergence between six Lake Malawi cichlid species that show extensive phenotypic diversity despite having them extremely low DNA sequence divergence. These DMRs were enriched in transposons and were associated with the transcription changes of ecologically relevant genes that are related to energy expenditure and lipid metabolism in the cichlid's live [68].

An extreme situation can be observed in asexual species exhibiting a lack of genetic variation due to their reproductive system. A study on epigenetic polymorphism in the clonal fish *Chrosomus eos-neogaeus* from seven geographically distant lakes showed that they have an interindividual DNA methylation variability. Moreover, individuals could be regrouped according to their lake of origin based on their unique methylation profile, as individuals of a given lake are epigenetically similar [69,70]. Thorson et al. measured the genome-wide DNA methylation variation of asexual New Zealand freshwater snail *Potamopyrgus antipodarum* from distinct habitats (two lakes versus two rivers). Those snails have significant methylation signatures when one is comparing those of the lake versus those of the river populations [71]. Later, they examined the methylation variation among those in the lakes that differ in their environmental disturbance and pollution histories. Using an MeDIP-Seq analysis, they showed

the presence of site-specific differences in the DNA methylation between each of those lake populations [57]. These studies raise the question of the environmental implications in epigenetic variability, which is discussed later.

In most of the wild animal populations that have been examined to date, independently of the studied organisms or the molecular analysis that is being used, the DNA methylation variation is larger than the variation in allele frequencies within and among natural animal populations. This should not be surprising as epimutations can happen randomly, such as mutations, but they can also be triggered by environmental conditions and by the genotype itself. The epigenetic diversity that is found in a population is therefore the result of the combination of these three distinct sources. To determine the implications of the epigenetic processes in evolution, a major concern is to characterize the degree of autonomy between epigenetic and genetic variation and ultimately, the degree of phenotypic variation that can be explained only by the environmental or stochastic epimutations [72].

3. Correlation between Epigenetic and Genetic Variation in Natural Animal Populations

Based on their degree of autonomy from the underlying genotype, epialleles are categorized into three types: obligatory, which is completely dependent on the genetic variation; facilitated, which is directed or loosely potentiated by the genotype; pure, which is independent of the genetic variation and is generated by stochastic events or environmental changes [73]. To identify which epialleles categories are encountered in natural populations, the correlation between the genetic and epigenetic profiles can be estimated, mostly by using a Mantel test [50,63,74,75]. A significant (positive or negative) correlation suggests that the epigenetic and genetic variations are interdependent, which corresponds to the presence of obligatory epialleles. In contrast, the absence of a significant correlation suggests that the epigenetic variation can autonomously impact the phenotypic variation, by being totally or partly independent from genetic control.

Under laboratory conditions, epimutations are expected to be mostly obligatory. The lack of environmental fluctuations in the laboratory housing conditions does not promote environmentally induced epimutations and the selection of epimutation-sensitive alleles that

are responsible for alternative phenotypes that occur while experiencing environmental changes in natural conditions (see the Baldwin effect in "Section 5.2 Epigenetics and macroevolution of natural animal population"). In this case, epigenetic variation can be viewed as a phenotypic read-out downstream of the genotype, with a low environmental contribution. For example, Hu and Barrett reviewed the epigenetically encoded thermal plasticity in animals. Of the 14 studies, 13 included a putatively obligatory epigenetic variation that was underlying phenotypic plasticity, and only one was categorized as "unknown" [45]. In mice and humans, some studies have evaluated the association between epigenetic and genetic variation with a narrow-sense heritability, i.e., the ratio of additive genetic variance to the total phenotypic variance. It appears that genetic variation can explain an average of 7-34 % of all methylation variation [76–78]. In natural animal populations, although several studies have measured epigenetic and genetic variation, only a few of them have estimated the relationship between those variations. Of the 26 reviewed studies, 12 did not calculate a correlation coefficient between the genetic and epigenetic variation, eight studies found a non-significant correlation, and six studies obtained a significant correlation (Table 1). Moreover, few authors have linked a calculated correlation coefficient to Richards' epiallele categories. For instance, Liebl et al. obtained a significant negative correlation between the genetic and DNA methylation variation within seven populations of house sparrows (*Passer domesticus*) [79]. However, they predicted that all three kinds of epialleles could play a role in those populations as their design could not discriminate between the three categories. Despite the lack of direct connection between these categories and the genetic vs. epigenetic variation–correlation coefficient, calculating this coefficient can still help to estimate the relative importance of the genetic and epigenetic variation in the mechanisms that are facilitating population divergence, and to highlight the extent to which epigenetic variation is under genetic control [67,73].

Table 1. Overview of studies focusing on genetic and epigenetic diversity and correlation in natural animal populations.

Species	Genetic vs. Epigenetic Correlation	Epialleles Category	Ref.
Clonal fish (*Chrosomus eos-neogaeus*)	No significant correlation	Putatively pure or facilitated	[69]
Clonal fish (*Chrosomus eos-neogaeus*)	No significant correlation	Putatively pure or facilitated	[70]
Clonal fish (*Chrosomus eos-neogaeus*)	No significant correlation	Unknown	[80]
House sparrows (*Passer domesticus*) (Africa)	Significant negative correlation	Unknown	[79]
House sparrows (*Passer domesticus*) (Australia)	No significant correlation	Unknown	[74]
Red grouse (*Lagopus lagopus scotica*)	No significant correlation	Unknown	[81]
Bats	Significant positive correlation	Unknown	[66]

(*Rhinolophus pusillus, Hipposideros armiger* and *Miniopterus fuliginosus*)			
South African (Gansbaii) sandhopper (*Talorchestia capensis)*	No significant correlation	Putatively pure or facilitated	[50]
South African sandhopper (*Talorchestia capensis)*	Significant negative correlation	Putatively obligatory	[50]
Pacific oyster (*Crassostrea gigas*)	Significant positive correlation	Putatively obligatory	[67]
Crested anole (*Anolis cristatellus*)	Significant positive correlation	Putatively obligatory	[54]
Eastern oyster *(Crassostrea virginica)*	No significant correlation	Unknown	[53]
Fish *(Gobio occitaniae)*	Significant positive correlation	Unknown	[75]
Chinook salmon (*Oncorhynchus tshawytscha*)	No significant correlation	Unknown	[82]

Even though the obligatory epialleles' relevance is questioned regarding its evolutionary potential [69], an interesting new insight is that the epigenome can also influence the genome, thereby creating a significant relationship between their variation. Firstly, the epigenetic variation can regulate the active status of transposable elements (TEs). TEs are DNA sequences that can change their position within a genome. The epigenetic control of gene expression mostly originates from the regulation of TEs that are inserted near genes [83]. In fact, TEs are the major carriers of epigenetic marks and are subject to almost all epigenetic regulatory mechanisms in plants [84]. Interestingly, there is a great variability in the locations of the TEs, not only between different species, but also within populations. More than 90% of the TEs that are inserted at a specific genomic position are not present in all individuals within both animal [85] and plant [84] populations. Transposon insertion polymorphism between individuals may come from epigenetic variation and it results in genomic sequence variation. Secondly, DNA methylation variation can influence the mutation rates in repetitive elements which are known to regulate genome 3D folding and the establishment of heterochromatin, among other regulation mechanisms [86]. These repetitive elements are patterns of nucleic acids that occur as multiple copies in the DNA sequence, comprising TEs, simple sequence repeats (SSRs), and microsatellites, and these represent a major fraction of vertebrate genomes [87]. Some studies have highlighted the correlation between a decrease in the DNA methylation of specific repetitive elements and an increase in the copy number variations (CNVs), thus showing another possible epigenetic-to-genetic flow [88,89]. Finally, besides these specific genome components, epimutations may alter the global genome stability and modify the mutation rate of the DNA sequences throughout the genome. Methylated cytosines (mCs) in the CpG context seem to have a higher mutation rate than non-methylated ones [90], and this is in part because mCs are subject to spontaneous deamination [91]. This process, where an mC turns into a T,

occurs at a rate that is 10- to 50-fold higher than any other mutation is in humans [92]. The result of this hypermutability is a CpG depletion in the consistently methylated genomic regions. Yet, the amount of CpGs in a genome partly determines its epigenetic potential, which is defined as "the capacity for environmentally induced phenotypic change (i.e., plasticity) via epigenetic modifications to relevant genomic elements" [93]. CpGs are considered as the capacitors of phenotypic plasticity; the more CpGs an organism has, then the more facilitated their acclimation is via DNA methylation and gene expression regulation.

In summary, epigenetic and genetic changes most likely work in concert to regulate the gene expression and phenotypic variation of complex traits (Figure 1). The proportion of the genotype-independent and -dependent epigenetic variation reflects the underlying mechanisms of the natural animal population's evolutionary pathways to promote phenotypic variation [65]. New insights that we would like to highlight is that even though there is a significant correlation between epigenetic and genetic variation, epigenetic variation is not necessarily dependent on genetic variation. As has already been explained, the epigenome can influence the genome in different ways. Moreover, a significant correlation does not imply that there is a causal relationship. Geographical and ecological processes may create parallel evolutions of genetic and epigenetic structures, and thus, similar patterns that are without any functional link with each other. To better understand these dynamics, it is important to consider the mechanisms that are influencing both genetic and epigenetic diversity, and the processes that only act on epigenetic markers in wild animal populations.

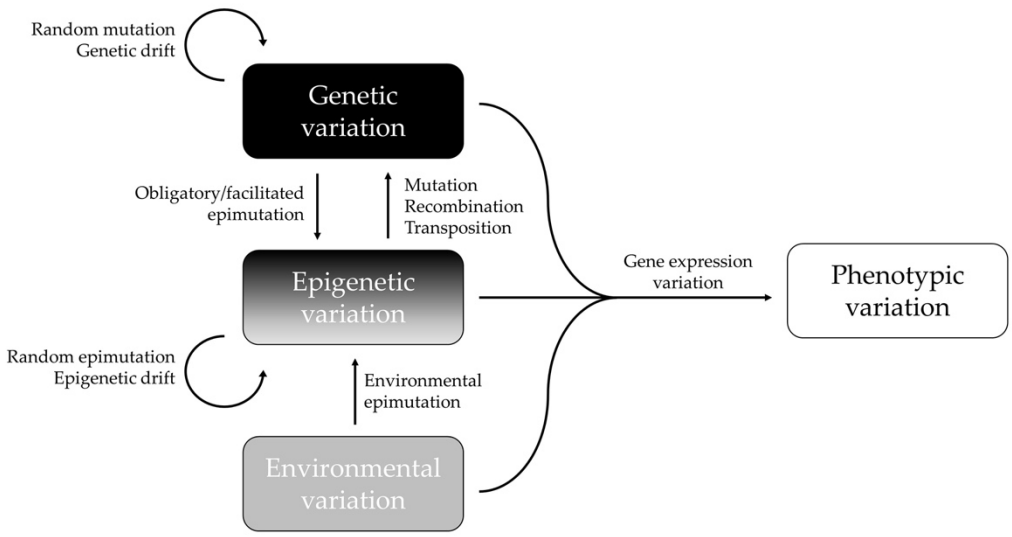


Figure 1. Interactions between epigenetic, genetic, environmental, and phenotypic variations. Epigenetic variation can depend upon the genotype (obligatory and facilitated epimutations), or it can be independent of the genotype (pure epimutations) and be generated by environmental changes or stochastic events (random epimutation/epigenetic drift). Adapted with permission from [47], 2022, Frédéric Silvestre.

4. Epigenetic Dynamics in Natural Animal Populations

4.1. Geographical and Ecological Processes Acting on Both Epigenetic and Genetic Diversities

If the epigenetic marks are stably inherited, similar processes that contribute to generating patterns of the genetic structure in natural populations can also act on the epigenetic variation [54,57]. Gene flow is an important mechanism for transferring alleles between populations, thus resulting in increasing the homogeneity among populations, and in increasing the genetic diversity within a population. Some factors can decrease the gene flow, thus generating genetic isolation and in some case, speciation. The gene flow is reduced in the species with low dispersal or mobility, that are living in fragmented habitats, are made of small populations, and are separated by a long distance. This geographically limited dispersal creates genetic differentiation, which is also called isolation by distance (IBD) [94]. Herrera et al. proposed a similar approach to measure the epigenetic IBD among individuals or among populations, and to use the spatial structure of their genetic diversity as a null model to investigate the processes that are shaping epigenetic variation in natural populations [95]. The variable level of transgenerational epigenetic inheritance and the capacity of epigenetic marks to be modified in response to environmental variation are the two factors explaining the possible differences between genetic and epigenetic diversity. In plants, this approach generally shows that there is a greater level of epigenetic IBD than genetic IBD, and there is a higher epigenetic similarity when this is compared to the amount of genetic similarity at the shortest distance, suggesting that both a significant TEI and a high responsiveness to the environmental local conditions are the major drivers of epigenetic spatial structure [95].

Besides the geographical distance, the ecological conditions are other landscape elements that can influence gene flow. Temperature, precipitation, humidity, elevation, substrate type, and vegetation density are all examples of the environmental factors that can also play a role in evolutionary processes like isolation by environment (IBE). IBE is defined as a pattern in which the degree of genetic differentiation increases with the environmental differences, independent of the geographic distance [96]. A variety of processes can generate genetic IBE, including natural and sexual selection against migrants from divergent environments and biased dispersal. IBD and IBE, besides acting on genetic structure, can also act on epigenetic diversity as

epialleles should be transferred between populations with gene flow [95]. DNA methylation divergence, genetic divergence, and reproductive isolation were investigated in eight pairs of geographically isolated species *Etheostoma* ('darters'), a diverse genus of North American freshwater fish [60]. The strongest reproductive barrier among darter species seems to be the behavioral reproductive isolation, i.e., a reduction in gene flow due to differences in mating behavior [97]. They found a significant relationship between behavioral isolation and interspecific epigenetic divergence, but not with genetic divergence. These results suggest that the strength of the behavioral isolation among the eight allopatric, phylogenetically independent species is predicted by epigenetic divergence [60]. Another study reported significant DNA methylation differentiation that is consistent with short-distance dispersal among great roundleaf bat populations in China [98]. These studies illustrate the strong relationship that may exist between epigenetic and isolation mechanisms as gene flow reduction occurs due to sexual selection or dispersal capacity, thus creating a higher epigenetic population structure than that of the genetic structure. A recent study compared spatial genetic and epigenetic variation based on single nucleotide polymorphisms (SNPs) and single methylation variants (SMVs) from eight populations of the Puerto Rican crested anole *Anolis cristatellus* that occupies a diverse range of habitats [54]. They found that the plots of the genetic and epigenetic IBD and IBE indicate that they have similar slopes, suggesting that the genetic and epigenetic variation may have shared responses to geographical and environmental factors. Interestingly, after controlling for the effects of the underlying genetic structure, there is still a relationship between the epigenetic and genetic structure, but they did find evidence for a strong pattern of genome-wide epigenetic IBE. This significant epigenetic IBE suggests that the epigenetic variation in *A. cristatellus* is not only attributable to the pattern of genetic variation, but that epigenetic differentiation is strongly correlated with environmental divergence. This is the first study of its kind, as the empirical demonstration of epigenetic IBE has been limited to only a few plant systems [63,95]. This difference between genetic and epigenetic IBE probably arises from the ecological processes that influence epigenetic but not genetic diversity.

4.2. Ecological Processes Increasing Epigenetic Diversity

Some processes can act on epigenetic but not genetic variation, thereby contributing to the extensive epigenetic diversity that exceeds that of the genetic variance, as described above. These pure methylation variations may be created by stochastic events (like random epimutations or epigenetic drift) and are also induced by environmental variation.

Random epimutations can arise at any time in the lifespan of the organism, and they are not induced by environmental factors. Accurately setting, erasing, and reproducing methylation patterns are complex processes involving a series of interconnected factors (reviewed in [47]). A first possible mechanism sustaining random epimutation refers to the imperfect fidelity of methylation replication. In eukaryotes, DNA methylation replication is catalyzed by the enzymes of the DNMT (DNA methyltransferase) family. DNMT1, also named as "the maintenance DNA methyltransferase", has an accuracy rate of ~95%, despite its regulatory mechanisms such as autoinhibition [47]. This defect of 5% inaccuracy can generate new DNA methylation patterns, especially since replication is required over the entire genome. A second mechanism underlying the random epimutations is *de novo* methylation during early-life stages. Epigenetic marks are placed at very specific times during the organism's development, namely gametogenesis and early embryogenesis. The patterns are set by other members of the methyltransferase family, namely DNMT3A and DNMT3B, which are also known as *de novo* methyltransferases. Due to mechanisms such as the imprinting of primordial germ cells and DNA methylation reprogramming, gametogenesis and early embryogenesis offer another window of susceptibility for random epigenetic alterations. Studies focusing on quantifying the epimutation rates have mainly focused on the model plant *Arabidopsis thaliana* [21,22,99]. The forward and backward CpG epimutation rates (i.e., methylation is gained or lost, respectively) were estimated to be 2.56×10^{-4} and 6.30×10^{-4} per generation per haploid methylome, respectively [22]. These estimates are similar to the rate that has been provided previously (4.46×10^{-4} by [21]), but they illustrate that methylation loss at the CpG is globally three times as likely as the methylation gain is. They also detailed the extent to which CpG epimutation rates depend on the genomic context, with the highest rates being found in gene bodies (forward: 3.48×10^{-4} and backward: 1.47×10^{-3}), and the lowest rates being found in transposable elements (forward: 3.24×10^{-4} and backward: 1.20×10^{-5}). Interestingly, a spontaneous error rate in methylation maintenance at the promoter CpG islands (both gains and losses) was measured to be 10^{-4} to 10^{-5} in vitro [100], which means that even the genome regions that are essential in gene expression regulation can be modified by random epimutations. This set of results contrasts with the spontaneous genetic mutation rate of 7×10^{-9} base substitutions per site per generation in *A. thaliana*, 2.3×10^{-10} in *C. elegans*, 3.4×10^{-10} in drosophila, and 5.0×10^{-11} in humans [101,102]. In other words, random epimutations can emerge at any time in the lifespan of the organism, with rates that are expected to be higher than the genetic mutation

rates. Some events have a high susceptibility for random epigenetic alterations including cell division, gametogenesis, and embryogenesis.

Epigenetic drift corresponds to the gradual changes in epigenetic patterns, and it is due to random epimutations. This neutral process is not directional as it creates both hyper- and hypomethylation. Moreover, drift is not uniform across the genome, and is variable between individuals of the same age. A meaningful drift example is age-related epigenetic drift. This uncoordinated accumulation of methylation variation creates a global DNA hypomethylation and degrades the transcriptional networks during aging [103]. This process is variable across the genome, may not occur homogeneously in all cells, and is variable between individuals of the same age. Thus, epigenetic drift leads to increased discordance between individual epigenomes across the lifespan of the organism. Conversely, some programmed changes in the methylation of specific CpG sites are consistently related to age between individuals of the same species. This programmed aging-associated epigenetic modification refers to the epigenetic clock [104]. The prevailing tendencies of these specific changes are the hypermethylation of the promoter sequences that are associated with CpG islands and the hypomethylation of CpG-poor genes. There is a strong correlation between the age and methylation levels of multiple CpG sites in individuals of the same species [103], whereby, their methylation status could be used as an epigenetic signature to estimate their biological age. Until recently, this “epigenetic clock” had only been developed in mammals, including humans, mice, whales, dogs, and wolves. A large international consortium recently compared thousands of methylation marks among 59 tissues and constructed highly accurate universal epigenetic clocks for 128 mammalian species [105]. Although very little is known about non-mammalian vertebrates, recent studies have also relied on DNA methylation repatterning during aging to develop such epigenetic clocks for a few fish species, including zebrafish, Japanese medaka, European seabass, Australian lungfish, Murray cod, and Mary River cod [106,107]. Thus, both epigenetic drift and the epigenetic clock contribute to time-related changes in DNA methylation, but in fundamentally different ways. In both cases, gene expression regulation by epigenetic mechanisms becomes gradually deregulated therefore leading to a diminished responsiveness to environmental stimuli. Ultimately, epigenetic drift could lead to a loss of cellular phenotypic plasticity [108]. Studies focusing on whether and how drift influences epigenetic marks in wild animal populations are very scarce. Recently, Venney et al. provided new evidence that drift could act on DNA methylation by highlighting

the correlation between microsatellites (considered as neutral genetic markers of genetic drift) and the differences in methylation among eight populations of Chinook salmon (*Oncorhynchus tshawytscha*) [82]. Despite there being a lack of studies focusing on epigenetic drift and random epimutations in wild animal populations, several field studies have explained the presence of large amounts of epigenetic diversity in contrast with the presence of smaller amounts of genetic diversity with the occurrence of mechanisms including stochastic epigenetic drift and epimutations in a wide range of animals [54,57,60,65–67,71,81].

Beside these processes, which are similar to those of genetics (genetic/epigenetic drift and random mutations/epimutations), another major mechanism can act specifically on epigenetics markers: environmentally induced epimutations. Unlike genetic variation, DNA methylation can be rapidly influenced by environmental variation, particularly when the organism is in the early developmental stages [109]. Some studies have investigated the influence of the environment in shaping the epigenome under different laboratory settings (e.g., temperature [110], diet [111], behavior [112], and chemicals [113]. However, they may not reflect the epigenetic processes that occur under field conditions with natural levels of environmental heterogeneity and complexity. Field studies on plant populations have shown that there are strong environmental effects on DNA methylation [30,63,95,114]. Similar results have been obtained for animals as population epigenetic studies have provided evidence of habitat-specific DNA methylation patterns in a wide range of wild animal species (e.g., [50,53,54]), especially between ecotypes, such as freshwater *vs.* marine three-spined sticklebacks *Gasterosteus aculeatus* [115], coastal *vs.* offshore common bottlenose dolphins *Tursiops truncates* [116], and lake *vs.* stream ecotypes of clonal fish *Chrosomus eos-neogaeus* [80]. These observations of environmentally induced epimutations are even more likely in habitats that are disturbed by urbanization and/or pollution, wherein DNA methylation variation could be driven by a variation in food availability and pollutant levels [55]. Guillette et al. have focused on the potential alterations in the epigenome of the American Alligator *Alligator mississippiensis* that live in contaminated (Lake Apopka—AP and Merritt Island—MI) and non-contaminated (Lake Woodruff—WO) lakes in Florida. They identified 85 differential DNA methylation regions between WO and AP, and 75 between WO and MI, showing that there are more epigenetic alterations in the species in the contaminated lakes compared to those in the species in the non-contaminated lake [109]. Similar results have been observed between asexual snails *Potamopyrgus antipodarum* living in rural lakes vs. urban lakes [57], between hatchery and

natural-origin steelheads *Oncorhynchus mykiss* [52], between baboons *Papio cynocephalus* that forage naturally in a savanna environment *vs.* baboons that have access to spatially concentrated human food scraps [117], and also between two closely related species of Darwin's finch living in rural *vs.* urban populations [56]. Regarding this last study, more interestingly, few of the DMRs between the rural and urban populations were found in the same DNA sequence regions in *G. fortis* and *G. fuliginosa.* This suggests that these species are responding to environmental changes in different ways, which correspond to a species-specific sensitivity to environmental variations, even when they were comparing the closely related species [56]. These studies show habitat-, population-, or species-specific DNA methylation patterns in a wide range of wild animal populations, indicating that local environmental factors may influence DNA methylation patterns among populations. Thus, environmentally induced epimutations could ultimately contribute to the extensive epigenetic diversity that is observed in the animal populations studies that are reported in this review. Moreover, the environmentally induced epigenetic variation between natural populations could be even greater in the case of isolation, especially isolation by environment, as described above. Ultimately, environmentally induced epimutations could lead to local adaptations if these marks are inherited across generations.

To date, we have found very few studies that evaluated the transgenerational inheritance of DNA methylation marks in natural animal populations encountering different environmental conditions. Wang et al. investigated the environmentally induced phenotypic variation, DNA methylation, as well as heritable epigenetic variations between intertidal and subtidal Pacific oysters *(Crassostrea gigas)* using WGBS. Their offspring F1 were produced and subjected to a common environment. There was a clear DNA methylation differentiation between the intertidal and subtidal oysters, as they identified 3012 differentially methylated genes (DMGs) in F0, and 3090 DMGs in F1. Moreover, the 1238 DMGs that were found in the F1 oysters were shared with those in the F0 generation, meaning that about 41% of the DMGs between the intertidal and subtidal oysters could be transmitted to the next generation. They also investigated the variation tendency in the 1238 inherited genes. Nearly 70% of the heritable DMGs had a consistent variation trend in response to the environments in the two generations. Finally, these DMGs were annotated, and they appeared to be involved in phosphorus, lipid, and protein metabolism, and in the regulation of GTPase activity, autophagosomes, and apoptosis [58]. This study highlighted the inherited environmentally induced methylation variation that may underlie the phenotypic divergence that is related to the heat stress between

intertidal and subtidal oysters across generations. A second study that was carried out by Hu et al. showed similar results by comparing the DNA methylation variation between marine and freshwater ecotypes of threespine sticklebacks (*Gasterosteus aculeatus*) using an RRBS technique. F0 fish were collected in marine and freshwater locations and maintained in a common garden. F1 and F2 subjects were generated by crossing the marine and freshwater wild-caught parents to explore their stable epigenetic variation and its underlying genetic basis across two generations of the marine-freshwater hybrid lines. Firstly, they identified 891 differentially methylated cytosines (DMCs) between the parental fish that were sampled from the marine versus the freshwater habitats. Then, they investigated the levels of intergenerationally stable methylation. They found that 94.8% (845 out of 891) of the DMCs between the ecotypes were identified as stable across generations, suggesting that this methylation divergence could play a role in facilitating their adaptation to different habitats. They also found a narrow-sense heritability of these stable DMCs, ranging from 24% to 35%, meaning that some of them are obligatory epimutations (under genetic control), while other are pure epimutations. Finally, their functional analysis identified several DMC-associated genes that are related to environmental variations such as salinity, osmosis, parasites, and diet [118]. Those two recent studies bring new insights into the extent to which variation in environmentally induced DNA methylation is stably transmitted across generations in wild animal populations, and they provide promising evidence for the adaptive mechanisms through which these transmitted epimutations occur.

To summarize, a population's epigenetic and genetic structures might be the consequences of the combination of ecological mechanisms that are in common with or specific to genetic and epigenetic dynamics (Figure 2). Gene flow can transfer both alleles and epialleles between different populations, thereby dealing with barriers such as geographical, environmental, or reproductive barriers. Stochastic events such as drift and mutations/epimutations also act on both genetic and epigenetic divergence, with there being a possible greater impact on epigenetic markers as the epimutation rates are expected to be higher than the genetic mutation rates are. A specificity of epigenetic markers is their responsiveness to environmental variation. It can create habitat-, population-, or species-specific DNA methylation patterns that may be transmitted to the next generation, resulting in among-population but also within-population variations, as individuals are likely to display different sensitivities to environmental stimuli. Taking these processes into account, the observed greater amount of epigenetic variability that

is seen when this is compared to the amount of genetic diversity might be caused by epigenetic drift and random or environmentally induced epimutations, whose effects are exacerbated in the situation of limited or insufficient gene flow to prevent divergence. This situation mainly occurs in species with a low dispersal or mobility, those that are living in fragmented habitats, are made of small populations, and are separated by a long distance, thus promoting genetic and epigenetic drift.

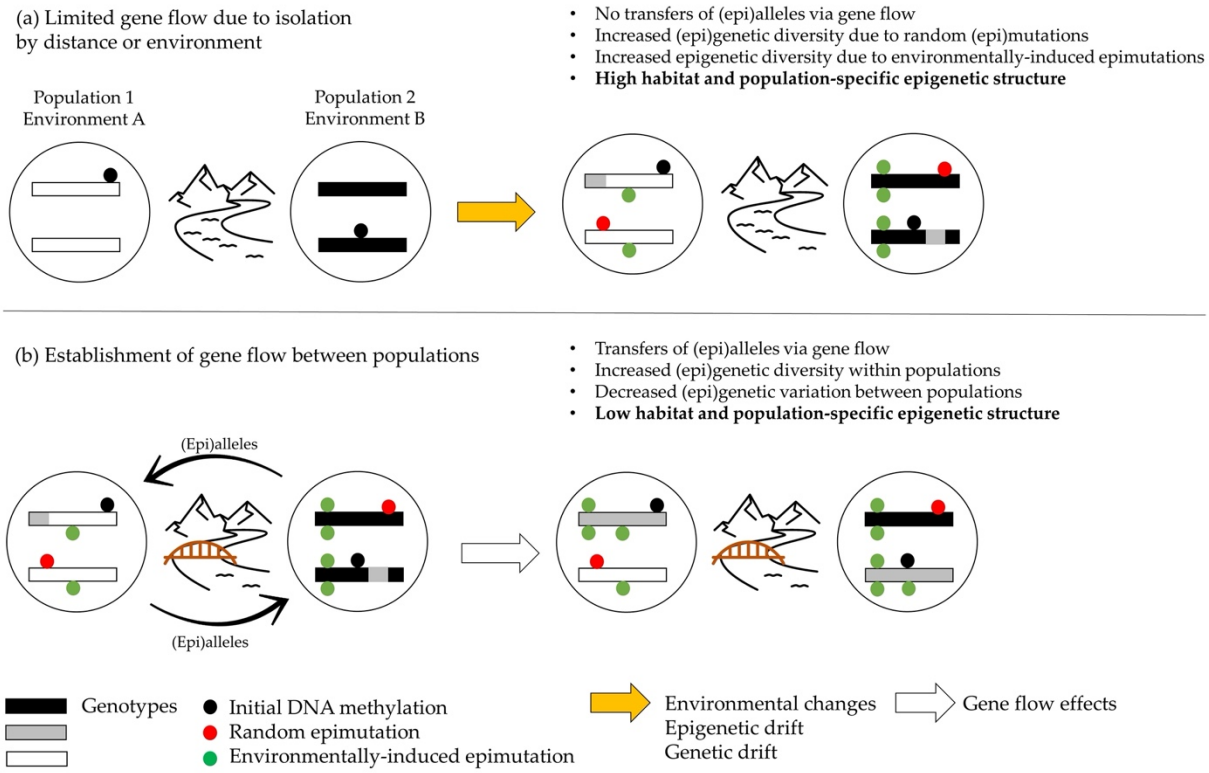


Figure 2. Epigenetic dynamics: ecological and geographical processes and their consequences on (epi)genetic diversity within populations and on (epi)genetic variation between populations. (**a**) Isolation by distance or by environment limits (epi)alleles transfer by gene flow. Stochastic events such as drift and (epi)mutations increase (epi)genetic diversity within populations over time. Environmentally induced epimutations create habitat and/or population-specific DNA methylation patterns that may be transmitted to the next generation, resulting in strong epigenetic structures. (**b**) As consequences of gene flow, (epi)genetic diversity increases within populations and (epi)genetic variation between populations decreases, thereby resulting in lower epigenetic structure that distinguished populations.

5. Epigenetic Variation as an Evolutionary Mechanism in Natural Populations

Natural selection acts on an organism's phenotypes to enhance their fitness. One of the basic principles of evolution is that phenotypic variation in a population derives from the accumulation of mutations in the DNA sequence which gradually accumulate over generations. However, the slow spread of genetic mutations does not explain all of the observed micro- and macroevolutions, and they cannot keep pace with the rapidly changing environment [66].

Unlike that of genetics, epigenetic inheritance can rapidly affect the population. As described above, epimutations can arise in response to an environmental modification on a much faster time scale, within a single generation than a single de novo genetic mutation in a single individual can. This neo-Lamarckian concept of acquired characteristic inheritance (also known as "soft inheritance"; [119]) and that of the neo-Darwinian evolution should not be seen as incompatible, but instead, they can form a unified theory for evolution which is named the Extended Evolutionary Synthesis (EES) [120]. This theory involves environmentally induced epimutations and an epigenetic transgenerational inheritance that alters the phenotypic variation, on which natural selection can act [121], among other concepts that are illustrated in Figure 3. Several studies on epigenetics in natural animal populations have showed environmentally induced epimutations, but none of them have focused on their evolutionary consequences, which could be the target of future studies. Despite this lack of evidence, the extended evolutionary synthesis theory provides new insights into the microevolution mechanisms for rapid evolutionary events such as an organism's local adaption to a new environment during introduction and invasive events in a fast-changing habitat, wherein stressors occur intermittently [121], but also, it provides new insights for macroevolution, including radiation and speciation [60].

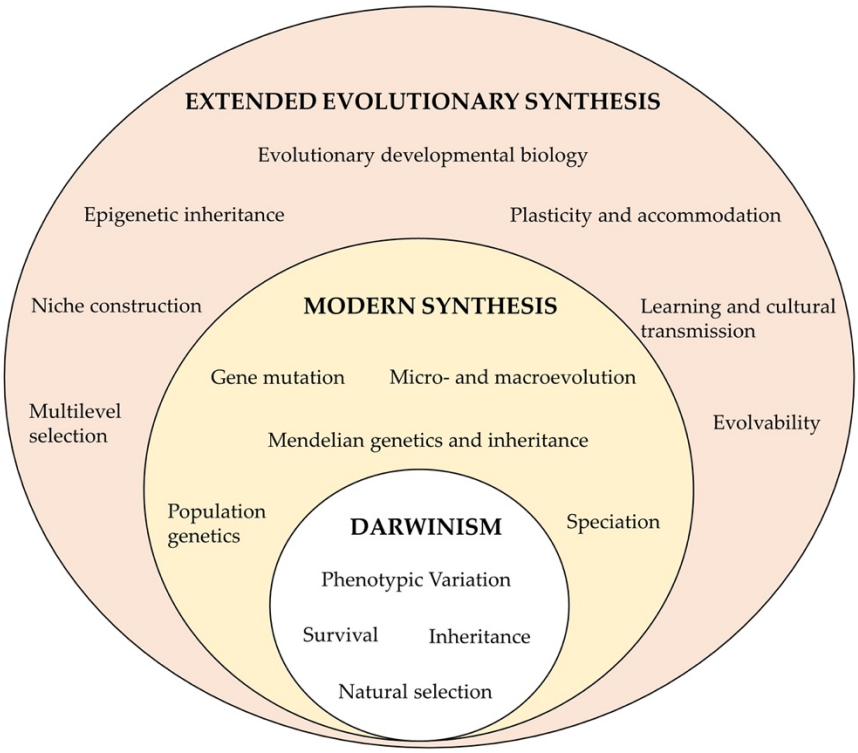


Figure 3. Schematic representation of key concepts included in the Extended Synthesis, illustrating the continuous expansion of evolutionary theories. Adapted with permission from [120], 2022, Massimo Pigliucci.

5.1. Epigenetics and microevolution of natural animal populations

Microevolution corresponds to the processes that lead to intraspecific evolutionary changes that occur over time within and among populations. Among these processes, local adaptation is considered to be one of the major mechanisms that is used to explain how organisms adapt to environmental variations or to a new habitat during introduction and invasive events [122]. It is the process by which organisms of the same species evolve and adapt towards different phenotypic optima depending on the local environment in which they live [87]. Studies on plants populations have demonstrated local adaptations that are related to epigenetic variation, close associations between epigenetic variants and environmental gradients in a variety of natural plant systems, and the role of local epigenetic adaptation during biological invasions (e.g., [63,123,124]). These studies have highlighted the importance of epimutations in local epigenetic adaptations.

Focusing on how epimutations regulate phenotypic traits during local adaptation might shed light on how animal species evolve and which evolutionary strategies they apply. On the one hand, environmentally induced epimutations can be associated to a sense-and-response system (i.e., phenotypic plasticity). As described previously in this review, recent studies have showed habitat-specific DNA methylation patterns in a large panel of species. In the previously detailed study of Wogan et al. on the DNA methylation variation in eight populations of the Puerto Rican crested anole *A. cristatellus*, they detected 95 single methylation variants (SMVs), thereby showing a significant relationship to climatic variables: 14 of these were significantly linked to the maximum temperature of the warmest month, and 25 were linked with temperature annual range. Moreover, all of the 95 SMVs were significantly correlated to precipitation seasonality [54]. This study indicates that DNA methylation variation can occur across the environmental gradients of the factors affecting methylation. Just like the plants' systems, environmentally induced epimutations could play an important role in the phenotypic plasticity, thereby leading to the local adaptation of animals. This strategy seems to be the best if the environmental variations are predictable. Baldanzi et al. investigated the levels of genetic and DNA methylation variation within and among populations of the sandhopper *Talorchestia capensis* from five localities along the South African coasts. Four populations showed significant negative relationships between their epigenetic and genetic diversity (corresponding

to a genome-dependent epimutation). The Gansbaai population, the exception, showed no correlation between the two patterns. Interestingly, the Gansbaai population is the only population that is found in a transition area with a high level of environmental changes. Environmentally induced epimutations in the individuals from Gansbaai could be a mechanism of their adaptation to these transitional environmental conditions [50].

On the other hand, random epimutations highlight the propensity to randomly diversify the phenotypes and these are supposed to be more advantageous when organisms encounter unpredictable environmental changes; a strategy that is referred to bet-hedging (i.e., organisms suffering decreased fitness under their normal conditions, but increased fitness under unexpected stressful conditions) [124]. Bet-hedging allows individuals of a population to present a panel of phenotypes including some with high fitness, ensuring the survival of a proportion of the population, whatever the current environmental conditions are. Most incidences of bet-hedging that have been so far highlighted are for prokaryotes, chordates, angiosperms, and arthropods (16 phyla, reviewed in [125]). To our knowledge, the only study in which the methylome of multicellular animals has been studied from a bet-hedging perspective is a study on a wild populations of clonal fish *Chrosomus eos-neogaeus* [80]. The authors of this study analyzed the DNA methylation polymorphism in *C. eos-neogaeus* between two types of environment: predictable (lakes) and unpredictable (intermittent streams) areas. They showed that the contribution of environmentally induced and stochastic epigenetic changes strongly differs between the predictable and unpredictable environments. Indeed, clonal fish that are found in predictable environments display environmentally induced epigenetic changes, whereas those living in unpredictable environments are characterized by a high contribution of random epimutations. Thus, pure epigenetic variation (environmentally induced or random) can be adaptive when the environment changes rapidly, thus being predictable or not.

Epigenetic mechanisms can be associated with another central phenomenon in evolutionary biology and population dynamics: the expansion of newly introduced populations, which is considered as a genetic paradox. These populations succeed when they spread out in a new environment, despite the fact that they are small, presumably not adapted to their new habitat, and encounter a significant decrease in genetic diversity which is associated with passing through a bottleneck [115]. Several natural animal population studies on invasive species have

showed that when the genetic diversity is low, epigenetic processes can maintain a high phenotypic variability via a compensatory mechanism between the epigenetic and genetic variation, which could explain their expansion ranges. These studies include observations of the asexual freshwater snail *Potamopyrgus antipodarum* [57,71], the pygmy mussel *Xenostrobus secures*, the tubeworm *Ficopomatus enigmaticus* [49], the mussels *Mytilus galloprovincialis* and *Xenostrobus securis* [126], and the house sparrow *Passer domesticus.* Regarding this latter species, studies have screened for the DNA methylation of the introduced house sparrows in Tampa (Florida) and the Nairobi (Kenya) populations [127], in several cities in Kenya [79], and in the Middle East [128]. Those populations encountered a recent founder effect, thereby reducing their genetic diversity. It turned out that each study obtained the same results: an excess of DNA methylation variation which was relative to genetic variation. Liebl et al. also identified a negative relationship between epigenetic and genetic diversity, which corresponded to a compensatory mechanism for reduced levels of genetic diversity [79]. However, a more recent study on the levels of epigenetic and genetic diversity across 15 sites in the introduced Australian house sparrow population failed to detect any correlation between the two profiles [74]. It suggested that epigenetic diversity is likely to compensate for low genetic diversity that occurs immediately after a bottleneck. A compensatory relationship may have been stronger in the earlier stages of the introduction, but this is now obscured by the genetic diversity recovery. Another insight involves the reversibility of the epigenetic markers. Epigenetic markers are highly dynamic, suggesting that the extent to which DNA methylation signatures are established and removed is variable over time. This study highlights the importance of incorporating history into population-wide epigenetic analysis.

5.2. Epigenetics and macroevolution of natural animal population

Macroevolution corresponds to the processes that lead to interspecific (or higher-rank taxa) evolutionary changes that occur over geologic time. For example, it includes adaptive radiation which is defined by a process in which organisms diversify from an ancestral species into multiple new forms, and this results in speciation. This process particularly occurs when new resources, new environmental niches, or new disturbance arise. In these situations, epigenetic variation is likely to play a role in the initial stages of ecological speciation by facilitating an adaptation to novel ecological environments via phenotypic plasticity. On the one hand, a significant environmental shift from a stable habitat to a novel, stable habitat should favour genetic assimilation [129]. During this process, environmental changes induce the epimutations

that are responsible for a new advantageous phenotype. This environmentally induced phenotype and its underlying epimutations are maintained across generations and these are subject to natural selection as an adaptive alternative. Over time, these environmentally induced epimutations are incrementally replaced with multiple advantageous genetic mutations through the process of natural selection. The epigenetic contribution to the phenotype decreases as the genetic contribution increases. Ultimately, the environmentally-induced phenotype becomes genetically encoded in the population due to the process of mutation selection, and the environmental signal, as well as the epigenetic marks that are no longer required to produce it [7]. It corresponds to a 'mutational assimilation' in which the mutations are facilitated by epigenetics [14]. This process requires that environmentally induced epimutations are inherited through generations and that the environment is stable for a period that is at least as long as the organism's generation time. This mechanism supports the theory that epigenetic variation precedes genetic variation, as it has the potential to accelerate genetic evolution [11]. On the other hand, a new habitat with fluctuating conditions selects for a high level of plasticity; a process that is named the Baldwin effect [130]. In this case, individuals can express alternative phenotypes due to an alternative methylation pattern established being on some sensitive alleles. These genes that are required for flexibility are selected, and their frequency will increase in the population. In this case, there is no inheritance of the DNA methylation marks. In summary, it is the flexibility of the phenotype that is selected, rather than the result of the flexibility itself. These two concepts of genetic assimilation and the Baldwin effect suggest a role for DNA methylation in the initiation of species divergence and radiation.

Moreover, field studies have showed that DNA methylation is also involved in the maintenance of species divergence. For example, Skinner et al. compared the epigenetic differences of five closely related species of Darwin's finches (*Geospiza fortis, G. fuliginosa, G. scandens, Camarhynchus parvulus,* and *Platyspiza crassirostris*) and tested the hypothesis that DNA methylation variation accumulates with phylogenetic distance. They obtained a significant correlation between the number of epigenetic variations and phylogenetic distance between the finches, but no significant between the genetic variants and the phylogenetic distance [65]. This study showed that epimutations appear continuously and accumulate over long periods of time (2–3 Myr). Another study on DNA methylation in fossilized steppe bison *Bison priscus* and bison fresh tissue has showed that there are stable patterns of methylation between ancient and contemporary DNA samples [131]. These findings suggest a role for DNA methylation, not

only in the initiation of radiation, but also in the maintenance of species divergence over evolutionary timescales, as epigenetic variations can persist over thousands of generations.

6. Future Research in Animal Population Epigenetics

The greatest challenge confronting populations epigenetics is to determine the role of natural epigenetic variation in adaptive evolution. Experimental field studies that are investigating this question in animals are in their first steps. Before considering epigenetics as an evolutionary mechanism, some characteristics have to be investigated or confirmed. First, despite the fact that epigenetic inheritance has been shown in laboratory studies, very few studies have focused on it in wild animal populations [58,118]. Epigenetically induced phenotypes can be transmitted to an organism's offspring if the epigenetic marks can resist resetting between generations, i.e., epigenetic reprogramming. This mechanism corresponds to an extensive erasing of epigenetic marks, and it occurs both in the germline and in the zygote immediately after fertilization in animals. The reprogramming process has been described in a few species such as mice [132], zebrafish [133], killifish [134], and medaka [135], but we still need to unravel the mechanisms of epigenetic reprogramming in more species in the wild, given its species-specific characteristics. Despite this barrier to transgenerational epigenetic transmission, emerging evidence has shown that pure (random and environmentally induced) epimutation inheritance may exist in animals. Considering the laboratory results, field studies such as those of Wang et al. [58] and Hu et al. [118] would offer a deeper understanding of epigenetic inheritance across individuals under natural conditions, particularly when exploring evolutionary scenarios in wild populations that are facing environmental variation.

A second feature to investigate is to what extent epigenetic variation is under genetic control. Unfortunately, the correlation between epigenetic and genetic variation in wild animal population studies have not been systematically evaluated. Yet, estimating this correlation is crucial to highlight the evolutionary relevance of epigenetic variation. Regarding studies that have calculated it, there were as many non-significant correlation coefficients as there were significant ones. These results contrast with similar studies in plants that mainly show a strong correlation between the patterns of epigenetic variation and the underlying genetic variants [45]. Otherwise, as genetic variation can blur the role of epigenetic variation, experimental systems in which the confounding effects of genetic variation have been controlled or reduced may be useful for isolating the contributions of epigenetic mechanisms in evolutionary processes. We

suggest that future studies could focus on a species with a known limited genetic variation. Researchers have used populations with a lack of genetic variation resulting from clonal reproduction (e.g., clonal fish, [69,70]) or bottlenecks following invasion (e.g., house sparrows, [79,127]). The mixed-mating reproduction system of the mangrove rivulus *Kryptolebias marmoratus* can be used to go even further into the analysis of epigenetic–genetic variations interaction. This system alternates between the cross-fertilization of a male and a hermaphrodite on the one hand, and self-fertilization (or selfing) hermaphrodites on the other hand, which is unique feature among vertebrates [136]. Consistent selfing naturally produces isogenic lineages [137]. Under laboratory conditions, a higher degree of methylation differentiation between genotypes than that between environments has been reported in two highly inbred strains [72]. They also pointed out that methylation differences between environments that are common to both strains mostly correspond to facilitated epialleles, suggesting the existence of a dynamic interaction between the genotype and the environment. For future studies on this species, we suggest the comparison of natural populations that exhibit a selfing rate gradient, and to investigate how epigenetic diversity varies among those populations with a different level of genetic diversity.

Thirdly, we suggest the use of concepts that have been developed in population genetics studies in their application to population epigenetics, while considering the non-mendelian inheritance and the environmental sensibility of epigenetics. The basic biostatistics of population genetics can be transferred to populations epigenetics to inspire a new index of epigenetic diversity and structure. Johnson and Kelly calculated the P_{ST}, the methylation analogue of Wright's F_{ST}, by subtracting the total variance in the methylation in all populations of Eastern oyster *Crassostrea virginica* from the variance within a single population and divided this by the variance that was in all of the populations ($P_{ST} = (\text{Variance}_{\text{Total}} - \text{Variance}_{\text{Sub}})/\text{Variance}_{\text{Total}}$) [53]. To take the analysis one step further, characterizing the total epigenetic variation is not sufficient to assess the capacity of an organism to respond to environmental changes. Distinguishing the different types of epimutations (i.e., randomly, genetically, or environmentally induced) might shed light on how organisms evolve in terms of plasticity or diversified bet-hedging adaptations. Field studies could analyse this partition of epigenetic variation as a population characteristic such as those found in population genetics, thereby expanding the molecular tool list to assess the evolutionary potential of populations.

Fourthly, population epigenetics can be a useful tool in conservation biology. The epigenome can be altered by biotic (e.g., parasitic or social) and abiotic (e.g., thermal or chemical) stressors, thereby creating a permanent epigenetic "foot-print" that is known as epigenetic memory [138]. These environmentally induced DNA methylation patterns can be considered as biomarkers to evaluate the past and present environmental stress events that are experienced by organisms, as there is evidence for epigenetic memory to be transgenerational [139]. To determine the chemical classes to which organisms have been exposed throughout their lifetime using epigenetic memory, more efforts are required in the identification of specific epimutations that are caused by these chemicals. Besides environmental toxicity safety assessments, a DNA methylation variation analysis can be relevant for improving translocations [140] and for studying the connectivity and clustering of wild populations [141]. As such, a DNA methylation study appears to be a promising tool in conservation biology.

Fifthly, the role of DNA methylation in allelic-specific expression (ASE) should be investigated in wild populations. In diploid organisms, genes are generally expressed from both alleles (biallelic expression), but there are exceptions wherein it occurs only from one allele (monoallelic expression), thereby creating an ASE at each involved gene locus. An ASE is the consequences of an epigenetic process that silences one of the parentally inherited alleles of a gene [142]. The most well-known examples of an ASE that is mediated by epigenetic mechanisms are genomic imprinting and X-chromosome inactivation [143]. Interestingly, random monoallelic expressions (RME) can also occur at the individual loci of autosomal genes, independently of the gene families [144]. Studies have showed that RME patterns are inherited during cell division [145,146], meaning that the earlier that this process occurs during development, the more cells and tissues that will express similar ASEs, and vice versa. This stochasticity that is provided by RME generates a wide diversity of gene expression and might confer many advantages such as generating cellular diversity or regulating gene dosage, as is observed in X-chromosome inactivation. As some cells could have advantageous combination of ASE patterns, it can also enhance the adaptability of organisms to environmental changes during development and throughout their life. Thus, the epigenetic regulation of allelic-specific expressions could create an expression imbalance that contributes to the generation of phenotypic variation among individuals.

Finally, regarding future research on animal populations, epigenetics complexity is worth noting. As detailed in the introduction, the genomic distribution of DNA methylation has been found in many clades of animals, but there are differences in how and where it occurs. Moreover, it is well established that different tissues have specific DNA methylation patterns within the organism, that there is an epigenetic dysregulation with age, and also an interaction between these two criteria as some studies show a tissue-specific effect of age on the epigenome [147,148]. A comparison of the studies characterizing DNA methylation diversity should therefore be interpreted with caution, although it is necessary to draw general lines on epigenetic variation in natural populations. Epigenetics multiplicity is also worth noting; while DNA methylation is the main studied epigenetic mechanism, RNA interference and histone modification are further mechanisms that must be included in the discussion about gene expression regulation generating phenotypic diversity.

7. Conclusions

DNA methylation diversity has been found to be a revealing parameter to characterize natural animal populations. Further studies on its dynamics, emergence, and subsequent implications in population fitness has become increasingly relevant, especially from evolutionary perspectives. The recent progress in ecological epigenetics allows a more complete understanding of how epigenetic diversity is modulated over time, which will be helpful for generating predictive models of the capacity of populations to adapt to environmental variation. Distinguishing random epimutations from environmentally induced ones and heritable epimutations from non-heritable ones may allow us to characterize the responses of organisms to environmental changes, as any variations in DNA methylation within a species might shed light on how they evolve. Although epigenetic studies in natural animal populations are relatively scarce and new, they highlight some important characteristics of DNA methylation that can be used in future research to investigate the link between epigenetic variation and phenotypic plasticity, and local adaptation and evolutionary mechanisms in the wild.

Author Contributions: All authors have read and agreed to the published version of the manuscript.

Funding: This review was supported by the FRS-FNRS (Fonds de la Recherche Scientifique) FRIA PhD grant attributed to V. Chapelle.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Glossary.

Term	Definition
Epigenetic mark	Chemical modifications to DNA, RNA, or proteins that influence chromatin state and gene expression. It includes DNA methylation, noncoding RNAs, and protein modifications (e.g., acetylation, deacetylation, ubiquitination, and histone methylation).
Epiallele	Locus presenting distinct epigenetic profiles due to differences in methylation or chromatin states.
Epimutation	Heritable change in gene activity that is not associated with a DNA mutation, but rather with the gain or loss of DNA methylation or other heritable modifications of chromatin.
Single methylation polymorphism (SMP)	Spontaneous variation in DNA methylation at base-pair resolutions that are due to errors in the maintenance of methylation states. The rates of SMP formation is at least four orders of magnitude greater than genetic mutations.
Epigenetic reprogramming	Erasure and remodelling of epigenetic marks such as DNA methylation during embryo development. Its purposes include the erasure and reestablishment of parental genomic imprints in germ cells, the erasure of epimutations, and the correct development of the embryo through the generation of totipotent or multipotent cells.
Epigenetic variation	Variation in epialleles which is studied among and/or within populations.
Epigenetic diversity	The amount of epigenetic variation within a population.
Transgenerational epigenetic inheritance (TEI)	Stable inheritance of epigenetic marks across multiple generations.
Epigenetic divergence	The process in which two or more populations of an ancestral species accumulate independent epimutations through time.
Epigenetic drift	Gradual changes in the epigenome that is due to random epimutations. This neutral process is not directional as it creates both hyper- and hypomethylation.
C_pG island	Short CpG-rich region of the genome characterized by at least 500 bp of DNA with a GC content ≥ 55%.
Epigenetic potential	The genomic capacity for environmentally induced phenotypic change (i.e., plasticity) via epigenetic modifications.
Phenotypic plasticity	Any change in an organism's phenotype in response to an environmental signal.
Neo-Lamarckism or Lamarckian inheritance	A theory of evolution based on the principle of soft inheritance, which refers to the inheritance of variations that are the result of non-genetic effects. It includes inheritance coming from evolutionary developmental biology, epigenetics, niche construction, and learning and cultural transmission. This theory is part of the extended evolutionary synthesis.
Extended evolutionary synthesis	A set of evolutionary theories including the modern synthesis (combination of Darwinian view of evolutionary change and Mendelian genetics) and soft inheritance (or Lamarckian inheritance). This theory is still under debate.

Table A2. Commonly used molecular techniques to evaluate DNA methylation diversity in field studies.

Technique	Description, Advantages, and Limitations
Methylation-sensitive amplified polymorphism (MSAP)	Modified from the amplified fragment length polymorphism (AFLP) technique, MSAP uses *Eco*RI (rare cutter) with either one or two methylation-sensitive isoschizomer restriction enzymes, *Hpa*II and *Msp*I (frequent-cutter), which recognize the same restriction site (5′-CCGG-3′) but have different cytosine methylation sensitivities. For each sample, MSAP analysis is performed using both *Eco*RI/*Hpa*II- and *Eco*RI/*Msp*I-digested samples. The resulting DNA fragments are ligated with linkers and PCR amplified. Such amplification produces a reduced population of fragments that are separated in denaturing polyacrylamide gels in order to compare the respective band patterns. This technique is useful for non-model species as it does not require a reference genome. It is one of the most commonly used methods for assessing DNA methylation changes in plants. However, the main disadvantage of MSAP is that it can only detect methylation on 5′-CCGG-3′.
Methylated DNA immunoprecipitation (MeDIP)-Seq	MeDIP is an enrichment-based purification technique that involves antibodies directed against mC or mCG to precipitate methylated DNA fragments. Differential DNA methylation regions are identified by comparing the coverage between groups of interest. Combining MeDIP with next-generation sequencing, it provides methylomes at typically 100-bp to 300-bp resolution.

	With the appropriate antibody, MeDIP is also able to detect hmC. MeDIP limitations include antibody quality and cross-reactivity, and relatively low-resolution level in comparison with bisulfite sequencing methods.
Reduced representation bisulfite sequencing (RRBS)	RRBS relies on digestion of genomic DNA with the enzyme MspI, which produces DNA fragments that begin and/or end with an informative CpG site (CpG-enriched genomic regions). Then, genomic DNA is treated with sodium bisulfite, which leaves methylated cytosines intact but converts unmethylated cytosines to uracil (and ultimately thymine after PCR). Amplification fragments are sequenced, allowing for the identification of methylated cytosines. This is an efficient and high-throughput technique due to its high definition since it produces genome-wide methylation profiles with single-nucleotide resolution. Compared to WGBS, it allows one to investigate larger numbers of individuals as it is more cost-effective, but it only provides limited genome coverage (5–10%) and is CpG island and promoter region-centric.
Whole-genome bisulfite sequencing (WGBS	WGBS combines the use of sodium bisulfite treatment and high-throughput DNA sequencing to produce genome-wide methylation profiles with single-nucleotide resolution. Unlike RRBS, it estimates all cytosines methylation levels (including CpG and non-CpG) across the genome, rather than CpG enriched genomic regions. This method is capable of testing approximately 90% of all cytosines in genomes studied to date but is cost prohibitive to sequence large numbers of individual samples.

References

1. Mameli, M. Nongenetic Selection and Nongenetic Inheritance. *Br. J. Philos. Sci.* **2004**, *55*, 35–71. https://doi.org/10.1093/bjps/55.1.35.
2. Danchin, É.; Charmantier, A.; Champagne, F.A.; Mesoudi, A.; Pujol, B.; Blanchet, S. Beyond DNA: Integrating Inclusive Inheritance into an Extended Theory of Evolution. *Nat. Rev. Genet.* **2011**, *12*, 475–486. https://doi.org/10.1038/nrg3028.
3. Allen, N.D.; Norris, M.L.; Surani, M.A. Epigenetic Control of Transgene Expression and Imprinting by Genotype-Specific Modifiers. *Cell* **1990**, *61*, 853–861. https://doi.org/10.1016/0092-8674(90)90195-K.
4. Miko, I. Phenotype Variability: Penetrance and Expressivity. *Nat. Educ.* **2008**, *1*, 137.
5. Youngson, N.A.; Whitelaw, E. Transgenerational Epigenetic Effects. *Annu. Rev. Genom. Hum. Genet.* **2008**, *9*, 233–257. https://doi.org/10.1146/annurev.genom.9.081307.164445.
6. Nicoglou, A.; Merlin, F. Epigenetics: A Way to Bridge the Gap between Biological Fields. *Stud. Hist. Philos. Sci. Part C Stud. Hist. Philos. Biol. Biomed. Sci.* **2017**, *66*, 73–82. https://doi.org/10.1016/j.shpsc.2017.10.002.
7. Angers, B.; Castonguay, E.; Massicotte, R. Environmentally Induced Phenotypes and DNA Methylation: How to Deal with Unpredictable Conditions until the next Generation and After. *Mol. Ecol.* **2010**, *19*, 1283–1295. https://doi.org/10.1111/j.1365-294X.2010.04580.x.
8. Greally, J.M. Population Epigenetics. *Curr. Opin. Syst. Biol.* **2017**, *1*, 84–89. https://doi.org/10.1016/j.coisb.2017.01.004.
9. Verhoeven, K.J.F.; Preite, V. Epigenetic Variation in Asexually Reproducing Organisms. *Evolution* **2014**, *68*, 644–655. https://doi.org/10.1111/evo.12320.
10. West-Eberhard, M.J. *Developmental Plasticity and Evolution*; Oxford University Press: New York, NY, USA, 2003; ISBN 978-0-19-802856-7.
11. West-Eberhard, M.J. Developmental Plasticity and the Origin of Species Differences. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 6543–6549. https://doi.org/10.1073/pnas.0501844102.
12. Robinson, B.W.; Dukas, R. The Influence of Phenotypic Modifications on Evolution: The Baldwin Effect and Modern Perspectives. *Oikos* **1999**, *85*, 582. https://doi.org/10.2307/3546709.
13. Grether, G.F. Environmental Change, Phenotypic Plasticity, and Genetic Compensation. *Am. Nat.* **2005**, *166*, E115–E123. https://doi.org/10.1086/432023.
14. Jablonka, E.; Lamb, M.J. The Inheritance of Acquired Epigenetic Variations. *J. Theor. Biol.* **1989**, *139*, 69–83. https://doi.org/10.1016/S0022-5193(89)80058-X.
15. West-Eberhard, M.J. Alternative Adaptations, Speciation, and Phylogeny (A Review). *Proc. Natl. Acad. Sci. USA.* **1986**, *83*, 1388–1392. https://doi.org/10.1073/pnas.83.5.1388.
16. Richards, E.J. Natural Epigenetic Variation in Plant Species: A View from the Field. *Curr. Opin. Plant Biol.* **2011**, *14*, 204–209. https://doi.org/10.1016/j.pbi.2011.03.009.
17. Vogt, G. Epigenetic Variation in Animal Populations: Sources, Extent, Phenotypic Implications, and Ecological and Evolutionary Relevance *J. Biosci.* **2021**, *46*, 24.
18. Burggren, W. Epigenetic Inheritance and Its Role in Evolutionary Biology: Re-Evaluation and New Perspectives. *Biology* **2016**, *5*, 24. https://doi.org/10.3390/biology5020024.
19. Kumar, D.; Thakur, M.K. Effect of Perinatal Exposure to Bisphenol-A on DNA Methylation and Histone Acetylation in Cerebral Cortex and Hippocampus of Postnatal Male Mice. *J. Toxicol. Sci.* **2017**, *42*, 281–289. https://doi.org/10.2131/jts.42.281.
20. Nguyen, T.; Li, G.E.; Chen, H.; Cranfield, C.G.; McGrath, K.C.; Gorrie, C.A. Maternal E-Cigarette Exposure Results in Cognitive and Epigenetic Alterations in Offspring in a Mouse Model. *Chem. Res. Toxicol.* **2018**, *31*, 601–611. https://doi.org/10.1021/acs.chemrestox.8b00084.

21. Schmitz, R.J.; Schultz, M.D.; Lewsey, M.G.; O'Malley, R.C.; Urich, M.A.; Libiger, O.; Schork, N.J.; Ecker, J.R. Transgenerational Epigenetic Instability Is a Source of Novel Methylation Variants. *Science* **2011**, *334*, 369–373. https://doi.org/10.1126/science.1212959.
22. van der Graaf, A.; Wardenaar, R.; Neumann, D.A.; Taudt, A.; Shaw, R.G.; Jansen, R.C.; Schmitz, R.J.; Colomé-Tatché, M.; Johannes, F. Rate, Spectrum, and Evolutionary Dynamics of Spontaneous Epimutations. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 6676–6681. https://doi.org/10.1073/pnas.1424254112.
23. Manikkam, M.; Guerrero-Bosagna, C.; Tracey, R.; Haque, M.M.; Skinner, M.K. Transgenerational Actions of Environmental Compounds on Reproductive Disease and Identification of Epigenetic Biomarkers of Ancestral Exposures. *PLoS ONE* **2012**, *7*, e31901. https://doi.org/10.1371/journal.pone.0031901.
24. Guerrero-Bosagna, C.; Morisson, M.; Liaubet, L.; Rodenburg, T.B.; de Haas, E.N.; Košťál, Ľ.; Pitel, F. Transgenerational Epigenetic Inheritance in Birds. *Environ. Epigenetics* **2018**, *4*, dvy008. https://doi.org/10.1093/eep/dvy008.
25. Bhandari, R.K.; vom Saal, F.S.; Tillitt, D.E. Transgenerational Effects from Early Developmental Exposures to Bisphenol A or 17α-Ethinylestradiol in Medaka, Oryzias Latipes. *Sci. Rep.* **2015**, *5*, 9303. https://doi.org/10.1038/srep09303.
26. Seong, K.-H.; Li, D.; Shimizu, H.; Nakamura, R.; Ishii, S. Inheritance of Stress-Induced, ATF-2-Dependent Epigenetic Change. *Cell* **2011**, *145*, 1049–1061. https://doi.org/10.1016/j.cell.2011.05.029.
27. Liew, Y.J.; Howells, E.J.; Wang, X.; Michell, C.T.; Burt, J.A.; Idaghdour, Y.; Aranda, M. Intergenerational Epigenetic Inheritance in Reef-Building Corals. *Nat. Clim. Chang.* **2020**, *10*, 254–259. https://doi.org/10.1038/s41558-019-0687-2.
28. Feil, R.; Fraga, M.F. Epigenetics and the Environment: Emerging Patterns and Implications. *Nat. Rev. Genet.* **2012**, *13*, 97–109. https://doi.org/10.1038/nrg3142.
29. Casier, K.; Boivin, A.; Carré, C.; Teysset, L. Environmentally-Induced Transgenerational Epigenetic Inheritance: Implication of PIWI Interacting RNAs. *Cells* **2019**, *8*, 1108. https://doi.org/10.3390/cells8091108.
30. Miryeganeh, M.; Saze, H. Epigenetic Inheritance and Plant Evolution. *Popul. Ecol.* **2019**, *62*, 17–27. https://doi.org/10.1002/1438-390X.12018.
31. Thiebaut, F.; Hemerly, A.S.; Ferreira, P.C.G. A Role for Epigenetic Regulation in the Adaptation and Stress Responses of Non-Model Plants. *Front. Plant Sci.* **2019**, *10*, 246. https://doi.org/10.3389/fpls.2019.00246.
32. Blouin, M.S.; Thuillier, V.; Cooper, B.; Amarasinghe, V.; Cluzel, L.; Araki, H.; Grunau, C. No Evidence for Large Differences in Genomic Methylation between Wild and Hatchery Steelhead (Oncorhynchus Mykiss). *Can. J. Fish. Aquat. Sci.* **2010**, *67*, 217–224. https://doi.org/10.1139/F09-174.
33. de Mendoza, A.; Lister, R.; Bogdanovic, O. Evolution of DNA Methylome Diversity in Eukaryotes. *J. Mol. Biol.* **2020**, *432*, 1687–1705. https://doi.org/10.1016/j.jmb.2019.11.003.
34. Zemach, A.; McDaniel, I.E.; Silva, P.; Zilberman, D. Genome-Wide Evolutionary Analysis of Eukaryotic DNA Methylation. *Science* **2010**, *328*, 916–919. https://doi.org/10.1126/science.1186366.
35. Rauluseviciute, I.; Drabløs, F.; Rye, M.B. DNA Hypermethylation Associated with Upregulated Gene Expression in Prostate Cancer Demonstrates the Diversity of Epigenetic Regulation. *BMC Med. Genom.* **2020**, *13*, 6. https://doi.org/10.1186/s12920-020-0657-6.
36. Spainhour, J.C.; Lim, H.S.; Yi, S.V.; Qiu, P. Correlation Patterns Between DNA Methylation and Gene Expression in The Cancer Genome Atlas. *Cancer Inf.* **2019**, *18*, 117693511982877. https://doi.org/10.1177/1176935119828776.
37. Feng, S.; Cokus, S.J.; Zhang, X.; Chen, P.-Y.; Bostick, M.; Goll, M.G.; Hetzel, J.; Jain, J.; Strauss, S.H.; Halpern, M.E.; et al. Conservation and Divergence of Methylation Patterning in Plants and Animals. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 8689–8694. https://doi.org/10.1073/pnas.1002720107.
38. Suzuki, M.M.; Bird, A. DNA Methylation Landscapes: Provocative Insights from Epigenomics. *Nat. Rev. Genet.* **2008**, *9*, 465–476. https://doi.org/10.1038/nrg2341.
39. Hon, G.C.; Rajagopal, N.; Shen, Y.; McCleary, D.F.; Yue, F.; Dang, M.D.; Ren, B. Epigenetic Memory at Embryonic Enhancers Identified in DNA Methylation Maps from Adult Mouse Tissues. *Nat. Genet.* **2013**, *45*, 1198–1206. https://doi.org/10.1038/ng.2746.
40. de Mendoza, A.; Hatleberg, W.L.; Pang, K.; Leininger, S.; Bogdanovic, O.; Pflueger, J.; Buckberry, S.; Technau, U.; Hejnol, A.; Adamska, M.; et al. Convergent Evolution of a Vertebrate-like Methylome in a Marine Sponge. *Nat. Ecol. Evol.* **2019**, *3*, 1464–1473. https://doi.org/10.1038/s41559-019-0983-2.
41. Potok, M.E.; Nix, D.A.; Parnell, T.J.; Cairns, B.R. Reprogramming the Maternal Zebrafish Genome after Fertilization to Match the Paternal Methylation Pattern. *Cell* **2013**, *153*, 759–772. https://doi.org/10.1016/j.cell.2013.04.030.
42. Rae, P.M.M.; Steele, R.E. Absence of Cytosine Methylation at C-C-G-G and G-C-G-C Sites in the RDNA Coding Regions and Intervening Sequences of Drosophila and the RDNA of Other Higher Insects. *Nucl. Acids. Res.* **1979**, *6*, 2987–2995. https://doi.org/10.1093/nar/6.9.2987.
43. Simpson, V.J.; Johnson, T.E.; Hammen, R.F. *Caenorhabditis Elegans* DNA Does Not Contain 5-Methylcytosine at Any Time during Development or Aging. *Nucl. Acids. Res.* **1986**, *14*, 6711–6719. https://doi.org/10.1093/nar/14.16.6711.
44. Xu, X.; Li, G.; Li, C.; Zhang, J.; Wang, Q.; Simmons, D.K.; Chen, X.; Wijesena, N.; Zhu, W.; Wang, Z.; et al. Evolutionary Transition between Invertebrates and Vertebrates via Methylation Reprogramming in Embryogenesis. *Natl. Sci. Rev.* **2019**, *6*, 993–1003. https://doi.org/10.1093/nsr/nwz064.
45. Hu, J.; Barrett, R.D.H. Epigenetics in Natural Animal Populations. *J. Evol. Biol.* **2017**, *30*, 1612–1632. https://doi.org/10.1111/jeb.13130.
46. Schmitz, R.J.; Schultz, M.D.; Urich, M.A.; Nery, J.R.; Pelizzola, M.; Libiger, O.; Alix, A.; McCosh, R.B.; Chen, H.; Schork, N.J.; et al. Patterns of Population Epigenomic Diversity. *Nature* **2013**, *495*, 193–198. https://doi.org/10.1038/nature11968.
47. Biwer, C.; Kawam, B.; Chapelle, V.; Silvestre, F. The Role of Stochasticity in the Origin of Epigenetic Variation in Animal Populations. *Integr. Comp. Biol.* **2020**, *60*, 1544–1557. https://doi.org/10.1093/icb/icaa047.
48. Oey, H.; Whitelaw, E. On the Meaning of the Word 'Epimutation.' *Trends Genet.* **2014**, *30*, 519–520. https://doi.org/10.1016/j.tig.2014.08.005.
49. Ardura, A.; Zaiko, A.; Morán, P.; Planes, S.; Garcia-Vazquez, E. Epigenetic Signatures of Invasive Status in Populations of Marine Invertebrates. *Sci. Rep.* **2017**, *7*, 42193. https://doi.org/10.1038/srep42193.

50. Baldanzi, S.; Watson, R.; McQuaid, C.D.; Gouws, G.; Porri, F. Epigenetic Variation among Natural Populations of the South African Sandhopper Talorchestia Capensis. *Evol. Ecol.* **2017**, *31*, 77–91. https://doi.org/10.1007/s10682-016-9877-9.
51. Whitaker, J.M.; Welsh, A.B.; Hondorp, D.W.; Boase, J.C.; Merovich, G.T.; Welsh, S.; Krueger, C. Variation in DNA Methylation Is Associated with Migratory Phenotypes of Lake Sturgeon *ACIPENSER FULVESCENS* in the St. Clair River, MI, USA. *J. Fish Biol.* **2018**, *93*, 942–951. https://doi.org/10.1111/jfb.13804.
52. Gavery, M.R.; Nichols, K.M.; Goetz, G.W.; Middleton, M.A.; Swanson, P. Characterization of Genetic and Epigenetic Variation in Sperm and Red Blood Cells from Adult Hatchery and Natural-Origin Steelhead, *Oncorhynchus Mykiss*. *G3 Genes|Genomes|Genet.* **2018**, *8*, 3723–3736. https://doi.org/10.1534/g3.118.200458.
53. Johnson, K.M.; Kelly, M.W. Population Epigenetic Divergence Exceeds Genetic Divergence in the Eastern Oyster *Crassostrea virginica* in the Northern Gulf of Mexico. *Evol. Appl.* **2020**, *13*, 945–959. https://doi.org/10.1111/eva.12912.
54. Wogan, G.O.U.; Yuan, M.L.; Mahler, D.L.; Wang, I.J. Genome-wide Epigenetic Isolation by Environment in a Widespread *Anolis* Lizard. *Mol. Ecol.* **2020**, *29*, 40–55. https://doi.org/10.1111/mec.15301.
55. Watson, H.; Powell, D.; Salmón, P.; Jacobs, A.; Isaksson, C. Urbanization Is Associated with Modifications in DNA Methylation in a Small Passerine Bird. *Evol. Appl.* **2021**, *14*, 85–98. https://doi.org/10.1111/eva.13160.
56. McNew, S.M.; Beck, D.; Sadler-Riggleman, I.; Knutie, S.A.; Koop, J.A.H.; Clayton, D.H.; Skinner, M.K. Epigenetic Variation between Urban and Rural Populations of Darwin's Finches. *BMC Evol. Biol.* **2017**, *17*, 183. https://doi.org/10.1186/s12862-017-1025-9.
57. Thorson, J.L.M.; Smithson, M.; Sadler-Riggleman, I.; Beck, D.; Dybdahl, M.; Skinner, M.K. Regional Epigenetic Variation in Asexual Snail Populations among Urban and Rural Lakes. *Environ. Epigenetics* **2019**, *5*, dvz020. https://doi.org/10.1093/eep/dvz020.
58. Wang, X.; Li, A.; Wang, W.; Zhang, G.; Li, L. Direct and Heritable Effects of Natural Tidal Environments on DNA Methylation in Pacific Oysters (Crassostrea Gigas). *Environ. Res.* **2021**, *197*, 111058. https://doi.org/10.1016/j.envres.2021.111058.
59. Flatscher, R.; Frajman, B.; Schönswetter, P.; Paun, O. Environmental Heterogeneity and Phenotypic Divergence: Can Heritable Epigenetic Variation Aid Speciation? *Genet. Res. Int.* **2012**, *2012*, 1–9. https://doi.org/10.1155/2012/698421.
60. Smith, T.A.; Martin, M.D.; Nguyen, M.; Mendelson, T.C. Epigenetic Divergence as a Potential First Step in Darter Speciation. *Mol. Ecol.* **2016**, *25*, 1883–1894. https://doi.org/10.1111/mec.13561.
61. Lira-Medeiros, C.F.; Parisod, C.; Fernandes, R.A.; Mata, C.S.; Cardoso, M.A.; Ferreira, P.C.G. Epigenetic Variation in Mangrove Plants Occurring in Contrasting Natural Environment. *PLoS ONE* **2010**, *5*, e10326. https://doi.org/10.1371/journal.pone.0010326.
62. Medrano, M.; Herrera, C.M.; Bazaga, P. Epigenetic Variation Predicts Regional and Local Intraspecific Functional Diversity in a Perennial Herb. *Mol. Ecol.* **2014**, *23*, 4926–4938. https://doi.org/10.1111/mec.12911.
63. Foust, C.M.; Preite, V.; Schrey, A.W.; Alvarez, M.; Robertson, M.H.; Verhoeven, K.J.F.; Richards, C.L. Genetic and Epigenetic Differences Associated with Environmental Gradients in Replicate Populations of Two Salt Marsh Perennials. *Mol. Ecol.* **2016**, *25*, 1639–1652. https://doi.org/10.1111/mec.13522.
64. Morán, P.; Pérez-Figueroa, A. Methylation Changes Associated with Early Maturation Stages in the Atlantic Salmon. *BMC Genet.* **2011**, *12*, 86. https://doi.org/10.1186/1471-2156-12-86.
65. Skinner, M.K.; Gurerrero-Bosagna, C.; Haque, M.M.; Nilsson, E.E.; Koop, J.A.H.; Knutie, S.A.; Clayton, D.H. Epigenetics and the Evolution of Darwin's Finches. *Genome Biol. Evol.* **2014**, *6*, 1972–1989. https://doi.org/10.1093/gbe/evu158.
66. Liu, S.; Sun, K.; Jiang, T.; Feng, J. Natural Epigenetic Variation in Bats and Its Role in Evolution. *J. Exp. Biol.* **2015**, *218*, 100–106. https://doi.org/10.1242/jeb.107243.
67. Zhang, X.; Li, Q.; Kong, L.; Yu, H. Epigenetic Variation of Wild Populations of the Pacific Oyster Crassostrea Gigas Determined by Methylation-Sensitive Amplified Polymorphism Analysis. *Fish Sci.* **2018**, *84*, 61–70. https://doi.org/10.1007/s12562-017-1154-5.
68. Vernaz, G.; Malinsky, M.; Svardal, H.; Du, M.; Tyers, A.M.; Santos, M.E.; Durbin, R.; Genner, M.J.; Turner, G.F.; Miska, E.A. Mapping Epigenetic Divergence in the Massive Radiation of Lake Malawi Cichlid Fishes. *Nat. Commun.* **2021**, *12*, 5870. https://doi.org/10.1038/s41467-021-26166-2.
69. Massicotte, R.; Whitelaw, E.; Angers, B. DNA Methylation: A Source of Random Variation in Natural Populations. *Epigenetics* **2011**, *6*, 421–427. https://doi.org/10.4161/epi.6.4.14532.
70. Massicotte, R.; Angers, B. General-Purpose Genotype or How Epigenetics Extend the Flexibility of a Genotype. *Genet. Res. Int.* **2012**, *2012*, 317175. https://doi.org/10.1155/2012/317175.
71. Thorson, J.L.M.; Smithson, M.; Beck, D.; Sadler-Riggleman, I.; Nilsson, E.; Dybdahl, M.; Skinner, M.K. Epigenetics and Adaptive Phenotypic Variation between Habitats in an Asexual Snail. *Sci. Rep.* **2017**, *7*, 14139. https://doi.org/10.1038/s41598-017-14673-6.
72. Berbel-Filho, W.M.; Rodríguez-Barreto, D.; Berry, N.; Garcia De Leaniz, C.; Consuegra, S. Contrasting DNA Methylation Responses of Inbred Fish Lines to Different Rearing Environments. *Epigenetics* **2019**, *14*, 939–948. https://doi.org/10.1080/15592294.2019.1625674.
73. Richards, E.J. Inherited Epigenetic Variation—Revisiting Soft Inheritance. *Nat. Rev. Genet.* **2006**, *7*, 395–401. https://doi.org/10.1038/nrg1834.
74. Sheldon, E.L.; Schrey, A.; Andrew, S.C.; Ragsdale, A.; Griffith, S.C. Epigenetic and Genetic Variation among Three Separate Introductions of the House Sparrow (*Passer domesticus*) into Australia. *R. Soc. Open Sci.* **2018**, *5*, 172185. https://doi.org/10.1098/rsos.172185.
75. Fargeot, L.; Loot, G.; Prunier, J.G.; Rey, O.; Veyssière, C.; Blanchet, S. Patterns of Epigenetic Diversity in Two Sympatric Fish Species: Genetic vs. Environmental Determinants. *Genes* **2021**, *12*, 107. https://doi.org/10.3390/genes12010107.
76. McRae, A.F.; Powell, J.E.; Henders, A.K.; Bowdler, L.; Hemani, G.; Shah, S.; Painter, J.N.; Martin, N.G.; Visscher, P.M.; Montgomery, G.W. Contribution of Genetic Variation to Transgenerational Inheritance of DNA Methylation. *Genome Biol.* **2014**, *15*, R73. https://doi.org/10.1186/gb-2014-15-5-r73.

77. Carja, O.; MacIsaac, J.L.; Mah, S.M.; Henn, B.M.; Kobor, M.S.; Feldman, M.W.; Fraser, H.B. Worldwide Patterns of Human Epigenetic Variation. *Nat. Ecol. Evol.* **2017**, *1*, 1577–1583. https://doi.org/10.1038/s41559-017-0299-z.
78. Orozco, L.D.; Morselli, M.; Rubbi, L.; Guo, W.; Go, J.; Shi, H.; Lopez, D.; Furlotte, N.A.; Bennett, B.J.; Farber, C.R.; et al. Epigenome-Wide Association of Liver Methylation Patterns and Complex Metabolic Traits in Mice. *Cell Metab.* **2015**, *21*, 905–917. https://doi.org/10.1016/j.cmet.2015.04.025.
79. Liebl, A.L.; Schrey, A.W.; Richards, C.L.; Martin, L.B. Patterns of DNA Methylation Throughout a Range Expansion of an Introduced Songbird. *Integr. Comp. Biol.* **2013**, *53*, 351–358. https://doi.org/10.1093/icb/ict007.
80. Leung, C.; Breton, S.; Angers, B. Facing Environmental Predictability with Different Sources of Epigenetic Variation. *Ecol. Evol.* **2016**, *6*, 5234–5245. https://doi.org/10.1002/ece3.2283.
81. Wenzel, M.A.; Piertney, S.B. Fine-Scale Population Epigenetic Structure in Relation to Gastrointestinal Parasite Load in Red Grouse (*Lagopus lagopus scotica*). *Mol. Ecol.* **2014**, *23*, 4256–4273. https://doi.org/10.1111/mec.12833.
82. Venney, C.J.; Sutherland, B.J.G.; Beacham, T.D.; Heath, D.D. Population Differences in Chinook Salmon (*Oncorhynchus tshawytscha*) DNA Methylation: Genetic Drift and Environmental Factors. *Ecol. Evol.* **2021**, *11*, 6846–6861. https://doi.org/10.1002/ece3.7531.
83. Slotkin, R.K.; Martienssen, R. Transposable Elements and the Epigenetic Regulation of the Genome. *Nat. Rev. Genet.* **2007**, *8*, 272–285. https://doi.org/10.1038/nrg2072.
84. Elbarbary, R.A.; Lucas, B.A.; Maquat, L.E. Retrotransposons as Regulators of Gene Expression. *Science* **2016**, *351*, aac7247. https://doi.org/10.1126/science.aac7247.
85. Schauer, S.N.; Carreira, P.E.; Shukla, R.; Gerhardt, D.J.; Gerdes, P.; Sanchez-Luque, F.J.; Nicoli, P.; Kindlova, M.; Ghisletti, S.; Santos, A.D.; et al. L1 Retrotransposition Is a Common Feature of Mammalian Hepatocarcinogenesis. *Genome Res.* **2018**, *28*, 639–653. https://doi.org/10.1101/gr.226993.117.
86. Liu, J.; Ali, M.; Zhou, Q. Establishment and Evolution of Heterochromatin. *Ann. N. Y. Acad. Sci.* **2020**, *1476*, 59–77. https://doi.org/10.1111/nyas.14303.
87. Platt, A.; Gugger, P.F.; Pellegrini, M.; Sork, V.L. Genome-Wide Signature of Local Adaptation Linked to Variable CpG Methylation in Oak Populations. *Mol. Ecol.* **2015**, *24*, 3823–3830. https://doi.org/10.1111/mec.13230.
88. Macia, A.; Muñoz-Lopez, M.; Cortes, J.L.; Hastings, R.K.; Morell, S.; Lucena-Aguilar, G.; Marchal, J.A.; Badge, R.M.; Garcia-Perez, J.L. Epigenetic Control of Retrotransposon Expression in Human Embryonic Stem Cells. *Mol. Cell. Biol.* **2011**, *31*, 300–316. https://doi.org/10.1128/MCB.00561-10.
89. Tang, M.-H.; Varadan, V.; Kamalakaran, S.; Zhang, M.Q.; Dimitrova, N.; Hicks, J. Major Chromosomal Breakpoint Intervals in Breast Cancer Co-Localize with Differentially Methylated Regions. *Front. Oncol.* **2012**, *2*, 197. https://doi.org/10.3389/fonc.2012.00197.
90. Makova, K.D.; Hardison, R.C. The Effects of Chromatin Organization on Variation in Mutation Rates in the Genome. *Nat. Rev. Genet.* **2015**, *16*, 213–223. https://doi.org/10.1038/nrg3890.
91. Duncan, B.K.; Miller, J.H. Mutagenic Deamination of Cytosine Residues in DNA. *Nature* **1980**, *287*, 560–561. https://doi.org/10.1038/287560a0.
92. Britten, R.J.; Baron, W.F.; Stout, D.B.; Davidson, E.H. Sources and Evolution of Human Alu Repeated Sequences. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 4770–4774. https://doi.org/10.1073/pnas.85.13.4770.
93. Kilvitis, H.J.; Hanson, H.; Schrey, A.W.; Martin, L.B. Epigenetic Potential as a Mechanism of Phenotypic Plasticity in Vertebrate Range Expansions. *Integr. Comp. Biol.* **2017**, *57*, 385–395. https://doi.org/10.1093/icb/icx082.
94. Wright, S. Isolation by distance. *Genetics* **1943**, *28*, 114–138. https://doi.org/10.1093/genetics/28.2.114.
95. Herrera, C.M.; Medrano, M.; Bazaga, P. Comparative Epigenetic and Genetic Spatial Structure of the Perennial Herb *Helleborus foetidus*: Isolation by Environment, Isolation by Distance, and Functional Trait Divergence. *Am. J. Bot.* **2017**, *104*, 1195–1204. https://doi.org/10.3732/ajb.1700162.
96. Wang, I.J.; Bradburd, G.S. Isolation by Environment. *Mol. Ecol.* **2014**, *23*, 5649–5662. https://doi.org/10.1111/mec.12938.
97. Mendelson, T.C.; Imhoff, V.E.; Venditti, J.J. The accumulation of reproductive barriers during speciation: postmating barriers in two behaviorally isolated species of darters (Percidae: Etheostoma). *Evolution* **2007**, *61*, 2596–2606. https://doi.org/10.1111/j.1558-5646.2007.00220.x.
98. Liu, S.; Sun, K.; Jiang, T.; Ho, J.P.; Liu, B.; Feng, J. Natural Epigenetic Variation in the Female Great Roundleaf Bat (*Hipposideros armiger*) Populations. *Mol. Genet. Genom.* **2012**, *287*, 643–650. https://doi.org/10.1007/s00438-012-0704-x.
99. Becker, C.; Hagmann, J.; Müller, J.; Koenig, D.; Stegle, O.; Borgwardt, K.; Weigel, D. Spontaneous Epigenetic Variation in the Arabidopsis Thaliana Methylome. *Nature* **2011**, *480*, 245–249. https://doi.org/10.1038/nature10555.
100. Yates, P.A.; Burman, R.; Simpson, J.; Ponomoreva, O.N.; Thayer, M.J.; Turker, M.S. Silencing of Mouse *Aprt* Is a Gradual Process in Differentiated Cells. *Mol. Cell. Biol.* **2003**, *23*, 4461–4470. https://doi.org/10.1128/MCB.23.13.4461-4470.2003.
101. Drake, J.W.; Charlesworth, B.; Charlesworth, D.; Crow, J.F. Rates of Spontaneous Mutation. *Genetics* **1998**, *148*, 1667–1686. https://doi.org/10.1093/genetics/148.4.1667.
102. Ossowski, S.; Schneeberger, K.; Lucas-Lledó, J.I.; Warthmann, N.; Clark, R.M.; Shaw, R.G.; Weigel, D.; Lynch, M. The Rate and Molecular Spectrum of Spontaneous Mutations in *Arabidopsis thaliana*. *Science* **2010**, *327*, 92–94. https://doi.org/10.1126/science.1180677.
103. Ashapkin, V.V.; Kutueva, L.I.; Vanyushin, B.F. Epigenetic Clock: Just a Convenient Marker or an Active Driver of Aging? In *Reviews on Biomarker Studies in Aging and Anti-Aging Research*; Guest, P.C., Ed.; Advances in Experimental Medicine and Biology; Springer International Publishing: Cham, Switzerland, 2019; Volume 1178, pp. 175–206; ISBN 978-3-030-25649-4.
104. Hernando-Herraez, I.; Evano, B.; Stubbs, T.; Commere, P.-H.; Jan Bonder, M.; Clark, S.; Andrews, S.; Tajbakhsh, S.; Reik, W. Ageing Affects DNA Methylation Drift and Transcriptional Cell-to-Cell Variability in Mouse Muscle Stem Cells. *Nat. Commun.* **2019**, *10*, 4361. https://doi.org/10.1038/s41467-019-12293-4.

105. Lu, A.T.; Fei, Z.; Haghani, A.; Robeck, T.R.; Zoller, J.A.; Li, C.Z.; Zhang, J.; Ablaeva, J.; Adams, D.; Almunia, J.; et al. Universal DNA methylation age across mammalian tissues. Preprint at *BioRxiv* **2021**. https://doi.org/10.1101/2021.01.18.426733.
106. Mayne, B.; Korbie, D.; Kenchington, L.; Ezzy, B.; Berry, O.; Jarman, S. A DNA Methylation Age Predictor for Zebrafish. *Aging* **2020**, *12*, 24817–24835. https://doi.org/10.18632/aging.202400.
107. Anastasiadi, D.; Piferrer, F. A Clockwork Fish: Age Prediction Using DNA Methylation-based Biomarkers in the European Seabass. *Mol. Ecol. Resour.* **2020**, *20*, 387–397. https://doi.org/10.1111/1755-0998.13111.
108. Li, Y.; Tollefsbol, T.O. Age-Related Epigenetic Drift and Phenotypic Plasticity Loss: Implications in Prevention of Age-Related Human Diseases. *Epigenomics* **2016**, *8*, 1637–1651. https://doi.org/10.2217/epi-2016-0078.
109. Guillette, L.J.; Parrott, B.B.; Nilsson, E.; Haque, M.M.; Skinner, M.K. Epigenetic Programming Alterations in Alligators from Environmentally Contaminated Lakes. *Gen. Comp. Endocrinol.* **2016**, *238*, 4–12. https://doi.org/10.1016/j.ygcen.2016.04.012.
110. Sheldon, C.C.; Conn, A.B.; Dennis, E.S.; Peacock, W.J. Different Regulatory Regions Are Required for the Vernalization-Induced Repression of *FLOWERING LOCUS C* and for the Epigenetic Maintenance of Repression. *Plant Cell* **2002**, *14*, 2527–2537. https://doi.org/10.1105/tpc.004564.
111. Feil, R. Environmental and Nutritional Effects on the Epigenetic Regulation of Genes. *Mutat. Res./Fundam. Mol. Mech. Mutagenesis* **2006**, *600*, 46–57. https://doi.org/10.1016/j.mrfmmm.2006.05.029.
112. Meaney, M.J. Maternal Care, Gene Expression, and the Transmission of Individual Differences in Stress Reactivity Across Generations. *Annu. Rev. Neurosci.* **2001**, *24*, 1161–1192. https://doi.org/10.1146/annurev.neuro.24.1.1161.
113. Voisin, A.-S.; Suarez Ulloa, V.; Stockwell, P.; Chatterjee, A.; Silvestre, F. Genome-Wide DNA Methylation of the Liver Reveals Delayed Effects of Early-Life Exposure to 17-α-Ethinylestradiol in the Self-Fertilizing Mangrove Rivulus. *Epigenetics* **2022**, *17*, 473–497. https://doi.org/10.1080/15592294.2021.1921337.
114. Richards, E.J. Population Epigenetics. *Curr. Opin. Genet. Dev.* **2008**, *18*, 221–226. https://doi.org/10.1016/j.gde.2008.01.014.
115. Artemov, A.V.; Mugue, N.S.; Rastorguev, S.M.; Zhenilo, S.; Mazur, A.M.; Tsygankova, S.V.; Boulygina, E.S.; Kaplun, D.; Nedoluzhko, A.V.; Medvedeva, Y.A.; et al. Genome-Wide DNA Methylation Profiling Reveals Epigenetic Adaptation of Stickleback to Marine and Freshwater Conditions. *Mol. Biol. Evol.* **2017**, *34*, 2203–2213. https://doi.org/10.1093/molbev/msx156.
116. Tatsch, A.; Proietti, M.; Zanini, R.; Fruet, P.; Secchi, E. Beyond Genetic Differences: Epigenetic Variation in Common Bottlenose Dolphins Tursiops Truncatus from Contrasting Marine Ecosystems. *Mar. Ecol. Prog. Ser.* **2021**, *671*, 219–233. https://doi.org/10.3354/meps13761.
117. Lea, A.J.; Altmann, J.; Alberts, S.C.; Tung, J. Resource Base Influences Genome-Wide DNA Methylation Levels in Wild Baboons (*Papio cynocephalus*). *Mol. Ecol.* **2016**, *25*, 1681–1696. https://doi.org/10.1111/mec.13436.
118. Hu, J.; Wuitchik, S.J.S.; Barry, T.N.; Jamniczky, H.A.; Rogers, S.M.; Barrett, R.D.H. Heritability of DNA Methylation in Threespine Stickleback (*Gasterosteus aculeatus*). *Genetics* **2021**, *217*, iyab001. https://doi.org/10.1093/genetics/iyab001.
119. Mayr, E. *The Growth of Biological Thought: Diversity, Evolution, and Inheritance*; Harvard University Press: Cambridge, MA, USA, 1982; ISBN 978-0-674-36446-2.
120. *Evolution, the Extended Synthesis*; Pigliucci, M., Müller, G., Konrad Lorenz Institute for Evolution and Cognition Research, Eds.; MIT Press: Cambridge, MA, USA, 2010; ISBN 978-0-262-51367-8.
121. Skinner, M.K.; Guerrero-Bosagna, C.; Haque, M.M. Environmentally Induced Epigenetic Transgenerational Inheritance of Sperm Epimutations Promote Genetic Mutations. *Epigenetics* **2015**, *10*, 762–771. https://doi.org/10.1080/15592294.2015.1062207.
122. Tigano, A.; Friesen, V. Genomics of Local Adaptation with Gene Flow. *Mol. Ecol.* **2016**, *25*, 2144–2164. https://doi.org/10.1111/mec.13606.
123. Xie, H.J.; Li, H.; Liu, D.; Dai, W.M.; He, J.Y.; Lin, S.; Duan, H.; Liu, L.L.; Chen, S.G.; Song, X.L.; et al. *ICE1* Demethylation Drives the Range Expansion of a Plant Invader through Cold Tolerance Divergence. *Mol. Ecol.* **2015**, *24*, 835–850. https://doi.org/10.1111/mec.13067.
124. Vogt, G. Stochastic Developmental Variation, an Epigenetic Source of Phenotypic Diversity with Far-Reaching Biological Consequences. *J. Biosci.* **2015**, *40*, 159–204. https://doi.org/10.1007/s12038-015-9506-8.
125. Simons, A.M. Modes of Response to Environmental Change and the Elusive Empirical Evidence for Bet Hedging. *Proc. R. Soc. B.* **2011**, *278*, 1601–1609. https://doi.org/10.1098/rspb.2011.0176.
126. Ardura, A.; Clusa, L.; Zaiko, A.; Garcia-Vazquez, E.; Miralles, L. Stress Related Epigenetic Changes May Explain Opportunistic Success in Biological Invasions in Antipode Mussels. *Sci. Rep.* **2018**, *8*, 10793. https://doi.org/10.1038/s41598-018-29181-4.
127. Schrey, A.W.; Coon, C.A.C.; Grispo, M.T.; Awad, M.; Imboma, T.; McCoy, E.D.; Mushinsky, H.R.; Richards, C.L.; Martin, L.B. Epigenetic Variation May Compensate for Decreased Genetic Variation with Introductions: A Case Study Using House Sparrows (*Passer domesticus*) on Two Continents. *Genet. Res. Int.* **2012**, 2012, 979751. https://doi.org/10.1155/2012/979751.
128. Riyahi, S.; Vilatersana, R.; Schrey, A.W.; Ghorbani Node, H.; Aliabadian, M.; Senar, J.C. Natural Epigenetic Variation within and among Six Subspecies of the House Sparrow, *Passer domesticus*. *J. Exp. Biol.* **2017**, *220*, 4016–4023. https://doi.org/10.1242/jeb.169268.
129. Young, R.L.; Badyaev, A.V. Evolution of Ontogeny: Linking Epigenetic Remodeling and Genetic Adaptation in Skeletal Structures. *Integr. Comp. Biol.* **2007**, *47*, 234–244. https://doi.org/10.1093/icb/icm025.
130. Simpson, G.G. The Baldwin Effect. *Evolution* **1953**, *7*, 110. https://doi.org/10.2307/2405746.
131. Llamas, B.; Holland, M.L.; Chen, K.; Cropley, J.E.; Cooper, A.; Suter, C.M. High-Resolution Analysis of Cytosine Methylation in Ancient DNA. *PLoS ONE* **2012**, *7*, e30226. https://doi.org/10.1371/journal.pone.0030226.
132. Reik, W.; Dean, W.; Walter, J. Epigenetic Reprogramming in Mammalian Development. *Science* **2001**, *293*, 1089–1093. https://doi.org/10.1126/science.1063443.

133. Mhanni, A.A.; McGowan, R.A. Global Changes in Genomic Methylation Levels during Early Development of the Zebrafish Embryo. *Dev. Genes. Evol.* **2004**, *214*, 412–417. https://doi.org/10.1007/s00427-004-0418-0.
134. Fellous, A.; Labed-Veydert, T.; Locrel, M.; Voisin, A.-S.; Earley, R.L.; Silvestre, F. DNA Methylation in Adults and during Development of the Self-Fertilizing Mangrove Rivulus, *Kryptolebias marmoratus*. *Ecol. Evol.* **2018**, *8*, 6016–6033. https://doi.org/10.1002/ece3.4141.
135. Wang, X.; Bhandari, R.K. DNA Methylation Dynamics during Epigenetic Reprogramming of Medaka Embryo. *Epigenetics* **2019**, *14*, 611–622. https://doi.org/10.1080/15592294.2019.1605816.
136. Costa, W.J.E.M.; Lima, S.M.Q.; Bartolette, R. Androdioecy in Kryptolebias Killifish and the Evolution of Self-Fertilizing Hermaphroditism. *Biol. J. Linn. Soc.* **2010**, *99*, 344–349. https://doi.org/10.1111/j.1095-8312.2009.01359.x.
137. Tatarenkov, A.; Earley, R.L.; Taylor, D.S.; Avise, J.C. Microevolutionary Distribution of Isogenicity in a Self-Fertilizing Fish (*Kryptolebias marmoratus*) in the Florida Keys. *Integr. Comp. Biol.* **2012**, *52*, 743–752. https://doi.org/10.1093/icb/ics075.
138. Mirbahai, L.; Chipman, J.K. Epigenetic Memory of Environmental Organisms: A Reflection of Lifetime Stressor Exposures. *Mutat. Res./Genet. Toxicol. Environ. Mutagenesis* **2014**, *764–765*, 10–17. https://doi.org/10.1016/j.mrgentox.2013.10.003.
139. Carvan, M.J.; Kalluvila, T.A.; Klingler, R.H.; Larson, J.K.; Pickens, M.; Mora-Zamorano, F.X.; Connaughton, V.P.; Sadler-Riggleman, I.; Beck, D.; Skinner, M.K. Mercury-Induced Epigenetic Transgenerational Inheritance of Abnormal Neurobehavior Is Correlated with Sperm Epimutations in Zebrafish. *PLoS ONE* **2017**, *12*, e0176155. https://doi.org/10.1371/journal.pone.0176155.
140. Crotti, M.; Yohannes, E.; Winfield, I.J.; Lyle, A.A.; Adams, C.E.; Elmer, K.R. Rapid Adaptation through Genomic and Epigenomic Responses Following Translocations in an Endangered Salmonid. *Evol. Appl.* **2021**, *14*, 2470–2489. https://doi.org/10.1111/eva.13267.
141. Rey, O.; Eizaguirre, C.; Angers, B.; Baltazar-Soares, M.; Sagonas, K.; Prunier, J.G.; Blanchet, S. Linking Epigenetics and Biological Conservation: Towards a *Conservation epigenetics* Perspective. *Funct. Ecol.* **2020**, *34*, 414–427. https://doi.org/10.1111/1365-2435.13429.
142. Ferguson-Smith, A.C. Genomic Imprinting: The Emergence of an Epigenetic Paradigm. *Nat. Rev. Genet.* **2011**, *12*, 565–575. https://doi.org/10.1038/nrg3032.
143. Lyon, M.F. Gene Action in the X-Chromosome of the Mouse (*Mus musculus* L.). *Nature* **1961**, *190*, 372–373. https://doi.org/10.1038/190372a0.
144. Eckersley-Maslin, M.A.; Thybert, D.; Bergmann, J.H.; Marioni, J.C.; Flicek, P.; Spector, D.L. Random Monoallelic Gene Expression Increases upon Embryonic Stem Cell Differentiation. *Dev. Cell* **2014**, *28*, 351–365. https://doi.org/10.1016/j.devcel.2014.01.017.
145. Jeffries, A.R.; Perfect, L.W.; Ledderose, J.; Schalkwyk, L.C.; Bray, N.J.; Mill, J.; Price, J. Stochastic Choice of Allelic Expression in Human Neural Stem Cells. *Stem Cells* **2012**, *30*, 1938–1947. https://doi.org/10.1002/stem.1155.
146. Reinius, B.; Mold, J.E.; Ramsköld, D.; Deng, Q.; Johnsson, P.; Michaëlsson, J.; Frisén, J.; Sandberg, R. Analysis of Allelic Expression Patterns in Clonal Somatic Cells by Single-Cell RNA–Seq. *Nat. Genet.* **2016**, *48*, 1430–1435. https://doi.org/10.1038/ng.3678.
147. Akintola, A.D.; Crislip, Z.L.; Catania, J.M.; Chen, G.; Zimmer, W.E.; Burghardt, R.C.; Parrish, A.R. Promoter Methylation Is Associated with the Age-Dependent Loss of N-Cadherin in the Rat Kidney. *Am. J. Physiol.-Ren. Physiol.* **2008**, *294*, F170–F176. https://doi.org/10.1152/ajprenal.00285.2007.
148. Thompson, R.F.; Atzmon, G.; Gheorghe, C.; Liang, H.Q.; Lowes, C.; Greally, J.M.; Barzilai, N. Tissue-Specific Dysregulation of DNA Methylation in Aging: Tissue-Specific Epigenetic Dysregulation with Aging. *Aging Cell* **2010**, *9*, 506–518. https://doi.org/10.1111/j.1474-9726.2010.00577.x.

Comments and transition

As insights of this review, DNA methylation diversity has been found to be a revealing parameter to characterize natural animal populations. The recent progress in ecological epigenetics allows a more complete understanding of how epigenetic diversity is modulated over time by genetics and environments, which will be helpful for generating predictive models of the capacity of populations to adapt to environmental variation. This review showed that there was as many obligatory than pure epimutations in wild populations depending on the studies and the species of interest. These results contrast with similar studies in plants that mainly show a strong correlation between patterns of epigenetic variation and underlying genetic variants (obligatory epimutations). Otherwise, as genetic variation can blur the role of epigenetic variation, studies in which confounding effects of genetic variation have been controlled or reduced may be useful for isolating the contributions of epigenetic mechanisms in evolutionary processes.

Researchers focused on populations with lack of genetic variation resulting from clonal reproduction or bottlenecks following invasion and showed substantial epigenetic diversity and habitat-specific methylome created by pure epimutations. The mix-mating reproduction system of the mangrove rivulus *Kryptolebias marmoratus* can be used to go even further into the analysis of epigenetic-genetic variations interaction. For the first time, this question can be addressed in a species naturally found under genetically-diverse or isogenic population, allowing us to cover a vast spectrum of genetic diversity configurations of a single species. The next article is a field work comparing epigenetic and behavioral variation in four wild rivulus populations encountering a gradient of genetic diversity, including a highly isogenic population.

CHAPTER 4: EPIGENETIC VARIABILITY IN ONE ISOGENIC LINEAGE REARED UNDER LABORATORY CONDITIONS

To investigate the question of random *versus* environmentally induced epimutations, we collected fish offspring from the Emerson Point Preserve population, and we ran an ecotoxicological experiment on it. We exposed them to methylmercury (MeHg) as it can create permanent transgenerational effects on DNA methylation and behaviors in the zebrafish (more detailed in the following article). Thus, we aim to distinguish potential environmentally (MeHg)-induced epimutations in the exposed groups, and natural rate of random epimutations in the control group of mangrove rivulus, and to link them to gene expression and behavioral variation. We exposed rivulus larvae to MeHg from 0 to 7 days post-hatching (dph), and evaluated immediate effects on DNA methylation, gene expression and behaviors at the end of the exposure, but also delayed effects in adult rivulus (90 dph). Early-life is recognized as a sensitive window during which the environment can have long-lasting effects on the organism phenotype later in life. Discovering out how environmental stressors influence phenotypic variance is crucial to understand animals' ability to acclimate to new environmental conditions during development and adulthood. This ecotoxicological study would highlight the level of random epimutations and the potential alternations of environmental changes on the methylation level of targeted genes related to personality in the mangrove rivulus by using a methylation gene-specific approach.

Aquatic Toxicology
Available online 4 March 2023, 106474

EARLY-LIFE EXPOSURE TO METHYLMERCURY INDUCES REVERSIBLE BEHAVIORAL IMPAIRMENTS AND GENE EXPRESSION MODIFICATIONS IN ONE ISOGENIC LINEAGE OF MANGROVE RIVULUS FISH KRYPTOLEBIAS MARMORATUS

V Chapelle[1*], J Lambert[1], T Deom[1], E Tessier[2], D Amouroux[2], F Silvestre[1]

[1]Laboratory of Evolutionary and Adaptive Physiology, Institute of Life, Earth, and the Environment, University of Namur, 61 Rue de Bruxelles, 5000 Namur, Belgium

[2]Université de Pau et des Pays de L'Adour, E2S UPPA, CNRS, Institut des Sciences Analytiques et de Physicochimie pour l'Environnement et les Matériaux, Pau, France

[*] Corresponding author. E-Mail address: valentine.chapelle@unamur.be. Postal address: 61 Rue de Bruxelles, 5000 Namur, Belgium.

Abstract:

Methylmercury (MeHg) is a ubiquitous bioaccumulative neurotoxicant present in aquatic ecosystems. It is known to alter behaviors, sensory functions and learning abilities in fish and other vertebrates. Developmental and early-life stages exposure to MeHg can lead to brain damage with immediate consequences on larvae behavior, but may also induce long term effects in adults after a detoxification period. Epigenetic mechanisms are a possible explanation for such delayed disruption. However, very little is known about developmental origin of behavioral impairment in adults due to early exposure to MeHg. The aim of this study is to assess whether early-life MeHg exposure induces immediate and/or delayed effects on behaviors, related genes expression and DNA methylation (one of epigenetic mechanisms). To reach this goal, newly hatched larvae of mangrove rivulus fish, *Kryptolebias marmoratus*, were exposed to two sub-lethal concentrations of MeHg (90 µg/L and 135 µg/L) for 7 days, and immediate and delayed effects were assessed respectively in 7 dph (days post-hatching) and 90 dph fish. This species naturally produces isogenic lineages due to its self-fertilizing reproduction system, which is unique among vertebrates. It allows to study how environment

stressors can influence organism's phenotype while minimizing genetic variability. As results, both MeHg exposures are associated with a decreased foraging efficiency and thigmotaxis, and a dose-dependent reduction in larvae locomotor activity. Regarding molecular analysis in larvae whole bodies, both MeHg exposures induced significant decreased expression of DNMT3a, MAOA, MeCP2 and NIPBL, and significant increase of GSS, but none of those genes underwent methylation changes in targeted CpGs. None of the significant behavioral and molecular impairments observed in 7-dph larvae were found in 90-dph adults, which highlight a distinction between immediate and delayed effects of developmental MeHg exposure. Our results suggest implications of aminergic system and its neurotransmitters, redox/methylation trade-off and possibly other epigenetic mechanisms in MeHg neurotoxicity underlying behavioral alterations in rivulus.

Key words: methylmercury, delayed effects, behaviors, DNA methylation, mangrove rivulus

Citation: Chapelle, V.; Lambert, J.; Deom, T.; Tessier, E.; Amouroux, D.; Silvestre, F. Early-life exposure to methylmercury induces reversible behavioral impairments and gene expression modifications in one isogenic lineage of mangrove rivulus fish *Kryptolebias marmoratus*. Aquatic Toxicology 2023, https://doi.org/10.1016/j.aquatox.2023.106474

Received 3 October 2022, Revised 28 February 2023, Accepted 2 March 2023, Available online 4 March 2023.

1. Introduction

Methylmercury (MeHg) is a neurotoxic contaminant generated through methylation of heavy metal mercury by anaerobic bacteria in aquatic environments. It enters the aquatic food web through the consumption of these bacteria by zooplankton, and undergoes bioaccumulation and biomagnification resulting in high concentrations in large predatory fish and other top predators including humans (Baeyens et al., 2003). It is established that MeHg crosses the blood-brain barrier and can induce brain damages, impaired neurological development and behaviors. In fish, MeHg exposure causes altered swimming activity and prey capture success (Mora-Zamorano et al., 2017; Samson, 2001; Xiaojuan Xu, 2012; Zhu et al., 2020), visual deficit (Carvan et al., 2017; Weber et al., 2008), learning and memory impairments (Smith et al., 2010; Xu et al., 2016). Several cellular mechanisms have been proposed for MeHg-induced neurotoxicity including oxidative stress (Carvan et al., 2017; Farina and Aschner, 2019), alterations of neuronal differentiation (Tamm et al., 2008), and disruption of glutamatergic and dopaminergic neurotransmitter systems (Faro et al., 2002).

Most recent studies of MeHg neurotoxicity have focused on adverse outcome pathways and behavioral effects of developmental and early-life stages exposure. MeHg exposure during these stages can cause delayed effects that can be observed later in life, but also transgenerational effects (Carvan et al., 2017; Xu et al., 2016). It has been suggested that delayed and transgenerational MeHg-altered phenotypes result from gene expression modification without changes to the underlying nucleotide sequence of DNA (i.e., epigenetic mechanism) (Carvan et al., 2017; Skinner, 2011). Generally, MeHg alterations of epigenome include downregulation of microRNA expression, reduced histone acetylation and increased histone methylation, and global DNA hypomethylation in brain (reviwed in Culbreth and Aschner, 2019). As such, epigenetic modifications have become an attractive mechanistic target for the impacts of environment on phenotypic variation. Even with this extensive compilation of cellular and molecular data, it is still challenging to link MeHg-induced behavioral impairments to specific molecular alterations, and to understand the underlying mechanisms of delayed behavioral effects of early-life stages exposure.

In the present study we used the mangrove rivulus *Kryptolebias marmoratus* as model system to determine immediate and delayed behavioral impairments and associated molecular mechanisms following an early exposure to MeHg. Mangrove rivulus is a powerful emerging

model vertebrate species in ecotoxicology due to its unique reproductive system: it is the only known vertebrate capable of self-fertilization, for which hermaphrodites can naturally produce highly homozygous and isogenic populations after a few generations (Costa et al., 2010). These characteristics allow scientists to drastically minimize genetic variation between individuals, and to focus on how epimutations and environment variation, such as pollutant exposure, influence phenotype. This is made possible thanks to the expanding repertoire of genetic and epigenetic data on mangrove rivulus (Kelley et al., 2016). Furthermore, rivulus is subject to MeHg exposure in its natural environment since mangroves are considered as potential MeHg hotspots due to their large amount of organic matter and their physico-chemical characteristics (Lei et al., 2019). Their location facilitates pollutant accumulation, as showed in Florida where Hg levels were elevated in mangrove transition zone compared to both the upstream canals and the open waters of Florida Bay (Rumbold et al., 2011). Total mercury (THg) concentrations in freshwater ecosystems range from 0.3 ng/L to 450 µg/L, with higher levels found downstream of pollution sources including mines and industrial discharges (Kidd and Batchelar, 2011). There are fewer data available on the distribution of mercury in mangrove waters, and even less on MeHg. THg concentrations in mangroves water ranged from 0.04 to 110 ng/L, with large temporal and spatial variations. In waters collected from mangrove in south Florida, USA, Bergamaschi et al. (2012) recorded 26 ng/L MeHg. However, MeHg transfer from water into the base of the food web (bioconcentration) and subsequent biomagnification in the aquatic food web leads to most of the MeHg in higher trophic levels (Wu et al., 2019). Mangrove rivulus is a predator and can accumulate MeHg through contaminated prey consumption including bivalves that showed 100 to 940 ng/g dw Hg concentration (Saha et al., 2006) and polychaetes containing 50 to 280 ng/g dw Hg (Alam et al., 2010) in mangrove ecosystems. To our knowledge, there is no available data on THg and MeHg concentrations in mangrove rivulus.

Our objective is to assess whether an early exposure to sublethal MeHg of newly hatched rivulus larvae exposed from 0 to 7 dph induces immediate and delayed effects on behaviors including locomotor activity, thigmotaxis (used as index of anxiety) and foraging efficiency in 7 dph and 90 dph rivulus, and to unravel underlying mechanisms of these alterations by investigating expression and methylation of related genes. We choose a long detoxification period of 83 days after 7 days of exposure at high concentration of MeHg to highlight delayed and potentially irreversible effects, and to reveal underlying mechanisms that could be too subtle at lower MeHg concentrations. We hypothesize that MeHg exposure during early life can induce lasting epimutations that could impact gene expression, leading to behavioral

impairments in rivulus larvae but also in adults. This is the first set of experiments that aimed to identify MeHg effects in mangrove rivulus, joining the restricted list of ecotoxicological studies on this fish species.

2. Materials and methods

2.1. Experimental fish procurement and methylmercury exposure

Mangrove rivulus used for this experiment are the 4th generation obtained from one single hermaphrodite to reduce genetic variation between individuals. This ancestor was sampled in 2019 in South Florida (Emerson Point Preserve, Palmetto, Florida, USA; N27°31'56.8", W82°37'46.4") by Kristy Marson, Ryan L. Earley, Frédéric Silvestre and Valentine Chapelle. It was transferred to the University of Namur where it generated a stock population raised in the laboratory of evolutionary and adaptive physiology (LEAP, University of Namur, Belgium), housed in 12 ± 1 parts per thousand (ppt) saltwater (Instant OceanTM sea salt), at 25 ± 1°C, 12:12 light:dark cycle and fed every day *ad libitum* with living *Artemia salina*. These housing conditions are kept for the rest of the experiment as control condition.

Eggs were collected and individually placed in plastic 24-wells microplates (Cellstar®) filled with 2 mL of 12 ± 1 ppt saltwater. Plates with eggs were housed in an incubator at 25 ± 1 °C, 12:12 light:dark cycle. Water was changed every day until hatching. Newly hatched autonomous larvae were randomly assigned and individually exposed to nominal methylmercury concentrations of 0 (control), 90, or 135 µg/L for 7 days in 12-wells microplates filled with 4 mL of exposure solution (Figure 1). Two of the twelve wells were dedicated to internal control. A total of 114 0-day post-hatching (dph) autonomous larvae were split into three exposure groups (Table 1).

Methylmercury solutions were obtained from lyophilized methylmercury chloride (CH3HgCl) purchased from Sigma-Aldrich® (ref: 442534). A stock solution of 135 mg/L MeHg in deionized water was kept for the entire experiment at 4°C in the dark. From this stock solution, 90 and 135 µg/L exposure solutions were newly produced every day. These two MeHg concentrations were chosen based on pre-test results as they were the two lowest sublethal MeHg concentrations having behavioral effects among 7 other concentrations (unpublished data). Every day during the 7-days exposure, each larva was fed with one drop of their exposure solution containing *Artemia salina* and half of the solution was renewed to maintain stable

MeHg concentrations. At the end of exposure, all larvae took the two behavioral tests described below. Then, they were separated in two different groups: 46 larvae were euthanized, measured, and conserved at -80°C for molecular analysis and 58 larvae were kept until 90 dph to study delayed effects of MeHg (Table 1). These 58 larvae were individually transferred into a « cleaning bath » filled with salt water (12 ± 1ppt at 25 ± 1 °C) and then to a glass jar filled with 200 mL of salt water and placed under the same physico-chemical conditions than control fish until 90 dph. Mature fish took the same behavioral tests they took at 7 dph, then were euthanized, measured and conserved at -80°C for molecular analysis.

All rivulus husbandry and experimental procedures were performed in accordance with the Belgian animal protection standards and were approved by the University of Namur Local Research Ethics Committee (UN 21 367 KE). The agreement number of the laboratory for fish experiments is LA1900048.

Table 1: Total number of mangroves rivulus used per condition and analysis. MeHg immediate effects are measured on 7-days post-hatching (dph) larvae, while MeHg delayed effects are measured on 90 dph adults.

Conditions	**Larvae**			**Adults**		
	Behaviors	Genes expression	Gene-specific methylation	Behaviors	Genes expression	Gene-specific methylation
Control	52	22	3	28	24	3
90 µg/L MeHg	31	12	3	16	13	3
135 µg/L MeHg	31	12	3	14	13	3
Total	114	46	9	58	50	9

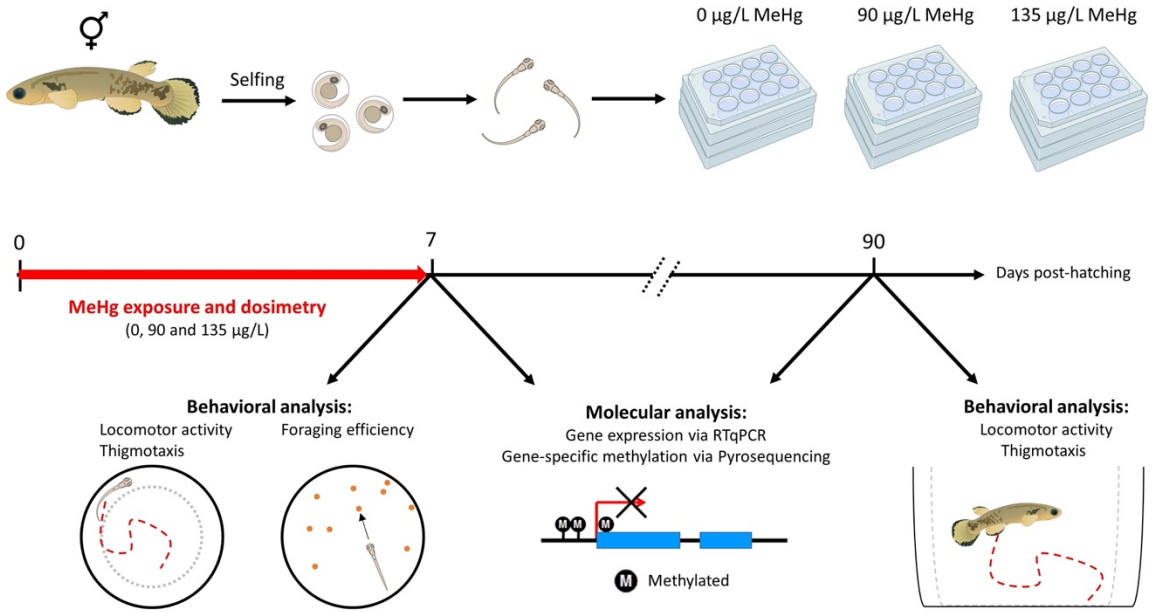


Figure 1: Experimental design: MeHg exposures, behavioral and molecular analysis timeline. Eggs were produced by self-fertilization of hermaphrodite individuals from one single ancestor. Newly hatched autonomous larvae were randomly assigned and individually exposed to nominal methylmercury concentrations of 0, 90, or 135 µg/L for 7 days. At 7 dph, the locomotor activity and thigmotaxis were analyzed on all of the 114 larvae, directly followed by the assessment of foraging efficiency. 46 larvae were euthanized, measured, and conserved at -80°C for gene expression and DNA methylation analysis and 58 larvae were kept until 90 dph to study delayed effects of MeHg.

2.2. Methylmercury and inorganic mercury speciation analysis

Methylmercury and inorganic mercury concentration were measured at different levels and time points. Firstly, newly prepared working solution of 90 and 135 µg/L of MeHg were sampled, stabilized by acidification (1% hydrochloric acid 37%) and stored at -20°C to further assess the actual MeHg concentrations to which larvae were exposed. Inorganic mercury (iHg) concentration was also measured to evaluate MeHg demethylation rate. Secondly, 6 randomly selected larvae of 7 dph were euthanized at the end of exposure to analyze MeHg and iHg concentration in their whole body (2 individuals per condition).

MeHg and iHg concentrations in working solutions and in larvae were determined using species-specific isotope dilution analysis and gas chromatography combined with inductively coupled plasma mass spectrometry (GC/ICP-MS, Thermo Scientific). Water samples were directly analyzed, following the methodology published elsewhere (Cavalheiro et al., 2016; Monperrus et al., 2005) whereas larvae were first microwave digested in alkaline solution

(Cavalheiro et al., 2014). Limits of detection (LOD) are 0.008 µg/L for MeHg and 0.011 µg/L for iHg in water, and 0.005 µg/g for MeHg and 0.008 µg/g for iHg in larvae.

2.3. Behavioral tests

At the end of MeHg exposure, all of the 114 rivulus larvae were transferred from 12-wells to 6-wells microplates to evaluate locomotor activity and thigmotaxis (the tendency to remain close to the walls, used as index of anxiety), directly followed by the assessment of foraging efficiency. Locomotor activity and thigmotaxis were monitored and analyzed by using DanioVision (Noldus Information Technology, Wageningen, Netherlands). After 5 minutes of acclimation, total distance moved and cumulative duration spent in wells center were recorded for 10 minutes and calculated using EthoVision software version 15.0 (Noldus Information Technology). After this behavioral test, we directly evaluated foraging efficiency by introducing 10 *Artemia salina* nauplii per rivulus. They were allowed to feed for 10 min during which the number of capture successes and fails were manually counted based on video-records. Regarding 90-dph adult rivulus, similar tests were used to evaluate delayed effects of MeHg but in bigger tanks filled with one liter of 12 ppt saltwater.

2.4. Tissue samplings for molecular analysis

After the first run of behavioral tests, 46 of the 114 larvae were immersed in 4 °C water for euthanasia. Their standard length was measured, and whole larvae were snap-frozen in liquid nitrogen and stored at −80 °C for subsequent molecular analyses. Regarding 90 dph adults, they were immersed in 4 °C water for euthanasia directly after the second run of behavioral tests. Their weight and standard length were measured. Death was ensured by decapitation. The brain was removed, snap-frozen in liquid nitrogen and stored at −80 °C for subsequent molecular analyses.

2.5. Genes of interest

Expression and methylation of several genes of interest were analyzed to assess MeHg immediate and delayed effects at the molecular level. Genes coding for Toll interacting protein (Tollip), monoamine oxidase A (MAOA) and methyl CpG binding protein 2 (MeCP2) were chosen for their roles in neuronal functions and/or personality traits (Baronio et al., 2022; Newman et al., 2005; Oguro et al., 2011; Pietri et al., 2013; Sallinen et al., 2009). Genes coding for DNA methyltransferase 3a (DNMT3a) and Nipped-B-like protein (NipBL) were chosen for

their roles in epigenetic mechanisms (Bell and Felsenfeld, 2000; Gao et al., 2019). Finally, we chose Glutathione synthetase (GSS) for its central role in glutathione biosynthesis pathway, and response to stressor such as heavy metals (Farina and Aschner, 2019).

2.6. Simultaneous DNA and RNA extractions

DNA and RNA were simultaneously extracted with the Quick-DNA/RNA™ Microprep Plus Kit from ZYMO research (ref: D7005) following the manufacturer's protocol. This purification allows us to obtain relative expression data and DNA methylation levels in the same individual. Briefly, whole larvae or adult brains were homogenized 2 minutes with bead beating, then incubated with proteinase K at 55°C for 30 minutes (brains) or 1 hour (larvae bodies). The supernatant was transferred to columns and washed multiple times to separately eluate approximately 20 µL of DNA and RNA. DNA and RNA concentrations were measured using the Nanodrop (Thermo Fisher, Waltham, MA), and their integrity was evaluated on a 1% agarose gel. DNA and RNA samples were stored at −80 °C for subsequent molecular analyses, respectively gene-specific DNA methylation analysis via pyrosequencing and relative gene expression analysis via RT-qPCR.

2.7. Gene expression by reverse transcription quantitative PCR (RT-qPCR)

DNase treatment was performed on RNA samples with Invitrogen DNA-free™ DNA Removal Kit from Thermo Fisher Scientific (ref: AM1906) before converting 500 ng per RNA samples into cDNA with the RevertAid RT Reverse Transcription Kit from Thermo Scientific™ (ref: K1691). Samples were stored at −20 °C during primer design and tests.

Primers were designed with AmplifX 2.1.1 and Primer blast (NCBI genome: ASM164957v2) using the exon-spanning method. Primers efficiencies were tested on cDNA pool of samples made from all MeHg conditions. 2.5 µL of 50x diluted cDNA was added to 2.5 µL of primers mix and 5 µL of SYBR green (Bio Rad®). Three technical replicates were established per dilution. SYBR green quantitative PCR was conducted on a StepOnePlus Real-Time PCR System® (Applied Biosystems). A melting curve and an end-point agarose gel electrophoresis followed by SYBR safe (Thermo Fisher) staining were used to check for accurate amplification of the target amplicon. Primer efficiencies were calculated according to the MIQE guidelines and accepted between 90 and 110% (see RTqpCR primers in Table A.1).

Relative expression of genes of interest was normalized with mean expression of two house-keeping genes: ubiquitin-conjugating enzyme E2 A (ube2a) and selenoprotein 15 (SEP15). These genes were selected after testing their overall stability value and their intragroup and intergroup variation. The relative gene expression (RGE) is the gene of interest expression level in comparison to the mean of housekeeping genes expression.

2.8. Gene-specific DNA methylation by pyrosequencing

Pyrosequencing relies on bisulfite treatment of the genomic DNA, converting unmethylated cytosine to uracil (thymine after PCR amplification) while methylated cytosines are refractory to the treatment. Bisulfite treatment was performed on DNA samples with EZ DNA Methylation kit from Zymo Research (ref: D5001). For optimized bisulfite conversion, we used 500 ng of genomic DNA and followed the manufacturer's protocol excepted for the last step of elution. We performed two elutions of 10 µL M-Elution Buffer instead of a single 10 µL elution to double our final elution volume.

PCR amplification was performed using PyroMark PCR Kit from Qiagen (ref: 978703) in 25 µL reaction volumes containing 1 µL of DNA template, 6 µL nuclease-free H2O, 12.5 µL of 2x PyroMark PCR Master Mix, 1 µL MgCl2, 2.5 µL 10x CoralLoad Concentrate, 2 µL forward and reverse primer mix (5 µM). We followed manufacturer's protocol for PCR conditions. Forward and reverse primers for PCR and sequencing primer for pyrosequencing were designed with software PyroMark Assay Design SW 2.0 on selected DNA sequences. Firstly, we highlighted CpG islands across each sequence including promotor region, exons and introns using MethPrimer 2.0. Then, we used Match™ to highlight smaller sequences containing binding sites of transcription factors (TF) whose functions are relevant to our study; central nervous system and sensory organs development, neuronal plasticity, oxidative and cytotoxic stress resistance. We selected sequences of approximately 150 bp, containing CpGs islands and TF binding sites in different regions including promotors, exons and introns (see Table A.2). Pyrosequencing was performed on the Pyromark Q24 instrument using PyroMark Q24 Advanced Reagents (Qiagen) according to the manufacturer's protocol, with a 15 µL input of PCR products. Pyrograms were manually interpreted and evaluated using the PyroMark Q24 software.

2.9. Data statistical analysis

Statistical analyses were performed with the Prism™ 8.0.2 (GraphPad software, Inc.). The normal distribution of standard length and molecular variables was analyzed with a Shapiro-Wilk normality test, and the homogeneity of variances was tested with a Barlett's test. If the normality and homogeneity were proved, we realized an ordinary one-way ANOVA followed by a Tukey's multiple comparisons test. Otherwise, if normality and/or homogeneity couldn't be proved, a nonparametric Kruskal-Wallis test was performed, followed by a Dunn's multiple comparison test. For all statistical analysis, p value was fixed to 0.05.

Regarding behavioral analysis, these measurements cannot be considered as independent because two measurements were collected per fish. As we have repeated measures, linear mixed models (LMMs) were used on behavioral variables using "lmer" function from "lme4" R package in R Studio (version 1.4.1103). MeHg condition and rivulus age were entered as fixed effects and individuals as random effects.

3. Results

3.1. Mercury speciation in water and larvae

Measured concentrations of MeHg in newly prepared working solutions of 90 and 135 µg/L (nominal concentrations) were 75.17 ± 2.17 and 120.24 ± 6.18 µg/L respectively (mean ± SD) (Table 2). We also observed in both MeHg exposure conditions low concentrations of iHg. MeHg content measured in the control group remains under the LOD of the GC/ICP-MS. In whole larvae exposed during 7 days to 0, 90 or 135 µg/L MeHg, we measured <LOD, 29.16 ± 0.86 and 47.62 ± 0.95 µg/g dw, respectively (mean ± SD). Significant concentrations of iHg were observed under these experimental conditions. By comparing measured MeHg concentrations in fresh working solutions and in whole larvae at the end of the exposure, we obtained MeHg bioconcentration factors of 388 and 396 in 90 µg/L and 135 µg/L MeHg conditions, respectively.

Table 2: Methylmercury (MeHg) and inorganic mercury (iHg) concentrations in fresh solutions used for exposures and in whole larvae at the end of the 7-days MeHg exposure. Limits of detection (LOD) are 0.008 µg/L for MeHg and 0.011 µg/L for iHg in water, and 0.005 µg/g for MeHg and 0.008 µg/g for iHg in larvae. Mean ± SD.

Fresh working solutions		
Nominal MeHg concentration (µg/L)	*Measured MeHg concentration (µg/L)*	*Measured iHg concentration (µg/L)*
0	< LOD	< LOD
90	75.17 ± 2.17	3.93 ± 0.41
135	120.24 ± 6.18	6.04 ± 1.86
Whole larvae (7-dph)		
Nominal MeHg concentration (µg/L)	*Measured MeHg concentration (µg/g dw)*	*Measured iHg concentration (µg/g dw)*
0	< LOD	< LOD
90	29.16 ± 0.86	38.55 ± 0.84
135	47.62 ± 0.95	40.95 ± 0.95

3.2. Standard length

Rivulus standard length were measured at the beginning of the experiment (1 dph rivulus), at the end of MeHg exposure (7 dph) and after the recovery period (90 dph). We didn't show any significant standard length differences between fish at the beginning of the experiment, and also no significant immediate or delayed effects of MeHg exposure on standard length (Table 3).

Table 3: Data monitoring of fish standard length (mm) at different ages. Dph = days post hatching. No significant differences were observed in 1-dph rivulus (Ncontrol = 52; N90 = 42; N135 = 43), 7-dph rivulus (Ncontrol = 24; N90 = 14; N135 = 14) or 90-dph rivulus (Ncontrol = 26; N90 = 16; N135= 13). Mean ± SD.

Age (dph)	Control	90 µg/L	135 µg/L
1	4.79 ± 0.26	4.65 ± 0.27	4.61 ± 0.24
7	5.30 ± 0.27	5.17 ± 0.21	5.15 ± 0.27
90	22.2 ± 1.12	21.5 ± 1.62	21 ± 1.87

3.3. Locomotor activity assessment

Linear mixed models (LMMs) showed a significant interaction between MeHg condition and rivulus age ($p < 0.01$). All MeHg-exposed larvae exhibited lower locomotor activity relative to control, with 250 ± 99.8 cm travelled by control rivulus, 159 ± 94.8 cm travelled by rivulus exposed to 90 µg/L MeHg and 82.6 ± 65.9 cm travelled by rivulus exposed to 135 µg/L MeHg (mean ± SD). We observed significant dose-dependent effect of MeHg, as activity was reduced by a factor of 1.6 and 3.0 in fish exposed to 90 and 135 µg/L MeHg, respectively, in comparison to controls larvae. On the opposite, no significant effect of MeHg was observed in 90 dph adults that showed activity reduction as larvae, with 141 ± 111 cm, 157 ± 106 cm and 174 ± 138 cm travelled by rivulus exposed to 0, 90 or 135 µg/L MeHg, respectively (Figure 2).

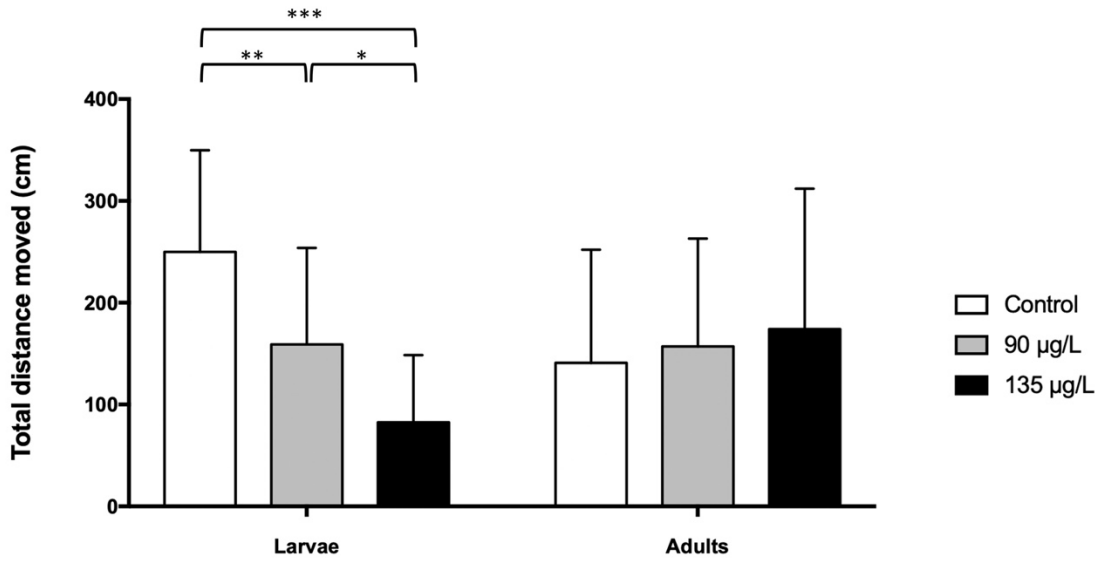


Figure 2: Total distance moved during 10 minutes of activity assessment test in 7-dph larvae and 90-dph adults exposed from 0 to 7 dph to different concentrations of methylmercury (MeHg). Linear-mixed models shows a significant interaction between MeHg conditions and rivulus age, with a dose-dependent immediate effects of MeHg in larvae (Ncontrol = 52; N90 = 31; N135 =31; *P <0.05; **P < 0.01; ***P < 0.001) and no significant delayed effects in adult rivulus (Ncontrol = 26; N90 = 16; N135 =14; *P <0.05; **P < 0.01; ***P < 0.001). Bar charts representing mean ± SD.

3.4. Thigmotaxis assessment

To evaluate thigmotaxis, we analyzed total time spent in arena central zone. All MeHg-exposed larvae spent significantly more time in the center of the well in comparison to the control, with 62.2 ± 49.7 s, 107 ± 102 s and 131 ± 100 s spent in the center of the arena by control, 90 µg/L exposed and 135 µg/L exposed rivulus, respectively (mean ± SD). MeHg-exposed larvae exhibit a lower thigmotaxis behavior than control larvae, corresponding to a less anxious state.

On the opposite, no significant effects of MeHg were observed in 90 dph adults, as control, 90 µg/L and 135 µg/L exposed rivulus spent 40.7 ± 58.4 s, 37.6 ± 61.2 s and 40.9 ± 65 s in the center of the arena (mean ± SD) (Figure 3).

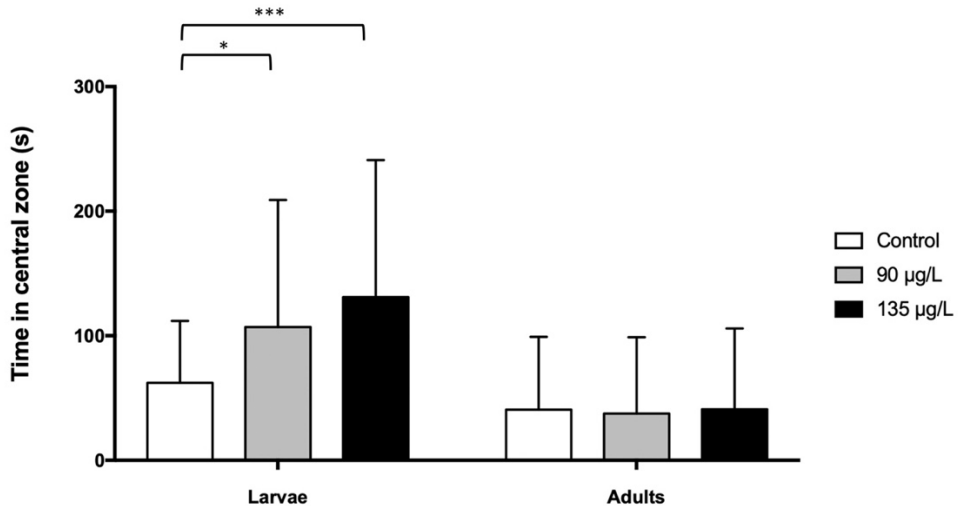


Figure 3: Time spent in the center of the arena during 10 minutes of thigmotaxis assessment test in 7-dph larvae and 90-dph adults exposed from 0 to 7 dph to different concentrations of methylmercury (MeHg). Results show a significant immediate effect of MeHg in larvae (Ncontrol = 52; N90 = 31; N135 =31; *P <0.05; **P < 0.01; ***P < 0.001) and no significant delayed effects in adult rivulus (Ncontrol = 26; N90 = 16; N135 =14; *P <0.05; **P < 0.01; ***P < 0.001). Bar charts representing mean ± SD.

3.5. Foraging efficiency

The foraging efficiency test on 10 *Artemia salina* nauplii per 7-dph larvae showed a significant decrease in the number of prey captures in both MeHg exposure groups, as control larvae caught 7.29 ± 2.13 preys, 90 µg/L exposed larvae caught 4.45 ± 3.42 preys and 135 µg/L exposed larvae caught 3.61 ± 3.17 preys in 10 minutes (mean ± SD, $p < 0.001$) (Figure 4A). MeHg-exposed larvae also tried less to capture preys in comparison with control, with 9.08 ± 3.09, 5.65 ± 3.57 and 4.84 ± 3.56 prey capture tried by control, 90 µg/L and 135 µg/L larvae, respectively (mean ± SD, $p < 0.001$). As exposed larvae caught less preys and also tried less to capture preys in comparison with control, percentage of prey capture success (prey capture/capture attempt) shows no significant differences between condition, with 83.1 ± 18.2 %, 74.8 ± 28.6 % and 69.6 ± 30.8 % of capture success for control, 90 µg/L and 135 µg/L larvae, respectively (mean ± SD) (Figure 4B).

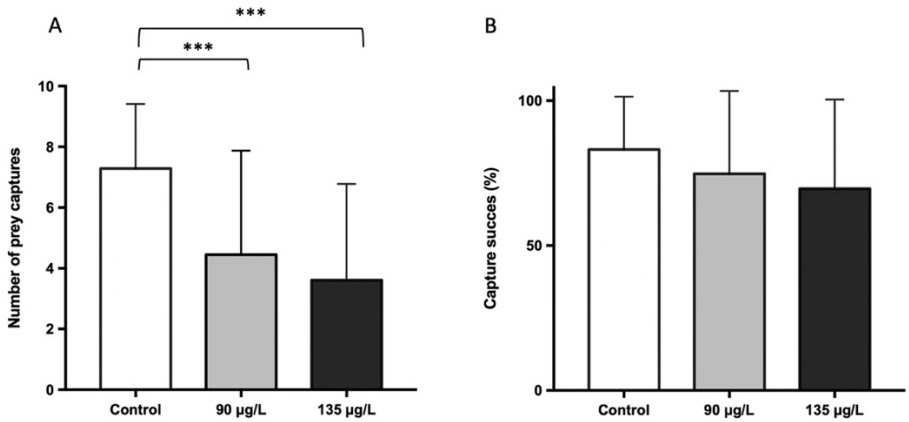


Figure 4: Immediate effects of MeHg exposure on foraging efficiency on 10 Artemia salina nauplii per 7-dph larvae. (A) Number of prey capture trials and (B) the prey capture success of rivulus larvae. A Kruskal-Wallis test showed a significant decrease in the number of capture trials for both groups exposed to MeHg but not significant effect of MeHg on prey capture success (NControl = 52; N90 = 31; N135 = 31; P <0.05; **P < 0.01; ***P < 0.001). Bar charts representing mean ± SD.

3.6. Gene expression

Relative expression analyses in whole larvae revealed a significant reduction in expression of DNMT3a ($p < 0.05$), NipBL ($p < 0.01$), MeCP2 ($p < 0.01$) and MAOA ($p < 0.01$) in 90 and 135 µg/L MeHg-exposed groups compared to controls (Figure 5A). We also highlighted a significant increased expression of GSS in larvae exposed to 90 and 135 µg/L MeHg, with a relative expression in control, 90 µg/L MeHg and 135 µg/L MeHg groups of 0.97 ± 0.32, 2.51 ± 1.36 and 2.57 ± 0.77 respectively (mean ± SD, $p < 0.001$) (Figure 5B). Finally, we did not show any significant effect of MeHg exposure on relative expression of Tollip. Relative expression analyses in brain of 90-dph adults exposed to MeHg from 0 to 7 dph showed no significant delayed effects of MeHg on expression of all genes of interest (Figure 6).

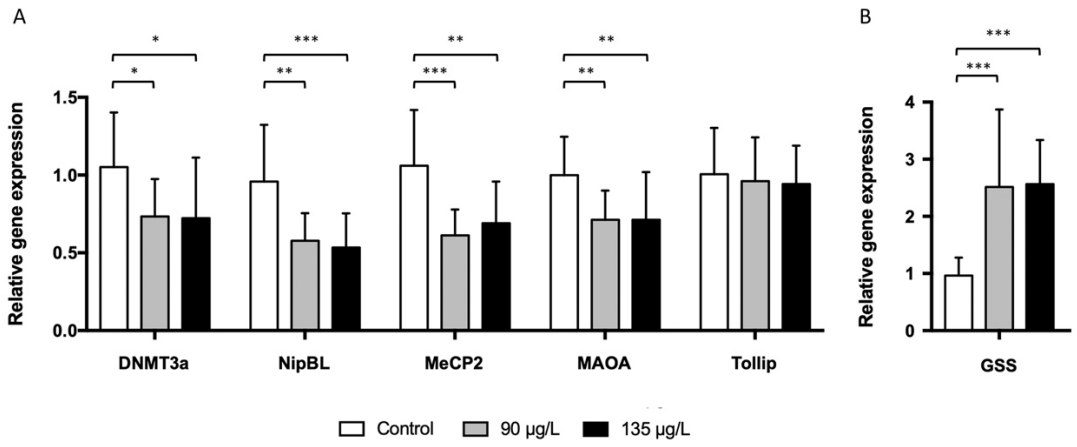


Figure 5: Relative gene expression (RGE) in whole body of 7-dph larvae rivulus in response to MeHg exposure from 0 to 7 dph. Results are presented as mean ± SEM. Housekeeping genes used are Ubiquitin-conjugating enzyme E2 A (UBE2A) and selenoprotein 15 (Sep15). (A) MeHg exposure creates a decreased expression of DNA methyltransferase 3a (DNMT3a), Nipped-B-like protein (NipBL), methyl CpG binding protein 2 (MeCP2), monoamine oxidase A (MAOA), and no significant effects on Toll interacting protein (Tollip). (B) MeHg exposure creates an increased expression of Glutathione synthetase (GSS) (NControl = 22; N90 = 11; N135 = 11; P <0.05; **P < 0.01; ***P < 0.001). Bar charts representing mean ± SD.

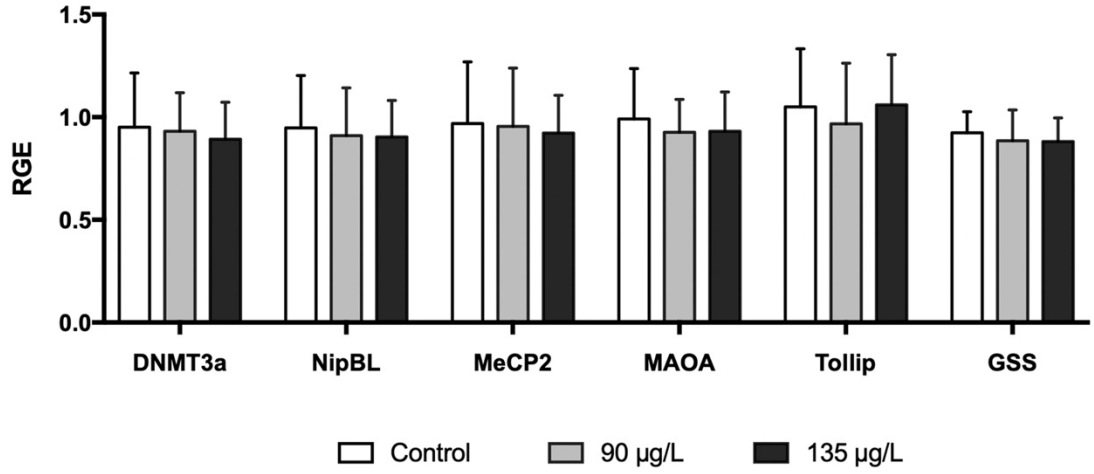


Figure 6: Relative gene expression (RGE) in brain of 90-dph rivulus in response to MeHg exposure from 0 to 7 dph. Results are presented as mean ± SEM. Housekeeping genes used are Ubiquitin-conjugating enzyme E2 A (UBE2A) and selenoprotein 15 (Sep15). No significant delayed effects were observed in adults rivulus on expression of DNA methyltransferase 3a (DNMT3a), Nipped-B-like protein (NipBL), methyl CpG binding protein 2 (MeCP2), monoamine oxidase A (MAOA), Toll interacting protein (Tollip) and Glutathione synthetase (GSS) (NControl = 24; N90 = 13; N135 = 13; P <0.05; **P < 0.01; ***P < 0.001). Bar charts representing mean ± SD.

3.7. Gene-specific DNA methylation

Analysis of gene-specific methylation in whole larvae showed no significant effects of MeHg on methylation of targeted CpGs in all genes of interest (Figure 7). We can observe a global high level of methylation of DNMT3a and GSS targeted CpGs, as larvae showed an average of 71.08 ± 2.58 % of methylation at targeted CpGs in DNMT3a, and 83.82 ± 1.79 of methylation at targeted CpGs in GSS. In the opposite, we can observe a global low level of methylation of MeCP2 and MAOA targeted CpGs, as larvae showed an average of 2.21 ± 0.82 % of methylation at targeted CpGs in MeCP2, and 3.60 ± 0.36 of methylation at targeted CpGs in MAOA. Finally, NIPBL targeted CpGs showed intermediate levels of methylation, with 23.65 ± 2.25 % of methylation (mean ± SD). The same analysis was performed on 90 dph adult brains to search for possible delayed effects of MeHg on targeted CpGs methylation level. We showed no significant effects of MeHg on targeted CpGs methylation in any genes of interest (Figure 8). As for larvae, we can observe a global high level of methylation of DNMT3a and GSS, a

global low level of methylation of MeCP2 and MAOA, and an intermediate level of methylation of NIPBL, with very similar values.

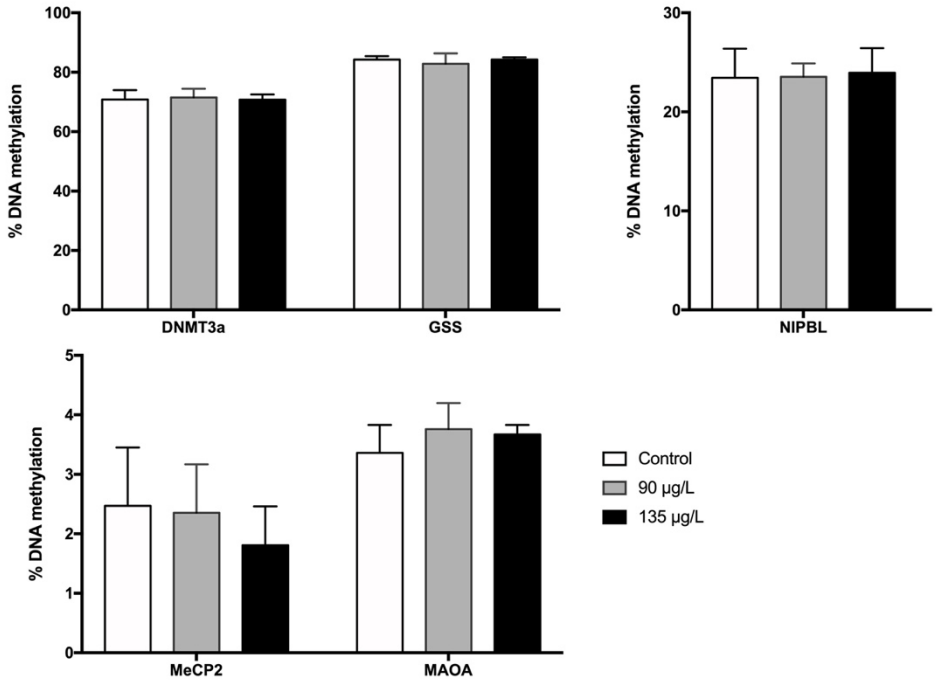


Figure 7: Mean percentage of methylation at targeted CpGs in DNMT3a, GSS, NIPBL, MECP2 and MAOA in whole body of 7-dph larvae rivulus exposed to MeHg from 0 to 7 dph (NControl = 3; N90 = 3; N135 = 3; $P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Bar charts representing mean ± SD.

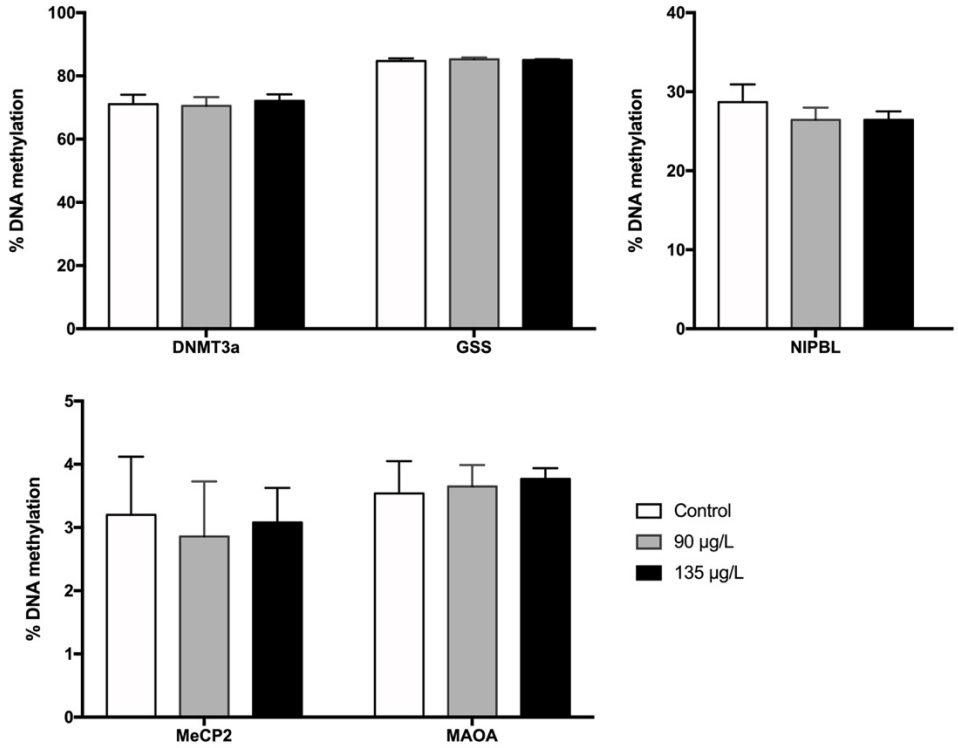


Figure 8: Mean percentage of methylation at targeted CpGs in DNMT3a, GSS, NIPBL, MECP2 and MAOA in brains of 90 dph rivulus exposed to MeHg from 0 to 7 dph (NControl = 3; N90 = 3; N135 = 3; P <0.05; **P < 0.01; ***P < 0.001). Bar charts representing mean ± SD.

4. Discussion

4.1. Mangrove ecosystems and bioconcentration

Mangrove rivulus is a relevant model species to assess MeHg exposure consequences as mangroves are considered as MeHg hotspots due to their large amount of organic matter and their physico-chemical characteristics, so as other wetland ecosystems (Lei et al., 2019). MeHg was found in water, sediments, and resident species of South Florida mangroves, as described in the introduction section. Our dosimetry results showed bioconcentration factors (BCF) of 388 and 396 in 90 µg/L and 135 µg/L MeHg conditions, respectively. Comparison of BCF with other studies is delicate as it is linked to bioavailability, which is influenced by different environmental factors such as dissolved organic matter, salinity, dissolved oxygen concentration and pH (Berzas Nevado et al., 2003; Kidd and Batchelar, 2011). BCF varies also according to organisms that allow the entry of MeHg into the aquatic food web (Wu et al., 2019). Mangrove rivulus are predators, with various terrestrial and aquatic invertebrates forming the bulk of the diet including gastropods, various crustaceans, dipterans, formicids, and juvenile fishes, sometimes from its own species (Taylor, 2012). In mangroves biota, the reported THg levels were 100 ng/g dw in snails and crabs (Cheng and Yap, 2015), 50 to 280 ng/g dw in polychaetes (Alam et al., 2010), 100 to 940 ng/g dw in bivalves (Saha et al., 2006) and 22 to 617 ng/g dw in fish from the Sundarbans mangrove systems in Bangladesh (Borrell et al., 2016). Into the wild, mangrove rivulus is thus not an entry of MeHg into the aquatic food web, but is rather at a higher trophic level where bioaccumulation occurs. It is worth noting that MeHg concentration in whole 7-dph larvae bodies exposed from 0 to 7 dph is the results of detoxification processes, as significant iHg concentrations were observed. Demethylation and detoxification processes are discussed further.

4.2. Immediate effects of MeHg on rivulus behaviors, physiology and ecology

We showed that MeHg exposures significantly decreased rivulus larvae locomotor activity in a dose dependent manner. This hypoactivity is consistent with studies carried out on other fish species such as zebrafish *Danio rerio* (Zhu et al., 2020), yellow perch *Perca flavescens* (Mora-Zamorano et al., 2017) and grass carp *Ctenopharyngodon idella* (Baldissera et al., 2020)

exposed to MeHg. In contrast to our results, MeHg can also create hyperactivity as observed in 6 dpf zebrafish exposed to 10 and 30 nM at ≤ 2 hpf for 24 hours (Mora-Zamorano et al., 2016). These differences in locomotor activity outcomes between but also within species may be attributable to MeHg concentrations, way of exposure (waterborne, diet, etc.), exposure duration and stages as some developmental stages are more critical than others.

Locomotor activity can influence foraging, dispersal and antipredator behaviors, which have important consequences on individual's fitness as it influences reproductive success, food access, survival and social interactions (Réale et al., 2007). In our study, we highlighted a significant decrease in the number of prey capture, but no significant effect on capture success (prey capture/capture attempt) in rivulus larvae. It means that MeHg-exposed larvae tried less to capture preys, but when they try, they succeed as well as the control ones. These results interpretation is complex as feeding behavior depends on different factors such as visual acuity, learning capacities, locomotor activity and growth. In our case, MeHg seemed to primarily reduce prey capture by larval activity alteration, which has a direct influence on prey encounter rates. As it has been reported that MeHg can create visual deficit (Carvan et al., 2017; Liu et al., 2016) and learning impairments (Xu et al., 2016), these causes of decreased foraging efficiency are not ruled out and should be deeper investigated.

Interestingly, MeHg reduced locomotor activity and prey capture in rivulus larvae, but has no significant immediate or delayed effects on growth. These results could be explained by energy allocation modification. MeHg induces detoxification responses that are energetically costly to exposed organisms (Handy et al., 1999). This energetic alteration could modify the energy allocation to other physiological processes, creating impairment of growth, reproduction, development and locomotor activity. Studies have associated locomotor alteration of zebrafish larvae (Andrade et al., 2016) and rainbow trout *Oncorhynchus mykiss* (Handy et al., 1999) with the energy needed to detoxification of carbendazim and cooper, respectively. Our results indicate that this possible energy allocation trade-off between MeHg detoxification processes and locomotor activity occurs without concomitant reductions in growth. As detailed in Handy et al. (1999), where they obtained similar results, locomotor activity of exposed fish could be reduced as a metabolic 'sparing effect' to maintain growth, activity being the major component of energy expenditure. This observed stable growth across MeHg conditions and time points is consistent with other ecotoxicological studies on mangrove rivulus where exposure to 20 µg/L

and 15 mg/L of β-N-Methylamino-L-alanine (BMAA) from 0 to 7 dph created no immediate effects on growth at 7 dph (Carion et al., 2018) and no delayed effects on growth at 50 and 170 dph (Carion et al., 2020). In contrast, mangrove rivulus exposed to 4 and 120 ng/L of 17-α-ethinylestradiol (EE2) from 0 to 28 dph were significantly smaller in standard length than the control group at 28 dph, but underwent compensatory growth and reached a similar size as controls at 90 dph (Voisin et al., 2016). These results provide insight into the diversity of energy allocation by mangrove rivulus under chemical stresses.

In addition to locomotor activity and prey capture reduction, MeHg exposure also creates thigmotaxis reduction in exposed larvae, as they spent significantly more time in the center of the arena. Thigmotaxis (or "wall hugging") is a validated index of anxiety that has been shown in several fish species (Dadda et al., 2010; Schnörr et al., 2012; Sharma et al., 2009) but has not yet been characterized in mangrove rivulus. Here we provide evidence that larval rivulus as young as 7 dph show thigmotaxic behavior as control larvae strongly avoid the center of the arena by spending most of their time close to the walls during the locomotor activity assessment (89.64 ± 8.29 %). These results are consistent with what was found in zebrafish larvae as they spent 79.10 ± 2.36% of the thigmotaxis test in the outer zone (Schnörr et al., 2012). These findings suggest that thigmotaxis develops early in rivulus life and appears to be already expressed in an adult-like manner in larvae as adults rivulus from our control group spent 93.22 ± 9.73 % of their time close to the walls during the locomotor activity assessment. We characterized one innate behavior of mangrove rivulus, that have a natural tendency to remain at the edge of its environment for shelter seeking, foraging and predator avoidance (Taylor, 2012). In our experiment, MeHg exposure decreased this anxiety-like behavior in rivulus larvae, altering the normal preference of the periphery in favor of the central area. This change induced by MeHg exposure may be a threat to the survival of these larvae in terms of predator avoidance and prey capture. Compiled at reduction of locomotor activity and prey capture, MeHg can significantly affect rivulus survival and fitness.

4.3. Mechanisms underlying immediate phenotypic effects of MeHg

Besides MeHg effects on rivulus behaviors and growth, we investigated possible underlying mechanisms by studying MeHg effects on expression of six genes and on their methylation level at targeted CpGs. Our hypothesis is that MeHg could modify these epigenetic marks,

creating environmentally-induced epimutations, which could explain gene expression modification induced by MeHg exposure.

In our study, we showed that MeHg exposure significantly decreased DNMT3a, NIPBL, MAOA and MECP2 expression, and increased GSS expression in larvae. However, there is no significant effect of MeHg on DNA methylation of targeted CpGs located in those genes. On the one hand, DNA methylation changes could occur at other locations in the genome, which is supported by the observed downregulation of DNMT3a expression in MeHg-exposed larvae, the enzyme responsible for de novo DNA methylation. One interesting perspective would be to use a Reduced Representation Bisulfite Sequencing (RRBS) or Whole Genome Bisulfite Sequencing (WGBS) for genome-wide analysis of DNA methylation modifications induced by MeHg exposure. On the other hand, changes in gene expression could be due to other epigenetic mechanisms altered by MeHg such as post-translational modification of histones, supported by the downregulation of NIPBL expression in MeHg-exposed larvae. NIPBL initiates deacetylation of lysine 9 of histone 3 (H3K9) and recruits histone deacetylases. This process of histone deacetylation allows histones to wrap the DNA more tightly and creates a compaction of nucleosome structure, preventing transcription (Gao et al., 2019). As MeHg exposure decreased NIPBL expression in rivulus larvae, it could facilitate the dissociation of DNA and relaxation of nucleosome structure, allowing various transcription factors to bind to DNA and activate gene transcription. The observed DNMT3a and NIPBL downregulations are in line with previous studies where MeHg exposure decreased histones H3 and H4 acetylation, decreased global DNA methylation and DNMTs activity (reviewed in Culbreth and Aschner, 2019). The functional consequences of these epigenetic modifications are not entirely understood. However, these examples show that epigenetic modifications are an attractive mechanistic target and illustrate the potential of MeHg to impact the epigenome.

As mentioned above, we highlighted an increased relative expression of Glutathione synthase (GSS) in MeHg-exposed larvae. MeHg is known to interact with either thiols or selenols groups including glutathione (GSH; a major thiol antioxidant). This complex formation, GS-HgCH3, is a common mechanism for MeHg metabolism and clearance from the body as it can be transported out of the cell and excreted in the bile (Dutczak and Ballatori, 1994). Increased MeHg exposure competes with redox buffering for available GSH, resulting in oxidative stress (Farina and Aschner, 2019). Moreover, clearance of MeHg is delayed due to this competition

for GSH, extending organism exposure duration. Pro-oxidative properties of MeHg could explain the observed behavioral impairments, as recent evidence suggests the involvement of brain oxidative damage in mercury-mediated motor dysfunction (Santana et al., 2019; Teixeira et al., 2019). It is worth noting that, according to the redox/methylation trade-off hypothesis, oxidative stress would result in global hypomethylation (Carvan, 2020; Farina and Aschner, 2019), as S-adenosylmethionine (methyl donor used by DNMTs to methylate DNA) and GSH share a common synthesis pathway from homocysteine.

MeHg exposure also modified expression of genes involved in neuronal functions and behaviors. MeHg-exposed larvae showed significant decrease of monoamine oxidase A (MAOA) relative expression. In fish, monoamine oxidase regulates levels of monoamine neurotransmitters such as dopamine, serotonin, and noradrenaline in the nervous tissue. These neurotransmitters are involved in neurofunctional behaviors such as fight-or-flight response, reward-motivated behaviors, attention, emotion, cognition and motor activity (Beyrouty et al., 2006; Newman et al., 2005). Enzymatic degradation catalyzed by MAO is thus required for proper neuronal development and behaviors (Beyrouty et al., 2006). It has been proposed that MeHg disrupts these pathways by reducing brain MAO activity, leading to an increased concentration of these neurotransmitters and ultimately creates neurotoxicity in various brain regions (Bridges et al., 2017; Sallinen et al., 2009). Reduction in MAO activity due to MeHg exposure has been associated with hyperactivity in fathead minnow *Pimephales promelas* (Bridges et al., 2017), but also with hypoactivity in Atlantic salmon *Salmo salar* (Berntssen et al., 2003). This implies that reduction in MAO activity could be a widespread mechanism of MeHg neurotoxicity, and that the behavioral consequences vary across studies. A study of loss of function in MAO supports the hypoactivity hypothesis, as *mao*−/− zebrafish showed decreased locomotor activity, but also abnormal serotonergic and dopaminergic systems (Baronio et al., 2022). An interesting perspective would be to evaluate dopamine, serotonin and noradrenaline concentrations in brain of MeHg-exposed rivulus to determine if this exposure would cause their accumulation in rivulus brain, which could be associated with the observed hypoactivity and reduced foraging efficiency.

We also showed that there is significant decrease of Methyl-CpG-binding protein 2 (MeCP2) relative expression in MeHg-exposed rivulus larvae. MeCP2 is a transcription factor that can act as gene expression repressor or activator (Guy et al., 2011). Once bound, MeCP2 modulates

gene expression by recruiting protein complexes involved in histone modification and chromatin remodeling (Skene et al., 2010). MeCP2 is found in high concentrations in neurons and is associated with maturation of the central nervous system and in forming synaptic contacts (Guy et al., 2011). Consequently, MeCP2 dysfunction impacts brain anatomy and neuronal structure and function, and can create several neurodevelopmental diseases associated with autism spectrum disorders (ASD), including Rett Syndrome (RTT). Patients with RTT suffer from pronounced alterations of their motor systems, learning disabilities and social behavioral deficits (Sansom et al., 2008; Weaving, 2005). This can be associated with autistic-like features, as dysfunction of the reward system and avoidance of tactile stimulation (Belmonte et al., 2004). MeCP2 knockout zebrafish had decreased locomotor activity and velocity, and has an impact on thigmotaxis as mutants tends to stay more in the arena center in comparison to wild type (Pietri et al., 2013). In our experiment, MeHg-exposed larvae showed decreased MeCP2 expression, thigmotaxis and locomotor activity, which is consistent with RTT phenotypes created in MeCP2 knockout zebrafish. Al-Mazroua et al. (2022) evaluated MeHg effects on autism-like behaviors in BTBR autistic mouse model. MeHg administration aggravated existing behavioral abnormalities in BTBR mice with increased stereotypic, repetitive, and impaired social behaviors. Interestingly, studies on MeCP2 knockout mice highlighted other dysfunctions, as defects in the different aminergic systems including the dopaminergic system (Panayotis et al., 2011). This is in line with the hypothesis that one of the MeHg neurotoxicity mechanisms involve aminergic system, by disturbing expression of genes regulating these pathways including MAOA and MeCP2. Disfunction of the aminergic system could create the observed behaviors alterations including autistic features (reduction of interest in environment exploration, avoidance of tactile stimulation from the wall, etc.).

4.4. Delayed effects and detoxification of MeHg

Immediate effects of MeHg on locomotor activity, prey capture, thigmotaxis and gene expression in 7 dph larvae are no longer observed later in life, after 83 days of recovery. Several studies evaluated reversibility of MeHg effects after a detoxification period in fish. MeHg exposure during fish embryogenesis can create delayed impairments in the feeding behavior, swimming activity, learning abilities, and a delayed mortality syndrome (Samson, 2001; Xiaojuan Xu, 2012), but can also create transitory impairments at similar concentrations (Weis and Weis, 1995). Post-hatching but still early developmental exposure can also have delayed consequences on swimming activity and related dopaminergic neurons functionality in

zebrafish (Huang et al., 2016). These studies highlight the complexity of MeHg effects that may be reversible/transitient in some cases, and irreversible in others. The delay between MeHg exposure and behavioral testing on 90 dph adults rivulus might have allowed time for protective and repair mechanisms to operate despite a relatively high exposure concentration. MeHg mechanisms of neurotoxicity could be countered later in life as the mercury is detoxified.

Detoxification of MeHg includes demethylation that takes place at different organs including gut and liver (Wang et al., 2017). In our study, detoxification by demethylation process is supported by our Hg species distribution, as 43 % and 54 % of THg (as the sum MeHg + iHg) were in the form of MeHg in 90 µg/L and 135 µg/L MeHg exposed larvae, respectively. These results are in line with the study of (Gonzalez et al., 2005) where MeHg represented 66% of THg in the liver of zebrafish, and decreased to 36% after 63 days of MeHg exposure. Once exposure ends, fish could enter in depuration stage and efficiently eliminate accumulated iHg. Wang et al. (2017) showed that during depuration of 30 days in the marine fish *Acanthopagrus schlegeli*, whole-body concentrations of iHg also decreased significantly to the same level to the beginning of their experiment. Our study showed that there could be a considerable amount of iHg generated from demethylation and accumulated by rivulus larvae during the exposure. During the recovery period, rivulus possibly eliminate accumulated iHg, which could progressively diminish MeHg toxic effects until significant effects observed in 7 dph rivulus disappear.

4.5. Mangrove rivulus as ecotoxicological model species

Mangrove rivulus is the only known self-fertilizing hermaphroditic vertebrate (Costa et al., 2010). Consistent self-fertilization is an extreme form of inbreeding and it consequently produces isogenic lineages. By naturally minimizing genetic noise in scientific studies, researchers can investigate how the environment influences the phenotype (Kelley et al., 2016). Moreover, this mating system provides an ideal model for the identification of true cause-effect relationships between the environment, the epigenome and the phenotype, including the role of epigenetic mechanisms in toxicity and ecotoxicity. A growing body of work on mangrove rivulus supports its value as a new model species for studying the potential lasting effects of environmental stressors through epigenetic modifications. Voisin et al. showed that early life exposure of rivulus to an endocrine disrupting compound, 17-α-ethinylestradiol, can induce delayed effects on the adult phenotype, proteome and epigenome (Voisin et al., 2021, 2016).

Carion et al. highlighted immediate effects of the neurotoxin β-N-Methylamino-L-alanine (BMAA) on rivulus larvae behaviors, and delayed effects of BMAA on expression of genes involved in glutamate turnover, intracellular dopamine levels and astrocyte protective mechanisms (Carion et al., 2020, 2018). These studies join ours in showing that the mangrove rivulus seems more resistant to pollutants, at least in terms of its behavioral phenotype and mortality compared to other fish species. In comparison, a delayed mortality syndrome was observed in zebrafish larvae after 72h of exposure to 15 µg/l MeHg (Samson, 2001) while no mortality was observed in our experiment. Mangrove rivulus may have more effective protective mechanisms that could come from its high plasticity, an essential prerequisite for adaptation to mangroves environmental conditions. At first sight, high phenotypic plasticity would be paradoxical considering rivulus reproductive system. Consistent selfing is an intense form of inbreeding and produces isogenic lineages with high homozygosity level, which reduces fitness by increasing the risk that deleterious and recessive alleles are expressed and exposed to selection or by reducing any benefits due to heterozygote advantage (Davenport, 1908). This phenomenon is known as inbreeding depression, and is often associated with an increased vulnerability to environmental stress (Fox and Reed, 2011). Such inbreeding-by-environment interaction might imply that inbred organisms have a lower capacity for adjusting their phenotype to environmental variation, corresponding to a reduced phenotypic plasticity (Reed et al., 2012). Several empirical studies showed alterations of developmental plasticity (Auld and Relyea, 2010) and phenotypic plasticity of morphological or life history traits (Schiegg et al., 2002; Swillen et al., 2015) due to inbreeding. This interplay between inbreeding depression, phenotypic plasticity and environmental variation should be further investigated to understand how declining natural populations respond to environmental stress. This paradox of robustness and inbreeding makes the rivulus an interesting ecotoxicological model to consider, as it can highlights protective mechanisms and phenotypic plasticity that are highly solicitated, while showing MeHg phenotypic effects encountered in other species but with a low genetic variability among individuals.

Another interesting characteristics of mangrove rivulus is its inter-individual variability in a large range of phenotypic traits. Although rivulus used in this study are from the same isogenic lineage, we still observe high individual differences within each condition. For instance, the total distance moved by rivulus larvae during the activity/thigmotaxis test has a coefficient of variation (CV) of 40 %, 60% and 80% in control, 90 µg/L and 135 µg/L MeHg group

respectively, with a significant difference between variances in control group vs 135µg/L group (F test, F = 2.29, DFn = 51, Dfd = 30, $p < 0.05$). In comparison, total distanced moved by AB line zebrafish exposed to 0 or 10 µg/L MeHg showed CV of 20% and 17%, respectively (Zhu et al., 2020). Thus, there is greater behavioral variability between rivulus generated by selfing from one unique ancestor than between zebrafish from AB line, which is known to have low level of genetic variability (Coe et al., 2009). Two main observations can be drawn from these rivulus activity data: there is a naturally high inter-individual variation in control group and this variation increases with MeHg concentrations. The increase of variance could be the first step in the adaptation of a population in a changing environment (Orlando and Guillette, 2001). As reviewed in Nikinmaa and Anttila (2019), variability should always be included as an endpoint in data analysis as it would bring new information about the responses of organisms to environmental contamination. Therefore, mangrove rivulus is naturally suited to investigate sources of phenotypic variation that are not genetic, as environmental influences, and environmentally-induced or random epigenetic modifications.

5. Conclusion

We used a new vertebrate model species, the mangrove rivulus fish, to investigate immediate and delayed effects of MeHg at different endpoints (behaviors, gene expression and DNA methylation). We reported for the first time that MeHg exposure during early life stages can significantly impair behaviors and gene expression in rivulus larvae, but we didn't find these effects after a detoxification period of several months. Although neurotoxic effects of MeHg on brain have been extensively studied, their underlying mechanisms are not fully understood. Based on our results, we suggested implications of aminergic system and its neurotransmitters, redox/methylation trade-off and other epigenetic mechanisms. It should be kept in mind that no single process can explain the multitude of effects observed in MeHg-exposed organisms. The use of natural isogenic lineages of mangrove rivulus in ecotoxicological studies could help overcome this challenge, as it permits explicit examination of environmental and epigenetic effects on the phenotype by reducing the genetic variation in the experiment.

CRediT authorship contribution statement

Valentine Chapelle: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Jérôme Lambert**: Methodology, Formal analysis. **Thomas Deom**: Investigation, Formal analysis. **Emmanuel Tessier**: Methodology, Formal analysis, Resources. **David Amouroux**: Resources. **Frédéric Silvestre**: Supervision, Methodology, Formal analysis, Resources, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that no competing interests exist.

Acknowledgments

This study was supported by the FRS-FNRS (Fonds de la Recherche Scientifique) FRIA PhD grant attributed to V. Chapelle. The authors want to thank the technical support by Océane Asensio (Institute of Analytical Sciences and Physico-Chemistry for Environment and Materials, Pau, France) for her contribution to inorganic mercury and methylmercury speciation We also thank Ryan L. Earley and Kristine M. Marson (Department of Biological Sciences, University of Alabama, USA) for their field assistance while sampling mangrove rivulus from Emerson Point Preserve to constitute our fish stock for this experiment.

APPENDIX

Table A.1: Newly designed primers for gene expression analysis via RTqPCR.

Gene	Gene ID	Forward primer sequence	Reverse primer sequence	Efficacity (%)
SEP15	108244479	TCAAGGGTCTTCAGATCAAGTACG	TCGGTGTTCCACTTGAGGATG	92,46
UBE2a	108231206	ACACCTTTTGAAGATGGAACCTT	GGTCGGACTCCAACGATTCTG	106,33
DNMT3a	108233456	TACACGAGATCAAAAAGAAGACCAGGA	CATCGCTCCTACGAAGAGAGGAT	96,31
NIPBL	108231013	CAAACTCTACTGTCCATGAACCCCA	CTGGGCTCCCATGAACTTGATTCT	91,76
TOLLIP	108251745	ACTGTCGGATCAGGCTGGGT	CGTCCATCGAAAACGCTCTCTCA	99,64
GSS	108233054	AGACTTCCTGCAAGAGGCTTTAGC	GTAGTCGGACCGATTGAGACCCA	108,63
MAOA	108241385	ACTGATACTCGAGGGAAAAGATCG	GCTCCATGATATGCGTTTGTGTG	96,36
MECP2	108229207	TAAGATGCCCTTTGGCAAGACA	GGACTTGGCAGGTGGAGTAG	99,40

Table A.2: Newly designed primers for gene-specific methylation analysis via pyrosequencing.

Gene	Gene ID	Forward primer sequence	Reverse primer sequence	Synthesis primer sequence	Target regions
DNMT3a	108233456	TGTGGATTATTTGGTTTATGAGATAATAG	/5Biosg/ACTACTTCTACACCAAATTTATACTCATTA	ATTTGGTTTATGAGATAATAGAT	Promotor
		AGGAAATTTGTTAAGTTTGATTTGTGTG	/5Biosg/AACTAACAACTACCCTAACACAAT	TTTTTATTTTTTTGTTAATAAATAT	Intron 1
		TGATATTTGGAGTGTATATTAGGTAGT	/5Biosg/ACCAAAACTTCAATCTACAACACAT	GGTAGTTAGGTATAAAGTTTTTA	Intron 2
		GGTGGAATTTTGTTGTTATAATGAGA	/5Biosg/TCTCCCAACCACATCACCCA	ATTTTTAGTTTTGTGTTTAATTAGG	Intron 4 - Exon 5
NIPBL	108231013	/5Biosg/GGGATTAGTTGTAAATTGATGATATAAA	CCCTAAAATTTCTTCTTCTTTTCTTTTAAC	TTTATTTACCTACACAAACCTA	Promotor
GSS	108233054	GTTATAGTTATTTTTGAGATGGTAAAGG	/5Biosg/ACAAAATCTATCACTTCCCACAAA	TTTTGAGATGGTAAAGGT	Promotor
MAOA	108241385	GTGTTGTTTTTAGTTTTATAGAGGGTAATA	/5Biosg/AACCTTTTCCCTCCAATATCAATATTTTCA	GGGTTTAAGTTAATTTTTAAT	Exon 1
MECP2	108229207	AAAAGGTAGTTGGTTTAAGAAGTTTATATA	/5Biosg/AATTTTATATTAAAAAAACTCCAAACATCT	GTTGGTTTAAGAAGTTTATATAT	Promotor
		GGGGAGGTGTGTAGTATAGATGTTTG	CAACCAATTTTACTCAACAACAC	AAATTATTTATTTAAATAAAAATGG	Intron 1
		GGGGAGGTGTGTAGTATAGATGTTTG	CAACCAATTTTACTCAACAACAC	GGGAATAAGTTATTAATTTAGGG	Exon 3

References

Alam, M.A., Gomes, A., Sarkar, S.K., Shuvaeva, O.V., Vishnevetskaya, N.S., Gustaytis, M.A., Bhattacharya, B.D., Godhantaraman, N., 2010. Trace Metal Bioaccumulation by Soft-bottom Polychaetes (Annelida) of Sundarban Mangrove Wetland, India and Their Potential Use as Contamination Indicator. Bull. Environ. Contam. Toxicol. 85, 492–496. https://doi.org/10.1007/s00128-010-0110-1

Al-Mazroua, H.A., Nadeem, A., Ansari, M.A., Attia, S.M., Albekairi, T.H., Bakheet, S.A., Alobaidi, A.F., Alhosaini, K., Alqarni, S.A., Ibrahim, K.E., Alsaad, A.M.S., Ahmad, S.F., 2022. Methylmercury chloride exposure exacerbates existing neurobehavioral and immune dysfunctions in the BTBR T+ Itpr3tf/J mouse model of autism. Immunol. Lett. 244, 19–27. https://doi.org/10.1016/j.imlet.2022.03.001

Andrade, T.S., Henriques, J.F., Almeida, A.R., Machado, A.L., Koba, O., Giang, P.T., Soares, A.M.V.M., Domingues, I., 2016. Carbendazim exposure induces developmental, biochemical and behavioral disturbance in zebrafish embryos. Aquat. Toxicol. 170, 390–399. https://doi.org/10.1016/j.aquatox.2015.11.017

Auld, J.R., Relyea, R.A., 2010. Inbreeding depression in adaptive plasticity under predation risk in a freshwater snail. Biol. Lett. 6, 222–224. https://doi.org/10.1098/rsbl.2009.0726

Baeyens, W., Leermakers, M., Papina, T., Saprykin, A., Brion, N., Noyen, J., De Gieter, M., Elskens, M., Goeyens, L., 2003. Bioconcentration and Biomagnification of Mercury and Methylmercury in North Sea and Scheldt Estuary Fish. Arch. Environ. Contam. Toxicol. 45, 498–508. https://doi.org/10.1007/s00244-003-2136-4

Baldissera, M.D., Souza, C.F., da Silva, A.S., Henn, A.S., Flores, E.M.M., Baldisserotto, B., 2020. Diphenyl diselenide dietary supplementation alleviates behavior impairment and brain damage in grass carp (Ctenopharyngodon idella) exposed to methylmercury chloride. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 229, 108674. https://doi.org/10.1016/j.cbpc.2019.108674

Baronio, D., Chen, Y.-C., Panula, P., 2022. Abnormal brain development of monoamine oxidase mutant zebrafish and impaired social interaction of heterozygous fish. Dis. Model. Mech. 15, dmm049133. https://doi.org/10.1242/dmm.049133

Bell, A.C., Felsenfeld, G., 2000. Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. Nature 405, 482–485. https://doi.org/10.1038/35013100

Belmonte, M.K., Cook, E.H., Anderson, G.M., Rubenstein, J.L.R., Greenough, W.T., Beckel-Mitchener, A., Courchesne, E., Boulanger, L.M., Powell, S.B., Levitt, P.R., Perry, E.K., Jiang, Y.H., DeLorey, T.M., Tierney, E., 2004. Autism as a disorder of neural information processing: directions for research and targets for therapy. Mol. Psychiatry 9, 646–663. https://doi.org/10.1038/sj.mp.4001499

Bergamaschi, B.A., Krabbenhoft, D.P., Aiken, G.R., Patino, E., Rumbold, D.G., Orem, W.H., 2012. Tidally Driven Export of Dissolved Organic Carbon, Total Mercury, and Methylmercury from a Mangrove-Dominated Estuary. Environ. Sci. Technol. 46, 1371–1378. https://doi.org/10.1021/es2029137

Berntssen, M.H.G., Aatland, A., Handy, R.D., 2003. Chronic dietary mercury exposure causes oxidative stress, brain lesions, and altered behavior in Atlantic salmon (Salmo salar) parr. Aquat. Toxicol. 65, 55–72. https://doi.org/10.1016/S0166-445X(03)00104-8

Berzas Nevado, J.J., García Bermejo, L.F., Rodríguez Martín-Doimeadios, R.C., 2003. Distribution of mercury in the aquatic environment at Almadén, Spain. Environ. Pollut. 122, 261–271. https://doi.org/10.1016/S0269-7491(02)00290-7

Beyrouty, P., Stamler, C.J., Liu, J.-N., Loua, K.M., Kubow, S., Chan, H.M., 2006. Effects of prenatal methylmercury exposure on brain monoamine oxidase activity and neurobehavior of rats. Neurotoxicol. Teratol. 28, 251–259. https://doi.org/10.1016/j.ntt.2005.12.007

Borrell, A., Tornero, V., Bhattacharjee, D., Aguilar, A., 2016. Trace element accumulation and trophic relationships in aquatic organisms of the Sundarbans mangrove ecosystem (Bangladesh). Sci. Total Environ. 545–546, 414–423. https://doi.org/10.1016/j.scitotenv.2015.12.046

Bridges, K., Venables, B., Roberts, A., 2017. Effects of dietary methylmercury on the dopaminergic system of adult fathead minnows and their offspring: Effects of MeHg on dopaminergic system of fatheads. Environ. Toxicol. Chem. 36, 1077–1084. https://doi.org/10.1002/etc.3630

Carion, A., Hétru, J., Markey, A., Suarez-Ulloa, V., Frédéric, S., 2018. Behavioral effects of the neurotoxin ß-N-methylamino-L-alanine on the mangrove rivulus (Kryptolebias marmoratus) larvae. J. Xenobiotics. https://doi.org/10.4081/xeno.2018.7820

Carion, A., Markey, A., Hétru, J., Carpentier, C., Suarez-Ulloa, V., Denoël, M., Earley, R.L., Silvestre, F., 2020. Behavior and gene expression in the brain of adult self-fertilizing mangrove rivulus fish (Kryptolebias marmoratus) after early life exposure to the neurotoxin β-N-methylamino-l-alanine (BMAA). NeuroToxicology 79, 110–121. https://doi.org/10.1016/j.neuro.2020.04.007

Carvan, M.J., 2020. Methylmercury induces transgenerationally transmissible epigenetic changes influencing zebrafish behavior, in: Behavioral and Neural Genetics of Zebrafish. Elsevier, pp. 493–510. https://doi.org/10.1016/B978-0-12-817528-6.00028-0

Carvan, M.J., Kalluvila, T.A., Klingler, R.H., Larson, J.K., Pickens, M., Mora-Zamorano, F.X., Connaughton, V.P., Sadler-Riggleman, I., Beck, D., Skinner, M.K., 2017. Mercury-induced epigenetic transgenerational inheritance of abnormal neurobehavior is correlated with sperm epimutations in zebrafish. PloS One 12, e0176155. https://doi.org/10.1371/journal.pone.0176155

Cavalheiro, J., Sola, C., Baldanza, J., Tessier, E., Lestremau, F., Botta, F., Preud'homme, H., Monperrus, M., Amouroux, D., 2016. Assessment of background concentrations of organometallic compounds (methylmercury, ethyllead and butyl- and phenyltin) in French aquatic environments. Water Res. 94, 32–41. https://doi.org/10.1016/j.watres.2016.02.010

Cheng, W.H., Yap, C.K., 2015. Potential human health risks from toxic metals via mangrove snail consumption and their ecological risk assessments in the habitat sediment from Peninsular Malaysia. Chemosphere 135, 156–165. https://doi.org/10.1016/j.chemosphere.2015.04.013

Coe, T.S., Hamilton, P.B., Griffiths, A.M., Hodgson, D.J., Wahab, M.A., Tyler, C.R., 2009. Genetic variation in strains of zebrafish (Danio rerio) and the implications for ecotoxicology studies. Ecotoxicology 18, 144–150. https://doi.org/10.1007/s10646-008-0267-0

Costa, W.J.E.M., Lima, S.M.Q., Bartolette, R., 2010. Androdioecy in Kryptolebias killifish and the evolution of self-fertilizing hermaphroditism: ANDRODIOECY IN KRYPTOLEBIAS KILLIFISH. Biol. J. Linn. Soc. 99, 344–349. https://doi.org/10.1111/j.1095-8312.2009.01359.x

Culbreth, M., Aschner, M., 2019. Methylmercury Epigenetics. Toxics 7, 56. https://doi.org/10.3390/toxics7040056

Dadda, M., Koolhaas, W.H., Domenici, P., 2010. Behavioral asymmetry affects escape performance in a teleost fish. Biol. Lett. 6, 414–417. https://doi.org/10.1098/rsbl.2009.0904

Davenport, C.B., 1908. Degeneration, albinism and inbreeding. Science 28, 454–454. https://doi.org/10.1126/science.28.718.454-b

Dutczak, W.J., Ballatori, N., 1994. Transport of the glutathione-methylmercury complex across liver canalicular membranes on reduced glutathione carriers. J. Biol. Chem. 269, 9746–9751. https://doi.org/10.1016/S0021-9258(17)36946-6

Farina, M., Aschner, M., 2019. Glutathione antioxidant system and methylmercury-induced neurotoxicity: An intriguing interplay. Biochim. Biophys. Acta BBA - Gen. Subj., The Biochemistry of Mercury toxicity 1863, 129285. https://doi.org/10.1016/j.bbagen.2019.01.007

Faro, L.R.F., do Nascimento, J.L.M., Alfonso, M., Durán, R., 2002. Mechanism of action of methylmercury on in vivo striatal dopamine release. Neurochem. Int. 40, 455–465. https://doi.org/10.1016/S0197-0186(01)00098-5

Fowler, K., Whitlock, M.C., 1999. The distribution of phenotypic variance with inbreeding. Evolution 53, 1143–1156. https://doi.org/10.1111/j.1558-5646.1999.tb04528.x

Fox, C.W., Reed, D.H., 2011. Inbreeding depression increases with environmental stress: an experimental study and meta-analysis. Evolution 65, 246–258. https://doi.org/10.1111/j.1558-5646.2010.01108.x

Gao, D., Zhu, B., Cao, X., Zhang, M., Wang, X., 2019. Roles of NIPBL in maintenance of genome stability. Semin. Cell Dev. Biol. 90, 181–186. https://doi.org/10.1016/j.semcdb.2018.08.005

Gonzalez, P., Dominique, Y., Massabuau, J.C., Boudou, A., Bourdineaud, J.P., 2005. Comparative Effects of Dietary Methylmercury on Gene Expression in Liver, Skeletal Muscle, and Brain of the Zebrafish (Danio rerio). Environ. Sci. Technol. 39, 3972–3980. https://doi.org/10.1021/es0483490

Guy, J., Cheval, H., Selfridge, J., Bird, A., 2011. The Role of MeCP2 in the Brain. Annu. Rev. Cell Dev. Biol. 27, 631–652. https://doi.org/10.1146/annurev-cellbio-092910-154121

Handy, R.D., Sims, D.W., Giles, A., Campbell, H.A., Musonda, M.M., 1999. Metabolic trade-off between locomotion and detoxification for maintenance of blood chemistry and growth parameters by rainbow trout (Oncorhynchus mykiss) during chronic dietary exposure to copper. Aquat. Toxicol. 47, 23–41. https://doi.org/10.1016/S0166-445X(99)00004-1

Huang, S.S.Y., Noble, S., Godoy, R., Ekker, M., Chan, H.M., 2016. Delayed effects of methylmercury on the mitochondria of dopaminergic neurons and developmental toxicity in zebrafish larvae (Danio rerio). Aquat. Toxicol. 175, 73–80. https://doi.org/10.1016/j.aquatox.2016.03.004

Kelley, J.L., Yee, M.-C., Brown, A.P., Richardson, R.R., Tatarenkov, A., Lee, C.C., Harkins, T.T., Bustamante, C.D., Earley, R.L., 2016. The Genome of the Self-Fertilizing Mangrove Rivulus Fish, Kryptolebias marmoratus : A Model for Studying Phenotypic Plasticity and Adaptations to Extreme Environments. Genome Biol. Evol. 8, 2145–2154. https://doi.org/10.1093/gbe/evw145

Kidd, K., Batchelar, K., 2011. Mercury, in: Fish Physiology. Elsevier, pp. 237–295. https://doi.org/10.1016/S1546-5098(11)31027-8

Lei, P., Zhong, H., Duan, D., Pan, K., 2019. A review on mercury biogeochemistry in mangrove sediments: Hotspots of methylmercury production? Sci. Total Environ. 680, 140–150. https://doi.org/10.1016/j.scitotenv.2019.04.451

Liu, Q., Klingler, R.H., Wimpee, B., Dellinger, M., King-Heiden, T., Grzybowski, J., Gerstenberger, S.L., Weber, D.N., Carvan, M.J., 2016. Maternal methylmercury from a wild-caught walleye diet induces developmental abnormalities in zebrafish. Reprod. Toxicol. 65, 272–282. https://doi.org/10.1016/j.reprotox.2016.08.010

Monperrus, M., Tessier, E., Veschambre, S., Amouroux, D., Donard, O., 2005. Simultaneous speciation of mercury and butyltin compounds in natural waters and snow by propylation and species-specific isotope dilution mass spectrometry analysis. Anal. Bioanal. Chem. 381, 854–862. https://doi.org/10.1007/s00216-004-2973-7

Mora-Zamorano, F.X., Klingler, R., Basu, N., Head, J., Murphy, C.A., Binkowski, F.P., Larson, J.K., Carvan, M.J., 2017. Developmental Methylmercury Exposure Affects Swimming Behavior and Foraging Efficiency of Yellow Perch (Perca flavescens) Larvae. ACS Omega 2, 4870–4877. https://doi.org/10.1021/acsomega.7b00227

Mora-Zamorano, F.X., Svoboda, K.R., Carvan, M.J., 2016. The Nicotine-Evoked Locomotor Response: A Behavioral Paradigm for Toxicity Screening in Zebrafish (Danio rerio) Embryos and Eleutheroembryos Exposed to Methylmercury. PLOS ONE 11, e0154570. https://doi.org/10.1371/journal.pone.0154570

Newman, T.K., Syagailo, Y.V., Barr, C.S., Wendland, J.R., Champoux, M., Graessle, M., Suomi, S.J., Higley, J.D., Lesch, K.-P., 2005. Monoamine oxidase A gene promoter variation and rearing experience influences aggressive behavior in rhesus monkeys. Biol. Psychiatry 57, 167–172. https://doi.org/10.1016/j.biopsych.2004.10.012

Nikinmaa, M., Anttila, K., 2019. Individual variation in aquatic toxicology: Not only unwanted noise. Aquat. Toxicol. 207, 29–33. https://doi.org/10.1016/j.aquatox.2018.11.021

Oguro, A., Kubota, H., Shimizu, M., Ishiura, S., Atomi, Y., 2011. Protective role of the ubiquitin binding protein Tollip against the toxicity of polyglutamine-expansion proteins. Neurosci. Lett. 503, 234–239. https://doi.org/10.1016/j.neulet.2011.08.043

Orlando, F.E., Guillette, J.L., 2001. A re-examination of variation associated with environmentally stressed organisms. APMIS 109, S178–S186. https://doi.org/10.1111/j.1600-0463.2001.tb05765.x

Panayotis, N., Pratte, M., Borges-Correia, A., Ghata, A., Villard, L., Roux, J.-C., 2011. Morphological and functional alterations in the substantia nigra pars compacta of the Mecp2-null mouse. Neurobiol. Dis. 41, 385–397. https://doi.org/10.1016/j.nbd.2010.10.006

Pietri, T., Roman, A.-C., Guyon, N., Romano, S.A., Washbourne, P., Moens, C.B., de Polavieja, G.G., Sumbre, G., 2013. The first mecp2-null zebrafish model shows altered motor behaviors. Front. Neural Circuits 7. https://doi.org/10.3389/fncir.2013.00118

Réale, D., Reader, S.M., Sol, D., McDougall, P.T., Dingemanse, N.J., 2007. Integrating animal temperament within ecology and evolution. Biol. Rev. 82, 291–318. https://doi.org/10.1111/j.1469-185X.2007.00010.x

Reed, D.H., Fox, C.W., Enders, L.S., Kristensen, T.N., 2012. Inbreeding-stress interactions: evolutionary and conservation consequences: Inbreeding-stress interactions. Ann. N. Y. Acad. Sci. 1256, 33–48. https://doi.org/10.1111/j.1749-6632.2012.06548.x

Rumbold, D.G., Evans, D.W., Niemczyk, S., Fink, L.E., Laine, K.A., Howard, N., Krabbenhoft, D.P., Zucker, M., 2011. Source Identification of Florida Bay's Methylmercury Problem: Mainland Runoff Versus Atmospheric Deposition and In situ Production. Estuaries Coasts 34, 494–513. https://doi.org/10.1007/s12237-010-9290-5

Saha, M., Sarkar, S.K., Bhattacharya, B., 2006. Interspecific variation in heavy metal body concentrations in biota of Sunderban mangrove wetland, northeast India. Environ. Int. 32, 203–207. https://doi.org/10.1016/j.envint.2005.08.012

Sallinen, V., Sundvik, M., Reenilä, I., Peitsaro, N., Khrustalyov, D., Anichtchik, O., Toleikyte, G., Kaslin, J., Panula, P., 2009. Hyperserotonergic phenotype after monoamine oxidase inhibition in larval zebrafish. J. Neurochem. 109, 403–415. https://doi.org/10.1111/j.1471-4159.2009.05986.x

Samson, J., 2001. Delayed effects of embryonic exposure of zebrafish (Danio rerio) to methylmercury (MeHg). Aquat. Toxicol. 51, 369–376. https://doi.org/10.1016/S0166-445X(00)00128-4

Sansom, D., Krishnan, V.H.R., Corbett, J., Kerr, A., 2008. Emotional and behavioral aspects of Rett syndrome. Dev. Med. Child Neurol. 35, 340–345. https://doi.org/10.1111/j.1469-8749.1993.tb11646.x

Santana, L.N. da S., Bittencourt, L.O., Nascimento, P.C., Fernandes, R.M., Teixeira, F.B., Fernandes, L.M.P., Freitas Silva, M.C., Nogueira, L.S., Amado, L.L., Crespo-Lopez, M.E., Maia, C. do S.F., Lima, R.R., 2019. Low doses of methylmercury exposure during adulthood in rats display oxidative stress, neurodegeneration in the motor cortex and lead to impairment of motor skills. J. Trace Elem. Med. Biol. 51, 19–27. https://doi.org/10.1016/j.jtemb.2018.09.004

Schiegg, K., Pasinelli, G., Walters, J.R., Daniels, S.J., 2002. Inbreeding and experience affect response to climate change by endangered woodpeckers. Proc. R. Soc. Lond. B Biol. Sci. 269, 1153–1159. https://doi.org/10.1098/rspb.2002.1966

Schnörr, S.J., Steenbergen, P.J., Richardson, M.K., Champagne, D.L., 2012. Measuring thigmotaxis in larval zebrafish. Behav. Brain Res. 228, 367–374. https://doi.org/10.1016/j.bbr.2011.12.016

Sharma, S., Coombs, S., Patton, P., de Perera, T.B., 2009. The function of wall-following behaviors in the Mexican blind cavefish and a sighted relative, the Mexican tetra (Astyanax). J. Comp. Physiol. A 195, 225–240. https://doi.org/10.1007/s00359-008-0400-9

Skene, P.J., Illingworth, R.S., Webb, S., Kerr, A.R.W., James, K.D., Turner, D.J., Andrews, R., Bird, A.P., 2010. Neuronal MeCP2 Is Expressed at Near Histone-Octamer Levels and Globally Alters the Chromatin State. Mol. Cell 37, 457–468. https://doi.org/10.1016/j.molcel.2010.01.030

Skinner, M.K., 2011. Environmental epigenetic transgenerational inheritance and somatic epigenetic mitotic stability. Epigenetics 6, 838–842. https://doi.org/10.4161/epi.6.7.16537

Smith, L.E., Carvan, M.J., Dellinger, J.A., Ghorai, J.K., White, D.B., Williams, F.E., Weber, D.N., 2010. Developmental selenomethionine and methylmercury exposures affect zebrafish learning. Neurotoxicol. Teratol. 32, 246–255. https://doi.org/10.1016/j.ntt.2009.09.004

Swillen, I., Vanoverbeke, J., De Meester, L., 2015. Inbreeding and adaptive plasticity: an experimental analysis on predator-induced responses in the water flea Daphnia. Ecol. Evol. 5, 2712–2721. https://doi.org/10.1002/ece3.1545

Tamm, C., Duckworth, J.K., Hermanson, O., Ceccatelli, S., 2008. Methylmercury inhibits differentiation of rat neural stem cells via Notch signalling. NeuroReport 19, 339–343. https://doi.org/10.1097/WNR.0b013e3282f50ca4

Taylor, D.S., 2012. Twenty-Four Years in the Mud: What Have We Learned About the Natural History and Ecology of the Mangrove Rivulus, Kryptolebias marmoratus? Integr. Comp. Biol. 52, 724–736. https://doi.org/10.1093/icb/ics062

Teixeira, F.B., Leão, L.K.R., Bittencourt, L.O., Aragão, W.A.B., Nascimento, P.C., Luz, D.A., Braga, D.V., Silva, M.C.F. da, Oliveira, K.R.M., Herculano, A.M., Maia, C.S.F., Lima, R.R., 2019. Neurochemical dysfunction in motor cortex and hippocampus impairs the behavioral performance of rats chronically exposed to inorganic mercury. J. Trace Elem. Med. Biol. 52, 143–150. https://doi.org/10.1016/j.jtemb.2018.12.008

Voisin, A.-S., Fellous, A., Earley, R.L., Silvestre, F., 2016. Delayed impacts of developmental exposure to 17-α-ethinylestradiol in the self-fertilizing fish Kryptolebias marmoratus. Aquat. Toxicol. 180, 247–257. https://doi.org/10.1016/j.aquatox.2016.10.003

Voisin, A.-S., Suarez Ulloa, V., Stockwell, P., Chatterjee, A., Silvestre, F., 2021. Genome-wide DNA methylation of the liver reveals delayed effects of early-life exposure to 17-α-ethinylestradiol in the self-fertilizing mangrove rivulus. Epigenetics 1–25. https://doi.org/10.1080/15592294.2021.1921337

Wang, X., Wu, F., Wang, W.-X., 2017. In Vivo Mercury Demethylation in a Marine Fish (Acanthopagrus schlegeli). Environ. Sci. Technol. 51, 6441–6451. https://doi.org/10.1021/acs.est.7b00923

Weaving, L.S., 2005. Rett syndrome: clinical review and genetic update. J. Med. Genet. 42, 1–7. https://doi.org/10.1136/jmg.2004.027730

Weber, D.N., Connaughton, V.P., Dellinger, J.A., Klemer, D., Udvadia, A., Carvan, M.J., 2008. Selenomethionine reduces visual deficits due to developmental methylmercury exposures. Physiol. Behav. 93, 250–260. https://doi.org/10.1016/j.physbeh.2007.08.023

Weis, J.S., Weis, P., 1995. Effects of embryonic exposure to methylmercury on larval prey-capture ability in the mummichog, fundulus heteroclitus. Environ. Toxicol. Chem. 14, 153–156. https://doi.org/10.1002/etc.5620140117

Wu, P., Kainz, M.J., Bravo, A.G., Åkerblom, S., Sonesten, L., Bishop, K., 2019. The importance of bioconcentration into the pelagic food web base for methylmercury biomagnification: A meta-analysis. Sci. Total Environ. 646, 357–367. https://doi.org/10.1016/j.scitotenv.2018.07.328

Xiaojuan Xu, 2012. Developmental methylmercury exposure affects avoidance learning outcomes in adult zebrafish. J. Toxicol. Environ. Health Sci. 4. https://doi.org/10.5897/JTEHS12.004

Xu, X., Weber, D., Martin, A., Lone, D., 2016. Trans-generational transmission of neurobehavioral impairments produced by developmental methylmercury exposure in zebrafish (Danio rerio). Neurotoxicol. Teratol. 53, 19–23. https://doi.org/10.1016/j.ntt.2015.11.003

Zhu, J., Tang, L., Qiao, S., Wang, Lijuan, Feng, Y., Wang, Li, Wu, Q., Ding, P., Zhang, Z., Li, L., 2020. Low-dose methylmercury exposure impairs the locomotor activity of zebrafish: Role of intestinal inositol metabolism. Environ. Res. 190, 110020. https://doi.org/10.1016/j.envres.2020.110020

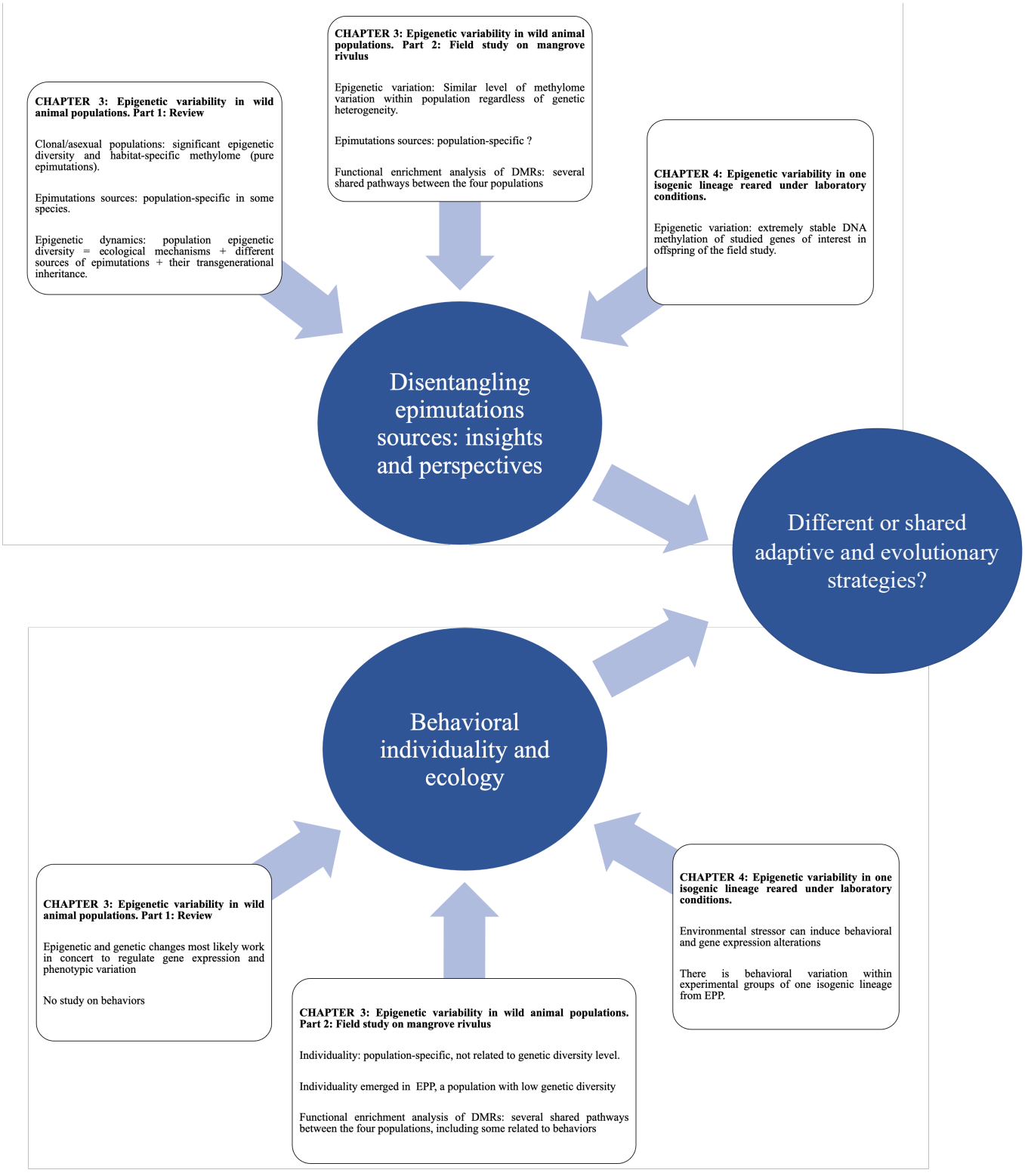
Comments and transition

Besides its ecotoxicological results, this study highlights some relevant characteristics of offspring of wild rivulus from Emerson Point Preserve. Firstly, the level of DNA methylation of studied genes of interest is extremely stable through time (7 dph larvae *versus* 90 dph adults rivulus) and experimental groups (0, 90 or 135 µg/L MeHg). Secondly, there is behavioral variation within experimental groups of offspring from Emerson Point Preserve isogenic lineage, with higher variation in exposed groups than in control group.

It suggests that there is less epigenetic variation under standardized experimental conditions than observed into the wild for the same genotype, supporting the effects of natural conditions (environmental heterogeneity, selection pressure, (epi)genetic drif,…) on epimutations. However, the major weakness of this study is the use of a gene-specific approach. The idea was to make an *a priori* approach with genes of interest coming out of the RRBS results from the field study. Unfortunately, due to delays in the RRBS analysis caused by the pandemic and logistical constraints, we had to start this experiment before the acquisition of RRBS results. This makes the comparison methylation results from the field and from this study to be taken with caution.

CHAPTER 5: GENERAL DISCUSSION AND PERSPECTIVES

The comparison of our three articles results can generate new insights to achieve our goal i.e. to determine the role of DNA methylation in adaptation and evolution of mangrove rivulus *Kryptolebias marmoratus*. Our discussion is based on three main points represented below.



1. Disentangling epimutations sources: insights and perspectives

1.1 Genetic and epigenetic diversity in wild populations

For the first time, epigenetic diversity can be assessed in a species naturally found under genetically-diverse and isogenic population, allowing us to cover a vast spectrum of genetic diversity configurations of a single species. Even though genetic diversity has not been evaluated with whole genome sequencing method, our microsatellites analysis allowed us to confirm the genetic diversity gradient across the four studied wild populations. Regarding our almost-clonal population, Emerson Point Preserve (EPP), we observed substantial epigenetic diversity, which raises the question of the environmental implications in epigenetic variability. This result is not unprecedented, as interindividual DNA methylation variability have been found in the clonal fish *Chrosomus eos-neogaeus* (Massicotte et al., 2011; Massicotte & Angers, 2012) and asexual New Zealand freshwater snail *Potamopyrgus antipodarum* (Thorson et al., 2017, 2019). In these studies, individuals could be regrouped according to their lake/river of origin based on their unique methylation profile, as individuals of a given site were epigenetically similar. Rivulus from EPP do cluster together when analyzed with other rivulus populations, joining this idea of site-specific methylome induced by environmental conditions. Regarding the three other rivulus populations, which are more genetically diverse, the distinction of genome-dependent and genome-independent epimutations is more complicated as different genotypes could respond to the same environmental context with contrasting DNA methylation levels. These facilitated epimutations have been shown in two mangrove rivulus lineages reared in different environments (Berbel-Filho et al., 2019). However, by combining data of genetic diversity, genetic distance, hierarchical clustering of rivulus methylome of the four wild populations and the laboratory experiment on EPP rivulus, we can highlight some interesting patterns that challenge the place of genetic sequence in epimutations sources.

One interesting result is that the level of epigenetic variation within all four populations is similar, regardless of genetic heterogeneity. Moreover, functional enrichment analysis of genes where high methylation differences emerge within each population (>70% diffmeth) showed shared pathways between the four populations, while a smaller proportion were population-specific. For example, among genes whose methylation varies considerably within all four populations, we found genes involved in visual perception and in the response to external

stimuli such as light. There are also genes involved in adult feeding behavior. This means that within each population, there is variation in the DNA methylation of genes involved in these pathways, regardless of genetic heterogeneity within population. This could be due to shared environmental variation within these populations, affecting the methylation of shared genes. It could also be due to epimutations affecting methylation-sensitive genes (see Baldwin effects in last section), or to non-random epimutations such as the epigenetic clock. How and why do populations with drastic differences in their reproductive and genetic characteristics show methylation differences of similar magnitude and affecting shared pathways? This question is addressed later under an evolutionary and adaptative point of view.

As detailed in the previous article (Chapter 3 section 2), there is a discontinuity between epigenetic and genetic distance among populations, and between epigenetic and genetic diversity within populations. For example, Emerson Point Preserve and Long Key have the highest F_{ST} and G_{ST} values, but still cluster together according to their epigenomes. These results suggest that obligatory epimutations, dependent of the underlying genetic sequence, are not the first driver of methylome patterns in the mangrove rivulus and that environmental conditions or random events could play an important role in the generation of methylome pattern. The substantial epigenetic diversity observed in EPP supports this hypothesis given its extremely low genetic diversity. Unlike Belizean populations, individuals from EPP and LK populations distinctly clustered according to their origin and could be regrouped according to their population based on their unique methylation profile. Epigenetic variation measured within EPP, as well as within the other populations, may result from plasticity due to the heterogeneity of environmental conditions within a given site, but also to stochastic epigenetic variation such as epigenetic drift.

As detailed in our review, epigenetic drift corresponds to the neutral and gradual changes in epigenetic patterns. A meaningful age-related epigenetic drift is the epigenetic clock (Hernando-Herraez et al., 2019). This uncoordinated accumulation of methylation variation creates a global DNA hypomethylation and degrades the transcriptional networks during aging (Ashapkin et al., 2019). This process is variable across the genome, may not occur homogeneously in all cells, but is a good indicator of aging. There is potential for the development of an epigenetic clock in fish as previous studies highlighted age-dependent hypomethylation of CpG sites in zebrafish (Shimoda et al., 2014), in Chinook salmon

(*Oncorhynchus tshawytscha*) (Venney et al., 2016) and in steelhead trout (*Oncorhynchus mykiss*) (Gavery et al., 2019). Recently, an epigenetic clock has been constructed for zebrafish *Danio rerio* (Mayne et al., 2020), medaka *Oryzias latipes* (Bertucci et al., 2021) and European seabass *Dicentrarchus labrax* (Anastasiadi & Piferrer, 2020). One interesting perspective would be to develop epigenetic clock to estimate age of wild caught mangrove rivulus, as age is an important factor in ecology, affecting reproductive maturity, behavior, reproductive success, demographic structure, species interactions and epigenome, among others. It would allow us to estimate rivulus age, and to determine if the observed epigenetic variation within and among wild rivulus populations is influenced by such non-random events, or mostly by environmental changes.

1.2 Random and environmentally-induced epimutations

To investigate the occurrence of random and/or environmentally-induced epimutations, another interesting perspective is to evaluate DNA methylation variation of EPP rivulus living in standardized and contrasting environmental conditions. Although the study of the effects of methylmercury (MeHg) on gene-specific methylation in the offspring of wild EPP rivulus showed no significant effect, these data obtained can be compared with our field study by focusing on methylation variation of the targeted genes with the same found in our RRBS analysis. One relevant result is the low variation of DNA methylation of the targeted genes (DNMT3a, GSS, NipBL, MeCP2, MAOA) across MeHg exposure groups and timepoints. No matter if rivulus were exposed to 0, 90 or 135 µg/L MeHg, and if it is at the end of the exposure (larvae of 7 dph) or at the end of the detoxification phase (adults of 90 dph), the mean differential methylation among genes is 6.40 ± 1.63 % methylation (mean ± SD) (Table 5). This epigenetic variation detected under standardized conditions is expected to represent random epigenetic modifications in the isogenic lineage, at least for the targeted genes.

Regarding the methylation data of these genes from RRBS analysis on wild EPP rivulus from the same isogenic lineage, we investigated methylation differences of CpGs found in the same genomic regions that the ones selected for the gene-specific analysis (however, not the exact same CpGs as there were none in common in both experiments). We grouped our data from RRBS according to location in each gene i.e. promotor region, exon 1, intron 1, etc. As results, we found higher methylation differences in brains of rivulus from the field than in brains of their offspring reared under standardized laboratory conditions. As a good example, CpGs

found in DNMT3a exons show differential methylation level of 40.03 ± 27.19 % among wild rivulus, and 14.44 ± 1.67 among laboratory-reared rivulus (Table 5).

The reduction in methylation variation observed between wild EPP rivulus and their offspring (forming an isogenic lineage) reared in controlled conditions suggests that environmental heterogeneity and epigenetic drift may account for the observed epigenetic variation within natural environments. Such reduction in variation could be attributed to the reversibly of environmentally-induced epimutations due to environmental homogeneity in laboratory, to the absence of transgenerational epigenetic inheritance due to developmental events such as DNA methylation reprogramming, or due to young age of EPP rivulus (7 and 90 dph) used in the laboratory experiment limiting the effects of stochastic events including epigenetic drift. These results are in accordance with the one obtained in similar studies on clonal organisms where they observed an epigenetic variation reduction in individuals transferred from natural to experimental conditions, which confirm the environmental influence on epigenetic variation and highlight the rapid epigenetic response of individuals following changes in environmental conditions (Leung et al., 2016). It is important to keep in mind that we compared offspring from different wild parents to offspring from a single wild parent. Using a single isogenic lineage in this comparison is essential as each genotype may have a different perception or interpretation of environmental signals. A recent study showed that the epigenetic response to a given environmental signal is influenced by the genotype in mangrove rivulus, corresponding to facilitated epimutations (Berbel-Filho et al., 2019). Thus, this interaction between environment and genotype can result in different environmentally induced epigenetic responses.

Table 5: Comparison of differential methylation of genes found in field and laboratory study on rivulus brains from Emerson Point Preserve.

	Field study (n = 20)[1]		Laboratory study (n = 15)[2]	
Genes	DiffMeth in exons (%)	DiffMeth in promotors (%)	DiffMeth in exons (%)	DiffMeth in promotors (%)
NIPBL	30.74 ± 12.16	**7.46 ± 7.40**	NA	**8.80 ± 2.82**
GSS	46.20 ± 25.01	NA	NA	3.40 ± 0.89
DNMT3a	**38.80 ± 26.33**	**15.16 ± 10.29**	**14.44 ± 1.67**	**8.00 ± 2.45**
MAOA	86.25 ± 18.56	**21.73 ± 16.07**	NA	**1.33 ± 0.87**
MeCP2	NA	NA	NA	2.44 ± 1.06

Note: Data correspond to the mean ± SD of differential methylation of CpGs among mangrove rivulus from EPP. CpGs are in exons or promotors of genes found in both field and laboratory studies. CpGs analyzed with RRBS (field study) are different than CpGs

analyzed with gene-specific approach of pyrosequencing (laboratory study), but in the same gene region. DiffMeth, differential methylation; NIPBL, Nipped-B-like protein; GSS, Glutathione synthetase; DNMT3a, DNA methyltransferase 3 alpha; MAOA, Monoamine oxydase A; MeCP2, methyl CpG binding protein 2.

[1] Field study: Data from 20 wild rivulus caught in Emerson Point Preserve (F0) from one isogenic lineage

[2] Laboratory study: Data from 15 offspring of one wild rivulus caught in Emerson Point Preserve and analyzed in field study.

To go further in the analysis of epimutation origins, we suggest proceeding to a combination of a field study followed by a common garden experiment with the same individuals, as previously done for the asexual fish *Chrosomus* eos-neogaeus (Leung et al., 2016). The common garden experiments allowed the elimination of most of the confounding factors (e.g., lineages found in different environmental conditions or genetic variation among individuals from distinct sites). Epigenetic variability can be measured on individuals reared in controlled conditions and compared to individuals from their respective sampled sites. With the mangrove rivulus, several lineages from the same population can be reared together, which can help us to distinguish if the observed epigenetic diversity on the field come from the environment, the genotype of their interaction. Moreover, comparing epigenetic diversity within an isogenic lineage on the field and in the common garden allow us to investigate the rate of random epimutations. Contrary to what has been done in our laboratory study, we strongly encourage investigating epigenetic variation with Reduced Representation Bisulfite Sequencing (RRBS) or Whole Genome Bisulfite Sequencing (WGBS).

Summary of "1. Disentangling epimutations sources: insights and perspectives"

- There is a discontinuity between epigenetic and genetic distance among rivulus populations, and between epigenetic and genetic diversity within populations.
- Pure epimutations (environmentally-induced and/or random) do occur in wild rivulus populations.
- There is less epigenetic variation under standardized experimental conditions than observed into the wild for the same genotype, supporting the role of environmental variation, random (epigenetic drift) and non-random (epigenetic clock) events in the generation of epimutations.
- Obligatory epimutations are not excluded as genetic and epigenetic distances match for Belizean populations.

- Perspectives to discern the proportion of obligatory, facilitated and pure epimutations:
  - Recording environmental data from the field
  - Experiment a common garden
  - Development of epigenetic clock for mangrove rivulus

2. Behavioral individuality and ecology

2.1 Behavioral variation arises in isogenic lineage reared in standardized environments, but not individuality

Variation in personality traits has been the focus of several laboratory studies on mangrove rivulus, which showed considerable variation in boldness (Edenbrow & Croft, 2011, 2013; James et al., 2018), aggressiveness (Edenbrow & Croft, 2012, 2013) and exploration (Edenbrow & Croft, 2011, 2013) in response to abiotic and biotic environmental changes. In our study on the immediate and delayed effects of methylmercury (MeHg), we confirm that behaviors or rivulus from EPP can be affected by an environmental stressor as we observed a decreased foraging efficiency and thigmotaxis, and a dose-dependent reduction in larvae locomotor activity that were reversible. Although rivulus used in this study were from the same isogenic lineage, we still observe high individual differences within each condition. For instance, the total distance travelled by rivulus larvae during the activity test had a coefficient of variation (CV) of 40 %, 60% and 80% in control, 90 µg/L and 135 µg/L MeHg group respectively. In comparison, total distanced moved by AB line zebrafish (known to have low level of genetic variability (Coe et al., 2009)) exposed to 0 or 10 µg/L MeHg showed CV of 20% and 17%, respectively (Zhu et al., 2020). Two main observations can be drawn from these rivulus activity data: there is a naturally high inter-individual variation in control group and this variation increases with MeHg concentrations. The increase of variance could be the first step in the adaptation of a population in a changing environment (Orlando & Guillette, 2001). As reviewed in Nikinmaa and Anttila (2019), variability should always be included as an endpoint in data analysis as it would bring new information about the responses of organisms to environmental contamination, and about the variation among individual sensibilities to stress. Therefore, mangrove rivulus is naturally suited to investigate sources of phenotypic variation that are not genetic, as environmental influences and environmentally-induced or random epigenetic modifications.

As supplementary analysis, we calculated repeatability of activity (total distance moved, TDM) and boldness (cumulative duration in internal zone, CDIZ) by comparing behaviors of rivulus at 7 and 90 dph (n = 26, same fish) of control group, as we observed a significant interaction between MeHg exposure condition and replicate (7 or 90 dph). There is no significant individuality in activity and boldness (Table 6), meaning that there is no intrinsic among-individual variation for these traits in these rivulus. However, the ancestors of these fish (F0, from the wild EPP population) did show individuality in boldness. This loss of significant among-individual behavioral variation is in line with the reduction in methylation variation observed between wild EPP rivulus and their offspring (forming an isogenic lineage) reared in controlled conditions. These results suggest that wild conditions (environmental heterogeneity, selection pressure, (epi)genetic drift, etc) may maintain epigenetic diversity but also individuality in rivulus population EPP, which is discussed in the next section.

Table 6: Linear mixed models for activity and boldness of mangrove rivulus from the control group of methylmercury experiment. These rivulus are the F2, F3 and F4 generations from F0 wild mangrove rivulus caught in Emerson Point Preserve.

	Activity (TDM)			**Boldness (CDIZ)**		
Fixed effects	*Estimates*	*CIs (95%)*	*df*	*Estimates*	*CIs (95%)*	*df*
(Intercept)	0.41 *	0.00 – 0.81	48.65	0.33	-0.06 – 0.72	49.00
Length	0.04	-0.30 – 0.38	47.99	0.10	-0.23 – 0.43	49.00
7 dph	*Reference*			*Reference*		
90 dph	-1.77	-9.11 – 5.58	47.94	-2.95	-10.14 – 4.23	49.00
Random effects						
σ^2	0.73			0.79		
$\tau_{00\ FishID}$	0.12			0.00		
ICC	0.14			0.00		
N_{FishID}	26			26		
Observations	52			52		
Repeatability	0.14			0.00		

Two linear mixed models with total distance moved (TDM) and cumulative duration in internal zone (CDIZ) as dependent variable included 'Replicate' (7 and 90 dph) and Length as fixed effects, and Fish ID as random effects. Conditional repeatability estimates the proportion of the total variance that is due to among-individual differences within control group while considering fixed effects. ** p<0.05 ** p<0.01 *** p<0.001.*

2.2 Individuality arises in isogenic but not in some genetically-diverse wild populations

Why and how consistent individual differences in behavior exist and persist in face of selection are important questions that are far to be solved. Behaviors influence the ability of an individual to act optimally in a specific situation and/or to an environmental factor, which makes it a relevant phenotype to investigate in ecology (Hertel et al., 2020). Studying behavioral individuality allow us to distinguish the part of total behavioral variance that is explained by intrinsic among-individual variation and to reduce the effect of reversible factors (environmental conditions, internal states) and intra-individual variation (behavioral plasticity and predictability). Genome sequence is considered as the main factor driving this intrinsic among-individual behavioral variation, but non-genetic factors have also recently been proposed as important drivers of individuality including prebirth developmental stochasticity, maternal effects and epigenetics (Bierbach et al., 2017; Laskowski et al., 2022; Vogt, 2015). In our field study on behavioral individuality within four wild rivulus populations encountering a genetic diversity gradient, we can investigate if individuality occurs in mangrove rivulus populations, and if genetic diversity is the main intrinsic factor driving this individuality. We found significant individuality of boldness in the almost-clonal population of EPP, meaning that among-individual variation in boldness in this population are not only driven by external factors or internal state (i.e. reversible variation), but also by non-genetic intrinsic variation among individuals. This individuality was found in the other Floridan population LK where there is more genetic diversity, but not in the most genetically-diverse populations in Belize. These results are the opposite of what would be observed if the main intrinsic driver of individuality in boldness was genetic diversity.

This individuality can arise from different sources. Firstly, rivulus can vary in their epigenetic marks, as confirmed with the RRBS analysis and detailed previously. Our main hypothesis is that epigenetic variation could be another source of behavioral individuality and should be considered as another intrinsic variation among individuals, as some DNA methylation marks can be irreversible and can even be transferred to the next generation (Bhandari et al., 2015; Guerrero-Bosagna et al., 2018; Liew et al., 2020). The responsiveness of environmentally-induced epimutations can help populations facing rapid, fluctuating environmental changes. This phenomenon is named epigenetic buffering, and could facilitate evolutionary rescue through heritability of environmentally induced phenotypes, reducing genetic loss and

increasing the probability of genetic mutation, all reviewed in (O'Dea et al., 2016). This phenomenon could help population with low genetic diversity such as EPP population to face environment changes. Secondly, later-in-life individuality can be strongly shaped by factors predating birth like maternal provisioning, epigenetics and pre-birth developmental stochasticity (Laskowski et al., 2022). Previous study on rivulus boldness revealed that the intensity of this trait increases during ontogeny and then stabilized at sexual maturity suggesting a developmental flexibility of these behavioral traits in this species (Chang et al., 2012). This flexibility could allow individuals to adapt to highly variable local environmental conditions in which rivulus evolves and therefore would be directly linked to temporal and spatial heterogeneity of the mangrove forests (Edenbrow & Croft, 2011). Recently, a study on genetically identical fish *Poecilia formosa* reared in identical environment showed that substantial behavioral individuality is already present at the very first day of life after birth and that these early signatures of individuality gradually strengthened over ontogeny and predict behavior up to at least ten weeks later (Laskowski et al., 2022). Thus, once individuality is triggered, it could set the starting point for further behavioral differentiation. One interesting perspective is to investigate if individuality is already present at the very first day of life after birth in mangrove rivulus, and how it evolves through the lifespan. As explained in the previous section, there is no individuality in boldness in the offspring of these fish reared under laboratory conditions. Expressing individuality in their behaviors would give the mangrove rivulus the capacity to survive in their highly complex and variable mangrove environment, which constitutes an evolutionary benefit. Individuality could be irreversible among rivulus within a generation, but reversible across generations (Kain et al., 2015). This is another hypothesis to investigate to understand why and how consistent individual differences in boldness exist and persist in face of selection.

On the opposite of Floridan populations, there is no individuality in genetically-diverse populations of Belize. It could be due to intra-individual variability that prevents the observation of consistent behavioral variation among individual. This variation is often attributed to behavioral plasticity and predictability. Behavioral plasticity corresponds to reversible changes in behavior in response to biotic and abiotic environmental conditions within the same individual (Dingemanse et al., 2010, reviewed in Stamps, 2016), which allows organisms to adjust their behavior along environmental gradients or over time. Even after accounting for consistent among-individual variation (i.e. differences in personality) and

behavioral plasticity (i.e. responsiveness to environmental/temporal change), it may still remain unexplained behavioral variability. This individual variation in residual within-individual variance corresponding to behavioral predictability (Westneat et al., 2015). One interesting perspective would be to perform more temporal replicates of behavioral tests to investigate the plasticity and predictability of mangrove rivulus behaviors with different genotypes to highlight a possible interaction between test parameters and to characterize rivulus reaction norm. Previous studies have indicated that intra-individual variation in behavioral traits can depend upon an individual's behavior, as bold three-spined sticklebacks *Gasterosteus aculeatus* are less plastic and more predictable than shy fish (Jolles et al., 2019). Another study on this species showed that behavioral plasticity was dependent upon the population of origin (high and low predation) (Bell & Stamps, 2004). As rivulus from EPP were significantly bolder than TC, we suggest testing the hypothesis that EPP rivulus are less plastic and more predictable than Twin Cayes rivulus, allowing the emergence of individuality in EPP but not in TC.

The results obtained in our laboratory and field studies can be discussed under an evolutionary and adaptive point of view. For example, among-individual variation in plasticity and predictability may be evolutionarily adaptive, e.g. diversification in bed-hedging (Biwer et al., 2020). In the next and last section, we discuss the insights that brought our epigenetic and behavioral results in the understanding of mangrove rivulus evolutionary mechanisms, and their possible variation across populations.

Summary of "2. Behavioral individuality and ecology"

- Behavioral variation arises in isogenic lineage reared in standardized environments.
- Individuality arises in wild isogenic population of rivulus (EPP), but not in their offspring reared in standardized environments.
- Natural conditions (environmental heterogeneity, selection pressure, (epi)genetic drift,…) may maintain individuality in rivulus population EPP.
- High intra-individual variation (plasticity and predictability) could prevent the emergence of individuality in genetically-diverse populations of mangrove rivulus.
- The reasons why and how consistent individual differences in behavior exist and persist in face of selection should be the focus of future studies.

- Perspectives:
  - Determine when individuality emerges in mangrove rivulus (already present at the very first day of life?) and how it evolves through the lifespan.
  - Investigate individuality inheritance and determine if individuality could be irreversible among rivulus within a generation, but reversible across generations.
  - Perform more temporal replicates of behavioral tests to investigate the plasticity and predictability of mangrove rivulus behaviors and to characterize rivulus reaction norm.

3. Different or shared adaptive and evolutionary strategies?

One interesting result coming from the field study is that the level of epigenetic variation within all four populations is similar, regardless of genetic heterogeneity. Moreover, the functional enrichment analysis of genes where differentially methylated cytosines occur within a population showed shared pathways between the four populations, while a smaller proportion were population-specific. **How and why populations with drastic differences in their reproductive and genetic characteristics show methylation differences of similar magnitude and affecting shared pathways?** Another result that makes us question the evolutionary strategies of mangrove rivulus is that individuality in behaviors seems to be related to the location rather than to the genetic diversity within a population, as it emerged in Floridan populations encountering drastic genetic diversity (H_e) differences level (H_e in EPP = 0.04; H_e in LK = 0.35), but not in Belizean populations (H_e in LC = 0.57; H_e in TC= 0.62). **How and why individuality persist in Floridan populations regardless of genetic diversity, but not in Belizean populations?** Different underlying adaptive and evolutionary strategies can help us to explain these patterns.

3.1 Phenotypic plasticity and diversifying bet-hedging

Phenotypic plasticity and diversified bet-hedging are two ecological models developed to describe how organisms maximize their fitness in changing environments (Philippi & Seger, 1989; Simons, 2011). Diversified bet-hedging strategy is based on the production of phenotypically variable offspring, irrespective of environmental conditions. Conserving

behavioral individuality across multiple generations even in the absence of considerable environmental variations would maximize survival chances of a population in case of environmental condition variation. In other words, diversified bet-hedging increases the likelihood that, at least, some individuals are well-adapted to the selection pressure of unpredictable environments. Through empirical studies, behavioral individuality has been reported to possibly reflect bet-hedging strategy (Carter et al., 2017; Kain et al., 2015). Kain et al. (2015) observed interindividual behavioral diversity (in light and temperature preference-dependent behaviors) in fruit flies *Drosophila melanogaster* that generated interindividual differences in survival and reproduction, reflecting a bet-hedging strategy. Moreover, this individuality was not heritable, which remind us of our results obtain for F0 and F2-F4 rivulus from EPP population, where individuality was found in F0 but not in their offspring. The presence of inter-individual variation in boldness and activity levels in mangrove rivulus reared under laboratory conditions could reflect a bet-hedging strategy to reduce risks and ensure the survival of some individuals of the population in case of rapid change of their environment. Random epimutations have been proposed to be among mechanisms underlying diversified bet-hedging strategy and may increase when organisms are exposed to environmental stresses (Rapp & Wendel, 2005). Furhter studies should investigate if random epimutations occur in mangrove rivulus reared in homogenous conditions and investigate if these epimutations could be the underlying mechanisms of behavioral variation among rivulus from the same isogenic lineage.

Into the field, the scenario is quite different. Following the perception of an environmental signal, specific genes may be epigenetically silenced or activated, resulting in a modified and environment-specific phenotype (Orlando & Guillette, 2001; Skinner et al., 2015; Skinner & Nilsson, 2021). Environmentally induced epigenetic variation has therefore been proposed to mediate phenotypic plasticity as they can shape phenotypic responses to environmental variation, facilitating adaptation, speciation, and adaptive radiation (Angers et al., 2010). Phenotypic plasticity is one of the processes underlying the general-purpose genotype (GPG) model (Stearns, 1989). It proposed that evolutionary success of organisms with low genetic diversity (asexual, clonal,…) could be possible via generalist lineages selected for their flexible phenotypes utilizing wide ecological niches. Such phenotypic flexibility enables a given genotype to be successful in many different and variable environments. As epigenetic variation potentially represents a molecular mechanism that can generate phenotypic plasticity, it can

also be part of the GPG model (Figure 18). Massicotte & Angers (2012) studied DNA methylation polymorphisms of individuals belonging to a single genetic lineage of the clonal diploid fish *Chrosomus eos-neogaeus* sampled in seven geographically distant lakes and showed that DNA methylation is a relevant molecular mechanism that contributes to phenotypic plasticity over variable environments in accordance with the GPG model. Methylation profiles allow the clustering of individuals in two distinct groups of populations among lakes, which was consistent with pH variation among the two epigenetic groups (Massicotte & Angers, 2012). In our case, each mangrove rivulus from the same isogenic lineage (mostly in EPP) presenting different epigenetic profiles could be seen as an acclimated epigenotype. It thus seems that these lineages have the potential to respond via DNA methylation variation when under variable environmental conditions. These lineages potentially have the capacity to colonize different environments and/or the ability to adjust following a perturbation in the environment.

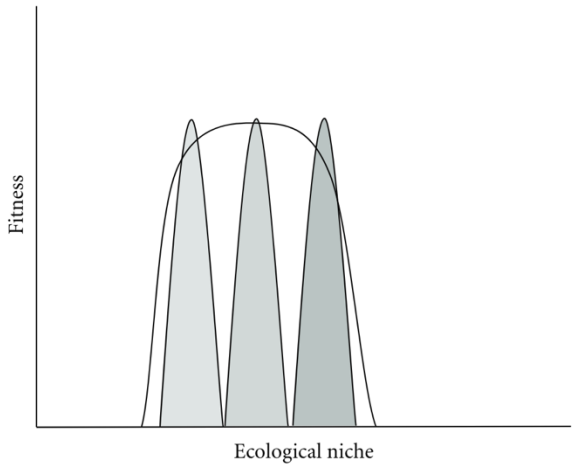


Figure 18: Graphic representation of the general-purpose genotype (GPG) model. Environmentally induced epigenotypes (grey distributions) extend the flexibility of a single genetic lineage (unfilled distribution), creating a wide ecological niche and a high fitness under variable environmental conditions. Adapted from Massicotte and Angers (2012).

Theoretical studies demonstrated that phenotypic plasticity would be selected for dealing with predictable environmental changes (Reed et al., 2010; Scheiner & Holt, 2012). Leung et al. (2016) analyzed the DNA methylation variation in wild populations of asexual fish *C. eos-neogaeus* between two types of environments: predictable (lakes) and unpredictable (intermittent streams) areas. Clonal fish that are found in predictable environments display environmentally induced epigenetic changes, whereas those living in unpredictable environments are characterized by a high contribution of random epimutations. They revealed that environmentally induced and random epimutations acts conjointly, while at a different

extent according to environmental uncertainty, suggesting that plasticity and random processes are complementary strategies. The difficulty of investigating this relationship between environmentally-induced and random epimutations (or between phenotypic plasticity and bet-hedging strategy) in wild populations of mangrove rivulus comes from the nature of its habitat. As detailed in the introduction, physico-chemical conditions of mangrove ecosystems are highly variable due to the alternation of tidal/rainfall flushing and drier periods through the day. However, this does not tell us anything about its unpredictability. The mangrove rivulus could have developed a phenotypic plasticity to face this extreme but predictable variability. Characterization of rivulus habitat (its variability and predictability) in EPP, LK, LC and TC would help us to understand **how and why individuality persist in Floridan populations regardless of genetic diversity, but not in Belizean populations.**

3.2 Baldwin effects, genetic assimilation and phenotypic convergence

Another driver of phenotypic plasticity is the underlying genetic sequence, as some alleles are more likely to encounter epimutations than others. Indeed, fluctuating environmental conditions may lead to the establishment of alternative methylation patterns on certain sensitive allele (Figure 19a). The frequency of advantageous methylation-sensitive alleles will therefore increase in subsequent generations, thereby increasing the number of individuals apt to react to environmental fluctuations. In this case, there is no inheritance of the methylation marks: it is the flexibility of the phenotype that is selected, rather than the result of the flexibility itself, a process named Baldwin effect (Simpson, 1953) (Figure 19b). DNA methylation modulates gene expression in response to the environment, while the genetic background provides heritability of the genes required for flexibility. The Baldwin effect could be advantageous in unstable or highly heterogeneous environments, such as mangrove ecosystems. Although very few different genotypes were found in the EPP rivulus population, these genotypes could have selected characteristics to be flexible and sensitive to environmentally-induced epimutations.

On the other hand, if environmentally induced phenotype and its underlying epimutations are maintained across generations, it can lead to genetic assimilation (Young & Badyaev, 2007) (Figure 19c). During this process, environmental changes induce the epimutations that are responsible for a new advantageous phenotype, on which natural selection acts. Over time, these environmentally induced epimutations are incrementally replaced with multiple

advantageous genetic mutations through the process of natural selection. The epigenetic contribution to the phenotype decreases as the genetic contribution increases. Ultimately, the environmentally-induced phenotype becomes genetically encoded in the population due to the process of mutation selection, and the environmental signal, as well as the epigenetic marks that are no longer required to produce it. It corresponds to a 'mutational assimilation' in which the mutations are facilitated by epigenetics. This complex mechanism supports the theory that epigenetic variation precedes genetic variation and is reviewed in Danchin et al. (2019).

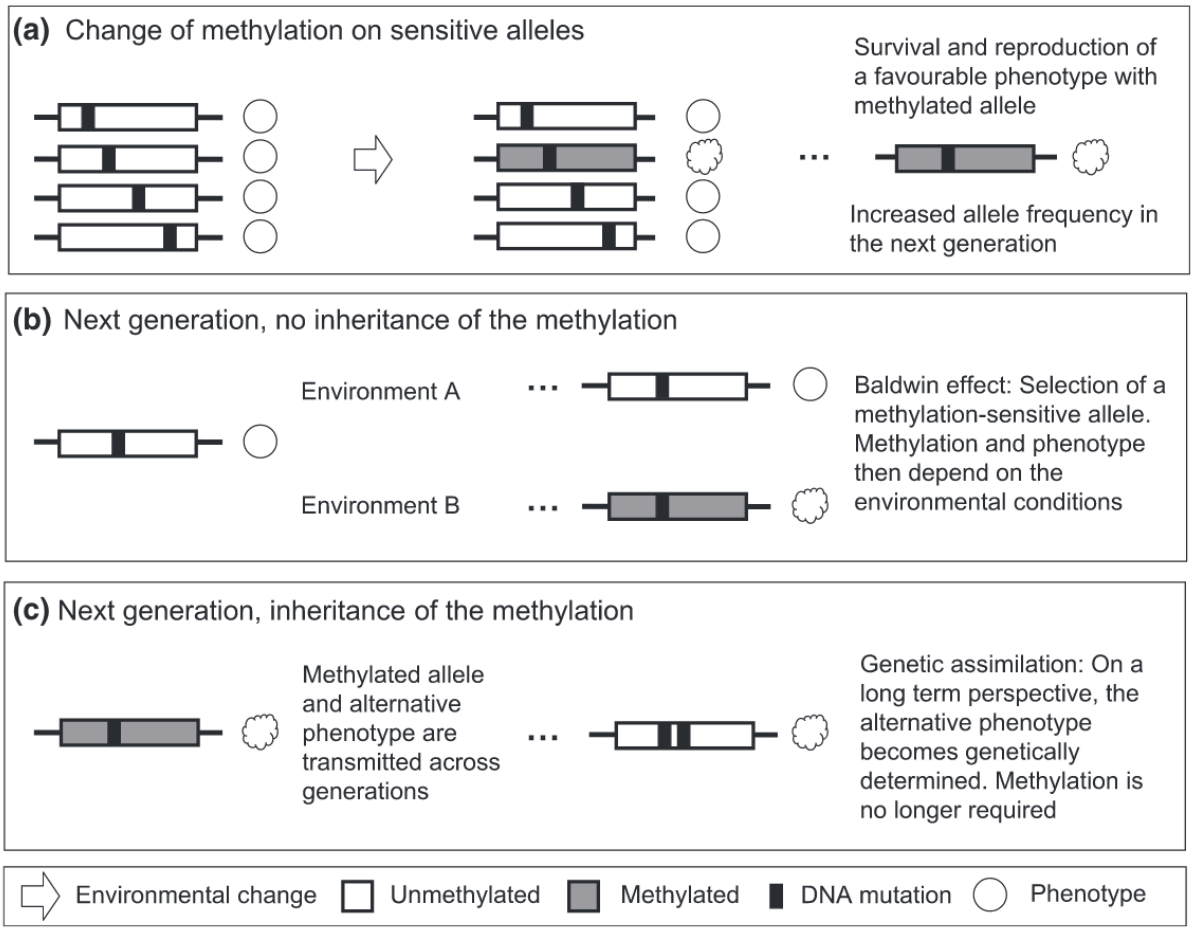
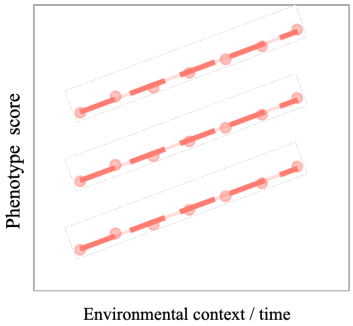


Figure 19: Consequences of the inheritance (or not) of methylation marks following an environmental change. (a) As a consequence of a given environmental change, the methylation of some alleles will be modified depending on their sensitivity. If the resulting alternative phenotype is advantageous, the frequency of this allele will increase in the next generation as a result of natural selection. (b) In the next generation, in the absence of inheritance of the new methylation marks, the initial phenotype will be restored in environment A whereas the alternative phenotype may reappear in environment B (for the same reason it appeared in A) because the selected allele is more sensitive to environmentally induced epigenetic variation. (c) In the next generation, in the presence of inheritance of the new methylation marks, the alternative phenotype is maintained for several generations until a mutation replaces the effect of methylation. From Angers et al., (2010)

The fact that alleles and epialleles can create the same phenotype is an important concept to include in our discussion. This process refers to phenotypic convergence and is mostly described in medical biology. For example, a study on the implications of the GAD1 gene in schizophrenia showed that there is a convergence between environmental and genetic factors in methylation-related remodeling of the GAD1 gene regulatory region. Several environmental factors (maternal stress during pregnancy, maternal immune activation during pregnancy, and low postpartum maternal care) can cause hypermethylation of the GAD1 gene regulatory region. Hypermethylation of the GAD1 gene regulatory region can also be caused by certain genetic variants (e.g., rs3749034, a schizophrenia-risk single nucleotide polymorphism). Together, these epigenetic changes have been associated with impaired neuronal synchronization and inhibition and the subsequent emergence of behavioral and cognitive deficits (Richetto & Meyer, 2021). Similar convergence of DNA methylation variation and somatic mutations has been observed in brain tumors (Mazor et al., 2015). We can use this concept of phenotypic convergence to explain **why populations with drastic differences in their genetic characteristics show methylation differences affecting the same pathways.**

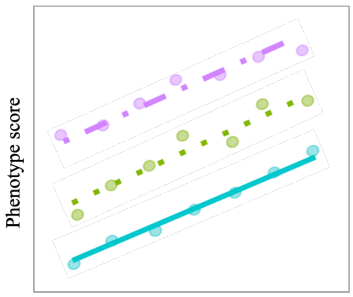
Considering all these concepts, the figure below groups the hypotheses to be tested to better characterize the role DNA methylation variation in the adaptation and evolution of mangrove rivulus *Kryptolebias marmoratus* (Figure 20).

Emerson Point Preserve



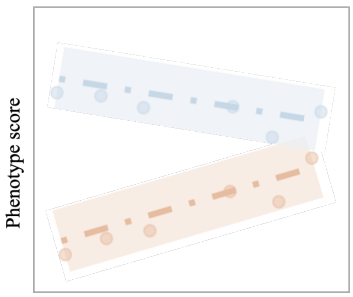
- Individuality and substantial epigenetic diversity
- Epigenetic buffering ? (environmentally-induced epimutations underly phenotypic plasticity)
- Baldwin effect ? (selection of flexible and methylation-sensitive genotypes)
- Rapid and reversible response to environmental fluctuations ? (less DNA methylation and behavioral variation in lab than into the wild
- Phenotypic convergence with LK, LC, TC leading to shared differentially methylated pathways ? (origin in EPP : epigenetic diversity)

Long Key



- Individuality and substantial epigenetic diversity
- Genetic assimilation ? (from epigenetically-determined to genetically determined phenotypes)
- Phenotypic convergence with EPP, LC, TC leading to shared differentially methylated pathways ? (origin in LK : epigenetic and/or genetic diversity)

Twin Cayes or Long Caye



- Substantial epigenetic diversity but no individuality
- High intra-individual variation (high plasticity and low predictability) ?
- Phenotypic convergence with EPP and LK leading to shared differentially methylated pathways ? (origin in TC and LC : epigenetic and/or genetic diversity)

Figure 20: Summary of adaptive and evolutionary concepts to investigate to better characterize the role DNA methylation variation in the adaptation of the mangrove rivulus Kryptolebias marmoratus. Lines color corresponds to different genotypes.

CHAPTER 6: CONCLUSIONS

The extended evolutionary synthesis emphasized the effect of epigenetic mechanisms as an additional source of phenotypic variation as it has been demonstrated to generate heritable phenotypic variation independent of genetic sequence alterations, in response to environmental changes or randomly, and hence can contribute to evolutionary changes. Along this thesis, we addressed multiple questions on DNA methylation variation i.e. epimutations: what is the extent of DNA methylation in wild animal populations? How epigenetic variation balances with genetic variation in wild populations of mangrove rivulus encountering genetic diversity gradient? What are the origins of epimutations in mangrove rivulus? Are they population-specific? Whether and how an environmental stressor induces epimutations in an isogenic lineage of mangrove rivulus?

The ecological and biological characteristics of the mangrove rivulus make it an attractive model species to answer these questions. Our work furnished new hypotheses about the epimutations sources in mangrove rivulus populations, how behavioral individuality arises among rivulus and what could be the underlying adaptive and evolutionary strategies. **DNA methylation diversity has been found to be a revealing parameter to characterize wild rivulus populations (strong epigenetic structure, and possibly population-specific epimutations sources), the environmental conditions they face (complex/natural habitats of standardized/laboratory conditions) and the evolutionary pathways they follow.** Mangrove rivulus can be useful to investigate adaptive and evolutionary processes mediated by epigenetic mechanisms such as epigenetic buffering, phenotypic plasticity, bet-hedging strategy, Baldwin effect, phenotypic convergence and genetic assimilation.

This thesis also reinforces the idea of studying behavioral variation and individuality in mangrove rivulus as a targeted phenotypic output, as we have shown surprising and original results in both field and laboratory studies. There is behavioral variation among fish from the same isogenic lineage reared in standardized environment, individuality among fish from a wild population with extremely low genetic diversity, but no individuality in more genetically-diverse wild populations.

This thesis provides a framework to further assess the potential of organisms to respond to environmental changes. Future studies on the mangrove rivulus could confirm our results and may provide deeper insights about the role of DNA methylation in organisms adaptation and evolution including field study recording a maximum of environmental data, common garden experiment with several isogenic lineages, transgenerational experiment and development of epigenetic clock for mangrove rivulus. These results could bring new useful insights for generating predictive models of the capacity of populations to adapt to environmental variation through DNA methylation changes.

REFERENCES

Alam, M. A., Gomes, A., Sarkar, S. K., Shuvaeva, O. V., Vishnevetskaya, N. S., Gustaytis, M. A., Bhattacharya, B. D., & Godhantaraman, N. (2010). Trace Metal Bioaccumulation by Soft-bottom Polychaetes (Annelida) of Sundarban Mangrove Wetland, India and Their Potential Use as Contamination Indicator. *Bulletin of Environmental Contamination and Toxicology*, *85*(5), 492-496. https://doi.org/10.1007/s00128-010-0110-1

Anastasiadi, D., & Piferrer, F. (2020). A clockwork fish : Age prediction using DNA methylation-based biomarkers in the European seabass. *Molecular Ecology Resources*, *20*(2), 387-397. https://doi.org/10.1111/1755-0998.13111

Angers, B., Castonguay, E., & Massicotte, R. (2010). Environmentally induced phenotypes and DNA methylation : How to deal with unpredictable conditions until the next generation and after. *Molecular Ecology*, *19*(7), 1283-1295. https://doi.org/10.1111/j.1365-294X.2010.04580.x

Ardura, A., Clusa, L., Zaiko, A., Garcia-Vazquez, E., & Miralles, L. (2018). Stress related epigenetic changes may explain opportunistic success in biological invasions in Antipode mussels. *Scientific Reports*, *8*(1), 10793. https://doi.org/10.1038/s41598-018-29181-4

Ardura, A., Zaiko, A., Morán, P., Planes, S., & Garcia-Vazquez, E. (2017). Epigenetic signatures of invasive status in populations of marine invertebrates. *Scientific Reports*, *7*(1), 42193. https://doi.org/10.1038/srep42193

Ashapkin, V. V., Kutueva, L. I., & Vanyushin, B. F. (2019). Epigenetic Clock : Just a Convenient Marker or an Active Driver of Aging? In P. C. Guest (Éd.), *Reviews on Biomarker Studies in Aging and Anti-Aging Research* (Vol. 1178, p. 175-206). Springer International Publishing. https://doi.org/10.1007/978-3-030-25650-0_10

Avise, J. (2008). *Clonality : The Genetics, Ecology, and Evolution of Sexual Abstinence in Vertebrate Animals*. Oxford University Press, USA.

Avise, J. C., & Tatarenkov, A. (2015). Population genetics and evolution of the mangrove rivulus Kryptolebias marmoratus, the world's only self-fertilizing hermaphroditic vertebrate : Genetics and evolution of a selfing killifish. *Journal of Fish Biology*, *87*(3), 519-538. https://doi.org/10.1111/jfb.12741

Baeyens, W., Leermakers, M., Papina, T., Saprykin, A., Brion, N., Noyen, J., De Gieter, M., Elskens, M., & Goeyens, L. (2003). Bioconcentration and Biomagnification of Mercury and Methylmercury in North Sea and Scheldt Estuary Fish. *Archives of Environmental Contamination and Toxicology*, *45*(4), 498-508. https://doi.org/10.1007/s00244-003-2136-4

Bell, A. M., & Aubin-Horth, N. (2010). What can whole genome expression data tell us about the ecology and evolution of personality? *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1560), 4001-4012. https://doi.org/10.1098/rstb.2010.0185

Bell, A. M., Hankison, S. J., & Laskowski, K. L. (2009). The repeatability of behavior : A meta-analysis. *Animal Behavior*, *77*(4), 771-783. https://doi.org/10.1016/j.anbehav.2008.12.022

Bell, A. M., & Stamps, J. A. (2004). Development of behavioral differences between individuals and populations of sticklebacks, Gasterosteus aculeatus. *Animal Behavior*, *68*(6), 1339-1348. https://doi.org/10.1016/j.anbehav.2004.05.007

Berbel-Filho, W. M., Berry, N., Rodríguez-Barreto, D., Rodrigues Teixeira, S., Garcia de Leaniz, C., & Consuegra, S. (2020). Environmental enrichment induces intergenerational behavioral and epigenetic effects on fish. *Molecular Ecology*, *29*(12), 2288-2299. https://doi.org/10.1111/mec.15481

Berbel-Filho, W. M., Rodríguez-Barreto, D., Berry, N., Garcia De Leaniz, C., & Consuegra, S. (2019). Contrasting DNA methylation responses of inbred fish lines to different rearing environments. *Epigenetics*, *14*(10), 939-948. https://doi.org/10.1080/15592294.2019.1625674

Bertucci, E. M., Mason, M. W., Rhodes, O. E., & Parrott, B. B. (2021). *The aging DNA methylome reveals environment-by-aging interactions in a model teleost* [Preprint]. Genomics. https://doi.org/10.1101/2021.03.01.433371

Bhandari, R. K., vom Saal, F. S., & Tillitt, D. E. (2015). Transgenerational effects from early developmental exposures to bisphenol A or 17α-ethinylestradiol in medaka, Oryzias latipes. *Scientific Reports*, *5*(1), 9303. https://doi.org/10.1038/srep09303

Bierbach, D., Laskowski, K. L., & Wolf, M. (2017). Behavioral individuality in clonal fish arises despite near-identical rearing conditions. *Nature Communications*, *8*(1), 15361. https://doi.org/10.1038/ncomms15361

Biro, P. A., Beckmann, C., & Stamps, J. A. (2010). Small within-day increases in temperature affects boldness and alters personality in coral reef fish. *Proceedings of the Royal Society B: Biological Sciences*, *277*(1678), 71-77. https://doi.org/10.1098/rspb.2009.1346

Biwer, C., Kawam, B., Chapelle, V., & Silvestre, F. (2020). The Role of Stochasticity in the Origin of Epigenetic Variation in Animal Populations. *Integrative and Comparative Biology*, *60*(6), 1544-1557. https://doi.org/10.1093/icb/icaa047

Blanc, M., Antczak, P., Cousin, X., Grunau, C., Scherbak, N., Rüegg, J., & Keiter, S. H. (2021). The insecticide permethrin induces transgenerational behavioral changes linked to transcriptomic and epigenetic alterations in zebrafish (Danio rerio). *Science of The Total Environment*, *779*, 146404. https://doi.org/10.1016/j.scitotenv.2021.146404

Both, C., Dingemanse, N. J., Drent, P. J., & Tinbergen, J. M. (2005). Pairs of Extreme Avian Personalities Have Highest Reproductive Success. *Journal of Animal Ecology*, *74*(4), 667-674.

Breed, M. D., & Moore, J. (2021). *Animal Behavior*. Academic Press.

Burggren, W. (2016). Epigenetic Inheritance and Its Role in Evolutionary Biology : Re-Evaluation and New Perspectives. *Biology*, *5*(2), 24. https://doi.org/10.3390/biology5020024

Carere, C., & Maestripieri, D. (2013). *Animal Personalities : Behavior, Physiology, and Evolution*. University of Chicago Press.

Carion, A., Hétru, J., Markey, A., Suarez-Ulloa, V., & Frédéric, S. (2018). Behavioral effects of the neurotoxin b-N-methylaminoL-alanine on the mangrove rivulus (Kryptolebias marmoratus) larvae. *Journal of Xenobiotics*. https://doi.org/10.4081/xeno.2018.7820

Carion, A., Markey, A., Hétru, J., Carpentier, C., Suarez-Ulloa, V., Denoël, M., Earley, R. L., & Silvestre, F. (2020). Behavior and gene expression in the brain of adult self-fertilizing mangrove rivulus fish (Kryptolebias marmoratus) after early life exposure to the neurotoxin β-N-methylamino-l-alanine (BMAA). *NeuroToxicology*, *79*, 110-121. https://doi.org/10.1016/j.neuro.2020.04.007

Carter, G. G., Farine, D. R., & Wilkinson, G. S. (2017). Social bet-hedging in vampire bats. *Biology Letters*, *13*(5), 20170112. https://doi.org/10.1098/rsbl.2017.0112

Carvan, M. J., Kalluvila, T. A., Klingler, R. H., Larson, J. K., Pickens, M., Mora-Zamorano, F. X., Connaughton, V. P., Sadler-Riggleman, I., Beck, D., & Skinner, M. K. (2017). Mercury-induced epigenetic transgenerational inheritance of abnormal neurobehavior is correlated with sperm epimutations in zebrafish. *PloS One*, *12*(5), e0176155. https://doi.org/10.1371/journal.pone.0176155

Casier, K., Boivin, A., Carré, C., & Teysset, L. (2019). Environmentally-Induced Transgenerational Epigenetic Inheritance : Implication of PIWI Interacting RNAs. *Cells*, *8*(9), 1108. https://doi.org/10.3390/cells8091108

Chang, C., Li, C.-Y., Earley, R. L., & Hsu, Y. (2012). Aggression and Related Behavioral Traits : The Impact of Winning and Losing and the Role of Hormones. *Integrative and Comparative Biology*, *52*(6), 801-813. https://doi.org/10.1093/icb/ics057

Coe, T. S., Hamilton, P. B., Griffiths, A. M., Hodgson, D. J., Wahab, M. A., & Tyler, C. R. (2009). Genetic variation in strains of zebrafish (Danio rerio) and the implications for ecotoxicology studies. *Ecotoxicology*, *18*(1), 144-150. https://doi.org/10.1007/s10646-008-0267-0

Cole, E. F., & Quinn, J. L. (2014). Shy birds play it safe : Personality in captivity predicts risk responsiveness during reproduction in the wild. *Biology Letters*, *10*(5), 20140178. https://doi.org/10.1098/rsbl.2014.0178

Conrad, J. L., Weinersmith, K. L., Brodin, T., Saltz, J. B., & Sih, A. (2011). Behavioral syndromes in fishes : A review with implications for ecology and fisheries management. *Journal of Fish Biology*, *78*(2), 395-435. https://doi.org/10.1111/j.1095-8649.2010.02874.x

Costa, W. J. E. M., Lima, S. M. Q., & Bartolette, R. (2010). Androdioecy in Kryptolebias killifish and the evolution of self-fertilizing hermaphroditism : ANDRODIOECY IN KRYPTOLEBIAS KILLIFISH. *Biological Journal of the Linnean Society*, *99*(2), 344-349. https://doi.org/10.1111/j.1095-8312.2009.01359.x

Culbreth, M., & Aschner, M. (2019). Methylmercury Epigenetics. *Toxics*, *7*(4), Art. 4. https://doi.org/10.3390/toxics7040056

D'Addario, C., Di Francesco, A., Pucci, M., Finazzi Agrò, A., & Maccarrone, M. (2013). Epigenetic mechanisms and endocannabinoid signalling. *FEBS Journal*, *280*(9), 1905-1917. https://doi.org/10.1111/febs.12125

Danchin, E., Pocheville, A., Rey, O., Pujol, B., & Blanchet, S. (2019). Epigenetically facilitated mutational assimilation : Epigenetics as a hub within the inclusive evolutionary synthesis. *Biological Reviews*, *94*(1), 259-282. https://doi.org/10.1111/brv.12453

Davis, W. P., Taylor, S. D., & Turner, B. J. (1995). Does the Autecology of the Mangrove Rivulus Fish (Rivulus Marmoratus) Reflect a Paradigm for Mangrove Ecosystem Sensitivity? *Bulletin of Marine Science*, *57*(1), 208-214.

de Mendoza, A., Hatleberg, W. L., Pang, K., Leininger, S., Bogdanovic, O., Pflueger, J., Buckberry, S., Technau, U., Hejnol, A., Adamska, M., Degnan, B. M., Degnan, S. M., & Lister, R. (2019). Convergent evolution of a vertebrate-like methylome in a marine sponge. *Nature Ecology & Evolution*, *3*(10), 1464-1473. https://doi.org/10.1038/s41559-019-0983-2

de Mendoza, A., Lister, R., & Bogdanovic, O. (2020). Evolution of DNA Methylome Diversity in Eukaryotes. *Journal of Molecular Biology*, *432*(6), 1687-1705. https://doi.org/10.1016/j.jmb.2019.11.003

Dingemanse, N. J., Kazem, A. J. N., Réale, D., & Wright, J. (2010). Behavioral reaction norms : Animal personality meets individual plasticity. *Trends in Ecology & Evolution*, *25*(2), 81-89. https://doi.org/10.1016/j.tree.2009.07.013

Dochtermann, N. A., & Dingemanse, N. J. (2013). Behavioral syndromes as evolutionary constraints. *Behavioral Ecology*, *24*(4), 806-811. https://doi.org/10.1093/beheco/art002

Edenbrow, M., & Croft, D. P. (2011). Behavioral types and life history strategies during ontogeny in the mangrove killifish, Kryptolebias marmoratus. *Animal Behavior*, *82*(4), 731-741. https://doi.org/10.1016/j.anbehav.2011.07.003

Edenbrow, M., & Croft, D. P. (2012). Kin and familiarity influence association preferences and aggression in the mangrove killifish Kryptolebias marmoratus. *Journal of Fish Biology*, *80*(3), 503-518. https://doi.org/10.1111/j.1095-8649.2011.03181.x

Edenbrow, M., & Croft, D. P. (2013). Environmental and genetic effects shape the development of personality traits in the mangrove killifish *Kryptolebias marmoratus*. *Oikos*, *122*(5), 667-681. https://doi.org/10.1111/j.1600-0706.2012.20556.x

Ellison, A., Rodríguez López, C. M., Moran, P., Breen, J., Swain, M., Megias, M., Hegarty, M., Wilkinson, M., Pawluk, R., & Consuegra, S. (2015). Epigenetic regulation of sex ratios may explain natural variation in self-fertilization rates. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1819), 20151900. https://doi.org/10.1098/rspb.2015.1900

Ellison, A., Wright, P., Taylor, D. S., Cooper, C., Regan, K., Currie, S., & Consuegra, S. (2012). Environmental diel variation, parasite loads, and local population structuring of a mixed-mating mangrove fish. *Ecology and Evolution*, *2*(7), 1682-1695. https://doi.org/10.1002/ece3.289

Eva, J., & Lamb, M. (2008). Soft inheritance : Challenging the Modern Synthesis. *Genetics and Molecular Biology - GENET MOL BIOL*, *31*. https://doi.org/10.1590/S1415-47572008000300001

Farina, M., & Aschner, M. (2019). Glutathione antioxidant system and methylmercury-induced neurotoxicity : An intriguing interplay. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *1863*(12), 129285. https://doi.org/10.1016/j.bbagen.2019.01.007

Faro, L. R. F., do Nascimento, J. L. M., Alfonso, M., & Durán, R. (2002). Mechanism of action of methylmercury on in vivo striatal dopamine release. *Neurochemistry International*, *40*(5), 455-465. https://doi.org/10.1016/S0197-0186(01)00098-5

Fellous, A., Earley, R. L., & Silvestre, F. (2019a). The Kdm/Kmt gene families in the self-fertilizing mangrove rivulus fish, Kryptolebias marmoratus, suggest involvement of histone methylation machinery in development and reproduction. *Gene*, *687*, 173-187. https://doi.org/10.1016/j.gene.2018.11.046

Fellous, A., Earley, R. L., & Silvestre, F. (2019b). Identification and expression of mangrove rivulus (Kryptolebias marmoratus) histone deacetylase (HDAC) and lysine acetyltransferase (KAT) genes. *Gene*, *691*, 56-69. https://doi.org/10.1016/j.gene.2018.12.057

Fellous, A., Labed-Veydert, T., Locrel, M., Voisin, A.-S., Earley, R. L., & Silvestre, F. (2018). DNA methylation in adults and during development of the self-fertilizing mangrove rivulus, *Kryptolebias marmoratus*. *Ecology and Evolution*, *8*(12), 6016-6033. https://doi.org/10.1002/ece3.4141

Feng, S., Cokus, S. J., Zhang, X., Chen, P.-Y., Bostick, M., Goll, M. G., Hetzel, J., Jain, J., Strauss, S. H., Halpern, M. E., Ukomadu, C., Sadler, K. C., Pradhan, S., Pellegrini, M., & Jacobsen, S. E. (2010). Conservation and divergence of methylation patterning in plants and animals. *Proceedings of the National Academy of Sciences*, *107*(19), 8689-8694. https://doi.org/10.1073/pnas.1002720107

Ferguson-Smith, A. C. (2011). Genomic imprinting : The emergence of an epigenetic paradigm. *Nature Reviews Genetics*, *12*(8), 565-575. https://doi.org/10.1038/nrg3032

Gavery, M. R., Nichols, K. M., Berejikian, B. A., Tatara, C. P., Goetz, G. W., Dickey, J. T., Van Doornik, D. M., & Swanson, P. (2019). Temporal Dynamics of DNA Methylation Patterns in Response to Rearing Juvenile Steelhead (Oncorhynchus mykiss) in a Hatchery versus Simulated Stream Environment. *Genes*, *10*(5), Art. 5. https://doi.org/10.3390/genes10050356

Gilbert, S. F., Opitz, J. M., & Raff, R. A. (1996). Resynthesizing Evolutionary and Developmental Biology. *Developmental Biology*, *173*(2), 357-372. https://doi.org/10.1006/dbio.1996.0032

Glastad, K. M., Hunt, B. G., & Goodisman, M. A. (2014). Evolutionary insights into DNA methylation in insects. *Current Opinion in Insect Science*, *1*, 25-30. https://doi.org/10.1016/j.cois.2014.04.001

Goll, M. G., & Bestor, T. H. (2005). EUKARYOTIC CYTOSINE METHYLTRANSFERASES. *Annual Review of Biochemistry*, *74*(1), 481-514. https://doi.org/10.1146/annurev.biochem.74.010904.153721

Guerrero-Bosagna, C., Morisson, M., Liaubet, L., Rodenburg, T. B., de Haas, E. N., Košťál, Ľ., & Pitel, F. (2018). Transgenerational epigenetic inheritance in birds. *Environmental Epigenetics*, *4*(2). https://doi.org/10.1093/eep/dvy008

Harrington, R. W. (1971). How Ecological and Genetic Factors Interact to Determine When Self-Fertilizing Hermaphrodites of Rivulus marmoratus Change into Functional Secondary Males, with a Reappraisal of the Modes of Intersexuality among Fishes. *Copeia*, *1971*(3), 389-432. https://doi.org/10.2307/1442438

He, S., Sun, H., Lin, L., Zhang, Y., Chen, J., Liang, L., Li, Y., Zhang, M., Yang, X., Wang, X., Wang, F., Zhu, F., Chen, J., Pei, D., & Zheng, H. (2017). Passive DNA demethylation preferentially up-regulates pluripotency-related genes and facilitates the generation of induced pluripotent stem cells. *Journal of Biological Chemistry*, *292*(45), 18542-18555. https://doi.org/10.1074/jbc.M117.810457

Hernando-Herraez, I., Evano, B., Stubbs, T., Commere, P.-H., Jan Bonder, M., Clark, S., Andrews, S., Tajbakhsh, S., & Reik, W. (2019). Ageing affects DNA methylation drift and transcriptional cell-to-cell variability in mouse muscle stem cells. *Nature Communications*, *10*(1), 4361. https://doi.org/10.1038/s41467-019-12293-4

Hertel, A. G., Niemelä, P. T., Dingemanse, N. J., & Mueller, T. (2020). A guide for studying among-individual behavioral variation from movement data in the wild. *Movement Ecology*, *8*(1), 30. https://doi.org/10.1186/s40462-020-00216-8

Hon, G. C., Rajagopal, N., Shen, Y., McCleary, D. F., Yue, F., Dang, M. D., & Ren, B. (2013). Epigenetic memory at embryonic enhancers identified in DNA methylation maps from adult mouse tissues. *Nature Genetics*, *45*(10), 1198-1206. https://doi.org/10.1038/ng.2746

Hu, J., & Barrett, R. D. H. (2017). Epigenetics in natural animal populations. *Journal of Evolutionary Biology*, *30*(9), 1612-1632. https://doi.org/10.1111/jeb.13130

Huneman, P., & Walsh, D. M. (2017). *Challenging the Modern Synthesis : Adaptation, Development, and Inheritance*. Oxford University Press.

Ito, S., Shen, L., Dai, Q., Wu, S. C., Collins, L. B., Swenberg, J. A., He, C., & Zhang, Y. (2011). Tet Proteins Can Convert 5-Methylcytosine to 5-Formylcytosine and 5-Carboxylcytosine. *Science*, *333*(6047), 1300-1303. https://doi.org/10.1126/science.1210597

James, W. R., Styga, J. M., White, S., Marson, K. M., & Earley, R. L. (2018). Phenotypically plastic responses to predation threat in the mangrove rivulus fish (Kryptolebias marmoratus) : Behavior and morphology. *Evolutionary Ecology*, *32*(5), 453-468. https://doi.org/10.1007/s10682-018-9952-5

Jang, H. S., Shin, W. J., Lee, J. E., & Do, J. T. (2017). CpG and Non-CpG Methylation in Epigenetic Gene Regulation and Brain Function. *Genes*, *8*(6), Art. 6. https://doi.org/10.3390/genes8060148

Jolles, J. W., Briggs, H. D., Araya-Ajoy, Y. G., & Boogert, N. J. (2019). Personality, plasticity and predictability in sticklebacks : Bold fish are less plastic and more predictable than shy fish. *Animal Behavior*, *154*, 193-202. https://doi.org/10.1016/j.anbehav.2019.06.022

Jolles, J. W., Fleetwood-Wilson, A., Nakayama, S., Stumpe, M. C., Johnstone, R. A., & Manica, A. (2014). The role of previous social experience on risk-taking and leadership in three-spined sticklebacks. *Behavioral Ecology*, *25*(6), 1395-1401. https://doi.org/10.1093/beheco/aru146

Jones, K. A., & Godin, J.-G. J. (2010). Are fast explorers slow reactors? Linking personality type and anti-predator behavior. *Proceedings of the Royal Society B: Biological Sciences*, *277*(1681), 625-632. https://doi.org/10.1098/rspb.2009.1607

Kain, J. S., Zhang, S., Akhund-Zade, J., Samuel, A. D. T., Klein, M., & Bivort, B. L. (2015). Variability in thermal and phototactic preferences in *Drosophila* may reflect an adaptive bet-hedging strategy. *Evolution*, *69*(12), 3171-3185. https://doi.org/10.1111/evo.12813

Kelley, J. L., Yee, M.-C., Brown, A. P., Richardson, R. R., Tatarenkov, A., Lee, C. C., Harkins, T. T., Bustamante, C. D., & Earley, R. L. (2016). The Genome of the Self-Fertilizing Mangrove Rivulus Fish, *Kryptolebias marmoratus* : A Model for Studying Phenotypic Plasticity and Adaptations to Extreme Environments. *Genome Biology and Evolution*, *8*(7), 2145-2154. https://doi.org/10.1093/gbe/evw145

Kidd, K., & Batchelar, K. (2011). Mercury. In *Fish Physiology* (Vol. 31, p. 237-295). Elsevier. https://doi.org/10.1016/S1546-5098(11)31027-8

Kilvitis, H. J., Hanson, H., Schrey, A. W., & Martin, L. B. (2017). Epigenetic Potential as a Mechanism of Phenotypic Plasticity in Vertebrate Range Expansions. *Integrative and Comparative Biology*, *57*(2), 385-395. https://doi.org/10.1093/icb/icx082

Kim, B.-M., Mirbahai, L., Mally, A., Kevin Chipman, J., Rhee, J.-S., & Lee, J.-S. (2016). Correlation between the DNA methyltransferase (Dnmt) gene family and genome-wide 5-methylcytosine (5mC) in rotifer, copepod, and fish. *Genes & Genomics*, *38*(1), 13-23. https://doi.org/10.1007/s13258-015-0333-y

Kim, S.-H., Kang, Y.-K., Koo, D.-B., Kang, M.-J., Moon, S.-J., Lee, K.-K., & Han, Y.-M. (2004). Differential DNA methylation reprogramming of various repetitive sequences in mouse preimplantation embryos. *Biochemical and Biophysical Research Communications*, *324*(1), 58-63. https://doi.org/10.1016/j.bbrc.2004.09.023

Laland, K. N., Uller, T., Feldman, M. W., Sterelny, K., Müller, G. B., Moczek, A., Jablonka, E., & Odling-Smee, J. (2015). The extended evolutionary synthesis : Its structure, assumptions and predictions.

Proceedings of the Royal Society B: Biological Sciences, *282*(1813), 20151019. https://doi.org/10.1098/rspb.2015.1019

Laskowski, K. L., Bierbach, D., Jolles, J. W., Doran, C., & Wolf, M. (2022). The emergence and development of behavioral individuality in clonal fish. *Nature Communications*, *13*(1), 6419. https://doi.org/10.1038/s41467-022-34113-y

LeBlanc, D. M., Wood, C. M., Fudge, D. S., & Wright, P. A. (2010). A Fish Out of Water : Gill and Skin Remodeling Promotes Osmo- and Ionoregulation in the Mangrove Killifish Kryptolebias marmoratus. *Physiological and Biochemical Zoology*, *83*(6), 932-949. https://doi.org/10.1086/656307

Lei, P., Zhong, H., Duan, D., & Pan, K. (2019). A review on mercury biogeochemistry in mangrove sediments : Hotspots of methylmercury production? *Science of The Total Environment*, *680*, 140-150. https://doi.org/10.1016/j.scitotenv.2019.04.451

Leung, C., Breton, S., & Angers, B. (2016). Facing environmental predictability with different sources of epigenetic variation. *Ecology and Evolution*, *6*(15), 5234-5245. https://doi.org/10.1002/ece3.2283

Liew, Y. J., Howells, E. J., Wang, X., Michell, C. T., Burt, J. A., Idaghdour, Y., & Aranda, M. (2020). Intergenerational epigenetic inheritance in reef-building corals. *Nature Climate Change*, *10*(3), 254-259. https://doi.org/10.1038/s41558-019-0687-2

Lins, L. S. F., Trojahn, S., Sockell, A., Yee, M.-C., Tatarenkov, A., Bustamante, C. D., Earley, R. L., & Kelley, J. L. (2018). Whole-genome sequencing reveals the extent of heterozygosity in a preferentially self-fertilizing hermaphroditic vertebrate. *Genome*, *61*(4), 241-247. https://doi.org/10.1139/gen-2017-0188

Lyon, M. F. (1961). Gene Action in the X-chromosome of the Mouse (Mus musculus L.). *Nature*, *190*(4773), 372-373. https://doi.org/10.1038/190372a0

Mackiewicz, M., Tatarenkov, A., Perry, A., Martin, J. R., Elder, J. F., Bechler, D. L., & Avise, J. C. (2006). Microsatellite Documentation of Male-Mediated Outcrossing between Inbred Laboratory Strains of the Self-Fertilizing Mangrove Killifish (Kryptolebias Marmoratus). *Journal of Heredity*, *97*(5), 508-513. https://doi.org/10.1093/jhered/esl017

Magnhagen, C., & Bunnefeld, N. (2009). Express your personality or go along with the group : What determines the behavior of shoaling perch? *Proceedings of the Royal Society B: Biological Sciences*, *276*(1671), 3369-3375. https://doi.org/10.1098/rspb.2009.0851

Manikkam, M., Guerrero-Bosagna, C., Tracey, R., Haque, Md. M., & Skinner, M. K. (2012). Transgenerational Actions of Environmental Compounds on Reproductive Disease and Identification of Epigenetic Biomarkers of Ancestral Exposures. *PLoS ONE*, *7*(2), e31901. https://doi.org/10.1371/journal.pone.0031901

Marson, K. M., Taylor, D. S., & Earley, R. L. (2019). Cryptic Male Phenotypes in the Mangrove Rivulus Fish, Kryptolebias marmoratus. *The Biological Bulletin*, *236*(1), 13-28. https://doi.org/10.1086/700697

Massicotte, R., & Angers, B. (2012). General-Purpose Genotype or How Epigenetics Extend the Flexibility of a Genotype. *Genetics Research International*, *2012*, 1-7. https://doi.org/10.1155/2012/317175

Massicotte, R., Whitelaw, E., & Angers, B. (2011). DNA methylation : A source of random variation in natural populations. *Epigenetics*, *6*(4), 421-427. https://doi.org/10.4161/epi.6.4.14532

Mathot, K. J., Wright, J., Kempenaers, B., & Dingemanse, N. J. (2012). Adaptive strategies for managing uncertainty may explain personality-related differences in behavioral plasticity. *Oikos*, *121*(7), 1009-1020. https://doi.org/10.1111/j.1600-0706.2012.20339.x

Mayne, B., Korbie, D., Kenchington, L., Ezzy, B., Berry, O., & Jarman, S. (2020). A DNA methylation age predictor for zebrafish. *Aging*, *12*(24), 24817-24835. https://doi.org/10.18632/aging.202400

Mazor, T., Pankov, A., Johnson, B. E., Hong, C., Hamilton, E. G., Bell, R. J. A., Smirnov, I. V., Reis, G. F., Phillips, J. J., Barnes, M. J., Idbaih, A., Alentorn, A., Kloezeman, J. J., Lamfers, M. L. M., Bollen, A. W., Taylor, B. S., Molinaro, A. M., Olshen, A. B., Chang, S. M., … Costello, J. F. (2015). DNA Methylation and Somatic Mutations Converge on the Cell Cycle and Define Similar Evolutionary Histories in Brain Tumors. *Cancer Cell*, *28*(3), 307-317. https://doi.org/10.1016/j.ccell.2015.07.012

Mhanni, A. A., & McGowan, R. A. (2004). Global changes in genomic methylation levels during early development of the zebrafish embryo. *Development Genes and Evolution*, *214*(8). https://doi.org/10.1007/s00427-004-0418-0

Mitchell, D. J., & Biro, P. A. (2017). Is behavioral plasticity consistent across different environmental gradients and through time? *Proceedings of the Royal Society B: Biological Sciences*, *284*(1860), 20170893. https://doi.org/10.1098/rspb.2017.0893

Mora-Zamorano, F. X., Klingler, R., Basu, N., Head, J., Murphy, C. A., Binkowski, F. P., Larson, J. K., & Carvan, M. J. (2017). Developmental Methylmercury Exposure Affects Swimming Behavior and Foraging Efficiency of Yellow Perch (Perca flavescens) Larvae. *ACS Omega*, *2*(8), 4870-4877. https://doi.org/10.1021/acsomega.7b00227

Mourabit, S., Edenbrow, M., Croft, D. P., & Kudoh, T. (2011). Embryonic development of the self-fertilizing mangrove killifish Kryptolebias marmoratus. *Developmental Dynamics*, *240*(7), 1694-1704. https://doi.org/10.1002/dvdy.22668

Nespolo, R. F., & Franco, M. (2007). Whole-animal metabolic rate is a repeatable trait : A meta-analysis. *Journal of Experimental Biology*, *210*(11), 2000-2005. https://doi.org/10.1242/jeb.02780

Nikinmaa, M., & Anttila, K. (2019). Individual variation in aquatic toxicology : Not only unwanted noise. *Aquatic Toxicology*, *207*, 29-33. https://doi.org/10.1016/j.aquatox.2018.11.021

O'Dea, R. E., Noble, D. W. A., Johnson, S. L., Hesselson, D., & Nakagawa, S. (2016). The role of non-genetic inheritance in evolutionary rescue : Epigenetic buffering, heritable bet hedging and epigenetic traps. *Environmental Epigenetics*, *2*(1), dvv014. https://doi.org/10.1093/eep/dvv014

Orlando, F. E., & Guillette, J. L. (2001). A re-examination of variation associated with environmentally stressed organisms. *APMIS*, *109*(S103), S178-S186. https://doi.org/10.1111/j.1600-0463.2001.tb05765.x

Philippi, T., & Seger, J. (1989). Hedging one's evolutionary bets, revisited. *Trends in Ecology & Evolution*, *4*(2), 41-44. https://doi.org/10.1016/0169-5347(89)90138-9

Pigliucci, M., Müller, G., & Konrad Lorenz Institute for Evolution and Cognition Research (Éds.). (2010). *Evolution, the extended synthesis*. MIT Press.

Poey, F. (1880). Revisio piscium cubensium. *Sociedad ESpañola de Historia Natural*.

Potok, M. E., Nix, D. A., Parnell, T. J., & Cairns, B. R. (2013). Germline epigenetics, and reprogramming in zebrafish early embryos. *Epigenetics & Chromatin*, *6*(1), O23. https://doi.org/10.1186/1756-8935-6-S1-O23

Prokopuk, L., Western, P. S., & Stringer, J. M. (2015). Transgenerational epigenetic inheritance : Adaptation through the germline epigenome? *Epigenomics*, *7*(5), 829-846. https://doi.org/10.2217/epi.15.36

Rae, P. M. M., & Steele, R. E. (1979). Absence of cytosine methylation at C-C-G-G and G-C-G-C sites in the rDNA coding regions and intervening sequences of Drosophila and the rDNA of other higher insects. *Nucleic Acids Research*, *6*(9), 2987-2995. https://doi.org/10.1093/nar/6.9.2987

Rapp, R. A., & Wendel, J. F. (2005). Epigenetics and plant evolution. *New Phytologist*, *168*(1), 81-91. https://doi.org/10.1111/j.1469-8137.2005.01491.x

Rauluseviciute, I., Drabløs, F., & Rye, M. B. (2020). DNA hypermethylation associated with upregulated gene expression in prostate cancer demonstrates the diversity of epigenetic regulation. *BMC Medical Genomics*, *13*(1), 6. https://doi.org/10.1186/s12920-020-0657-6

Ravichandran, M., Jurkowska, R., & Jurkowski, T. (2017). Target specificity of mammalian DNA methylation and demethylation machinery. *Organic & Biomolecular Chemistry*, *16*. https://doi.org/10.1039/C7OB02574B

Réale, D., & Festa-Bianchet, M. (2003). Predator-induced natural selection on temperament in bighorn ewes. *Animal Behavior*, *65*(3), 463-470. https://doi.org/10.1006/anbe.2003.2100

Réale, D., Reader, S. M., Sol, D., McDougall, P. T., & Dingemanse, N. J. (2007). Integrating animal temperament within ecology and evolution. *Biological Reviews*, *82*(2), 291-318. https://doi.org/10.1111/j.1469-185X.2007.00010.x

Reed, T. E., Waples, R. S., Schindler, D. E., Hard, J. J., & Kinnison, M. T. (2010). Phenotypic plasticity and population viability : The importance of environmental predictability. *Proceedings of the Royal Society B: Biological Sciences*, *277*(1699), 3391-3400. https://doi.org/10.1098/rspb.2010.0771

Reik, W., Dean, W., & Walter, J. (2001). Epigenetic Reprogramming in Mammalian Development. *Science*, *293*(5532), 1089-1093. https://doi.org/10.1126/science.1063443

Richards, C. L., Bossdorf, O., & Pigliucci, M. (2010). What Role Does Heritable Epigenetic Variation Play in Phenotypic Evolution? *BioScience*, *60*(3), 232-237. https://doi.org/10.1525/bio.2010.60.3.9

Richards, E. J. (2006). Inherited epigenetic variation—Revisiting soft inheritance. *Nature Reviews Genetics*, *7*(5), 395-401. https://doi.org/10.1038/nrg1834

Richetto, J., & Meyer, U. (2021). Epigenetic Modifications in Schizophrenia and Related Disorders : Molecular Scars of Environmental Exposures and Source of Phenotypic Variability. *Biological Psychiatry*, *89*(3), 215-226. https://doi.org/10.1016/j.biopsych.2020.03.008

Rumbold, D. G., Evans, D. W., Niemczyk, S., Fink, L. E., Laine, K. A., Howard, N., Krabbenhoft, D. P., & Zucker, M. (2011). Source Identification of Florida Bay's Methylmercury Problem : Mainland Runoff Versus Atmospheric Deposition and In situ Production. *Estuaries and Coasts*, *34*(3), 494-513. https://doi.org/10.1007/s12237-010-9290-5

Russo, V. E. A., Martienssen, R. A., & Riggs, A. D. (Éds.). (1996). *Epigenetic mechanisms of gene regulation*. Cold Spring Harbor Laboratory Press.

Saha, M., Sarkar, S. K., & Bhattacharya, B. (2006). Interspecific variation in heavy metal body concentrations in biota of Sunderban mangrove wetland, northeast India. *Environment International*, *32*(2), 203-207. https://doi.org/10.1016/j.envint.2005.08.012

Sakakura, Y., Soyano, K., Noakes, D. L. G., & Hagiwara, A. (2006). Gonadal morphology in the self-fertilizing mangrove killifish, Kryptolebias marmoratus. *Ichthyological Research*, *53*(4), 427-430. https://doi.org/10.1007/s10228-006-0362-2

Samson, J. (2001). Delayed effects of embryonic exposure of zebrafish (Danio rerio) to methylmercury (MeHg). *Aquatic Toxicology*, *51*(4), 369-376. https://doi.org/10.1016/S0166-445X(00)00128-4

Scarsella, G. E., Gresham, J. D., & Earley, R. L. (2018). Relationships between external sexually dimorphic characteristics and internal gonadal morphology in a sex-changing fish. *Journal of Zoology*, *305*(2), 133-140. https://doi.org/10.1111/jzo.12546

Scheiner, S. M., & Holt, R. D. (2012). The genetics of phenotypic plasticity. X. Variation versus uncertainty. *Ecology and Evolution*, *2*(4), 751-767. https://doi.org/10.1002/ece3.217

Schlichting, C. D., & Wund, M. A. (2014). Phenotypic plasticity and epigenetic marking : An assessment of evidence for genetic accommodation. *Evolution*, *68*(3), 656-672. https://doi.org/10.1111/evo.12348

Schmitz, R. J., Schultz, M. D., Lewsey, M. G., O'Malley, R. C., Urich, M. A., Libiger, O., Schork, N. J., & Ecker, J. R. (2011). Transgenerational Epigenetic Instability Is a Source of Novel Methylation Variants. *Science*, *334*(6054), 369-373. https://doi.org/10.1126/science.1212959

Seong, K.-H., Li, D., Shimizu, H., Nakamura, R., & Ishii, S. (2011). Inheritance of Stress-Induced, ATF-2-Dependent Epigenetic Change. *Cell*, *145*(7), 1049-1061. https://doi.org/10.1016/j.cell.2011.05.029

Shimoda, N., Izawa, T., Yoshizawa, A., Yokoi, H., Kikuchi, Y., & Hashimoto, N. (2014). Decrease in cytosine methylation at CpG island shores and increase in DNA fragmentation during zebrafish aging. *AGE*, *36*(1), 103-115. https://doi.org/10.1007/s11357-013-9548-5

Sih, A., Stamps, J., Yang, L. H., McElreath, R., & Ramenofsky, M. (2010). Behavior as a Key Component of Integrative Biology in a Human-altered World. *Integrative and Comparative Biology*, *50*(6), 934-944. https://doi.org/10.1093/icb/icq148

Simons, A. M. (2011). Modes of response to environmental change and the elusive empirical evidence for bet hedging. *Proceedings of the Royal Society B: Biological Sciences*, *278*(1712), 1601-1609. https://doi.org/10.1098/rspb.2011.0176

Simpson, G. G. (1953). The Baldwin Effect. *Evolution*, *7*(2), 110. https://doi.org/10.2307/2405746

Simpson, V. J., Johnson, T. E., & Hammen, R. F. (1986). *Caenorhabditis elegans* DNA does not contain 5-methylcytosine at any time during development or aging. *Nucleic Acids Research*, *14*(16), 6711-6719. https://doi.org/10.1093/nar/14.16.6711

Sinn, D. L., Gosling, S. D., & Moltschaniwskyj, N. A. (2008). Development of shy/bold behavior in squid : Context-specific phenotypes associated with developmental plasticity. *Animal Behavior*, *75*(2), 433-442. https://doi.org/10.1016/j.anbehav.2007.05.008

Skinner, M. K. (2011). Environmental epigenetic transgenerational inheritance and somatic epigenetic mitotic stability. *Epigenetics*, *6*(7), 838-842. https://doi.org/10.4161/epi.6.7.16537

Skinner, M. K., Guerrero-Bosagna, C., & Haque, M. M. (2015). Environmentally induced epigenetic transgenerational inheritance of sperm epimutations promote genetic mutations. *Epigenetics*, *10*(8), 762-771. https://doi.org/10.1080/15592294.2015.1062207

Skinner, M. K., & Nilsson, E. E. (2021). Role of environmentally induced epigenetic transgenerational inheritance in evolutionary biology : Unified Evolution Theory. *Environmental Epigenetics*, *7*(1), dvab012. https://doi.org/10.1093/eep/dvab012

Slotkin, R. K., & Martienssen, R. (2007). Transposable elements and the epigenetic regulation of the genome. *Nature Reviews Genetics*, *8*(4), 272-285. https://doi.org/10.1038/nrg2072

Smith, L. E., Carvan, M. J., Dellinger, J. A., Ghorai, J. K., White, D. B., Williams, F. E., & Weber, D. N. (2010). Developmental selenomethionine and methylmercury exposures affect zebrafish learning. *Neurotoxicology and Teratology*, *32*(2), 246-255. https://doi.org/10.1016/j.ntt.2009.09.004

Soto, C. G., Leatherland, J. F., & Noakes, D. L. G. (1992). Gonadal histology in the self-fertilizing hermaphroditic fish *Rivulus marmoratus* (Pisces, Cyprinodontidae). *Canadian Journal of Zoology*, *70*(12), 2338-2347. https://doi.org/10.1139/z92-314

Spainhour, J. C., Lim, H. S., Yi, S. V., & Qiu, P. (2019). Correlation Patterns Between DNA Methylation and Gene Expression in The Cancer Genome Atlas. *Cancer Informatics*, *18*, 117693511982877. https://doi.org/10.1177/1176935119828776

Stamps, J. A. (2016). Individual differences in behavioral plasticities. *Biological Reviews*, *91*(2), 534-567. https://doi.org/10.1111/brv.12186

Stamps, J., & Groothuis, T. G. G. (2010). The development of animal personality : Relevance, concepts and perspectives. *Biological Reviews*, *85*(2), 301-325. https://doi.org/10.1111/j.1469-185X.2009.00103.x

Stearns, S. C. (1989). The Evolutionary Significance of Phenotypic Plasticity. *BioScience*, *39*(7), 436-445. https://doi.org/10.2307/1311135

Stedman, E., & Stedman, E. (1950). Cell Specificity of Histones. *Nature*, *166*(4227), Art. 4227. https://doi.org/10.1038/166780a0

Suzuki, M. M., & Bird, A. (2008). DNA methylation landscapes : Provocative insights from epigenomics. *Nature Reviews Genetics*, *9*(6), 465-476. https://doi.org/10.1038/nrg2341

Tamm, C., Duckworth, J. K., Hermanson, O., & Ceccatelli, S. (2008). Methylmercury inhibits differentiation of rat neural stem cells via Notch signalling. *NeuroReport*, *19*(3), 339-343. https://doi.org/10.1097/WNR.0b013e3282f50ca4

Tatarenkov, A., Earley, R. L., Perlman, B. M., Scott Taylor, D., Turner, B. J., & Avise, J. C. (2015). Genetic Subdivision and Variation in Selfing Rates Among Central American Populations of the Mangrove Rivulus, Kryptolebias marmoratus. *Journal of Heredity*, *106*(3), 276-284. https://doi.org/10.1093/jhered/esv013

Tatarenkov, A., Earley, R. L., Taylor, D. S., & Avise, J. C. (2012). Microevolutionary Distribution of Isogenicity in a Self-fertilizing Fish (Kryptolebias marmoratus) in the Florida Keys. *Integrative and Comparative Biology*, *52*(6), 743-752. https://doi.org/10.1093/icb/ics075

Tatarenkov, A., Gao, H., Mackiewicz, M., Taylor, D. S., Turner, B. J., & Avise, J. C. (2007). Strong population structure despite evidence of recent migration in a selfing hermaphroditic vertebrate, the mangrove killifish (Kryptolebias marmoratus). *Molecular Ecology*, *16*(13), 2701-2711. https://doi.org/10.1111/j.1365-294X.2007.03349.x

Tatarenkov, A., Lima, S. M. Q., Earley, R. L., Berbel-Filho, W. M., Vermeulen, F. B. M., Taylor, D. S., Marson, K., Turner, B. J., & Avise, J. C. (2017). Deep and concordant subdivisions in the self-fertilizing mangrove killifishes (Kryptolebias) revealed by nuclear and mtDNA markers. *Biological Journal of the Linnean Society*, *122*(3), 558-578. https://doi.org/10.1093/biolinnean/blx103

Tatarenkov, A., Ring, B. C., Elder, J. F., Bechler, D. L., & Avise, J. C. (2010). Genetic Composition of Laboratory Stocks of the Self-Fertilizing Fish Kryptolebias marmoratus : A Valuable Resource for Experimental Research. *PLOS ONE*, *5*(9), e12863. https://doi.org/10.1371/journal.pone.0012863

Taylor, D. S. (1990). Adaptive Specializations of the Cyprinodont Fish Rivulus Marmoratus. *Florida Scientist*, *53*(3), 239-248.

Taylor, D. S. (2000). Biology and Ecology of Rivulus Marmoratus : New Insights and a Review. *Florida Scientist*, *63*(4), 242-255.

Taylor, D. S. (2012). Twenty-Four Years in the Mud : What Have We Learned About the Natural History and Ecology of the Mangrove Rivulus, Kryptolebias marmoratus? *Integrative and Comparative Biology*, *52*(6), 724-736. https://doi.org/10.1093/icb/ics062

Thorson, J. L. M., Smithson, M., Beck, D., Sadler-Riggleman, I., Nilsson, E., Dybdahl, M., & Skinner, M. K. (2017). Epigenetics and adaptive phenotypic variation between habitats in an asexual snail. *Scientific Reports*, *7*(1), 14139. https://doi.org/10.1038/s41598-017-14673-6

Thorson, J. L. M., Smithson, M., Sadler-Riggleman, I., Beck, D., Dybdahl, M., & Skinner, M. K. (2019). Regional epigenetic variation in asexual snail populations among urban and rural lakes. *Environmental Epigenetics*, *5*(4), dvz020. https://doi.org/10.1093/eep/dvz020

Tinbergen, N. (1963). On aims and methods of Ethology. *Zeitschrift Für Tierpsychologie*, *20*(4), 410-433. https://doi.org/10.1111/j.1439-0310.1963.tb01161.x

Turko, A. J., Earley, R. L., & Wright, P. A. (2011). Behavior drives morphology : Voluntary emersion patterns shape gill structure in genetically identical mangrove rivulus. *Animal Behavior*, *82*(1), 39-47. https://doi.org/10.1016/j.anbehav.2011.03.001

Turko, A. J., Tatarenkov, A., Currie, S., Earley, R. L., Platek, A., Taylor, D. S., & Wright, P. A. (2018). Emersion behavior underlies variation in gill morphology and aquatic respiratory function in the amphibious fish *Kryptolebias marmoratus*. *The Journal of Experimental Biology*, *221*(8), jeb168039. https://doi.org/10.1242/jeb.168039

Turner, B. J., Fisher, M. T., Taylor, D. S., Davis, W. P., & Jarrett, B. L. (2006). Evolution of 'maleness' and outcrossing in a population of the self-fertilizing killifish,. *Evolutionary Ecology Research*, *8*(8), 1475-1486.

Tuscher, J. J., & Day, J. J. (2019). Multigenerational epigenetic inheritance : One step forward, two generations back. *Neurobiology of Disease*, *132*, 104591. https://doi.org/10.1016/j.nbd.2019.104591

van der Graaf, A., Wardenaar, R., Neumann, D. A., Taudt, A., Shaw, R. G., Jansen, R. C., Schmitz, R. J., Colomé-Tatché, M., & Johannes, F. (2015). Rate, spectrum, and evolutionary dynamics of spontaneous epimutations. *Proceedings of the National Academy of Sciences*, *112*(21), 6676-6681. https://doi.org/10.1073/pnas.1424254112

Venney, C. J., Johansson, M. L., & Heath, D. D. (2016). Inbreeding effects on gene-specific DNA methylation among tissues of Chinook salmon. *Molecular Ecology*, *25*(18), 4521-4533. https://doi.org/10.1111/mec.13777

Vogt, G. (2015). Stochastic developmental variation, an epigenetic source of phenotypic diversity with far-reaching biological consequences. *Journal of Biosciences*, *40*(1), 159-204. https://doi.org/10.1007/s12038-015-9506-8

Vogt, G. (2017). Facilitation of environmental adaptation and evolution by epigenetic phenotype variation : Insights from clonal, invasive, polyploid, and domesticated animals. *Environmental Epigenetics*, *3*(1), dvx002. https://doi.org/10.1093/eep/dvx002

Vogt, G., Huber, M., Thiemann, M., van den Boogaart, G., Schmitz, O. J., & Schubart, C. D. (2008). Production of different phenotypes from the same genotype in the same environment by developmental variation. *Journal of Experimental Biology*, *211*(4), 510-523. https://doi.org/10.1242/jeb.008755

Voisin, A.-S., Fellous, A., Earley, R. L., & Silvestre, F. (2016). Delayed impacts of developmental exposure to 17-α-ethinylestradiol in the self-fertilizing fish Kryptolebias marmoratus. *Aquatic Toxicology*, *180*, 247-257. https://doi.org/10.1016/j.aquatox.2016.10.003

Voisin, A.-S., Suarez Ulloa, V., Stockwell, P., Chatterjee, A., & Silvestre, F. (2021). Genome-wide DNA methylation of the liver reveals delayed effects of early-life exposure to 17-α-ethinylestradiol in the self-fertilizing mangrove rivulus. *Epigenetics*, 1-25. https://doi.org/10.1080/15592294.2021.1921337

Wang, X., & Bhandari, R. K. (2019). DNA methylation dynamics during epigenetic reprogramming of medaka embryo. *Epigenetics*, *14*(6), 611-622. https://doi.org/10.1080/15592294.2019.1605816

Webber, Q. M. R., Laforge, M. P., Bonar, M., Robitaille, A. L., Hart, C., Zabihi-Seissan, S., & Vander Wal, E. (2020). The Ecology of Individual Differences Empirically Applied to Space-Use and Movement Tactics. *The American Naturalist*, *196*(1), E1-E15. https://doi.org/10.1086/708721

Weber, D. N., Connaughton, V. P., Dellinger, J. A., Klemer, D., Udvadia, A., & Carvan, M. J. (2008). Selenomethionine reduces visual deficits due to developmental methylmercury exposures. *Physiology & Behavior*, *93*(1), 250-260. https://doi.org/10.1016/j.physbeh.2007.08.023

West-Eberhard, M. J. (1986). Alternative adaptations, speciation, and phylogeny (A Review). *Proceedings of the National Academy of Sciences*, *83*(5), 1388-1392. https://doi.org/10.1073/pnas.83.5.1388

Westneat, D. F., Wright, J., & Dingemanse, N. J. (2015). The biology hidden inside residual within-individual phenotypic variation : The biology of residual phenotypic variance. *Biological Reviews*, *90*(3), 729-743. https://doi.org/10.1111/brv.12131

Wolf, M., & Weissing, F. J. (2012). Animal personalities : Consequences for ecology and evolution. *Trends in Ecology & Evolution*, *27*(8), 452-461. https://doi.org/10.1016/j.tree.2012.05.001

Wright, P. A. (2012). Environmental Physiology of the Mangrove Rivulus, Kryptolebias marmoratus, A Cutaneously Breathing Fish That Survives for Weeks Out of Water. *Integrative and Comparative Biology*, *52*(6), 792-800. https://doi.org/10.1093/icb/ics091

Wu, P., Kainz, M. J., Bravo, A. G., Åkerblom, S., Sonesten, L., & Bishop, K. (2019). The importance of bioconcentration into the pelagic food web base for methylmercury biomagnification : A meta-analysis. *Science of The Total Environment*, *646*, 357-367. https://doi.org/10.1016/j.scitotenv.2018.07.328

Wu, S.-F., Zhang, H., Hammoud, S. S., Potok, M., Nix, D. A., Jones, D. A., & Cairns, B. R. (2011). DNA Methylation Profiling in Zebrafish. In H. W. Detrich, M. Westerfield, & L. I. Zon (Éds.), *Methods in Cell Biology* (Vol. 104, p. 327-339). Academic Press. https://doi.org/10.1016/B978-0-12-374814-0.00018-5

Xiaojuan Xu. (2012). Developmental methylmercury exposure affects avoidance learning outcomes in adult zebrafish. *Journal of Toxicology and Environmental Health Sciences*, *4*(5). https://doi.org/10.5897/JTEHS12.004

Xu, X., Li, G., Li, C., Zhang, J., Wang, Q., Simmons, D. K., Chen, X., Wijesena, N., Zhu, W., Wang, Z., Wang, Z., Ju, B., Ci, W., Lu, X., Yu, D., Wang, Q., Aluru, N., Oliveri, P., Zhang, Y. E., … Liu, J. (2019). Evolutionary transition between invertebrates and vertebrates via methylation reprogramming in embryogenesis. *National Science Review*, *6*(5), 993-1003. https://doi.org/10.1093/nsr/nwz064

Xu, X., Weber, D., Martin, A., & Lone, D. (2016). Trans-generational transmission of neurobehavioral impairments produced by developmental methylmercury exposure in zebrafish (Danio rerio). *Neurotoxicology and Teratology*, *53*, 19-23. https://doi.org/10.1016/j.ntt.2015.11.003

Young, R. L., & Badyaev, A. V. (2007). Evolution of ontogeny : Linking epigenetic remodeling and genetic adaptation in skeletal structures. *Integrative and Comparative Biology*, *47*(2), 234-244. https://doi.org/10.1093/icb/icm025

Zemach, A., McDaniel, I. E., Silva, P., & Zilberman, D. (2010). Genome-Wide Evolutionary Analysis of Eukaryotic DNA Methylation. *Science*, *328*(5980), 916-919. https://doi.org/10.1126/science.1186366

Zhu, J., Tang, L., Qiao, S., Wang, L., Feng, Y., Wang, L., Wu, Q., Ding, P., Zhang, Z., & Li, L. (2020). Low-dose methylmercury exposure impairs the locomotor activity of zebrafish : Role of intestinal inositol metabolism. *Environmental Research*, *190*, 110020. https://doi.org/10.1016/j.envres.2020.110020

ACKNOWLEDGMENTS

First, I would like to thank my supervisor Frédéric Silvestre. After being my professor and my master thesis advisor, you helped me to write my FNRS project which was the starting point of this adventure. Thanks to your trust and your enthusiasm, I learned and did a lot of different things during these four years! Thank you for giving me the opportunity to participate to scientific congresses, and to do the unforgettable field missions in Florida and Belize. I hope you will have the opportunity to return there, and to accomplish your future research projects. There are a lot of them! You are an excellent advisor, you helped me at every step of my thesis, I could always count on you. I wish you to have more time to do research with your team.

I would like to thank the members of my thesis committee for their advice, willingness to help and availability. Ryan, I will never forget our moments spent in your natural habitat, the mangroves. I can say the same about Kristy Marson, who was even more excited than you were about going out to sample rivulus. I discovered a side of research I didn't know about, and I loved it! I would like to thank my co-supervisor Caroline, and also Victoria, Alice and Sofia for the interest you manifested in our research and for your good advices. Thank you all for being present at the important steps of my PhD thesis.

Thank you to my URBE colleagues, I will remember the good atmosphere during the numerous Happy Hours, Christmas dinners or during simple talks in the office. I would particularly like to thank Alessandra, Antoine Wittttorski, Ivan, Anthony, Jérôme and Thibaut who became much more than colleagues, and my dear witches Mathilde and Mahaut. You were the reason I got up in a good mood in the morning to come to the lab.

Thank you to my family and my friends who always supported me even if they did not quite understand what I was doing. I will miss the question “what are you exactly doing?” or "how are your fish?" knowing that I had not worked with it for a long time. A special thanks to my Dudu for encouraging and supporting me through this journey. Even if you are sometimes tactless, you have always been there during my “PhD bad times”.

And finally, all this would not have been possible without the financial support of several organizations. I would like to thank the FRS-FNRS (Belgian National Fund for Scientific Research) for funding these 4 years of PhD. Thanks to grants accorded by Institute of life, earth and environment (ILEE) and UNamur research counsel, I was able to carry out several field missions as well as participate to international conferences to disseminate my results, which is the ultimate goal of all research.