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The evolutionary potential of DNA methylation in the gonads of two populations of the mangrove rivulus (*Kryptolebias marmoratus*) and its developmental reprogramming

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Academic year of 2022-2023

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Definition of epigenetics

The developmental biologist Conrad H. Waddington (1905-1975) invented the word "epigenetics" to summarize a novel branch of biology which focuses on the links between gene and protein expression **(Waddington, 2012)**. In 1957, Waddington proposed the epigenetic landscape, where a ball symbolizing a cell, could follow different paths depending on the roughness of the surface (meaning intra- and extracellular environmental influences) **(Figure 1) (Waddington, 1957; Goldberg *et al.*, 2007)**. Although the general organization of DNA was roughly understood quite early in the middle of the 20th century, the booming of epigenetics arrived much later during the 1990s and 2000s with the flow of cloning and biochemical techniques permitting the identification of specific enzymes, writers, and erasers of epigenetic marks **(Peixoto *et al.*, 2020)**. The recent advances in sequencing technologies as well as the development of techniques profiling epigenetic marks and chromatin accessibility (using reagents applicable in any species) have rocketed epigenomic studies in multiple animal species **(Sadler, 2023)**. Ultimately, this allows to enlighten the vast epigenomic mechanisms utilized across the evolutionary tree **(Sadler, 2023)**, and the importance and complexity of the eukaryotic epigenome is now recognized **(Lowdon *et al.*, 2016)**.

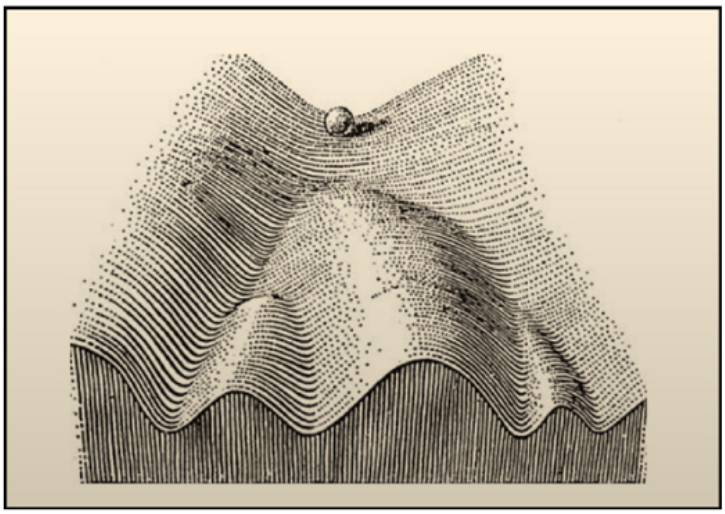

Figure 1: Waddington's epigenetic landscape. The epigenetic landscape represents the process of cellular decision-making during its development. The ball represents the cell, which can take various permitted trajectories leading to diverse outcomes or cell fates **(Goldberg *et al.*, 2007, reprinted from Waddington, 1957)**.

The definition of epigenetics has narrowed in recent years to genome-associated mechanisms of non-DNA sequence-based inheritance **(Perez & Lehner, 2019)**. In other words, epigenetics can be defined as mitotically and/or meiotically heritable changes in gene expression that can't be explained by changes in the gene sequence, therefore epigenetic variation doesn't alter the underlying nucleotide sequence **(Russo *et al.*, 1996; Youngson & Whitelaw, 2008; Lamka *et al.*, 2022)**. Such changes are mainly represented by DNA methylation, histone modifications and small RNA regulation, mechanisms implicated in processes such as cellular differentiation and development, behaviors, metabolism, morphology, and physiological phenotypes for example, by affecting gene expression as well as protein synthesis **(Nicoglou & Merlin, 2017; Perez & Lehner, 2019; Lamka *et al.*, 2022)**. However, there are numerous other epigenetic fields and mechanisms that have already been discovered such as: histone-related acetylation, histone methylation, post-translational histone modifications such as histone ubiquitination/ADP ribosylation/sumoylation, and epitranscriptomics (non-coding RNA, RNA methylation) **(Peixoto *et al.*, 2020)**.

The most studied and known epigenetic markers, DNA methylation (5mC, for the methylation of the 5th carbon of a cytosine nucleobase) and post-translational histone modifications, were quickly identified following the resolution of the DNA double helix structure **(Peixoto *et al.*, 2020)**. As a matter of fact, DNA methylation was first described in 1965 **(Craddock & Magee, 1965; Scarano *et al.*, 1965)**.

The regulatory role of epigenetics in living organisms

It has long been assumed that heritable variation due to DNA sequence differences (in other words genetic variation), allows populations of organisms to be hardy and adaptable to extreme environmental conditions **(Kilvitis *et al.*, 2014)**. Natural selection acts on the variation among different genotypes, ultimately changing the genetic composition of a population **(Kilvitis *et al.*, 2014)**. On the one hand, there is gripping evidence about the importance of genetic polymorphisms, but on the other hand evidence is also growing that epigenetic mechanisms (*e.g.*, DNA methylation and chromatin modifications) can affect ecologically important traits even if there is an absence of genetic variation **(Figure 2) (Kilvitis *et al.*, 2014)**. Therefore, there is an increasing interest in the causes and consequences of epigenetic variation (particularly DNA methylation, histone modifications and non-coding RNAs) in natural populations **(Husby, 2022; Laine *et al.*, 2022)**.

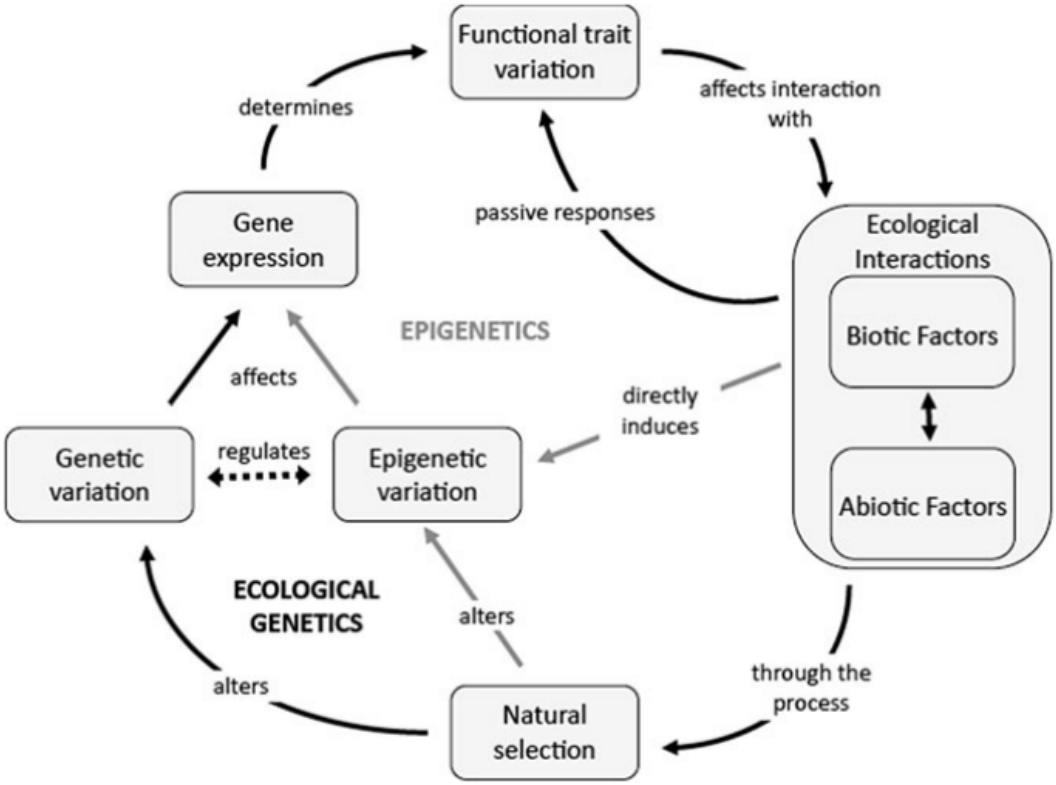

Figure 2: Interaction between genetic processes (in black), epigenetic ones (in grey), and the environment. The focus on functional trait variation emphasizes the need for data making the bridge between epigenetic loci and specific ecologically relevant phenotypes. The inclusion of abiotic and biotic factors in ecological interactions is highlighted, both factors can play roles in maintaining and creating epigenetic variation **(Kilvitis *et al.*, 2014)**.

Life-history traits, physiology, behavior, morphology, and sexual phenotype are greatly shaped by the interaction of genes with the environment, and many of these traits seem to co-vary **(Earley *et al.*, 2012)**. Organisms adapt to different environments by selection of the most suitable phenotypes from genetic variation or by phenotypic plasticity, which is the ability of a single genotype to produce diverse phenotypes in different environments **(Vogt, 2022)**. Phenotypic plasticity is an important mechanism allowing populations to face environmental changes **(Hu & Barrett, 2017)**. It has been recognized for a long time now that natural populations have genetic variation extending to plasticity, and growing recent evidence suggest that epigenetic variation may also contribute to shape phenotypic responses **(Hu & Barrett, 2017)**. All animals have evolved solutions to manage their genomes, enabling the efficient organization of meters of DNA strands in the nucleus, allowing specific regulation of gene expression while keeping transposable elements suppressed **(Sadler, 2023)**. It appears that epigenetic modifications are central to these accomplishments **(Sadler, 2023)**. For a given genotype, the epigenetic variation could be environmentally induced or occur randomly **(Angers *et al.*, 2020)**. In this extent, some strategies have been developed by organisms to face environmental

fluctuations such as phenotypic plasticity (environmentally induced) and diversified bet-hedging (relying on random occurrence, and is defined as a risk-spreading strategy exhibited by isogenic populations which evolved in unpredictably changing environments **(Slatkin, 1974; De Jong *et al.*, 2011)**). In addition, random variations can also represent a clue of developmental stability and can thus be used to assess how organisms deal with stressful environmental conditions **(Angers *et al.*, 2020)**.

Epigenetic processes manage gene expression and their products in a real-time perspective, allowing a single genome to display different phenotypes **(Angers *et al.*, 2020)**. Studies tackling phenotypic plasticity have demonstrated that epigenetic variation can play a primary role in an organism's response to environmental fluctuations, because epigenetic marks can be directly affected by the environment **(Chapelle & Silvestre, 2022)**. Notably, case studies revealed habitat-specific epigenetic marks maintained over subsequent years, suggesting the existence of epigenetic ecotypes **(Vogt, 2022)**. Formulated differently, epigenetic variations induced by the environment can arbitrate phenotypic plasticity and local adaptation because they permit the organisms to adapt to the environmental conditions by increasing the phenotypic possibilities of a genotype without any genetic sequence modification **(Angers *et al.*, 2010; Verhoeven & Preite, 2014)**. Hence, epigenetics can be seen as the 'missing link' between environmental and phenotypic variations **(Herrel *et al.*, 2020)**. Environmentally induced epimutations and their corresponding gene expression changes procure an ideal mean for directional and fast adaptation to changing or novel conditions because they can synchronously alter phenotypes in a population **(Daxinger & Whitelaw, 2010; Vogt, 2022)**.

The evolutionary potential of epigenetic mechanisms

Understanding the evolutionary implications of epigenetics and how its mechanisms participate to phenotypic variability is currently a great challenge in evolutionary biology **(Navarro-Martín *et al.*, 2020; Chapelle & Silvestre, 2022)**. Studies focusing on genetic variation and the manipulation of environmental conditions have confirmed that genotype, environment, and their interaction contribute to the variability of phenotypes, which is a key prerequisite for evolution by natural selection **(Chapelle & Silvestre, 2022)**. The Modern Evolutionary Synthesis assumes that genetic diversity is the only source of heritable variation in natural populations **(Mayr & Provine, 1998; Mameli, 2004)**. More specifically, random mutations can justify this genetic variability and therefore promote evolution by means of natural selection **(Mayr & Provine, 1998)**. Nonetheless, increasing evidence shows that genetic variation isn't the only source of heritable phenotypic diversity, and that epigenetic variation can also participate to heritable changes within populations, thus driving rapid evolution **(Bossdorf *et al.*, 2008; Skinner, 2015)**. It is nowadays admitted that genetic variation isn't the only source of phenotypic variation that can be inherited across generations, this is because only a small proportion of variance in complex traits can be explained by genetic variance **(Mameli, 2004)**. In addition to being another source of phenotypic variation, epigenetic variation can precede genetic adaptation *via* genetic accommodation (*i.e.*, quantitative genetic change in the frequency of genes affecting the regulation or form of a new trait **(West-Eberhard, 2003, 2005)**), therefore reversing the common model of evolution from a genotype-to-phenotype to a phenotype-to-genotype circuit **(West-Eberhard, 1986; Jablonka & Lamb, 1989)**.

When compared with genetic variation, epigenetic variation is more prompt to have higher spontaneous rates of mutation as well as enhanced sensitivity reaction to environmental stimuli, possibly providing the raw material for phenotypic selection when genetic variation is limited **(Drake *et al.*, 1998; Ossowski *et al.*, 2010; Becker *et al.*, 2011; Schmitz *et al.*, 2011; Zhang *et al.*, 2013)**. Hence, an evolutionary potential of epigenetic variation can be proposed due to its autonomy from genetic variation, in addition to its transgenerational stability (inherited *via* non-mendelian processes) **(Hu & Barrett, 2017; Chapelle & Silvestre, 2022)**. For example, there is a degree of autonomy of DNA methylation variation from genetics, eventually illustrating

its added value in evolutionary mechanisms **(Chapelle & Silvestre, 2022)**. Some ecological processes act on epigenetic variation and patterns, other act on both epigenetic and genetic structures. Therefore, it is important to consider these processes to comprehend the current patterns of genetic and epigenetic variation, in addition to the past and future dynamics of populations' epigenetics **(Chapelle & Silvestre, 2022)**.

The extended theory of evolution includes epigenetic variation as an evolutionary mechanism in natural populations **(Figure 3) (Schrey *et al.*, 2012; Chapelle & Silvestre, 2022)**. More precisely, epigenetic variation might contribute to population microevolution (*i.e.*, rapid evolutionary events which are adaptations to a novel environment during introduction and interfering events in rapidly changing habitats and when stressors are present) and to population macroevolution (encompassing speciation and radiation) **(Chapelle & Silvestre, 2022)**. Being able to differentiate the random epimutations from environmentally induced ones, and also the heritable epimutations from the non-heritable ones, could allow the characterization of the responses of organisms to environmental changes **(Herrel *et al.*, 2020; Chapelle & Silvestre, 2022)**. This is because any variation in DNA methylation within a species may indeed enlighten how these organisms evolve in this given environment **(Chapelle & Silvestre, 2022)**. Therefore, recent progress in ecological epigenetics allows a more complete comprehension of how epigenetic diversity is modulated over time, this will be handy for generating predictive models of the capacity of organisms and populations to adapt to environmental variation and changing climates **(Hu & Barrett, 2017; Chapelle & Silvestre, 2022)**.

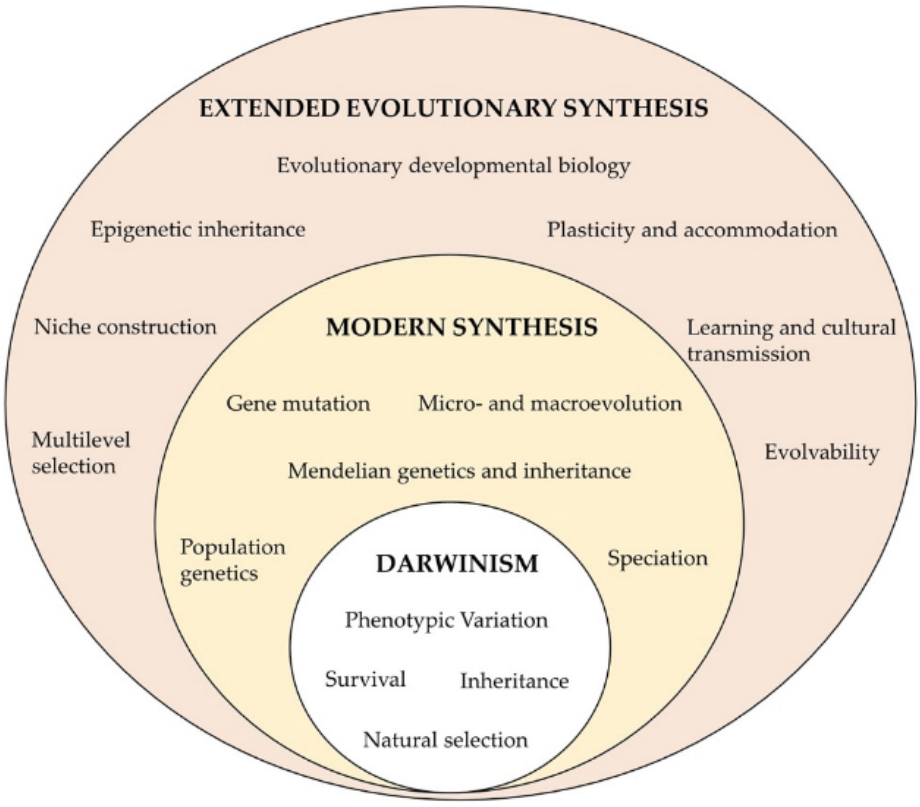

Figure 3: Schematic representation of key concepts that are incorporated within the Extended Synthesis. It demonstrates the continuous expansion of evolutionary theories **(Chapelle & Silvestre, 2022)**.

Special focus on DNA methylation

Cytosine methylation is a DNA modification that is crucial for vertebrate development and procures a plastic but still stable information in addition to the DNA code **(Ortega-Recalde & Hore, 2019)**. DNA methylation is the most broadly characterized epigenetic mechanism, both in plants and animals **(Ambrosi *et al.*, 2017; De Mendoza *et al.*, 2020)**. This stable epigenetic mechanism has important roles in the normal functioning of a cell and thus also in disease etiology for example **(Chatterjee *et al.*, 2017)**. DNA methylation occurs

across all taxa of life, and is primarily referring to the transfer of a methyl group to position 5 of cytosine residues, forming 5-methylcytosine (5mC) in prokaryotes and eukaryotes **(Zemach *et al.*, 2010; Ambrosi *et al.*, 2017)**. In animals, it concerns the addition of a methyl group to a cytosine within CpG dinucleotides **(Lister & Ecker, 2009; Feng *et al.*, 2010; Law & Jacobsen, 2010; Ambrosi *et al.*, 2017; Chapelle & Silvestre, 2022)**. The distribution of DNA methylation across the genome has been studied in many clades of animals, however differences appear in how and where this epigenetic mechanism occurs. More precisely, in vertebrates, the extent and the pattern of DNA methylation is well conserved throughout species, DNA methylation indeed takes place nearly across the whole genome, with 70-80% of cytosines included in CpG dinucleotides being methylated **(Feng *et al.*, 2010)**. DNA methylation levels vary widely among eukaryotes as there is a very limited methylation in ecdysozoan protostomes for example and high methylation in CpGs of vertebrates, whereas invertebrates, fungi and plants have a mosaic methylation, marked by interspersed methylated and unmethylated domains **(Gowher *et al.*, 2000; Suzuki & Bird, 2008; Chapelle & Silvestre, 2022)**. Often, DNA methylation (5mC) of regulatory regions is associated with gene down-regulation or silencing, however it isn't always the case **(Feng *et al.*, 2010; Spainhour *et al.*, 2019; Peixoto *et al.*, 2020; Rauluseviciute *et al.*, 2020)**. Gene bodies including introns and exons are consistently methylated, whereas CpGs in the gene promoter regions are commonly poorly methylated **(Suzuki & Bird, 2008; Hon *et al.*, 2013)**. Gene body methylation is positively correlated with transcriptional activity in the majority of animal species **(De Mendoza *et al.*, 2020)**. As it is typically associated with transcriptional repression, DNA methylation may thus also impact compaction of chromatin (heterochromatin maintenance) and hence transcriptional activity of a genomic region by directly interfering with DNA binding of the transcriptional machinery, or throughout a methyl-DNA-binding domain (MBD) protein such as MeCP2 (recruitment of MeCP2 to methylated CpG and its implication in gene repression) **(Kriaucionis & Bird, 2003; Lindeman *et al.*, 2010; Liang *et al.*, 2011)**. 5mC is the most stable epigenetic marker and is consequently of great biological significance because of its role in genome stability **(Maloisel & Rossignol, 1998)**, X-chromosome inactivation **(Heard & Disteche, 2006)**, imprinting **(Biémont, 2010)**, gene expression, and development **(Jaenisch & Bird, 2003)**.

Several well-known DNA methylation regulatory proteins have been identified, for example, the formation of 5mC is a reaction catalyzed by many enzymes, the DNA (cytosine-5)-methyltransferases (DNMTs) with three conserved proteins, DNMT1, DNMT3A and DNMT3B, characterized in vertebrates **(Figure 4) (Goll & Bestor, 2005; Campos *et al.*, 2012; Ambrosi *et al.*, 2017)**. The DNA methyl transferase 1 (DNMT1) is the first described writer of 5mC, it is the maintenance enzyme, restoring DNA methylation following DNA replication, thus supporting the transmission of this epigenetic mark to future daughter cells during mitosis **(Goll & Bestor, 2005; Ambrosi *et al.*, 2017; Peixoto *et al.*, 2020)**. DNMT1 was the first identified eukaryotic methyltransferase, and its corresponding gene (*DNMT1*) is essential for transposon silencing, imprinted gene regulation, and X inactivation, showing that the DNA methylation writing is involved in a myriad of biological processes **(Peixoto *et al.*, 2020)**. Moreover, DNMT3A and DNMT3B explain the acquisition of DNA methylation on both strands of DNA, independently of DNA replication, in other words, they catalyze *de novo* methylation **(Okano *et al.*, 1999; Goll & Bestor, 2005; Ambrosi *et al.*, 2017)**. This phenomenon is very important for gene expression regulation during embryonic development as well as aberrant gene repression in numerous diseases such as cancers **(Okano *et al.*, 1999)**. Another well-known DNA methylation regulatory protein is the TET (Ten-Eleven Translocation 1, 2 and 3) family, which is an eraser (DNA demethylase) of 5mC **(Ito *et al.*, 2010)**. In addition to 5mC, there is another modification, DNA 5-hydroxymethylation (5hmC), which has recently been characterized in vertebrate genomes **(Zhao & Chen, 2013)**, and is catalyzed by those TET enzymes *via* oxidation of 5mC marks **(Tahiliani *et al.*, 2009)**.

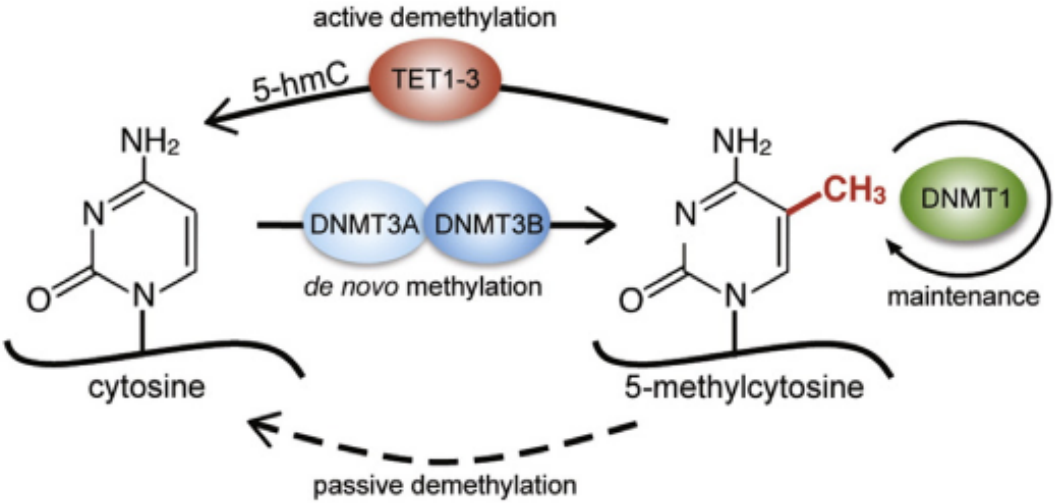

Figure 4: Key actors in the DNA methylation pathway. In eukaryotes, DNA methylation mostly occurs at the fifth carbon atom of cytosine bases. Its deposition is catalyzed by the *de novo* DNMTs, DNMT3A and DNMT3B. Methylation patterns are conserved by the maintenance DNMT, DNMT1, during replication. Passive DNA demethylation is considered to be achieved throughout cell division in the absence of DNMT1 maintenance activity. Inversely, active removal encompasses the mammalian TET1-3 proteins capable of converting 5-methylcytosine to its oxidized derivative 5-hydroxymethylcytosine (5hmC) (and even further to 5-formylcytosine and 5-carboxylcytosine, not shown here). These modifications are removed through DNA repair processes or are passively lost during replication. Furthermore, DNA repair processes are implicated in the direct removal of methylated cytosines **(Ambrosi *et al.*, 2017)**.

Epigenetic mechanisms such as DNA methylation, added to genetic variation, might greatly contribute to heritable phenotypic diversity in populations **(Fellous *et al.*, 2018)**. DNA methylation variation is autonomous from genetic variation in wild animal populations, therefore this methylation variation is relevant as an evolutionary mechanism, considering the extended evolutionary synthesis **(Chapelle & Silvestre, 2022)**. In fact, DNA methylation diversity could inform parameters usable for characterizing natural animal populations **(Chapelle & Silvestre, 2022)**.

Description of the model organism, the mangrove rivulus (*Kryptolebias marmoratus*)

Another obstacle that must be overcome to study the role of epigenetic variation in evolution is the fact that DNA sequence differences among individuals can rarely be excluded to explain heritability of phenotypes **(Fellous *et al.*, 2018)**. Therefore, a clear understanding of the role of epigenetic variation in evolution can better be achieved in individuals that are genetically identical while displaying a range of heritable phenotypes in nature **(Heard & Martienssen, 2014)**. Recent studies on the whole-genome scale of differently adapted clonal animals and plants showed that epigenetic mechanisms such as histone modifications, DNA methylation and non-coding RNAs are among the molecular pathways carrying phenotypic plasticity, hence epigenetic variation is used to stably adapt to diverse environments **(Vogt, 2022)**. In vertebrates, such model species are scarce, but it exists: it is the mangrove rivulus **(Figure 5) (Fellous *et al.*, 2018)**.

Figure 5: The mangrove rivulus (*Kryptolebias marmoratus*) is a new model fish species reproducing by androdioecy. In nature, hermaphrodites (foreground) coexist with males (background), however females have never been observed **(Biwer *et al.*, 2020; Photo credit: Frédéric Silvestre)**.

K. marmoratus is a small fish natively found in mangrove ecosystems located in a large geographic range including the Florida peninsula and the associated Keys, the Caribbean, Central America, Bahamas islands, South America, and perhaps portions of Cuba and Puerto Rico **(Scott Taylor, 2000; Tatarenkov *et al.*, 2011; Scott Taylor, 2012; Avise & Tatarenkov, 2015)**. *K. marmoratus* is closely affiliated to the red mangrove forests, *Rhizophora mangle*, exhibiting wet-dry seasonal alternation and semidiurnal tides, with very high tides in fall and very low tides during spring and summer **(Ellison *et al.*, 2012)**. The mangrove rivulus demonstrates many adaptations to living in microhabitats such as ephemeral pools and crab burrows, both showing important variation in salinity, ammonia, temperature, and oxygen **(Ellison *et al.*, 2012)**.

The mangrove rivulus, together with its putative sister species, *Kryptolebias hermaphroditus*, is the only known vertebrate able to self-fertilize **(Harrington, 1961; Tatarenkov *et al.*, 2009; Costa, 2011)**. Specifically, this self-fertilizing hermaphroditic vertebrate can produce offspring genetically similar to both parents and all siblings by sexual reproduction. This is called selfing, and in long term it results in individuals with completely homozygous genotypes, therefore producing "clones" **(Earley *et al.*, 2012; Tatarenkov *et al.*, 2012)**. The selfing rate is variable, depending on the ratio of males in the population, thus many isogenic lineages (lineages genetically identical) exist in natural populations, with each of them being genetically distinct **(Biwer *et al.*, 2020)**. In other words, the frequency of outcrossing in a population is thinly correlated with local frequencies of males, proposing that males are one of the factors influencing outcrossing **(Tatarenkov *et al.*, 2015)**. As such, genetically diverse individuals can mainly be found for example in populations from Belize, whereas numerous isogenic lineages can be found in populations from Florida as an example **(Tatarenkov *et al.*, 2015; Chapelle, 2023)**. In nature, hermaphrodites coexist with a low proportion of males (mostly fewer than 5%, there are primary and secondary males), however there are no females, constituting a rare androdioecious mixed reproductive system (alternative selfing and outcrossing with males) **(Scott Taylor, 2012; Ellison *et al.*, 2015)**. Hermaphrodites can lay unfertilized eggs, thus an outcrossing with males is possible but still less frequent than selfing. Nevertheless, most of the time, hermaphrodites self-fertilize by internal fecundation and numerous generations of exclusive selfing give rise to natural isogenic strains **(Mackiewicz *et al.*, 2006; Tatarenkov *et al.*, 2012)**. Self-fertilizing favors reproductive success when mate availability is scarce, however it renders populations more vulnerable to environmental change by reducing genetic variability **(Ellison *et al.*, 2015)**. This mixed-breeding strategy uniquely found in *K. marmoratus* might allow species to balance these duties, requiring a system for regulating sexual identity **(Ellison *et al.*, 2015)**. *K. marmoratus* is the best-known naturally self-fertilizing hermaphroditic vertebrate,

which affords the opportunity to work with genetically identical individuals to investigate the phenotypic effects (phenotypic plasticity) and the regulation of development and reproduction of epigenetic variance **(Fellous *et al.*, 2018, 2019a)**. Individuals with identical genotypes can be studied in many different environments, at any level during ontogeny or adulthood **(Earley *et al.*, 2012)**. Additionally, rivulus populations are characterized by a high genotypic diversity (which isn't common among clonal vertebrates), allowing for the assessment of variation among genotypes (by studying patterns of covariance among traits and reaction norms) **(Earley *et al.*, 2012)**. Furthermore, this species exhibits high phenotypic plasticity (possibly helping to prevail environmental challenges) within and between isogenic lineages **(Scott Taylor, 2000)** in life history traits (sex ratio and fecundity) **(Grageda *et al.*, 2005)**, in sexual phenotype **(Fellous *et al.*, 2018)** and in embryogenesis (diapause) **(Mesak *et al.*, 2015)**. The mangrove rivulus is very sensitive to environmental change, both during development and adulthood **(Earley *et al.*, 2012)**. On the one hand embryos of the mangrove rivulus reared at low temperature (18°C) can develop directly as primary males, and on the other hand adult hermaphrodites might undergo sex change to become secondary males **(Harrigton, 1961; Turner *et al.*, 2006; Earley *et al.*, 2012)**. Hence, **Ellison *et al.* (2015)** suggested that natural variation in self-fertilization rates among populations may be explained through epigenetic regulation (more precisely DNA methylation) of sex ratios **(Ellison *et al.*, 2015)**. In accordance with the "general-purpose genotype model" (stating that evolutionary success of isogenic lineages may be possible *via* generalist individuals selected for their plastic phenotypes in a broad range of ecological niches **(Massicotte & Angers, 2012)**), and the "genetic paradox of invasions" (stating that small invasive groups are able to conquer new environments never encountered before and proliferate despite the scarcity of genetic variation **(Sax & Brown, 2000; Estoup *et al.*, 2016)**), epigenetic mechanisms may allow the expression of a variety of phenotypes among genetically identical individuals in response to environmental cues **(Fellous *et al.*, 2018; Biwer *et al.*, 2020; Vogt, 2022)**.

Together with its biological characteristics, *K. marmoratus* is a very promising model to tackle key questions in ecotoxicology, physiology, behavior, and evolutionary biology, allowing researchers to distinguish genetic from epigenetic contributions to phenotypic plasticity, and also to study ecological and evolutionary epigenetics **(Ellison *et al.*, 2015; Voisin *et al.*, 2016; Fellous *et al.*, 2018, 2019a, 2019b; Biwer *et al.*, 2020; Chapelle & Silvestre, 2022)**. In addition to expressing phenotypic plasticity early in development, the mangrove rivulus also lays large eggs allowing easy identification of embryonic stages and has important capacities for physiological adaptation to challenging mangrove environments **(Fellous *et al.*, 2018)**. These three additional characteristics may together make the mangrove rivulus an important aid for advancing the comprehension of the epigenetic machinery **(Fellous *et al.*, 2018)**.

Epigenetic reprogramming, a first step towards transgenerational epigenetic inheritance (TEI)

Theoretical overview of TEI and epigenetic reprogramming

DNA is a reliable information transfer system because of the accuracy of DNA replication **(Perez & Lehner, 2019)**. Inheritance of genomic DNA underlies the vast majority of biological inheritance, however it has been commonly though for decades now that additional epigenetic information can be transmitted to future generations **(Bošković & Rando, 2018)**. It is indeed increasingly apparent that animals not only transmit DNA but also a variety of other molecules, such as proteins, RNA, and metabolites, to their descendants *via* gametes, therefore transferring information between generations **(Perez & Lehner, 2019)**. Currently, it remains unclear to what extent these molecules transport information throughout generations and whether this information changes related to the generation environment and physiological state (it is known that this information can be altered following change in the physiological and environmental conditions of previous generations) **(Perez & Lehner, 2019)**. It has been discovered that there is a multidimensional nature of the

inherited information carried by the epigenome and the characterization of its intra- and intergenerational dynamics have deeply changed the understanding of the functioning of biological organisms and the origins of phenotypic diversity **(Herrel *et al.*, 2020)**. Numerous mechanisms have been proposed to direct non-DNA sequence-based inheritance, and these can either be genome-associated (*e.g.*, synchronized modifications of DNA and histones) or genome-independent (*e.g.*, microbiome transfer) **(Skinner, 2008)**. Non-DNA sequence-based inheritance of information occurs in numerous species and is important for physiology and development (for example, a major purpose of epigenetic inheritance is to keep the repression of repetitive elements) **(Perez & Lehner, 2019; Burton & Greer, 2022)**. Furthermore, it transmits information about gene expression programs to the offspring **(Perez & Lehner, 2019)**. A controverse right now is the extent to which transmitted epigenetic information is shaped by the environment and physiology, and if this process can ever be adaptive **(Perez & Lehner, 2019; Donelson *et al.*, 2018)**. Moreover, non-DNA sequence-based inheritance also varies in generational span, with inheritance from one generation to a probable indefinite number. Although DNA-based information transfer is of high fidelity, other mechanisms are less robust, resulting in divergences in the durations of reliable information transfer **(Rando & Verstrepen, 2007)**. Therefore, it is quite intimidating to comprehend the evolutionary and ecological roles of non-genetic inheritance due to the complexity and diversity of epigenetic mechanisms **(Adrian-Kalchhauser *et al.*, 2020)**. However, three general features of non-genetic inheritance systems can be proposed: they are functionally interdependent with (rather than separate from) DNA sequence; epigenetic elements are probabilistic, interactive regulatory factors; and there is a phylogenetical and operational variation between precise mechanisms **(Adrian-Kalchhauser *et al.*, 2020)**.

There is confusion about two distinctions that are often misunderstood: firstly, genetic (thus DNA-based) *versus* epigenetic mechanisms of inheritance, and secondly environmentally responsive *versus* unresponsive phenomena **(Perez & Lehner, 2019)**. It is known that inheritance of environmentally acquired traits can be mediated by genetic inheritance. Stable long-term transcriptional repression can be fulfilled by an inherited epigenetic memory, in that case it may be unresponsive to the environment and physiology **(Ashe *et al.*, 2012)**. A current question is whether epigenetic mechanisms can procure a heritable and potentially adaptive memory of previous environmental exposure **(Heard & Martienssen, 2014)**. It is contestable that few well-established transgenerational effects are adaptive in the sense of preparing future generations for facing altered environmental conditions **(Perez & Lehner, 2019)**. More precisely, adaptive transgenerational effects could be imaginable for species such as *Caenorhabditis elegans* exhibiting short lifecycles with respect to environmental changes, but these transgenerational effects would be unlikely for long-lived animals such as humans **(Perez & Lehner, 2019)**.

In general, epigenetic marks are cleared and re-established each generation, nonetheless some reports in several model organisms show that at certain loci in the genome, this clearing isn't complete, and this is referred to as transgenerational epigenetic inheritance (TEI) **(Daxinger & Whitelaw, 2010)**. Some epigenetic marks may be stably inherited throughout generations *via* TEI **(Chapelle & Silvestre, 2022)**, as it has been reported in many plants, animals (*e.g.*, fish **(Kelley *et al.*, 2021)**, birds **(Guerrero-Bosagna *et al.*, 2018)**, and mammals **(Manikkam *et al.*, 2012)**) and invertebrate taxa **(Liew *et al.*, 2020)**. All together, these findings are suggesting that an environmental event in one generation could affect the phenotype in subsequent generations **(Daxinger & Whitelaw, 2010)**. Transgenerational effects refer exclusively to phenomena that could not be assigned to direct effects of a specific stimulus on the affected organism **(Perez & Lehner, 2019)**. As an example, an environmental trigger can directly affect a gestating embryo, and therefore the already-formed oocytes within a female embryo, in mammals **(Skinner, 2008; Heard & Martienssen, 2014)**. As such, only altered phenotypes happening in the second in the case of male transmission or third in the case of female transmission generation in mammals after a stimulus, can accurately be defined as transgenerational inheritance. Conversely, effects occurring in shorter timescales are defined as parental or intergenerational

(Figure 6) (Perez & Lehner, 2019; Tuscher & Day, 2019; Burton & Greer, 2022). A parental effect over a single generation can act *via* several mechanisms associated with phenotypic consequences. However, there is little evidence nowadays for multigenerational memory of physiological alterations following environmental fluctuations **(Bošković & Rando, 2018; Perez & Lehner, 2019)**. An important note is that regardless of the species, parental experiences are more likely to predict environmental conditions than the ones of more distant ancestors **(Perez & Lehner, 2019; Burton & Greer, 2022)**. Following this statement, adaptive effects seem more relevant in the context of intergenerational rather than transgenerational paradigms. Interestingly, many described intergenerational effects share mechanisms with transgenerational ones **(Ortega-Recalde & Hore, 2019; Perez & Lehner, 2019)**. Despite increasing popularity, the evidence for adaptive, environmentally responsive TEI is scarce, therefore assuming that TEI contributes to pre-adaptation of progeny to environmental conditions remains currently complicated **(Perez & Lehner, 2019)**. Currently, it seems that adaptive, environmentally responsive TEI, if it actually exists, is the exception rather than the rule. Nevertheless, epigenetic mechanisms can transfer information about ancestral state throughout generations, and even if the extent of this transmission is typically limited to a few generations, some cases, emerging from a loss of gene repression, can lead to longer-lasting memories **(Perez & Lehner, 2019)**.

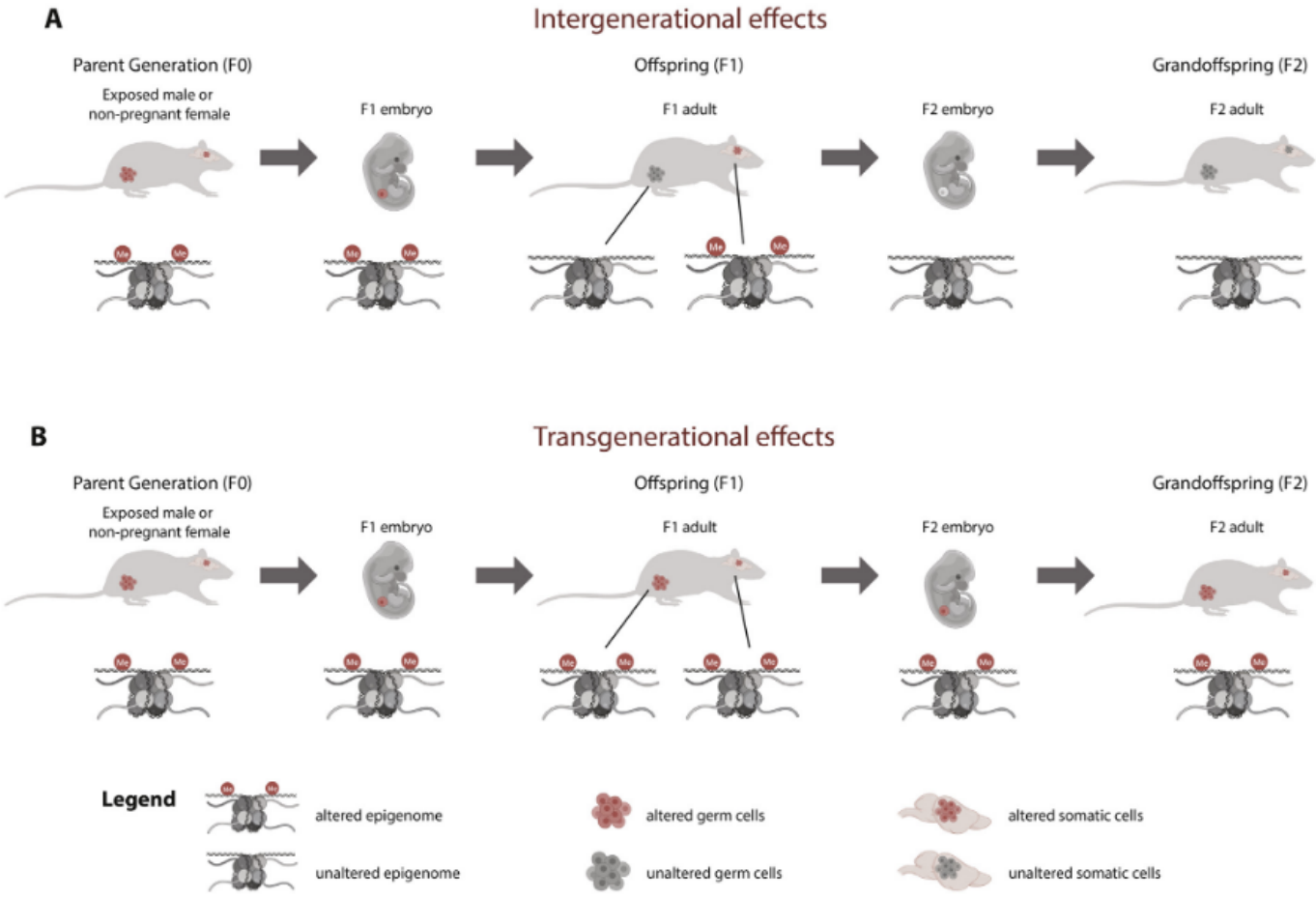

Figure 6: Intergenerational *versus* transgenerational inheritance. A) Intergenerational inheritance involves exposure of the parent generation (F0 male or non-pregnant female) to an environmental factor or external stimulus (*e.g.*, stress) leading to an epigenetic alteration in the parent and parental germline cells (F1, *e.g.*, sperm or oocytes). Epigenetic alterations may be observed in somatic tissues of F1 adults, however it doesn't persist in the F2 generation. B) Modifications acquired in the F0 generation need to be observed in the F1 and F2 generations (and even potentially beyond) for epigenetic effects to be considered as transgenerational. A pregnant female experiencing environmentally-induced epigenetic changes would have the pregnant mother (F0), the fetus (F1), and the fetal germline cells (F2), exposed to the external stimulus. In that case, intergenerational effects include transmission from F0 to F2, and only the persistence until F3 generation would be called a transgenerational effect **(Tuscher & Day, 2019)**.

Precise regulation of gene expression during gametogenesis and embryonic development across cell, tissue types and over time is primordial **(Fellous *et al.*, 2019a)**. DNA methylation memory establishment, maintenance and erasure is carefully balanced by molecular regulation, which is highly conserved among vertebrates. A direct consequence of retaining epigenetic memory in the form of 5mC is the enhanced potential for transgenerational epigenetic inheritance **(Ortega-Recalde & Hore, 2019)**. Currently, DNA methylation dynamics remain underexplored in most vertebrate lineages, thus the extent of information transmitted to the offspring by epigenetic modification is possibly underestimated **(Ortega-Recalde & Hore, 2019)**. One of the most remarkable features of DNA methylation is that it can be mitotically and/or meiotically heritable **(Wu & Morris, 2001)**, in addition to the fact that it can be in some circumstances transgenerationally inherited **(Jablonka & Raz, 2009)**. TEI is of significant importance in order to understand the role of epigenetics in evolution even if most epigenetic mutations have been reported to be neutral or deleterious **(Heard & Martienssen, 2014)**. One of the main hurdles that epimutations must overcome to be inherited is DNA methylation reprogramming, which happens twice: once in the germline and another time in the early embryo **(Grossniklaus *et al.*, 2013; Fellous *et al.*, 2018)**. Reprogramming can be defined as an erasure of epigenetic marks required for correct development of the embryo as well as the establishment of DNA methylation patterns in the new individual **(Monk *et al.*, 1987)**. Nonetheless, little is known about reprogramming in most vertebrates **(Fellous *et al.*, 2018)**, except for mice **(Edwards *et al.*, 2017)** and a few studies on zebrafish **(*e.g.*, Fang *et al.*, 2013)**. DNA methylation is thought to play an important role throughout development, because it regulates cellular differentiation after reprogramming **(Voisin *et al.*, 2022)**. DNA methylation is thus considered to be particularly sensitive to environmental factors **(Dorts *et al.*, 2016)**. The evolutionary significance of the reprogramming event is questionable, it is hypothesized as a possible critical window arbitrating phenotypic plasticity as well as evolutionary adaptation to remarkably variable environments. It is thus possible to infer that TEI is a prerequisite in understanding the possible role of epigenetics in evolution **(Fellous *et al.*, 2018)**. Hence, understanding TEI, environmental cues and reprogramming is important to comprehend the plausible contribution of epigenetics in rapid evolution and adaptation **(Figure 7) (Fellous *et al.*, 2018; Navarro-Martín *et al.*, 2020)**.

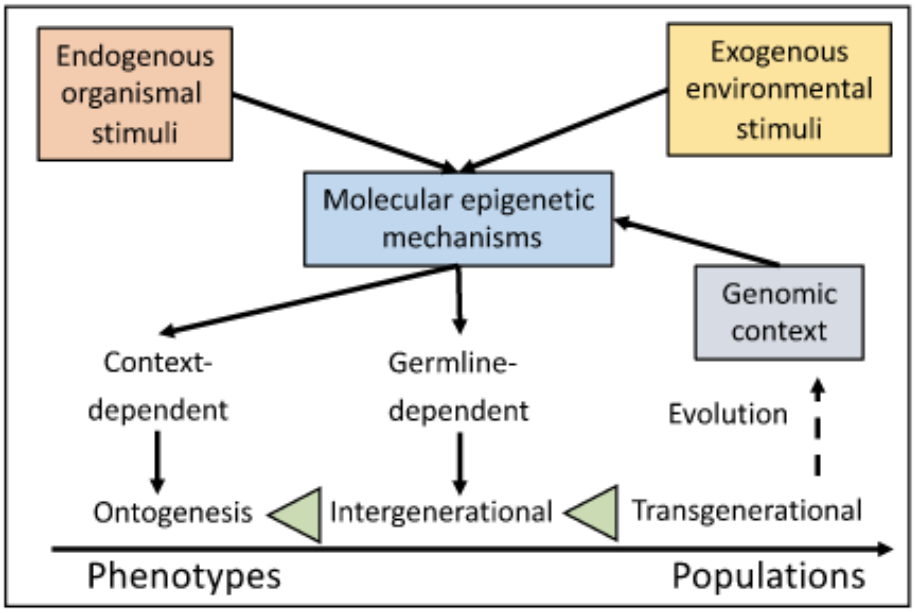

Figure 7: Molecular epigenetic mechanisms across temporal scales (Navarro-Martín *et al.*, 2020).

Finally, the concept of inclusive heritability can be proposed, which unifies genetic and non-genetic mechanisms of heritability, including all dimensions of inheritance such as the transmitted parental effect, the TEI, social variation and ecological variation **(Danchin *et al.*, 2011)**. It is increasingly accepted that epigenetics and more precisely TEI could be one of the missing factors for the understanding of phenomena that can't be explained by the DNA sequence alone **(Chapelle & Silvestre, 2022)**, like for example the

variance in expressivity (which is the degree to which complex trait expression diverges between individuals **(Miko, 2008)**) and the incomplete penetrance (stipulating that individuals of a given genotype express distinct phenotypes **(Allen *et al.*, 1990)**). Both of these phenomena are the result of an incomplete correlation between genotype and phenotype, and they might be partially justified by epigenetic mechanisms **(Chapelle & Silvestre, 2022)**.

What is already known about DNA methylation and its reprogramming in the mangrove rivulus?

The methylome of mangrove rivulus exhibits conserved vertebrate characteristics, because like mammals and other teleost fish, the genomes of *K. marmoratus* have high levels of DNA methylation genome-wide in the CpG dinucleotide context **(Chapelle, 2023)**. Notably, the average proportion of methylated cytosines in a CpG context was 65.11%, in brain tissue. Concerning the distribution of cytosine methylation, gene bodies (including introns and exons) are typically methylated, whereas CpGs in the gene promoter regions are lowly methylated without any significant differences between the populations studied **(Chapelle, 2023)**. Lastly, cytosine analysis of brains identified a total of 29664 CpGs covered by all individuals analyzed (n=134) with 10x coverage, while less shared cytosines in livers were discovered, with 14070 CpGs covered by all individuals (n=134) with 10x coverage **(Chapelle, 2023)**. CpG global DNA methylation levels in diverse organs of *K. marmoratus* have been investigated by **Fellous *et al.* (2018)** using the LUminometric Methylation Assay (LUMA) **(Table 1)**. They discovered that global DNA methylation varies at CpG sites in adult tissues, significantly diverging between male testes (87.2%) and hermaphrodite ovotestes (79.6%) (described for the DC4 lineage, which comes from Dove Creek in the Florida Keys) **(Fellous *et al.*, 2018)**. **Ellison *et al.* (2015)** have inspected the role of DNA methylation as a regulatory system for sex-ratio modulation in *K. marmoratus*. They discovered that there is apparently a significant interaction between sexual identity (hermaphrodite or male), temperature and methylation patterns when two selfing lines (both from Belize) are exposed to distinct temperatures during development **(Ellison *et al.*, 2015)**. Therefore, an epigenetic mechanism regulated by temperature modulates sexual identity in the mangrove rivulus, procuring a plausible widespread mechanism by which environmental change may influence selfing rates **(Ellison *et al.*, 2015)**.

(B)

Tissue	DNA methylation Mean% (±SEM) (Males)	DNA methylation Mean% (±SEM) (Hermaphrodites)	Sidak's multiple comparisons test (between male and hermaphrodite organs)	Sidak's multiple comparisons test (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

Table 1: CpG global methylation levels in different organs of *Kryptolebias marmoratus* using the LUminometric Methylation Assay. Results of a Sidak's post hoc test after a two-way ANOVA ($p < 0.05$) on adult tissues are shown. Different letters demonstrate tissues with significantly distinct mean DNA methylation **(Fellous *et al.*, 2018)**.

Fellous *et al.* (2018) have investigated genes potentially encoding DNA methyltransferases (DNMTs), MeCP2 and TET proteins, which have shown specific regulation in adult gonad and brain, as well as during early embryogenesis **(Fellous *et al.*, 2018)**. The conserved domains and expression profiles of these proteins suggest that they play important roles during reproduction and development **(Fellous *et al.*, 2018)**. The high evolutionary conservation and expression patterns of DNMT, MeCP2 and TET proteins in this fish encourage their biological roles in gametogenesis as well as in development **(Fellous *et al.*, 2018)**. Moreover, plausible histone lysine demethylases (Kdm) and methyltransferases (Kmt) were identified by **Fellous *et al.* (2019b)** for the mangrove rivulus. They found out that the expression pattern of *Kdm* and *Kmt* during embryonic

development was peaking in the gastrula stage whereas a reduction was observed in later embryogenesis. Kdm and Kmt expression profiles and domain conservation inform that they may play important roles during development, neurogenesis and gametogenesis **(Fellous *et al.*, 2019b)**.

Fellous *et al.* (2018) also mention that it is known for the mangrove rivulus that after fertilization, there is an immediate decrease in DNA methylation, regressing down to 15.8% in the gastrula stage followed by a re-establishment to 70.0% by stage 26 (when the liver is forming) **(Fellous *et al.*, 2018)**. A specific pattern of CpG DNA methylation reprogramming is characterized for this fish during embryogenesis **(Figure 8)**, which is happening particularly later, lasting longer and is more dramatic than reported at the same embryonic stages of other studied vertebrates (notably in comparison to zebrafish **(Figure 9)**), and is associated with active mRNA expression of DNA methylation/demethylation enzyme machinery **(Fellous *et al.*, 2018)**.

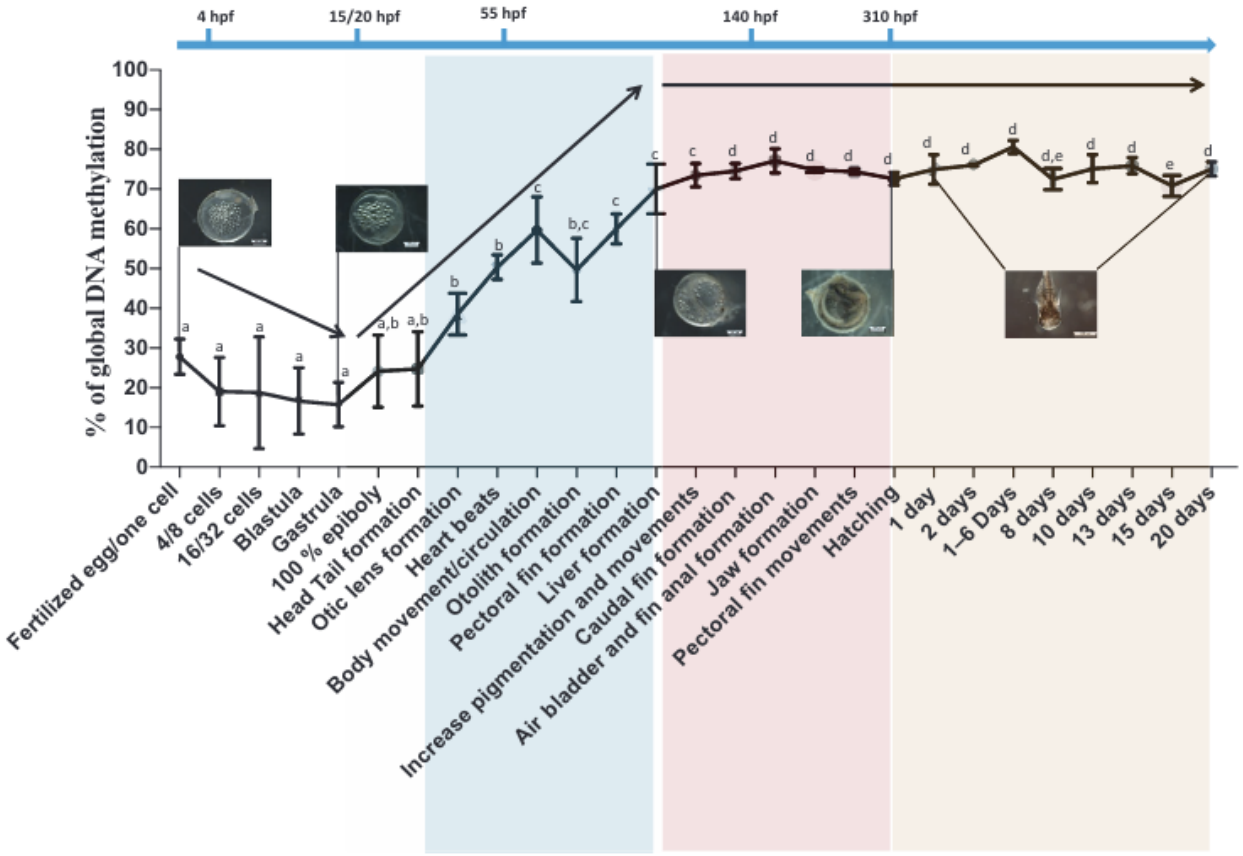

Figure 8: CpG DNA methylation levels throughout the embryonic development of *Kryptolebias marmoratus*. The results shown are given for a quantity of 600 ng of genomic DNA using the LUminometric Methylation Assay (LUMA) and are given for the corresponding stage name. The x axis represents the developmental stages. P-values are given for Tukey's post hoc test after one-way ANOVA, if $p < 0.05$ threshold. Means that are labeled with a similar letter don't display statistically significant differences. Hpf= hours post-fertilization **(Fellous *et al.*, 2018)**.

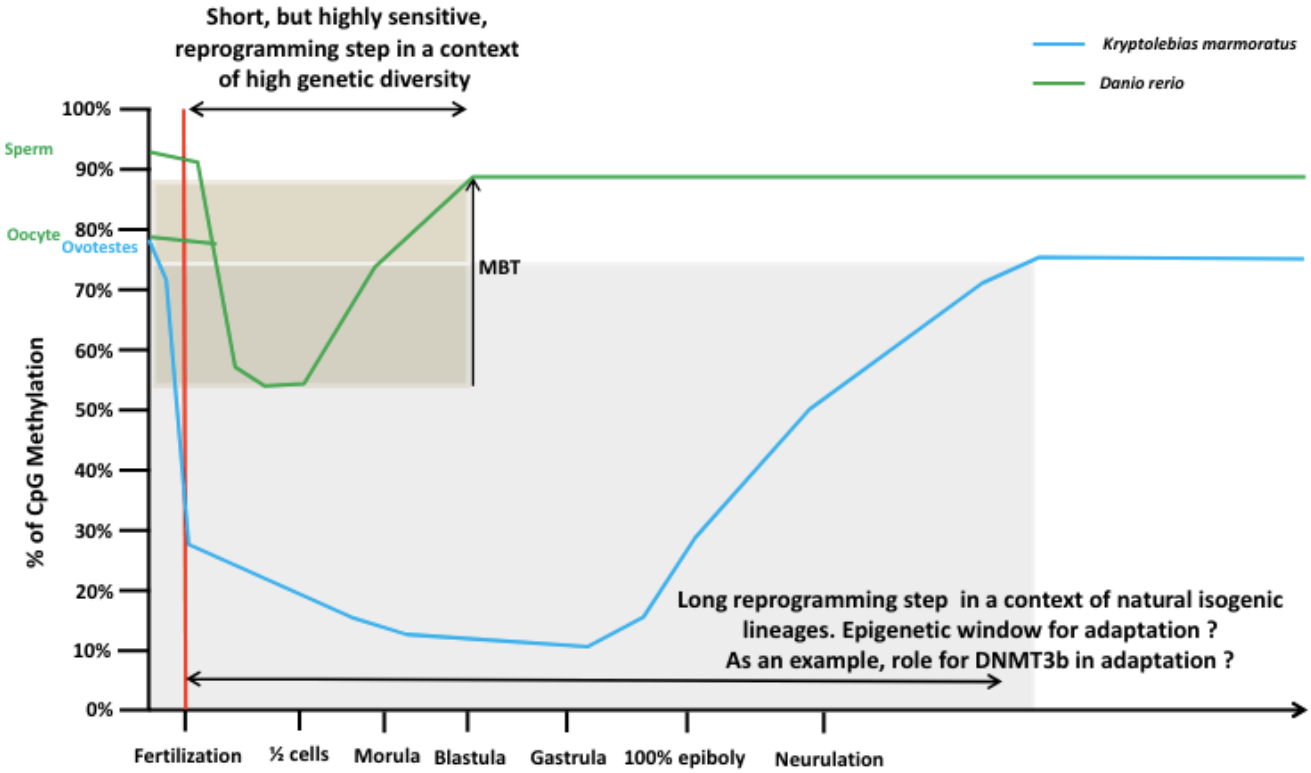

Figure 9: Dynamic of DNA methylation during early mangrove rivulus and zebra fish development. MBT= Mid Blastula Transition **(Fellous *et al.*, 2018)**.

Objectives of this master's thesis

This master's thesis is aiming to study the contribution of DNA methylation to the evolution and adaptation of the mangrove rivulus by examining the DNA methylation pattern variation within and between *K. marmoratus* populations reared under standardized laboratory conditions.

The precedent paragraphs of this work highlight important insights concerning regulatory and evolutionary roles of epigenetics that would need further investigation. Firstly, many studies have focused on understanding the implication of epigenetic variation towards phenotypic variation, either in genetically diverse populations or in clonal populations. The mangrove rivulus brings the exceptional opportunity to work on both cases (genetically diverse *versus* clonal populations) because of its natural characteristics. This ultimately allows for the comparison of populations covering a wide range of genetic diversity. Hence, studying DNA methylation in populations exhibiting a gradient of genetic diversity, from isogenic lineages to genetically distinct individuals, would help trying to answer the following general evolutionary and ecological relevant question: How can epigenetic variation come to a balance with genetic variation in populations of the mangrove rivulus exhibiting a gradient of genetic diversity between wild populations?

In order to tackle such an evolutionary and ecological question regarding epigenetics, this master's thesis is divided into two parts, both studying two populations of the mangrove rivulus **(Figure 10)**. The first population is coming from Florida and is called Emerson Point Preserve (EPP), which is characterized to have a low genetic diversity between the fish, therefore more isogenic fish are expected. The other population comes from Belize and is called Twin Cays (TC), where the fish exhibit a higher genetic variability between the individuals, thus less isogenic fish are awaited **(Tatarenkov *et al.*, 2015; Chapelle, 2023)**.

To investigate the balance between epigenetic and genetic variations in these distinct populations and to add additional knowledge on how these variations allow *K. marmoratus* to be able to live in its fluctuating wild environment, the first part of this master's thesis will focus on assessing the level of DNA methylation in the gonads among EPP and TC populations of this fish. The gonads are of crucial significance for studying an

evolutionary question, since a transgenerational aspect of epigenetic marks would be needed to correctly talk about evolution **(Fellous *et al.*, 2018)**. It is important to note that all the gonad samples of these two populations come from hermaphroditic fish. The resulting research question would be:

- Are there any differences in the pattern of DNA methylation in the gonads between these two populations of *K. marmoratus*?

These first results on the gonads of the mangrove rivulus will procure a quantitative insight on the DNA methylation pattern of this tissue between the two distinct populations. Once these results at hand, it could be very interesting thereafter to compare them with the DNA methylation results previously acquired from the liver and the brain of the same populations (work previously realized by **Chapelle (2023)**). Thus, another research question is emerging:

- Is the pattern of DNA methylation in the gonads of these two populations different than the one in the brain and liver tissues?

This analysis will effectively permit to obtain a complete knowledge of the DNA methylation pattern in a variety of tissues from EPP and TC populations, therefore possibly comforting our understanding of the epigenetic role in the evolution and ecology of *K. marmoratus*.

The hypotheses proposed for this first part of the master's thesis are the following:

- The null hypothesis for the first research question would propose that there are no differences in DNA methylation between the gonads of the two populations, therefore DNA methylation variation wouldn't act as an evolutionary potential in these populations of the mangrove rivulus
- The alternative hypothesis, which would actually be expected, stipulates that there is indeed a differential DNA methylation pattern between the gonads of EPP and TC populations, possibly contributing to an evolutionary potential between these two populations of *K. marmoratus*, eventually helping to face the challenges of these populations to their daily environmental pressures
- The null hypothesis for the second research question would be that there isn't any differential DNA methylation pattern between the brain, the liver, and the gonads of EPP and TC populations, therefore assuming that there isn't any differential contribution of DNA methylation in the diverse tissues of *K. marmoratus*, thus there isn't a tissue-specific role of DNA methylation towards achieving evolutionary and ecological success of EPP and TC populations in their respective environment
- The alternative hypothesis, which is once again expected, proposes that there is indeed a differential DNA methylation pattern between the three tissues of those two populations of *K. marmoratus*, demonstrating that a tissue-specific DNA methylation in genetically distinct populations procures evolutionary and ecological advantages regarding the environmental fluctuations

To reinforce the results from the first part of the master's thesis and this research on the balance between epigenetic and genetic variations in the two populations of the mangrove rivulus, the second part of this master's thesis studies the epigenetic reprogramming of three distinct stages of the embryonic development of *K. marmoratus*, with the idea to enrich the understanding of TEI. The three embryonic stages chosen are the gastrula, the OL (Otic Lens formation) and lastly the 27^{th} stages of development. Eggs laid by fish of EPP and TC populations will be picked for the analysis of epigenetic reprogramming throughout the three stages mentioned above. The epigenetic reprogramming will help to understand if DNA methylation marks at precise stages of *K. marmoratus* development are transmitted and expressed or not, effectively giving insights on the

epigenetic roles, even at the basic stages of life, that enable in part mangrove rivulus populations with distinct genetic background to proliferate in their natural environment. Thus, the research question is the following:

- Is there a differential pattern of DNA methylation in the three stages of embryonic development between EPP and TC populations?

The hypotheses for this second part of the master's thesis are:

- The null hypothesis here would be that there are no differences in DNA methylation between the different stages of embryonic development in the two populations exhibiting a distinct genetic background, showing that there isn't a specific role of an epigenetic reprogramming throughout successive developmental stages of the mangrove rivulus in order to procure a heritable and potentially adaptive memory of previous environmental exposure
- The alternative hypothesis, also expected throughout this work, would be that there is indeed a differential DNA methylation pattern between these developmental stages in EPP and TC populations, affirming that environmental characteristics and the genetic background of a population effectively impact the heritability and potentially adaptive memory of previous environmental exposure

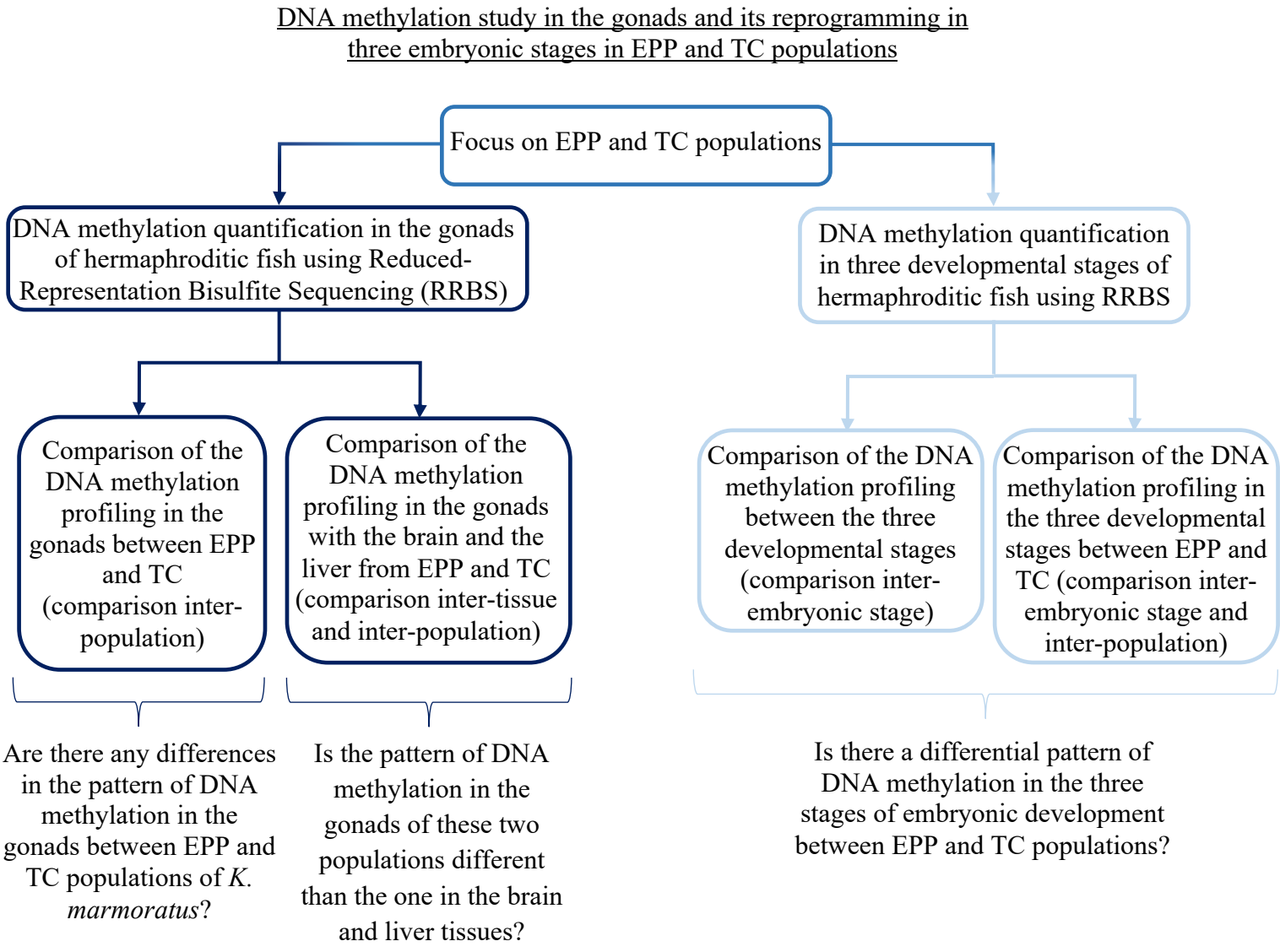

Figure 10: Schematic representation of the objectives and research questions approached in this master's thesis. The first part of this work focuses on gonad methylation (left part of the schema) whereas the second part of this master's thesis tackles DNA methylation reprogramming in three distinct embryonic stages (right part of the schema).

Techniques that will be used to measure DNA methylation

The field of molecular biology is rapidly advancing with the introduction of new powerful technologies, sequencing methods and analysis software being constantly developed **(Chatterjee *et al.*, 2017; Laine *et al.*, 2022)**. Tools that are regularly and originally developed for conducting research on humans and model species are now commonly used in ecological and evolutionary research **(Laird, 2010; Laine *et al.*, 2022)**. Studying ecological epigenetics is currently challenging and especially for vertebrate systems, because it requires technical expertise, hurdles with analyses and their interpretation, and limitations in obtaining sufficiently high sample sizes **(Laine *et al.*, 2022)**. It is important not to neglect the limitations of the experimental setup, technology, and analyses, because it may influence the reliability and reproducibility as well as the extent to which unbiased conclusions can be drawn from such studies **(Laine *et al.*, 2022)**.

Accurate measurements of normal and altered DNA methylation patterns are critical to understand their role in regulating gene expression as well as cell phenotype **(Chatterjee *et al.*, 2017)**. In the expanding field of epigenetics, there are numerous methods available to determine the methylation status of DNA samples **(Kurdyukov & Bullock, 2016)**. Over the last decade, impressive progress has been made in developing methodologies to investigate DNA methylation. Moreover, the availability of next-generation sequencing has enabled the profiling of methylation marks at an unprecedented scale **(Chatterjee *et al.*, 2017)**. Many methods that were once used to study locus-specific methylation have now been upgraded to a genome-wide scale using high-throughput sequencing or array platforms **(Laird, 2010)**. Because of the considerable number of techniques at hand, researchers are facing the challenge of assessing the goodness and the limitations of each technique and therefore selecting the appropriate method for their analysis that is best suited to answering a specific biological question still seems to be an arduous task **(Kurdyukov & Bullock, 2016; Chatterjee *et al.*, 2017)**.

This master's thesis studies the mangrove rivulus, therefore a vertebrate species. It is known that in vertebrates, the DNA methylation is mainly occurring in CpG dinucleotides **(Feng *et al.*, 2010)**, these latter will thus be the target of the analysis. Because the candidate genes in the gonads and the different embryos aren't known, differentially methylated regions are thus searched, allowing for a bisulfite conversion with an enrichment for CpG rich regions. From then on, bisulfite sequencing can be chosen as the optimal technique, more precisely considering Reduced-Representation Bisulfite Sequencing (RRBS) **(Kurdyukov & Bullock, 2016)**. With RRBS, only a fraction of the genome is sequenced **(Meissner *et al.*, 2005; Lee *et al.*, 2014)**. Enrichment of CpG-rich regions in RRBS is achieved by isolation of short fragments after MspI digestion which recognizes CCGG sites, cutting both methylated and unmethylated sites **(Kurdyukov & Bullock, 2016)**. By doing so, it ensures isolation of ~85% of CpG islands in the human genome for instance **(Kurdyukov & Bullock, 2016)**.

Sodium bisulfite treatment of DNA converts unmethylated cytosine residues to uracile *via* deamination, these uracile residues will be read as thymines for PCR amplification. However, this treatment leaves 5-methylcytosine residues unchanged, thus they remain read as cytosines **(Figure 11) (Kurdyukov & Bullock, 2016; Chatterjee *et al.*, 2017; Pulix & Plagge, 2020; Laine *et al.*, 2022)**. Furthermore, sodium bisulfite treatment doesn't allow the distinction between 5-methylcytosine and 5-hydroxymethylcytosine **(Rodger *et al.*, 2014)**.

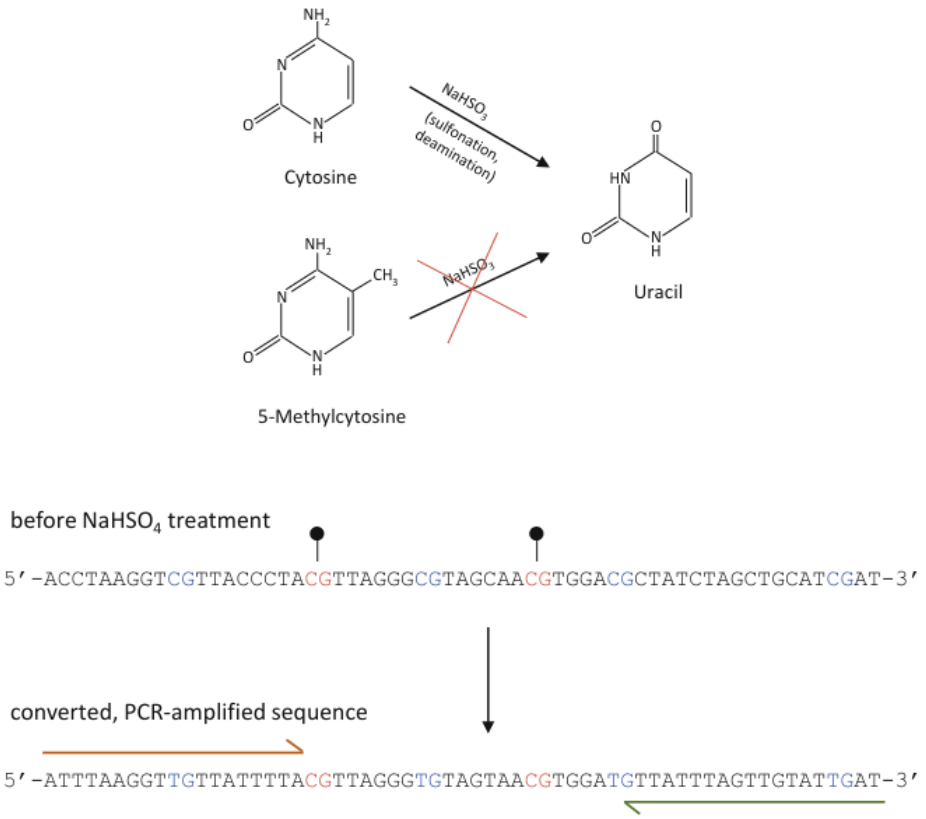

Figure 11: Bisulfite conversion of genomic DNA. The treatment with $NaHSO_3$ provokes the deamination of cytosine into uracile residues. Nonetheless, this conversion won't happen if the cytosine is methylated or hydroxymethylated in the carbon 5 position. Therefore, after a bisulfite treatment, the sense and antisense strands of the original double stranded DNA are no longer complementary. The following PCR amplification of a strand will now head towards an exchange of uracil residues to thymines and the DNA sequence is then characterized by an abundance of A and T. Importantly, only the methylated sites will remain as cytosine residues. Moreover, the primers for bisulfite sequencing have to be complementary to the converted sequence (orange and green arrows). Lastly, black lollipops represent methylated CpG sites. N.B: $NaHSO_4$ in the sentence "before $NaHSO_4$ treatment" should be written as $NaHSO_3$, as sulfite and not sulfate is used **(Pulix & Plagge, 2020)**.

Most current studies on ecological or evolutionary questions demand sample sizes that exceed the sample sizes used **(Lea *et al.*, 2017)**, highlighting the need for approaches targeting reduced and biased representation of the genome **(Laine *et al.*, 2022)**. Thus, RRBS, like other reduced representation approaches, makes use of restriction enzyme(s) that nonrandomly cut DNA at, or close to, the recognition sequence of the chosen restriction enzyme(s) **(Laine *et al.*, 2022)**. Concerning library preparation, genomic DNA is digested with a methylation insensitive restriction enzyme to enrich for CpG sites in the digested fragments **(Chatterjee *et al.*, 2017)**. The restriction enzyme MspI, which will be used in this work, cuts DNA in CG-rich areas often in coding regions, defining the fragments used for methylation analysis, therefore RRBS requires a reduced number of reads to acquire a modest coverage of a reproducible fraction of genome-wide CpG sites **(Stockwell *et al.*, 2014; Laine *et al.*, 2022)**. These sites are often enriched for promotor regions in vertebrate systems, where CpG methylation is known to impact gene expression **(Laine *et al.*, 2016)**. This makes RRBS cost-effective and also permits to avoid conducting analyses on hundreds of thousands of CpG sites expected to have no functional significance **(Sun *et al.*, 2015)**. Furthermore, in RRBS, enzymes exhibiting distinct properties and recognition sites may be combined to optimize the number and coverage of CpG sites (or the representation of any other targeted subset of the genome) and the average sequencing depth **(Laine *et al.*, 2022)**. Also, a size-selection step, generally of 40-220 bp, is performed on the digested fragments, with each fragment containing at least one CpG site. This reduces the requirement of extensive sequencing. This method therefore enriches for CpG sites, CpG islands, gene promoters, and gene bodies **(Chatterjee *et al.*, 2017)**. Nonetheless, the representation of repeat elements as well as enhancers is low in RRBS **(Chatterjee *et al.*, 2017)**. When using RRBS methods, the definition of a region needs to be done carefully because the whole

genome isn't represented (it is reduced), and the use of restriction enzymes generates biases towards certain features of the genome, such as CpG islands **(Meissner *et al.*, 2008)**.

RRBS availability as a kit comes with Illumina sequencing technology, and the coverage of RRBS is ~60% for promoters, 85% for CpG islands (~1.5 x 10^6 CpGs), and the CpG coverage is 13%, that is 4 million CpGs covered **(Meissner *et al.*, 2005; Lee *et al.*, 2014)**. Its sensitivity varies, as the sequencing depth correlates with the amount of DNA, although 20x coverage is recommended. RRBS has a great specificity, and the amount of starting material is quite low, 10 ng ~ 1 µg of DNA input with a single base resolution **(Meissner *et al.*, 2005; Lee *et al.*, 2014)**.

For techniques such as RRBS, where millions of CpG sites are studied, investigation of every single CpG site as an independent unit of analysis can considerably increase the false discovery rate **(Chatterjee *et al.*, 2017)**. This is due to the fact that variation at single sites is greater than the one of a contig of sites since the relatively lower coverage per site increases the sampling-based variation **(Ehrlich & Lacey, 2013)**. In addition, from a statistical point of view, the number of pieces of information available for a single-CpG site is lower than for a region, hence a comparison of a single site is less sturdy than that of a region **(Chatterjee *et al.*, 2017)**. For that reason, a common approach for large bisulfite sequencing data is to study larger regions and identify differentially methylated regions or DMRs **(Figure 12) (Laine *et al.*, 2022; Chatterjee *et al.*, 2017)**. Nevertheless, another commonly used approach which is quite simple as well is to analyze each CpG site in the studied samples and then identify differentially methylated sites, defined as differentially methylated positions (DMPs) or differentially methylated CpGs (DMCs) **(Laine *et al.*, 2022; Chatterjee *et al.*, 2017)**.

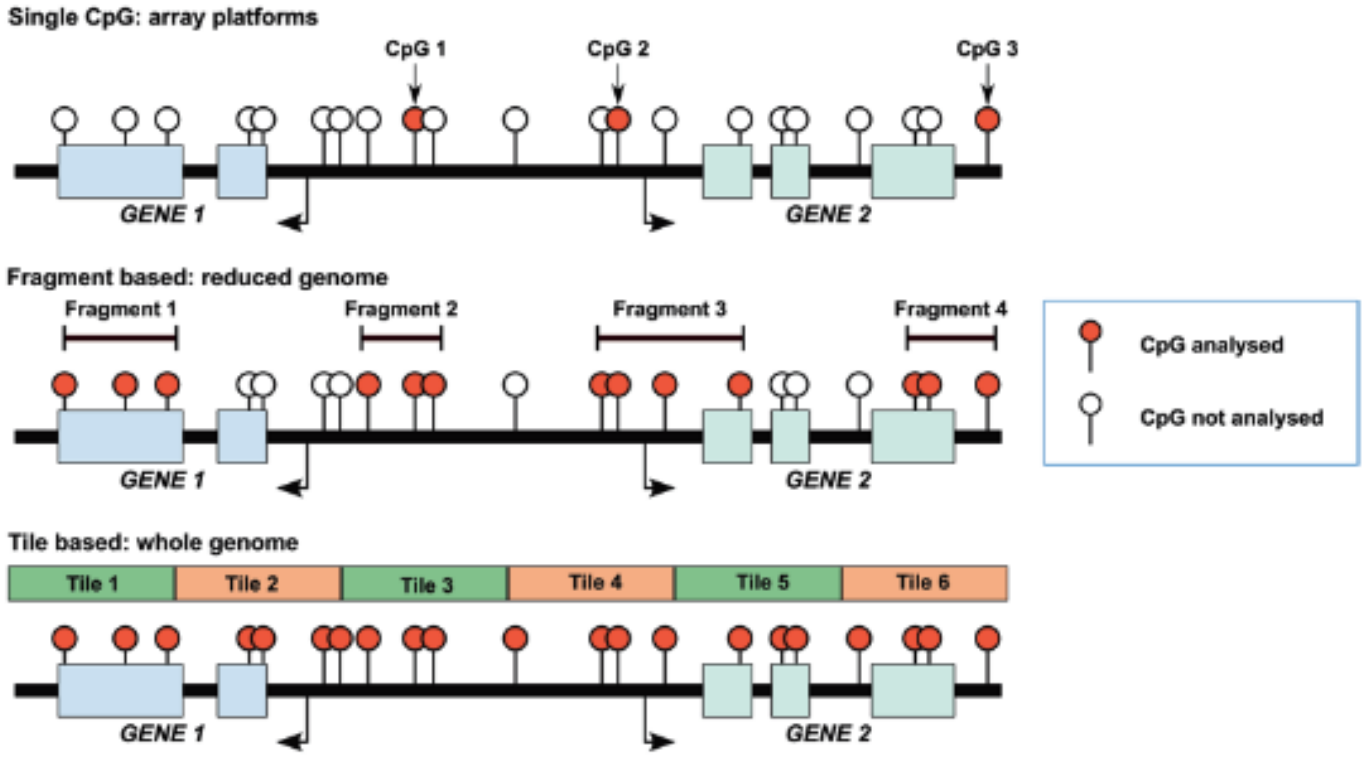

Figure 12: Major analysis approaches for genome-wide DNA methylation analysis. Numerous approaches exist for analyzing differential methylation between distinct groups and conditions, and these approaches differ based on the unit of analysis: the upper part of the figure represents the single CpG site approach independently analyzing each CpG site in investigated samples; the middle part of the figure shows RRBS and its MspI-digested fragments that can be used as the unit of analysis; lastly the lower part of the figure represents a common approach for large bisulfite sequencing data that investigates regions with fixed size genomic windows. It is also possible to use sliding windows following user-specified criteria **(Chatterjee *et al.*, 2017)**.

For single CpG analysis, a widely used tool is methylKit **(Akalin *et al.*, 2012)**, which is an R package applying a Fisher's exact test or logistic regression to calculate p-values, which are adjusted to q-values for multiple

test correction using a SLIM approach **(Wang *et al.*, 2011)**. The tool methylKit based on R incorporates logistic regression-based DMC analysis, sample clustering and visualization **(Akalin *et al.*, 2012)**. MethylKit will also be used for DMR analysis, using tiles (regions) of CpGs.

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