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Characterization of the brain molecular bases of personalities and assessment of their developmental plasticity in response to environmental stressors using an emerging model fish species, the mangrove rivulus, *Kryptolebias marmoratus*

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Laboratory of Evolutionary and Adaptive Physiology

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assessment of their developmental plasticity in response to environmental
stressors using an emerging model fish species, the mangrove rivulus,
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A dissertation submitted by
Alessandra CARION
In partial fulfillment of the requirements
for the degree of PhD in Biological Sciences
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Summary

The widespread existence of personalities across the animal kingdom suggests an evolutionary relevance. The consistent between-individual differences in behavior over time and across contexts, also called personality traits or behavioral individualities, influence various parameters such as food access, social interactions, predator avoidance and, ultimately, the fitness of organisms which corresponds to the ability to survive to reproductive age, find a mate and produce offspring. Although animal behavior constitutes the interactive link between an organism and its environment, behavioral individualities are largely interconnected with ecological dynamics and population evolution. Nowadays, organisms have to face (rapid) changing environmental conditions mostly related to human impacts. 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 changes can influence phenotypic variability is crucial to understand individual traits and animal's ability to acclimate to new environmental conditions during development and adulthood. Hence, the general objective of this thesis was to investigate the developmental plasticity of behavioral traits and the sequence of key molecular events leading to behavioral modifications that permit the organisms to cope with new environmental conditions. A unique vertebrate expressing very low to no genetic variability between lineages was used in this thesis providing an incredible model to identify the genetic and environmental sources of phenotypic variability. Hermaphrodites of the mangrove rivulus fish, *Kryptolebias marmoratus*, can self-fertilize and naturally produces highly homozygous and isogenic individuals within lineages.

The first chapter of this thesis aimed to characterize boldness and aggressiveness personality traits and the non-genetic mechanisms generating between-individual behavioral variability in hermaphrodites reared in the same controlled conditions. Variability in behaviors expressed by fish was observed despite the absence of genetic and environmental variations. The brain protein expression pattern and DNA methylation landscape in adult fish, globally profiled using respectively label-free quantitation (LFQ) and Reduced Representation Bisulfite Sequencing (RRBS) workflows, provided a list of candidate genes that might be associated to variations in the bold/shy or the aggressive/non-aggressive continuums, some through methylation changes. Proteomic as well as RRBS results have shown no proteins/genes similar to both

behavioral continuums indicating the implication of different pathways in the settlement of boldness and aggressiveness. Proteomic results revealed that the bold/shy continuum mostly impacted amino acid metabolic processes as well as structural and cytoskeleton proteins. The aggressive/non-aggressive continuum mostly influenced proteins involved in the central nervous system development, maintenance, plasticity and neurotransmission. The most significant differentially methylated fragment (DMF) between bold and shy fish was situated in the SKI gene body. The difference in methylation level of this gene could indicate potential difference in neurogenesis shaping boldness personality trait. With a methylation change of 40% between aggressive and non-aggressive fish, the toll-interacting protein coding gene controlling proinflammatory reaction in response to injury supports, with other significant DMFs, a correlation between the aggressive level and the fish immune response, as it has been discovered in humans. 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 change. This concept is known as the bet-hedging strategy, an evolutionary strategy in which a single genotype produces a distribution 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.

The second chapter was dedicated to understand the developmental plasticity of personality traits in *K. marmoratus* submitted to environmentally relevant stimuli during their development (the presence of a conspecific and low salinity environment) by investigating as well the effects on life history traits (growth and reproduction) and brain protein expression profiles. These two stimuli differentially affected fish growth and reproduction as well as the expression of some proteins in their brain, although the exposure stopped a long time before sampling. Social interactions during development induced expression changes of 43 proteins among which, the reticulon 1, the syntaxin-binding protein, the glutaminase, and the sodium-dependent neutral amino acid transporter revealed impacts on vesicle transport and neuronal activity. Low salinity exposure changed the expression of 15 proteins with the prothymosin alpha and the lipoamide acyltransferase indicated that the low salinity increased the lipid metabolism and modulated the immune response. However, no effects of stimuli were detected on fish behaviors. The camouflage of stimuli effects on behavior by the elevated behavioral

variability between individuals, the low environmental influence in the determinism of behavior or the choice of stimuli not eliciting strong enough effects were amongst the hypotheses raised and discussed in this chapter.

Those results led to the last experiments carried out during this thesis : exposing fish to an expected more intense stress, a neurotoxin, highly suspected to impact fish behavior, and investigate its immediate and delayed effects. This neurotoxin is the β -N-methylamino-L-alanine (BMAA), produced by cyanobacteria, diatoms and dinoflagellates naturally present in *K. marmoratus* environment and related to the development of neurodegenerative diseases in humans such as Parkinson's, amyotrophic lateral sclerosis' and Alzheimer's diseases. First, locomotion and prey capture behaviors in the rivulus larvae were investigated after one-week exposure to 2 sublethal BMAA doses (20 μ g/L and 15 mg/L) on newly hatched larvae. BMAA significantly increased the maximum velocity as well as the number of failures and trials for capturing preys revealing potential movement and synaptic signaling impairments. Second, the delayed effects of 2-weeks BMAA exposure to both sublethal concentrations were assessed on life history traits, behavioral traits and on the relative expression of 7 genes in fish brain known to be BMAA targets or involved in neurotransmission and/or personality traits. Although no effects on growth, reproduction and behavioral traits were detected, BMAA induced a significant increase of the expression of CaM and MAOA genes at 20 μ g/L BMAA compared to the control group. A significant decrease of expression was observed between this lowest BMAA dose and 15 mg/L for DRD4, MAOA and CaM genes. Our results suggest disruption of glutamate turnover, intracellular dopamine depletion and the potential activation of astrocyte protective mechanisms indicating potential long lasting effects of BMAA affecting phenotypic traits with aging.

According to the results gathered along this project, we could conclude that the mangrove rivulus behavioral traits seem to be submitted to low environmental influence even faced to neurotoxic compounds. Personality traits are possibly less plastic to stimuli occurring during rivulus development due to their influence on organism fitness. The hypothetic low environmental determinism of boldness and aggressiveness would avoid trait homogeneity within population to ensure rivulus acclimation and adaptation to new environmental conditions that may occur in adults, hypothesis highly plausible knowing how extreme the environmental conditions in mangrove habitats can be. Our results also suggest a bet-hedging strategy favoring the maintenance of traits diversity

within isogenic population of the mangrove rivulus through epigenetic mechanisms such as DNA methylation.

Résumé (français)

La présence répandue des traits de personnalité au sein du règne animal reflète leur importance évolutive. En effet, les différences de comportement entre individus qui sont constantes dans le temps et à travers différentes situations, aussi appelées traits de personnalité ou individualités comportementales, influencent divers paramètres tels que l'accès à la nourriture, les interactions sociales, les comportements d'antiprédation et *in fine*, le fitness des organismes qui représente la capacité d'un organisme à survivre jusqu'à maturité sexuelle, trouver un partenaire et produire une descendance. Bien que tout comportement animal soit défini comme le lien interactif entre un organisme et son environnement, les traits de personnalité sont largement interconnectés à l'écologie et à l'évolution d'une population. De nos jours, les organismes doivent faire face à des changements (rapides) de leurs conditions environnementales suite, entre autres, aux influences humaines. Le début de la vie d'un organisme est reconnu comme étant une fenêtre sensible au cours de laquelle l'environnement peut avoir des effets durables sur son phénotype et ce, jusqu'à l'âge adulte. Découvrir comment les changements environnementaux peuvent influencer la variabilité phénotypique est crucial pour comprendre les traits individuels et la capacité des organismes à s'acclimater à de nouvelles conditions environnementales pendant le développement et à l'âge adulte.

L'objectif général de cette thèse est donc d'étudier la plasticité développementale des traits de personnalité et la séquence des événements moléculaires clés conduisant à des modifications comportementales permettant aux organismes de faire face aux nouvelles conditions environnementales. Un vertébré unique, exprimant une variabilité génétique très faible voire nulle entre lignées, a été utilisé au cours de cette thèse, fournissant un modèle incroyable pour identifier les sources génétiques et environnementales de la variabilité phénotypique. Ce modèle est un poisson appelé le rivulus des mangroves, *Kryptolebias marmoratus*, comprenant des individus hermaphrodites capables de s'autoféconder et produisant dès lors des individus hautement homozygotes et naturellement isogéniques au sein d'une même lignée.

Le premier chapitre de cette thèse visait à caractériser les traits de personnalité d'audace et d'agressivité, ainsi que les mécanismes non génétiques générant de la variabilité comportementale entre des hermaphrodites élevés dans des conditions environnementales identiques. De la variabilité comportementale a été observée malgré l'absence de variations environnementales et génétiques entre les poissons testés. Les

analyses des profils d'expression protéique et des profils de méthylation de l'ADN du cerveau des poissons adultes (via respectivement les techniques de « label-free quantification » (LFQ) et de « Reduced Representation Bisulfite Sequencing » (RRBS)) ont fourni une liste de gènes candidats susceptibles d'être associés à des variations comportementales au niveau de l'axe audacieux/timide et de l'axe agressif/non-agressif, via entre autre, des changements de méthylation. Les résultats protéomiques et RRBS n'ont montré aucun(e) protéine/gène semblable aux deux continuums comportementaux, indiquant l'implication de différents mécanismes dans l'établissement de l'audace et de l'agressivité. Les résultats protéomiques ont révélé que le continuum audacieux/timide affectait principalement les processus métaboliques des acides aminés ainsi que les protéines structurelles et cytosquelettiques. Le continuum agressif/non-agressif a quant à lui, principalement influencé les protéines impliquées dans le développement, le maintien et la plasticité du système nerveux central ainsi que la neurotransmission. Le fragment différentiellement méthylé (FDM) le plus significatif détecté entre les poissons audacieux et timides était situé dans le corps du gène SKI. La différence du niveau de méthylation de ce gène pourrait indiquer une neurogenèse différenciée déterminant le niveau du caractère audacieux. Avec un changement de méthylation de 40% entre les poissons agressifs et non agressifs, le gène codant pour la protéine toll contrôlant la réaction pro-inflammatoire en réponse à une blessure, soutient, avec d'autres FDMs significatifs identifiés, une corrélation entre le niveau d'agressivité et la réponse immunitaire du poisson, comme cela été découvert chez l'homme. Conserver de l'individualité comportementale à travers plusieurs générations, même en l'absence de variations environnementales considérables, permettrait de maximiser les chances de survie d'une population en cas de changement des conditions environnementales. Ce concept est connu sous le nom de « bet-hedging strategy », stratégie évolutive dans laquelle un seul génotype produit une distribution de phénotypes parmi les descendants dans le but d'accroître la probabilité que certains individus soient bien adaptés à la pression de sélection due à des changements environnementaux imprévisibles.

Le deuxième chapitre fut consacré à comprendre la plasticité développementale des traits de personnalité chez *K. marmoratus* soumis à des stimuli pertinents d'un point de vue environnemental (présence d'un congénère ou d'une faible salinité) au cours de leur développement, en examinant également leurs effets sur les traits d'histoire de vie

(croissance et reproduction) et profils d'expression protéique du cerveau. Ces deux stimuli ont affecté différemment la croissance et la reproduction des poissons ainsi que l'expression de certaines protéines dans leur cerveau, bien que l'exposition se soit arrêtée longtemps avant l'échantillonnage des cerveaux. Les interactions sociales au cours du développement ont induit des modifications d'expression de 43 protéines, parmi lesquelles le réticulon 1, la protéine de liaison à la syntaxine, la glutaminase et le transporteur d'acide aminé neutre dépendant du sodium révélant des effets sur le transport de vésicules et l'activité neuronale. L'exposition à une faible salinité a modifié l'expression de 15 protéines dont la prothymosine alpha et la lipoamide acyltransférase indiquant qu'une faible salinité augmente le métabolisme des lipides et module la réponse immunitaire. Cependant, aucun effet des stimuli n'a été détecté sur le comportement des poissons. Le camouflage des effets sur le comportement suite à la variabilité comportementale élevée entre individus, la faible influence environnementale sur le déterminisme du comportement ou le choix des stimuli ne produisant pas d'effets suffisamment forts font partie des hypothèses soulevées et discutées dans ce chapitre.

Ces résultats ont conduit aux dernières expériences menées au cours de cette thèse: exposer les poissons à un stress plus intense, une neurotoxine dont l'impact sur le comportement des poissons est fortement suspecté, et étudier les effets immédiats et retardés de cette exposition. Cette neurotoxine est la β -N-méthylamino-L-alanine (BMAA), produite par les cyanobactéries, diatomées et dinoflagellés, présente naturellement dans l'environnement de *K. marmoratus* et liée au développement de maladies neurodégénératives chez l'homme telles que la maladie de Parkinson, la sclérose latérale amyotrophique et la maladie d'Alzheimer. Premièrement, les comportements de locomotion et de capture des proies chez le rivulus ont été étudiés après une exposition d'une semaine à 2 doses sublétales de BMAA (20 μ g/L et 15 mg/L) chez des larves fraîchement écloses. La BMAA a significativement augmenté la vitesse maximale de déplacement ainsi que le nombre d'essais et d'échecs de capture de proies révélant de potentielles altérations du mouvement et de la signalisation synaptique. Deuxièmement, les effets retardés d'une exposition des larves fraîchement écloses durant 2 semaines aux deux concentrations sublétales de BMAA ont été évalués sur les traits d'histoire de vie, les traits de comportement et l'expression relative de 7 gènes dans le cerveau des poissons adultes, gènes connus comme étant la cible de la BMAA ou

impliqués dans la neurotransmission et/ou les traits de personnalité. Bien qu'aucun effet sur la croissance, la reproduction et les traits comportementaux n'ait été détecté, la BMAA a induit une augmentation significative de l'expression des gènes CaM et MAOA à 20 µg/L BMAA comparé au groupe contrôle. Une diminution significative d'expression a été observée entre cette plus faible dose de BMAA et 15 mg/L pour les gènes DRD4, MAOA et CaM. La perturbation du renouvellement du glutamate, l'appauvrissement intracellulaire en dopamine et l'activation potentielle des mécanismes de protection des astrocytes sont les effets suspectés indiquant que la BMAA pourrait affecter les caractères phénotypiques sur le long terme, effets potentiellement visible avec le vieillissement.

Selon les résultats recueillis au cours de ce projet, nous pourrions conclure que les traits de comportement du rivulus de mangrove semblent être soumis à une faible influence environnementale, même en présence de composés neurotoxiques. Les traits de personnalité, en raison de leur influence sur le fitness des organismes, sont peut-être moins plastiques en réponse à des stimuli présents pendant le développement du rivulus. L'hypothétique déterminisme environnemental faible des traits d'audace et d'agressivité éviterait d'avoir une homogénéité comportementale au sein de la population afin d'assurer l'acclimatation et l'adaptation du rivulus aux nouvelles conditions environnementales pouvant survenir chez les adultes, hypothèse hautement plausible au regard des conditions environnementales extrêmes rencontrées dans les mangroves. Nos résultats suggèrent également une stratégie évolutive de « bet-hedging » favorisant le maintien de la diversité des traits au sein de la population isogénique du rivulus des mangroves par le biais de mécanismes épigénétiques tels que la méthylation de l'ADN.

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LIST OF ABBREVIATIONS AND ACRONYMS

1-DE	one-dimensional gel electrophoresis
2D-DIGE	2D difference in-gel electrophoresis
2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
AD	Alzheimer's disease
Agg	aggressive
ALS	amyotrophic lateral sclerosis
ASPD	antisocial personality disorders
AUC	area under the curve
AVT	Arginine vasotocin
BCP	1-Bromo-3-chloropropane
BDNF	Brain derived neurotrophic factor
BMAA	β -N-methylamino-L-alanine
BP	biological processes
BPD	borderline personality disorder
CaM	Calmodulin
CaMKII	Calmodulin kinase II
CC	Cellular compartment
cDNA	complementary DNA
Cer	ceramide
CG	compensatory growth
CNS	central nervous system
CpG	Cytosine followed by a Guanine
CTCF	CCCTC-binding factor
DAP	Differentially abundant protein
dCas9	deactivated Cas9
DDA	data-dependent acquisition
DHF	dihydrofolate
DHFR	Dihydrofolate reductase
DMF	differentially methylated fragment
DNAme	DNA methylation
DNMT	DNA methyltransferase
DOHaD	Developmental Origin of Health and Disease
Dph	days post hatching
DRD4	dopamine receptor 4
ELS	Early-life stages
Epd	Ependymine
ER	endoplasmic reticulum
FC	Fold change
FDR	False discovery rate
FMR	fragile X mental retardation

GABAA	γ -Aminobutyric acid A
GLM	Global linear mixed model
glula	glutamine synthetase a
GO	Gene Ontology
GOI	gene of interest
GS	Glutamine synthetase
Hb α 4	Haemoglobin α 4 subunit
HDAC	histone desacetylase
HKG	housekeeping gene
Kat	Lysine Acetyltransferases
Kdm	Histone Lysine Demethylases
Kmt	Histone Lysine Methyltransferases
LC	liquid chromatography
LFQ	Label-free quantitation
LMM	Linear mixed model
MAOA	monoamine oxidase A
MBP	methylated DNA binding protein
MeCP2	Methyl CpG Binding Protein 2
MF	molecular function
MHC1	Major histocompatibility complex Class I
MoTiPhe	Molecular tissue-specific phenotype
mRNA	messenger RNA
MS	mass spectrometry
Nagg	non-aggressive
NC	neurotoxic compound
ncRNA	non-coding RNA
ND	neurodegenerative disease
NSC	neuronal stem cell
PBCP	planetary boundaries chemical pollution
PCA	Principal Component Analysis
PD	Parkinson's disease
PDC	parkinsonism dementia complex
PDI	protein disulfide isomerases
ppt	parts per thousand
PSD	postsynaptic density complex
PTM	posttranslational modifications
QC	quality control
R	repeatability
R/time	conditional repeatability
R1	replicate number 1
R2	replicate number 2
R3	replicate number 3
R _A	agreement repeatability
R _c	consistency repeatability
R _E	enhanced repeatability
ROS	reactive oxygen species

RRBS	Reduced Representation Bisulfite Sequencing
RTN4	reticulon 4
SAM	S-adenosyl-L-methionine
SC	spectral counting
SERT	Serotonin transporter
SL	Sphingolipids
SNP	single nucleotide polymorphism
STR	Short tandem repeat
STXBP	syntaxin binding protein
syn	synuclein
TAD	topological associated domain
TDS	temperature-dependent sex determination
TET	Ten-Eleven Translocation protein
THF	tetrahydrofolate
tRNA	transfer RNA
Ve	environmental variability
Vg	genetic variability
VGLUT	glutamate vesicular transporter
Vgxe	interaction between genetic and environmental variability
Vp	phenotypic variability

GENERAL INTRODUCTION

1. Context of the study

Behavior constitutes the central core of the interactions between an organism and its environment. Behavior influences and is affected by development, physiology, environmental factors, ecological dynamics and evolution (Sih et al., 2010). Understanding these interactions is a major challenge of biology, particularly in the actual context of rapid human-related environmental changes. Personality traits are behaviors of particular interest for behavioral ecologists due to their high influence on organisms fitness and therefore on ecological and evolutionary processes. Individuals expressing their own behavior, concept commonly called behavioral individuality, is known to develop from genetic sources, environmental influence and their interactions. But recent studies emphasized the effect of a third “random” source generating variability between individuals, whose epigenetic mechanisms are part of, even in the absence of genetic diversity and environmental changes (Bierbach et al., 2017; Freund et al., 2013). Identifying the origin of the random variability is a challenge regarding the reduction of both genetic and environmental variability. A new valuable vertebrate model presents a reproductive strategy that allows to naturally produce clones and therefore to linger on molecular sources of behavioral variability, the mangrove rivulus, *Kryptolebias marmoratus*. Beyond answering fundamental questions of the molecular bases of personality traits, investigating how these behaviors respond to factors and stressors occurring during their development is crucial to better understand the developmental plasticity of personality traits and how organisms can cope with environmental changes. Developmental period constitutes a sensitive window to environmental clues such as social interactions, environmental physico-chemical conditions or pollutants, particularly due to the vulnerability of the central nervous system.

In this context, the first chapter was dedicated to assess the presence of behavioral individuality among isogenic individuals and characterize the brain molecular bases of boldness and aggressiveness personality traits in the mangrove rivulus. The molecular mechanisms underlying their developmental plasticity after exposure to social interactions or low salinity by combining measures of life-history

traits throughout the development and the brain molecular phenotype (proteome) in the adults were assessed (Chapter 2). To go further in the investigation of developmental plasticity of behavioral traits, a last experience was performed to investigate the immediate and lasting effects of a neurotoxin on rivulus behavior associated with changes of gene expression (Chapter 3). This neurotoxin is of great interest due to its causal relation to neurodegenerative diseases in humans such as Alzheimer disease, Parkinson disease and amyotrophic lateral sclerosis.

In this introduction, the general concepts of animal behavior as well as personality traits in the animal kingdom will first be described (section 2). In this section, particular attention will be dedicated to a concept known as behavioral syndrome and the origin and evolution of personalities. Then, the concept of phenotypic plasticity will be discussed from an historical point of view with the evolution of the central dogma of molecular biology (section 3). The stakes of omic approaches to investigate molecular bases of personalities and developmental plasticity will be examined with a focus on LFQ proteomics and DNA methylation analysis (section 4). Finally, the ecology, life cycle, reproductive characteristics, behavior and the genetic and epigenetic characteristics of the model organism used as well as its potential for this study will be presented (section 5).

2. Animals' behaviors

2.1. General concepts

Animal behavior has always interested human populations. 30,000 years-old prehistoric paintings of hunting scenes across the world testify of this widespread interest of humans. This began with interest in animal behavior to understand preys habits in the environment and therefore to improve their hunting. With the development of agriculture animals were domesticated and used. The first scientific descriptions of animal behaviors in the 19th century were using anecdotes and anthropomorphic views. The first exception arrived in 1859 with Charles Darwin who described animal behavior with an evolutionary point of view wondering how natural selection might have shaped rudimentary behaviors into sophisticated ones (Wyatt, 2017).

Behavior 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). Animal behavior is a complex concept which has been defined by Levitis et al. (2009) as “*the internally coordinated responses (actions or inactions) of whole living organisms (individuals or groups) to internal and/or external stimuli, excluding responses more easily understood as developmental changes*”. With this definition, we understand how wide the panel of behaviors can be. A meerkat standing straight above his burrow; an octopus changing color for camouflage on a coral reef; a bee collecting nectar from a flower; all of these are examples of animal behavior. The study of animal behavior referred as ethology, is a discipline of biology (1859) expanded in the XXth century with, for example, the biologist and ornithologist Nikolaas Tinbergen who developed four questions to describe animal behavior or a simple animal feature (Wyatt, 2017): What is it for? How did it develop during the lifetime of the individual? What mechanisms control this behavior? How did it evolve over evolutionary time? These questions are still used in ethology, but the study of animal behavior mainly expanded in the last two decades when individual behavioral differences were considered as the target of animal behavior research and not as a background noise anymore (Conrad et al., 2011; Réale et al., 2010; Sih et al., 2004; Wolf & Weissing, 2012). The magnitude as well as the ecological and evolutionary importance of behavioral variation of non-human animals was increasingly appreciated by researchers (Roche et al., 2016). Increasing progress has been recently provided in the study of animal behavior in terms of genetic and molecular mechanisms (Bell and Aubin-Horth, 2010). The development of new molecular tools providing original approaches such as the study of proteomes, transcriptomes and genomes revolutionized the study of animal behavior.

2.2. Individuality in behavior: personality traits

Each organism has to deal successfully with the constant changes of its environment (spatial, temporal, social, physico-chemical, etc.) in order to survive and reproduce. Especially in the actual context of (rapid) changing environment, it would be essential for an organism to behave appropriately to new environmental conditions. Factors that strongly influence organism's fitness in a specific situation, which corresponds to organism ability to survive to reproductive age, find a mate (or not

necessarily...) and produce offspring, are highly submitted to natural selection (Brown et al., 2007). Behavioral phenotypes are obviously not an exception. Some particular behavioral phenotypes strongly influence social relationships, predator avoidance or food access, and therefore the organisms' fitness (Figure 1) (Burns, 2008; Réale et al., 2007). These behaviors are commonly called personality traits and are defined as ***“individual differences in behavior that are consistent both across time and across contexts”*** (J. A. Stamps and Groothuis, 2010). 5 common axes of personality traits have been described in animals: aggressiveness, sociability, boldness, activity and exploration. Consistent differences have been reported in many other behavioral tendencies, such as cooperativeness, docility, impulsivity, responsiveness to environmental stimuli, etc. (Gosling, 2001; Réale et al., 2007; A Sih et al., 2004). Personality traits combination shapes the temperament of an individual and animal personalities have been reported in many species across the animal kingdom (mollusks, arthropods, amphibians, reptiles, birds, fish and mammals) (Conrad et al., 2011; Courteney-Jones and Briffa, 2014; Gosling, 2001; Hulthen et al., 2014; Hurtado and Mabry, 2017; Niemelä et al., 2012a; Sih et al., 2004; Toms et al., 2010; Wolf and Weissing, 2012b). Amongst personality traits, boldness and aggressiveness are the most studied ones (Réale et al., 2007).

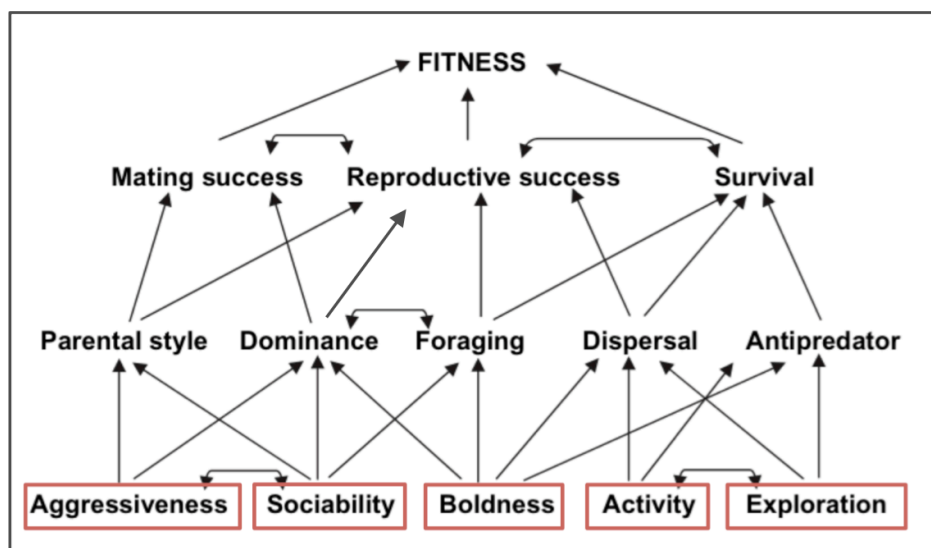


Figure 1 – Flow diagram illustrating the influence of the 5 main studied personality traits (red boxes) on component and composite traits leading to consequences on organism fitness. Adapted from Réale et al. (2007).

Boldness is described as the individual's reaction to a situation perceived as dangerous, the propensity of an organism to take risk in a given situation (Réale et al., 2007). Within a population each individual expresses a behavior in the “bold to shy” range and difference between individuals stays consistent across time and situations.

Aggressiveness is the propensity of organisms to be aggressive against an individual. It helps to defend against predators, to protect resources and territory (Ellison et al., 2013) and to maintain hierarchy in gregarious organisms allowing a better access to food and mating for the dominant (Ang and Manica, 2010). Even if the level of a behavioral trait such as aggressiveness can lead to a high energy demand or risk of injuries/death, it also provides benefits such as a better food access and reproductive success (Ruiz-Gomez & Huntingford, 2012).

Within a population, the amount and structure of variation between individuals can influence ecological and evolutionary processes as well as their interaction (Mcnamara and Leimar, 2010). Individual variation is also increasingly recognized by ecologists as a clue affecting intra- and interspecific competition and therefore the structure and dynamics of ecological networks (Sih et al., 2012). Adaptive personality research tends to discover and characterize the processes explaining the emergence of personality differences within and among species (Sih et al., 2015).

The study of animal personalities faces controversy and apprehensions from some researchers due to the lack of terminological consistencies, strong conceptual framework and empirical studies. The field research of animal personality is mostly theory-driven even though it is increasingly studied, and many terms describing closed concepts are used generating general misunderstanding for non-specialists (Carter et al., 2013; David and Dall, 2016). For detailed information about animal personality and terms commonly associated, please refer to the commentary of Roche et al. (2016).

2.3. Behavioral syndromes

Behavioral ecologists have noticed that some behavioral traits are positively or negatively correlated with each other in a range of animal taxa, including a variety of invertebrates, amphibians, reptiles, fish, birds, and mammals (Bell, 2005; Gosling, 2001; Kelleher et al., 2018; Sih et al., 2004; Sih and Watters, 2005; Waters et al., 2017). Two or more behavioral traits co-varying across contexts or situations form a behavioral syndrome (Conrad et al., 2011; Sih et al., 2004). Behavioral syndrome of personality

traits influences other behaviors such as foraging or reproductive behavior. However, the correlation between personality traits 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., 2004; Wolf and Weissing, 2012b). The early settlement of a behavioral syndrome during ontogenesis also limits the evolution of the behavioral traits. They thus evolve together in the same direction. The behavioral syndrome can thus be adaptive as well as maladaptive throughout organism life depending on the ecological context (Bell and Stamps, 2004; Sih and Bell, 2008).

For example, in the case of a positive correlation between boldness and aggressiveness, some individuals tend to be more aggressive and bold than others along time and across contexts within a population. These individuals are more likely to risk intra-specific battles but also to face a predator (Wolf et al., 2007). 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. However, a bold and aggressive individual is more easily exposed to dangerous situations (predators, parasites, etc.), which greatly increases the mortality risk. This example pictures how behavioral syndrome can be adaptive or maladaptive according the context and time. The presence of trade-offs between behavioral traits defines the limits of plasticity of each trait (Sih et al., 2004). Natural selection will thus select the most suitable trade-off in a specific environment (Conrad et al., 2011).

Some behavioral syndromes are more extensively studied such as the aggressiveness-boldness syndrome (A. Sih et al., 2004). The correlation between aggressiveness and boldness 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 between populations due to diverse factors such as the predation pressure (Bell and Stamps, 2004; Salonen and Peuhkuri, 2006). The first study discovering a correlation between behavioral traits in fish dates from 1976, where sticklebacks were used, with a special focus on their bold and aggressive behaviors (Huntingford, 1976). Ann Huntingford described a positive correlation

between territorial aggression of fish and their boldness towards a predator, without mentioning the term “syndrome”. Bell and Stamps (2004) described many years after a negative correlation in sticklebacks indicating potential differences between populations and/or experiments. Another study on the swordtail fish (*Xiphophorus multilineatus*) revealed that maternal investment influences the aggressive/bold syndrome settlement of the offspring. Sons of mothers reared with high quality diet expressed positive correlation within this syndrome while low quality diet did not. This study revealed that environment can modulate the settlement of syndromes (D’Amore et al., 2015). A last example concerns the model fish used in this thesis, the mangrove killifish (*kryptolebias marmoratus*). Edenbrow and Croft (2012) have found that correlation between boldness and aggression was only expressed by secondary males (hermaphrodites that turn into males - for more details, see the section 5 of the general introduction), indicating potential differences between sexes. As mentioned in the review of Sih et al. (2015), animal personalities and syndromes vary as a function of ecology.

2.4. Origin and evolution of personalities

Some studies tried to characterize the genetic bases of personality in human, model laboratory animals and domesticated species. They identified a few candidate genes related to personality traits such as the dopamine receptor 4 (DRD4) or the monoamine oxidase A (MAOA) (Table 1) (Checknita et al., 2015; Garamszegi et al., 2014a; Newman et al., 2005; Ni et al., 2007). Discovering the genetic architecture of the natural variation of personality traits, which are often polygenic (one phenotypic trait is governed by more than one gene) or submitted to pleiotropic effects (one gene influences two or more phenotypic traits) led research to roll away from traditional quantitative genetic approaches. In fact, personality traits raise many questions such as why individuals behave consistently over time and situations, and what maintains heritable variation in personality traits (Bell and Aubin-Horth, 2010). Whole genome sequencing techniques allows to investigate those questions at the genetic level (Table 2).

Increasing number of studies over the past 20 years focused on non-genetic bases of behavioral individuality. This research area emerged from observations of behavioral variability among genetically identical individuals reared in the same

laboratory/environmental conditions such as mice (Freund et al., 2013), insects (Schuett et al., 2011), pigs (Archer et al., 2003), and fish (Bierbach et al., 2017; Millot et al., 2014). The traditional equation representing the sources of phenotypic variability (V_p): " $V_p = V_g + V_e + V_{gxe}$ " with " V_g " and " V_e " respectively represent the genetic and environmental contributions to the phenotypic variability and " V_{gxe} " their interaction, seemed thus incomplete. Developmental variation such as maternal behavior, environmental effect, mitochondrial background and methylation-associated variability could induce accumulating differences over time potentially responsible of the behavioral variability persistence with the absence of genetic and environmental variability (Bierbach et al., 2017; Freund et al., 2013). Most of these studies used artificial clones or organisms with specific mode of reproduction such as parthenogenesis.

Table 1

Overview of genes implicated in boldness and aggressiveness personality traits (non-exhaustive list)

Genes (ID)	Aggressiveness	Boldness	Other personality traits	Species studied	References
Dopamine-receptor D4 (DRD4)	x			Great tit (<i>Parus major</i>)	(Verhulst et al., 2016)
			x (exploration)	Great tit (<i>Parus major</i>)	(Korsten et al., 2010)
		x		Hedge accentor (<i>Prunella modularis</i>)	(Holtmann et al., 2016)
		x	x (exploration)	collared flycatcher (<i>Ficedula albicollis</i>)	(Garamszegi et al., 2014a)
		x	x (exploration)	Yellow-crowned bishops (<i>Euplectes afer</i>)	(Mueller et al., 2014)
Monoamine oxidase A (MAOA)	x			Rhesus macaque (<i>Macaca mulatta</i>)	(Newman et al., 2005)
Calmodulin (CaM)		x		Rainbow trout (<i>Oncorhynchus mykiss</i>)	(Sneddon et al., 2005)
Ependymine (Epd)		x		Rainbow trout (<i>Oncorhynchus mykiss</i>)	(Sneddon et al., 2005)
γ -Aminobutyric acid A (GABAA)	x	x		Rainbow trout (<i>Oncorhynchus mykiss</i>)	(Miczek et al., 2003; Sneddon et al., 2005)
Arginine vasotocin (AVT)	x			Rainbow trout (<i>Oncorhynchus mykiss</i>)	(Backström and Winberg, 2009)
Serotonin transporter (SERT)		x		Hedge accentor (<i>Prunella modularis</i>)	(Holtmann et al., 2016)
Major histocompatibility complex Class I (MHC I)		x		Rainbow trout (<i>Oncorhynchus mykiss</i>)	(Sneddon et al., 2005)
Haemoglobin $\alpha 4$ subunit (Hb $\alpha 4$)		x		Rainbow trout (<i>Oncorhynchus mykiss</i>)	(Sneddon et al., 2005)
Brain derived neurotrophic factor (BDNF)		x (harm avoidance)	x (neuroticism)	Humans (<i>Homo sapiens</i>)	(Montag, 2014)

Table 2

Evolutionary questions about personality and the potential genetic mechanisms associated. Adapted from Bell and Aubin-Horth (2010).

Evolutionary question	Genetic mechanism
Why do individuals behave consistently?	Pleiotropy; cost of plasticity
What maintains heritable variation in personality traits?	Additive genetic variation underlying fitness-related personality traits
Can we compare personality axes across species?	Homologous genes/pathways underlying personality traits
How individuality in behavior can arise from natural isogenic individuals and be heritable?	?

3. Phenotypic plasticity

3.1. Central dogma of molecular biology

In 1958, the fundamental theory of molecular biology referred as the “central dogma of molecular biology” was presented by James Watson and Francis Crick, explaining the conversion and utilization of the genetic information (Figure 2). According to this dogma, DNA constitutes the stable and heritable genetic information that defines the totality of organism’s biological functions; organism’s interaction with the environment, its reproduction, nutrition, behavior, etc.. DNA can replicate (replication) or be transcribed to RNA (transcription), both molecules constituted of building blocs; the nucleic acids. RNA serves to translate the genetic information from DNA into proteins (translation), build from amino acids. This dogma described this relationship as unidirectional (from DNA to proteins) and biologists considered for a long time that the organism’s phenotypes was exclusively dependent on the genes (Van Holde and Zlatanova, 2018).

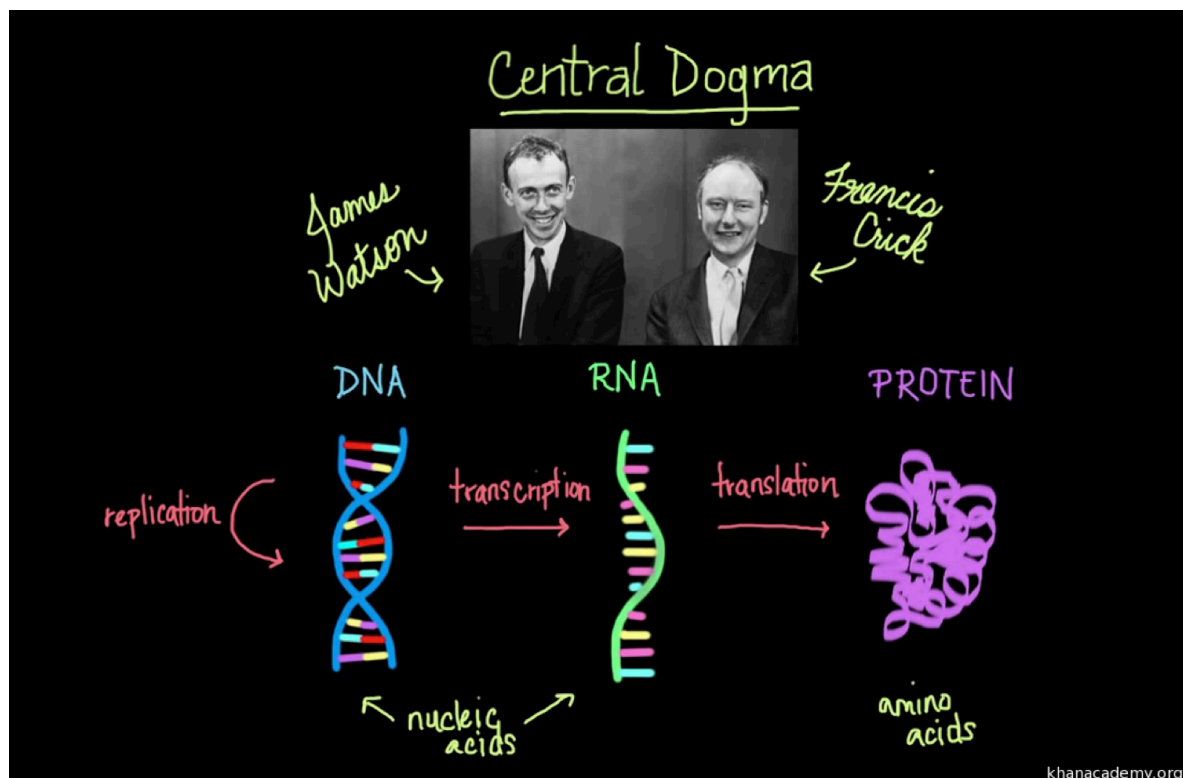


Figure 2 – Schematic illustration of the central dogma of molecular biology developed by James Watson and Francis Crick in 1958. The relationship between DNA, RNA and proteins is described as unidirectional. Adapted from Khanacademy.org.

3.2. Revised central dogma and implications for phenotypic plasticity

With the discoveries and improvement of new molecular techniques, the central dogma of molecular biology was implemented (Figure 3). The information flow was described as multidirectional in the revisited dogma. The discovery of reverse transcriptase enzymes revealed that RNA can be retrotranscribed into complementary DNA (cDNA) and that information coming from RNA can be integrated into the DNA sequence and replicated. This constituted the first violation of the central dogma in the 1970s (Coffin and Fan, 2016). The discovery of RNA viruses also went against the classic central dogma. RNA from viruses can be directly translated to proteins by the host cell machinery or serves as a template for another RNA strand that can be used for protein translation (Kolakofsky, 2015). Later, epigenomics highlighted 3 main epigenetic mechanisms that are able to generate various phenotypes from one genotype without any changes of the DNA sequence. The discovery of unchanged genetic information associated to phenotypic plasticity through molecular mechanisms such as DNA methylation, histone modifications and non-coding RNAs shook for good the initial thought of unidirectional flow of genetic information (detailed information about epigenetic mechanisms is provided in section 4) (Kilvitis et al., 2017; Norouzitallab et al., 2019).

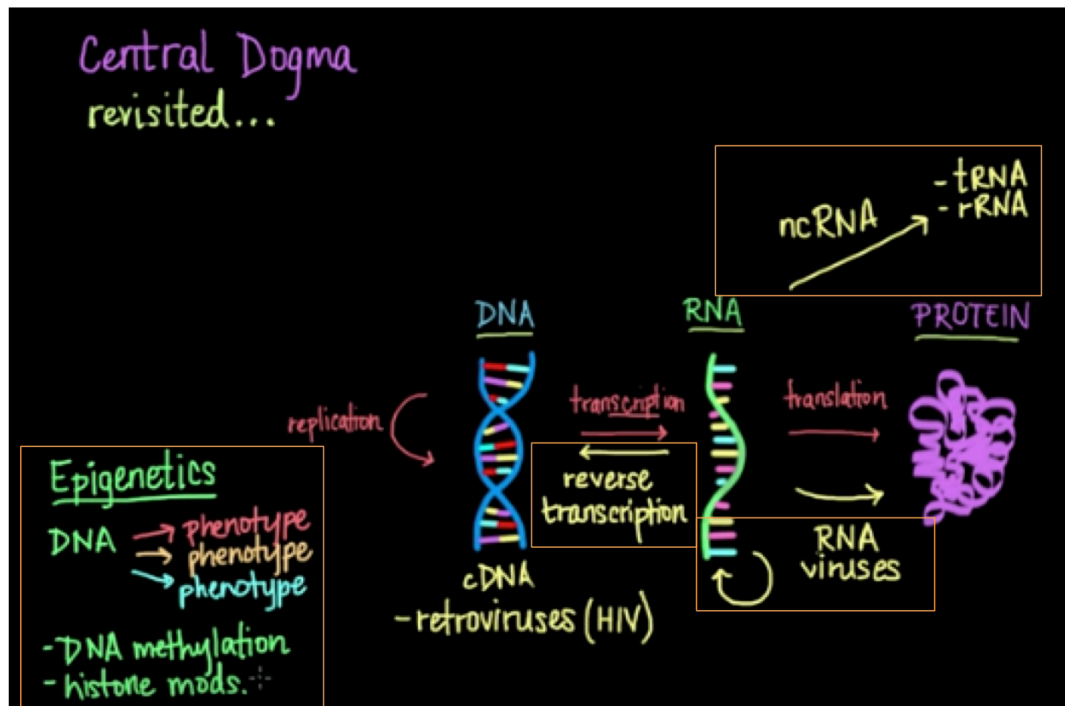


Figure 3 - Schematic illustration of the revisited central dogma of molecular biology. The relationship between DNA, RNA and proteins is no longer described as unidirectional with the discovery of reverse transcription allowing to incorporate information from RNA into the DNA sequences as well as the presence of RNA viruses that can directly lead to proteins. DNA methylation, histone modifications, non-coding RNA (ncRNA) constituting epigenetic modifications can modify phenotypes as well, without interfering with the DNA sequence. Adapted from Khanacademy.org.

However, the influence of epigenetic mechanisms on the phenotype of an organism is absent of the commonly used equation to represent factors determining the phenotype of an organism. Phenotypic plasticity refers to the capacity of one genotype to produce more than one phenotype in response to different environments. The phenotype of an organism is the outcome of its genotype (G), the environment in which it lives (E) and the interaction between the genotype and the environment (GxE) (Delcourt et al., 2017). If traits are not plastic, the phenotype is therefore exclusively function of the genome (Figure 4-A)(Karp, 2018; Li et al., 2017). The set of phenotypes expressed by an individual from a single genotype across a range of environments represents its reaction norm (Figure 4-B). Various genotypes can express different reaction norms even if they are exposed to the same environment. As well as individuals with the same genotype can express various phenotypes if they are exposed to different environment (Aubin-Horth, 2009).

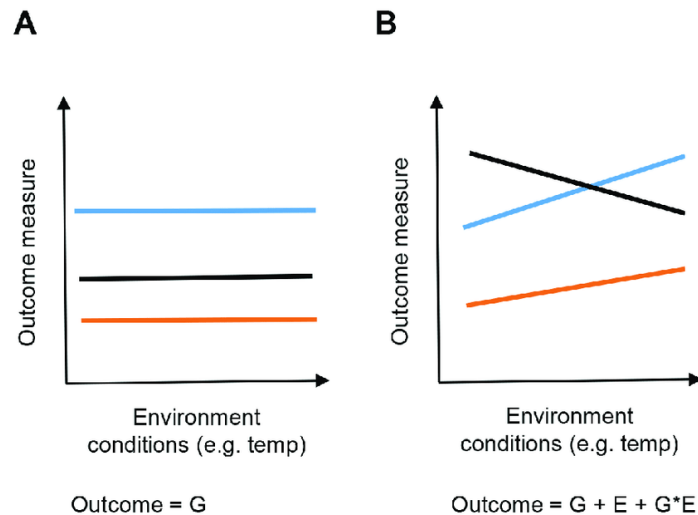


Figure 4 - Visualization of phenotypic plasticity. A schematic to demonstrate the effect of phenotypic plasticity on an outcome measure. Each line with a unique color represents a different genotype. (A) Behavior when there is no phenotypic plasticity showing an outcome that depends on the genotype but is independent of the environment. (B) Behavior when there is phenotypic plasticity showing that the outcome depends on the genotype, environment, and an interaction between the genotype and environment. The observed phenotype for an organism, for the majority of traits, is a function of the environment and the genotype. G, genotype; E, environment; temp, temperature. From (Karp, 2018).

Phenotypic plasticity can be subdivided into developmental plasticity and phenotypic flexibility (Figure 5). Developmental plasticity refers to the capacity of one genotype to produce diverse lasting changes to an actual phenotype due to past external experiences, stimuli or environmental conditions exposure during an individual's development (Crispo, 2007). The developmental plasticity is therefore an irreversible variation in the traits of individuals that results from environmental variation occurring during development. On the opposite, phenotypic flexibility is a reversible phenotypic transformation due to environmental influence over a shorter timescale than a lifetime on the phenotype (Piersma and Drent, 2003). Developmental plasticity constitutes a primordial part of the phenotypic plasticity by influencing organisms' fitness and on a broader scale population survival. During the early stages of development, the organisms are highly flexible and influenced by the environment. This period constitutes a sensitive window of organism life. Developmental plasticity drives many mechanisms such as immune system adaptation or learning capabilities (Minelli and Fusco, 2010; Nettle and Bateson, 2015) and leads to a persistent phenotype in adults (Beldade et al.,

2011). It can be expressed at different levels of body organization (extern or intern) such as morphology, behavior, changes of blood hormonal content, protein concentrations, modification of epigenetic marks leading to changes in genes expression, etc. A well-known example of developmental plasticity is the temperature-dependent sex determination (TDS) such as in most turtles and all species of crocodilians. For TDS species, the environment determines the sex after the fertilization, when temperature during early development irreversibly settles the phenotypic sex and can impact the sex ratio (Escobedo-Galván, 2013; Gilbert, 2000; Valenzuela et al., 2019). In a changing environment, developmental plasticity can be adaptive; leads the success of organisms in novel habitats and therefore allows organism's survival in the new environmental conditions (Fusco and Minelli, 2010).

Within a population, the mean and variance of a phenotype can be influenced by the plasticity expressed by individuals. The effect of plasticity on the phenotypic variance and average phenotype of a population depends on the temporal scale, genetic variability, and heterogeneity of environmental cues (Fordyce, 2006). The Figure 6 represents possible outcomes of plasticity on a population phenotype. An adaptive phenotypic plasticity can potentially contributes to genetic differentiation and speciation (Agrawal, 2001). When a population colonizes a new environment, plasticity is essential to survive and persist, influencing the strength and direction of natural selection, which impacts the phenotype frequency within the concerned population (Price, 2006; Price et al., 2003).

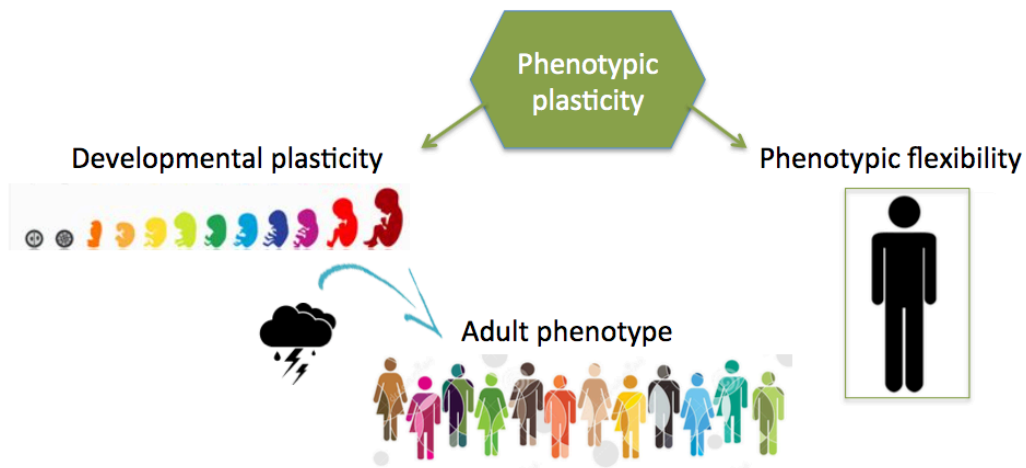


Figure 5 – Schematic illustration of the phenotypic plasticity subdivision into developmental plasticity and phenotypic flexibility. Phenotypic flexibility refers to reversible phenotypic changes due to the influence of the environment that can occur during developmental stages or in adult. On the opposite, the developmental plasticity is the irreversible variation in the traits of individuals that results from environmental variation occurring during development.

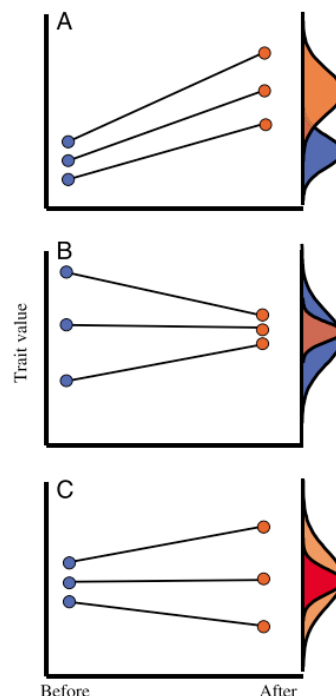


Figure 6 - Scenarios for change in mean and/or variance of a trait in a population between the constitutive phenotype expressed prior to an interaction (blue) and the induced phenotype following an interaction (red). (A) An increase in mean and variance of a trait. (B) Decrease in variance, mean unchanged. (C) Increase in variance, mean unchanged. From Fordyce (2006).

4. Omic studies

A recent concept representing "the large-scale study of all organizational levels of biological systems (genomics, transcriptomics, proteomics, metabolomics, etc.) is referred as the "system biology"." System biology aims to understand and predict the functioning of biological systems as a whole (Moore and Weeks, 2011). "Omics" consists of the system biology approaches that study all the components of a system without *a priori* using high-throughput techniques. They revolutionized biology by expanding and deepening our understanding of biological processes (Misra et al., 2018). "Omics" tools generate large-scale datasets, which combined with bioinformatic tools, provide the molecular signatures of an organelle, a tissue, an organ at a specific time or under a specific condition (Shi et al., 2019; Sukardi et al., 2010). Although the recent advances in "omics" techniques and associated data processing and analysis allow the understanding of molecular phenotypic changes associated to a specific investigation, integrated omics faces five main challenges with elevated amount of data to store and handle as well as the interpretation of results with the actual biological knowledge are among the biggest ones (Figure 7). Several "omics" tools exist that focus on specific levels of biological organization: metabolomics (metabolites profiling), proteomics (cell- and tissue-wide protein expression), transcriptomics (genomic-scale mRNA expression) and epigenomics (chromatin state and DNA chemical modifications). The combination of one or many of these techniques to the observations at the organism phenotypic level provides a deep picture of molecular bases of a particular phenotype.

Challenges in Integrated Omics

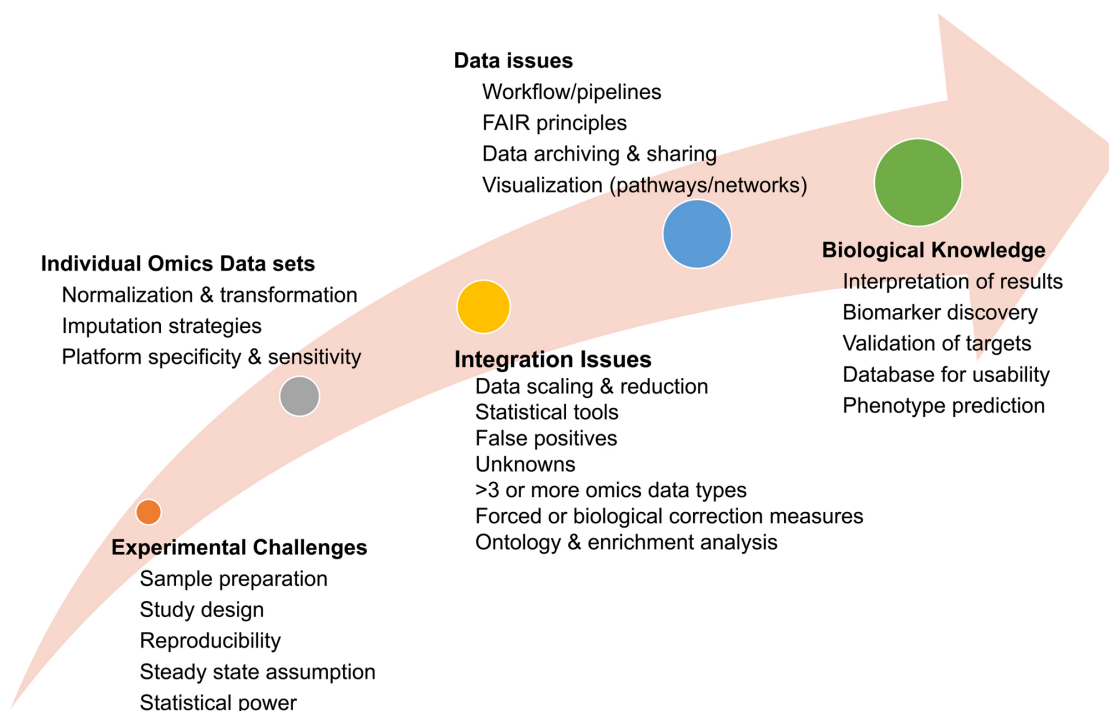


Figure 7 – Five challenges associated with integrated omics which encompass experimental challenges, individual omics datasets, integration tissues and biological knowledge. From Misra et al. (2018).

4.1. Proteomics

4.1.1. Proteomes: definition and stakes

Proteomics aims to characterize the proteomes; all proteins of a cell, an organelle, a tissue, an organ or an organism at a specific time and under defined conditions (Wilkins et al., 1996). The « proteome » was used and defined for the first time in the 1990s in order to have an equivalent concept to the « genome ». Proteomes are dynamic: they vary according to several parameters such as environmental conditions, tissue, developmental stages, etc. (Diz et al., 2012; Silvestre et al., 2012).

It was commonly assumed according to the central dogma of molecular biology that a direct correspondence exists between messenger RNA (mRNA) transcripts and the abundance of the proteins generated (Haider and Pal, 2013). Although the study of gene expression is very popular because it provides important information about gene expression at the mRNA level, many factors and regulatory steps exist between gene transcription and the resulting protein (Feder and Walser, 2005; Silvestre et al., 2012).

Alternative splicing, mRNA stability, translation, post-translational modifications and protein turnover can directly influence the protein abundance (Figure 8) (Diz et al., 2012). Following the different regulatory mechanisms involved between the transcription of a gene and the final protein, the level of mRNA is not necessarily correlated with protein abundance (Feder and Walser, 2005). In fact, the proportion of variation in protein abundance that can be explained by the abundance of mRNA was assessed at less than 0.5 (Feder and Walser, 2005). The expression of a same gene can produce several different proteins depending on various parameters such as environmental conditions. The proteome is more complex and larger than the transcriptome (Zubarev, 2013); hence the interest in focusing on proteomics - key link between the genotype and the organismal phenotype - which identify and quantify proteins, determine protein-protein interactions and detect post-translational modifications (Figure 9) (Diz et al., 2012; Silvestre et al., 2012). The proteome is considered as the cellular phenotype because the biochemical processes in cells relies mainly on the optimal functioning of proteins in both normal and stressful environmental conditions (Feder and Walser, 2005). "Cell fitness" therefore depends on proteins (Pan et al., 2009). The use of a combination of both transcriptomics and proteomics in several research fields of biology recently became very popular and provides better insight into complex biological pathways related to specific phenotypes (Schenk et al., 2019; Tica et al., 2018; Ye et al., 2017; Zhao et al., 2019). Despite the recent advances in proteomic technologies, actual proteomic techniques are limited and cannot detect the entire set of proteins from a cell or a tissue due to the wide dynamic range of the proteome that can reach 12 orders of magnitude such as in biofluids (e.g. plasma) (Surinova et al., 2011). Moreover, technologies in the field of proteomics face to the low detection issue of low-abundant proteins which therefore promotes the additional use of transcriptomics in studies (Zubarev, 2013).

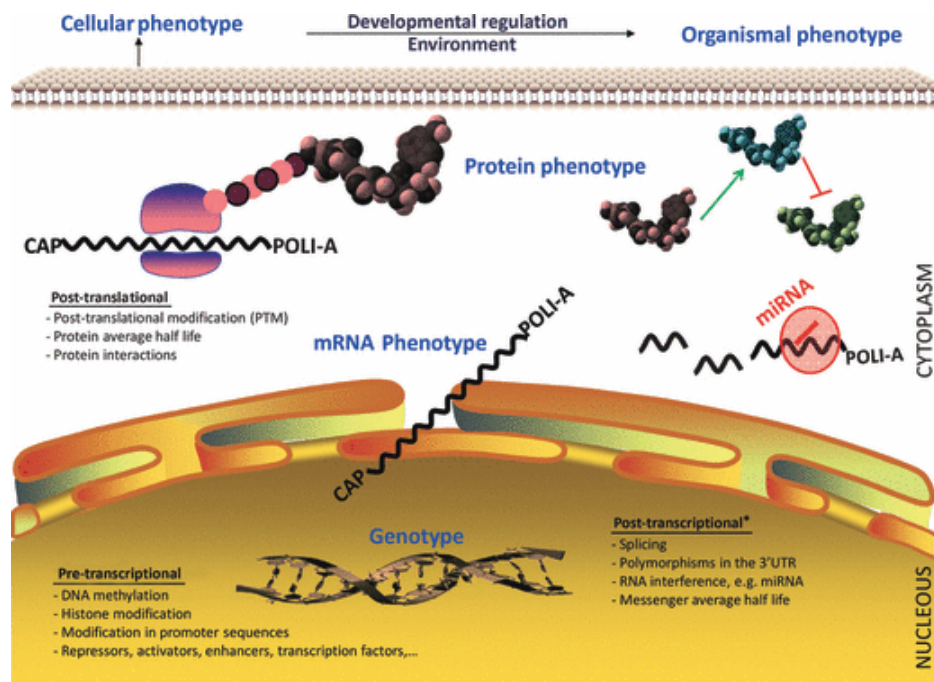


Figure 8 - Possible regulation steps causing discrepancies between the expression of a mRNA and its corresponding protein. First, the genome has to be ready to be transcribed. After transcription, several processes can change the levels of such an mRNA, or even its translation. Once the protein is synthesized, its expression levels can vary by interacting with other proteins or suffering different posttranslational modifications (PTM), and its average half-life can be also modified according the specific needs of the moment (see further details in Feder and Walser, 2005). In addition to these mechanisms, developmental processes and environment may cause proteomic differentiation across tissues and organs, necessary to understand the full phenotype complexity. Therefore, depending on which level we are working in, different relationships with the phenotype will be found. For simplicity, the potential influence of nongenetic (although inherited) mechanisms onto the phenotype (epigenetic, paternal effects, ecological inheritance, cultural inheritance, etc.) have not been fully considered in this figure (reviewed in Danchin et al., 2011). (*) Notice that posttranscriptional modifications could be produced at both nuclear and cytoplasmic levels. From (Diz et al., 2012)

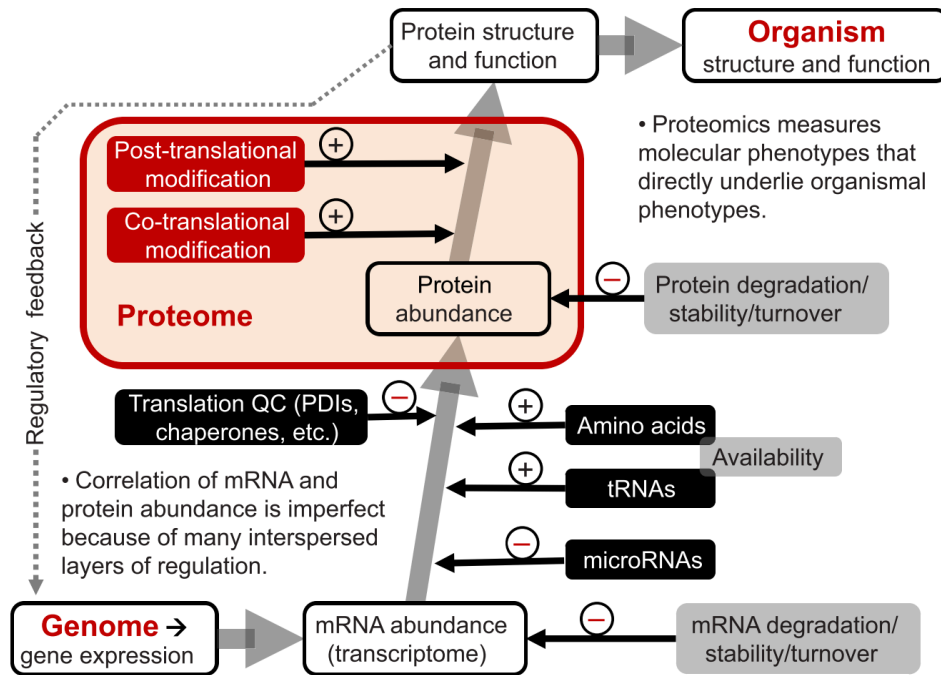


Figure 9 - Central position of the proteome within the genome to phenome continuum. Phenome represents the totality of observed phenotypes of an organism. Environmental signals (not shown) are integrated during each step along the genome to phenome continuum and phenotypic outcomes represent the result of complex genotype \times environment interactions (QC, quality control; PDI, protein disulfide isomerases). From Kültz (2015).

4.1.2. Proteomic approaches

The present section about proteomic approaches aims to picture a panel of proteomic techniques available, their characteristics and evolution. The shotgun approach used in this thesis is described in more details at the end of the section.

The functional proteomics aims to globally profile changes in protein abundances of biological systems, which requires sensitive and accurate tools; first, to identify proteins and, secondly, to quantify their abundances from a complex mixture (Old et al., 2005). Two major approaches of quantitative proteomics exist: label and label-free techniques that are gel-based or gel-free (Neilson et al., 2011).

Gel-based approaches were traditionally used such as one-dimensional (1-DE) gel electrophoresis, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and 2D difference in-gel electrophoresis (2D-DIGE) (using a set of fluorescent dyes) where protein abundance is determined by protein spot comparison followed by identification

using mass spectrometry (MS) (Figure 10)(Neilson et al., 2011). However, the technological advances in protein quantification at the MS level via gel-free shotgun techniques associated to gel-based limitations regarding reproducibility, multiplicity of proteins in a single spot, poor representation of low abundant proteins, highly acidic/basic proteins, issues with hydrophobic and extra-large proteins of gel-based techniques have reduced their use in proteomic studies depending on the biological question investigated (Abdallah et al., 2012; Aebersold and Mann, 2003).

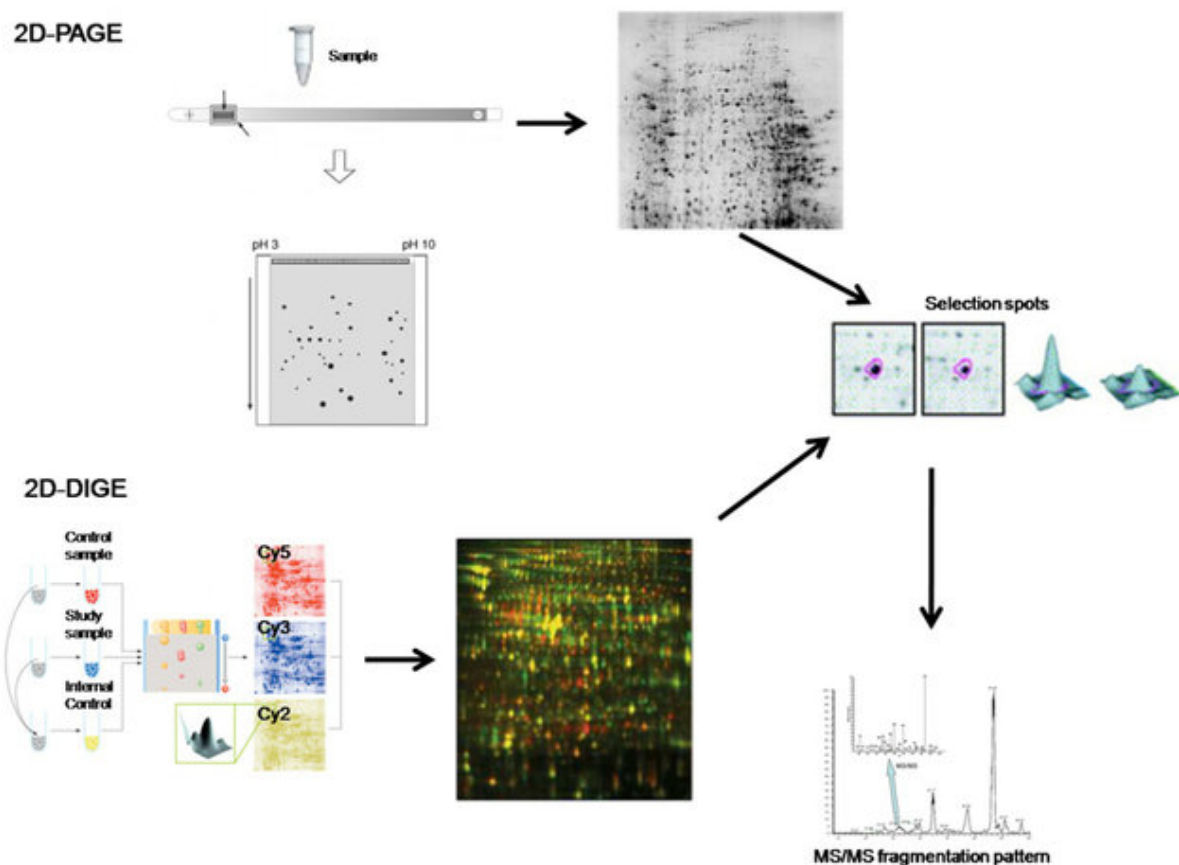


Figure 10 - Basic workflow of gel-based proteomic approaches. In 2D-PAGE, protein samples are separated according to their isoelectric point in a process termed isoelectric focusing, using gel strips with a fixed pH range. Then, the focused strip is placed on top of a polyacrylamide gel to allow proteins to separate according to their molecular weight during electrophoresis, generating a gel with protein spots. In 2D-DIGE, proteins from up to three samples are labelled with fluorescent dyes prior to their isoelectric focusing and subsequent gel electrophoresis. Gels are scanned with different wavelengths revealing spots and differences in expression between analysed samples. Protein spots of interest in both techniques are then excised, digested, and identified by MS. From Lopez-Camarillo and Aréchaga (2013).

Gel-free (MS-based) proteomic approaches allow both identification and quantification of proteins. Several labeling techniques exist such as SILAC (incorporation of stable isotope labels with amino acids in cell culture), iTRAQ (modifying peptides with isobaric tags) or ICAT (using isotope-coded affinity tags) (Gygi et al., 1999; Ong et al., 2002; Ross et al., 2004). The common steps of both label-based and label-free approaches through gel-free is the protein extraction followed by proteolysis into peptides. The distinct labeling of samples allows analyzing a mix of samples into the mass spectrometer in one run and calculates their relative quantification on the same mass spectrum by comparing adjacent peaks. With the MS-based label-free technique, each sample has to be individually MS analyzed, and comparing each corresponding mass spectrums allows to calculate the relative protein abundance (Figure 11). Both methods present pros and cons. Label-free requires high reproducibility between runs to find out the same peptide signatures between samples. Also, it provides higher quantitative proteome coverage and simpler sample preparation. On the contrary, despite the possibility to run multiple samples on the same mass spectrum with label-based methods, they are limited due to the labels availability and higher experimental variability (Neilson et al., 2011). The choice of the right proteomic tool depends on trade-off between the experimental design, the biological question, the availability of biological samples, the techniques limitations, and as usual, the price.

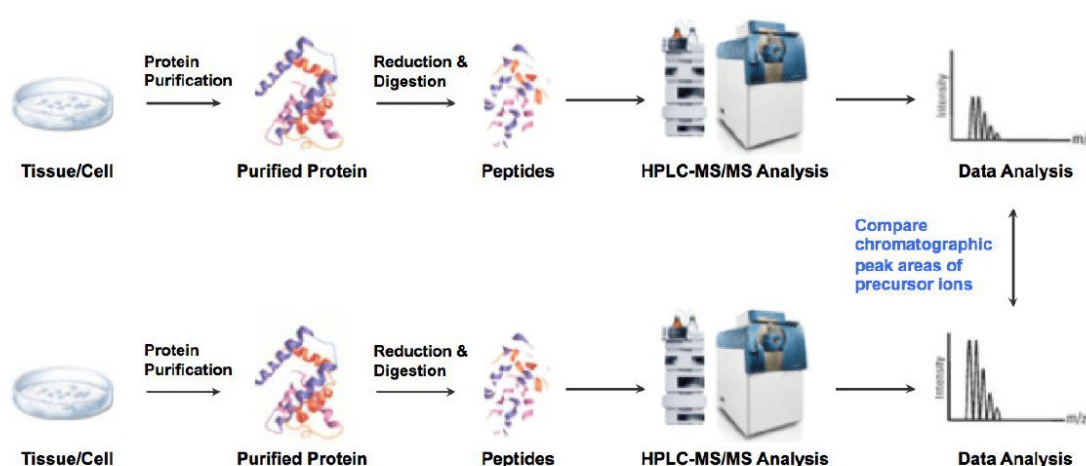


Figure 11 - Schematic label-free proteomic workflow. (<https://medium.com/@primebio/what-you-should-know-about-quantitative-proteomics-653a4ddd5e81>)

Shotgun proteomics, also called the bottom-up strategy of data-dependent acquisition (DDA) is a discovery method allowing the identification of proteins in complex mixtures commonly using a combination of liquid chromatography (LC) and MS (Figure 12). It provides a wider dynamic range and coverage, mostly for low-copy proteins and hydrophobic ones, compared to 2D-DIGE for example (Yates, 2013).

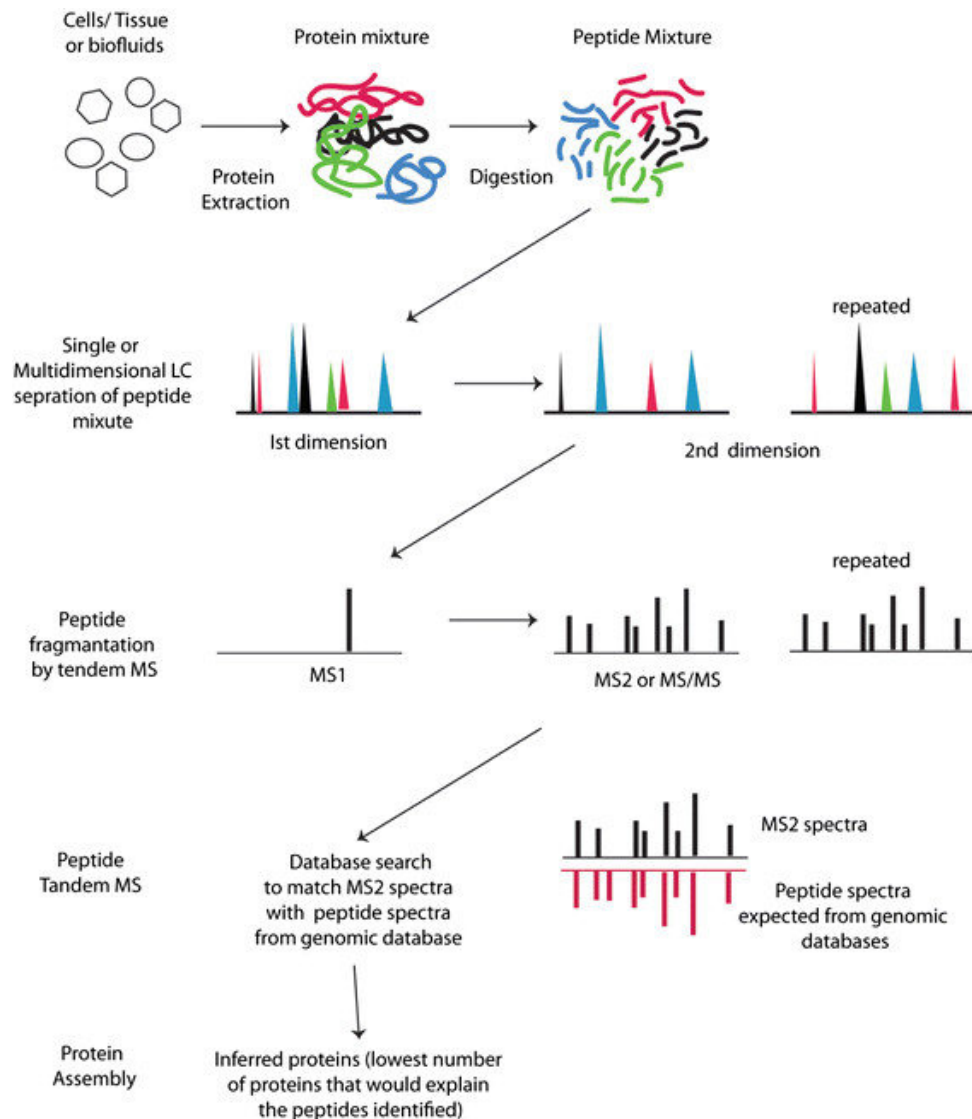


Figure 12 - Workflow of 'bottom-up' or shotgun proteomics. Protein extracts from cells, tissue or biofluids are prepared by mechanical (e.g., glass bead or homogenization) or chemical-based (precipitation, detergent solubilization) methods. Proteins are proteolytically digested into peptides, usually with trypsin, that are separated by 1D or 2D chromatographic separation. The final chromatographic step is performed in-line with the mass spectrometer. Two scan types are acquired: MS1 spectra contain intact peptide mass to charge (m/z) values; MS2 or tandem MS (MS/MS) spectra represent peptide fragment ion m/z values. Peptide MS1 and MS2 data are correlated with theoretical peptide m/z values with database search programs that use protein sequences as templates; parsimonious protein identifications with peptide matches are reported. From (Bhargava et al., 2014).

Proteins are first extracted from samples and digested. The high/ultra performance LC (HPLC/UPLC) permits to dissociate peptides before an electrospray ionization step, which vaporizes the sample and electrically charges it. In fact, the mass spectrometer sorts ions according to their mass to charge (m/z) ratio allowing to assess the molecular weight of chemical compounds. Then, ionized peptides generated are accelerated before passing through an electromagnetic field and ending their trajectories on a detector. Tandem mass spectrometry (MS/MS) is commonly used in gel-free proteomic analyses, which combines 2 analyzers in order to get a mass spectrum for both peptides (MS1) and fragments from the previously separated peptides (MS2). To obtain MS2, ionized peptides are individually selected by an acquisition software, then fragmented by passing through collision cells. The second run of MS provides a new spectrum of fragment ions. The MS-based peptide quantification and identification of shotgun-proteomics can be approached in two ways: by spectral counting (SC) or by calculating the area under the curve (AUC) from the mass spectrum (Figure 13) (Abdallah et al., 2012).

In shotgun proteomics, the protein identification procedure has two main steps: peptide identification and protein inference. The MS results allow to identify peptides by comparison with existing protein databases. MS/MS, by comparing fragments and peptides, permits to reconstruct the amino-acid sequence. The determination of original protein components of the sample is then possible via the analysis of sequence homology. Then, the quantification based on the spectral counting assumes that more abundant a peptide is, more it will be selected for fragmentation and will produce a higher abundance of MS/MS spectra (Liu et al., 2004). The signal intensities of individual peptides can be used to compare the relative abundance of proteins between samples as well. This measurement is linearly proportional to the concentration of the measured peptide (Bondarenko et al., 2002; Chelius and Bondarenko, 2002). Shotgun proteomics is a very good approach to get the most accurate picture of the proteome, and proteome changes occurring under different conditions. However, this approach is limited to the acquisition of differences in relative abundance. If the precise abundance of a smaller set of proteins is needed, it is necessary to use targeted proteomics techniques. For additional information about this technique and workflow, see Borràs and Sabidó, (2017).

As overviewed, a panel of methods exist in shotgun and targeted proteomics mostly thanks to the tremendous progress in mass spectrometry over the last 100 years (Yates, 2013). It is therefore necessary to first assess the biological question we want to answer, then examining the pros and cons of each techniques, evaluate the need of time, amount of samples, technology,... in order to choose the most suitable one (Graham et al., 2005).

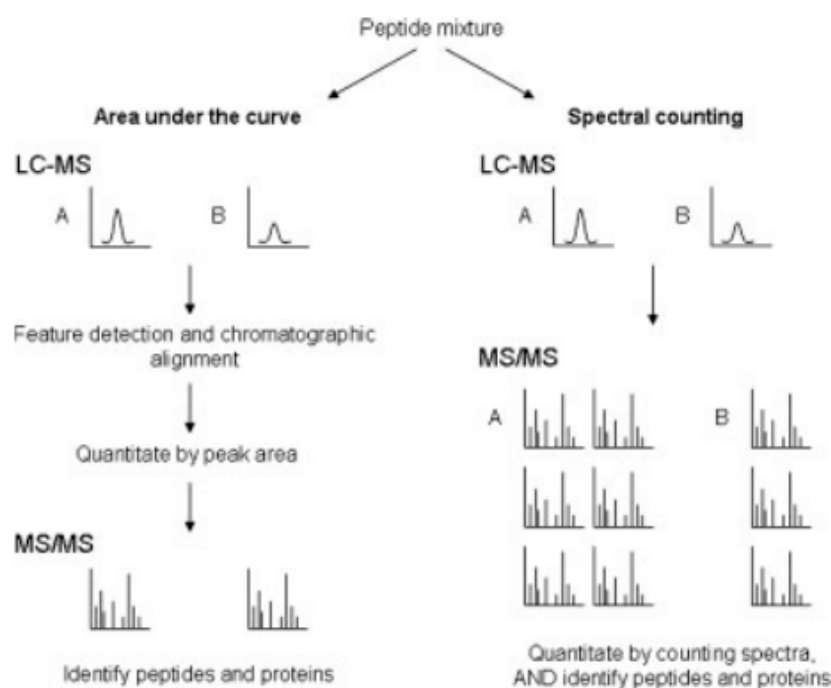


Figure 13 - Schematic representation of the two main approaches to quantify proteins from label-free proteomic workflow using 2 theoretical samples, A and B, expressing a two-fold change in expression. The area under the curve (AUC) quantitation is performed at the MS level and peptides found to have differential expression are identified by MS/MS either in a simultaneous or subsequent fragmentation step. Spectral counting quantitation and identification are performed simultaneously at the MS/MS level. Spectral counting computes abundance values from the number of times a peptide was successfully identified by tandem mass spectrometry (MS/MS) and compares these across experiments. Adapted from Neilson et al. (2011).

4.2. Epigenetics

4.2.1. History and concepts

With the tremendous increase of knowledge concerning molecular mechanisms underlying regulation of gene expression in eucaryotes, the definition of epigenetics has considerably evolved since its first use in 1939 by Conrad Waddington, when it referred to the study of, "*causal interactions between genes and their products, which brings the phenotype into being*" (Felsenfeld, 2014; Holliday, 1994). Today, epigenetics refers to the science that studies epigenetic modifications: "mitotically and/or meiotically heritable changes in gene expression that do not involve changes to the underlying DNA sequence" (Deans and Maggert, 2015; Riggs and Porter, 1996). Epigenetics therefore focuses on the regulation of gene expression independently of the genomic sequence. This type of study can be extended to the entire genome and is then called epigenomic. The complete history of epigenetics is available in the publication of Felsenfeld (2014).

The 3 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 RNA, and (3) DNA methylation (Chu et al., 2011; Riggs, 1975; Stedman and Stedman, 1950). New molecular aspects of epigenetic control have recently been discovered such as hydroxymethylation of cytosine and topological associated domains (TADs) that will be briefly described in the next paragraph as well (Day et al., 2013; Dixon et al., 2012).

4.2.2. Epigenetic mechanisms

4.2.2.1. Histone modifications

In eukaryotic cells, genomic DNA is hierarchically and dynamically arranged into a structure called chromatin (Figure 14). The DNA compaction into chromatin allows to fit the required genetic information into the cell nucleus (Hurd, 2010). The DNA (146 bp) wraps twice around the nucleosomal core particle; octamer made of 4 types of histones: H2A, H2B, H3 and H4 (Fischle et al., 2003). One of the primary roles of histones is to coil the DNA into a smaller volume. They are proteins with an amino-N-terminal tail that protrudes from the nucleosome and interacts with the adjacent one (Bannister and Kouzarides, 2011). The different nuclei are connected by a short extension of DNA. The structure of the nucleosome is further compacted by the attachment of the linker

histone protein H1 at the outside of the nucleosome forming the chromatosome. The nucleosomes fold up to form a 30 nm chromatin fiber. This fiber forms loops and condenses to finally give a chromosome (Hergeth and Schneider, 2015). Although the primary role of histone proteins was considered to be DNA packing, histones are mandatory to many biological processes such as the regulation of gene expression, repair of damaged DNA, DNA replication and combination, and heritable epigenetic regulation (Lennartsson and Ekwall, 2009; Norouzitallab et al., 2014; Siklenka et al., 2015). Chromatin responds to external cues through histone modifications patterns constituting a code that brings DNA to be compact (heterochromatin) or loose (euchromatin). Heterochromatin refers to the DNA silent form while euchromatin refers to the active one (Fischle et al., 2003).

Histones are commonly subjected to post-translational regulation taking place on their N-terminal tails through the addition of a chemical group such as acetyl, methyl or phosphate, which affects interactions between nucleosomes and therefore modifies the overall chromatin folding (Figure 15). These groups can regulate the recruitment of enzymatic complexes as well. The panel of histone modifications plays as a code influencing gene expression. For example, acetylation of histones is commonly associated with an increase in transcriptional activity and deacetylation with repression but the consequences depend on which histone carries the chemical modification (Norouzitallab et al., 2019).

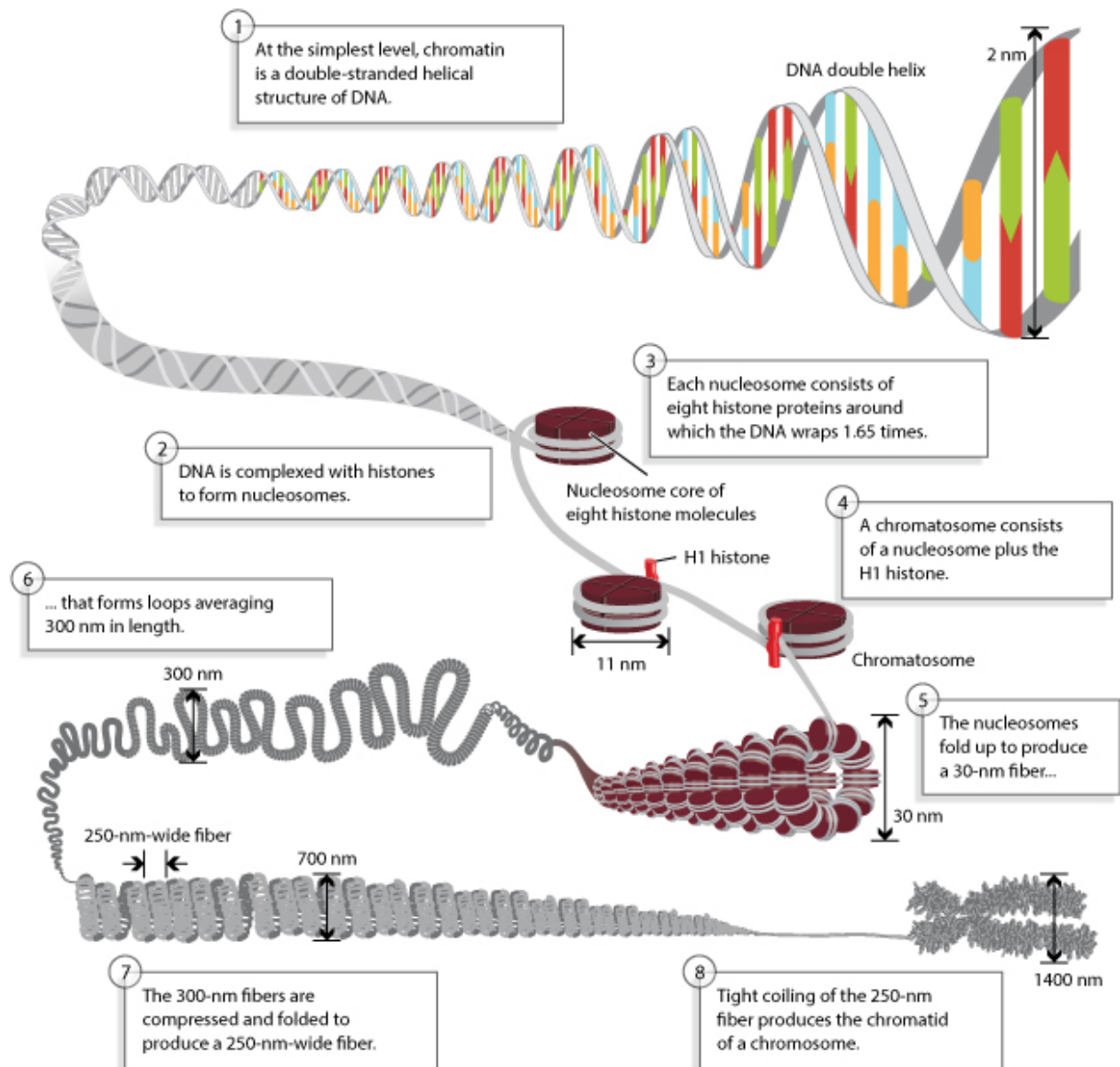


Figure 14 – The role of histones in the DNA organization. Adapted from Annunziato (2008).

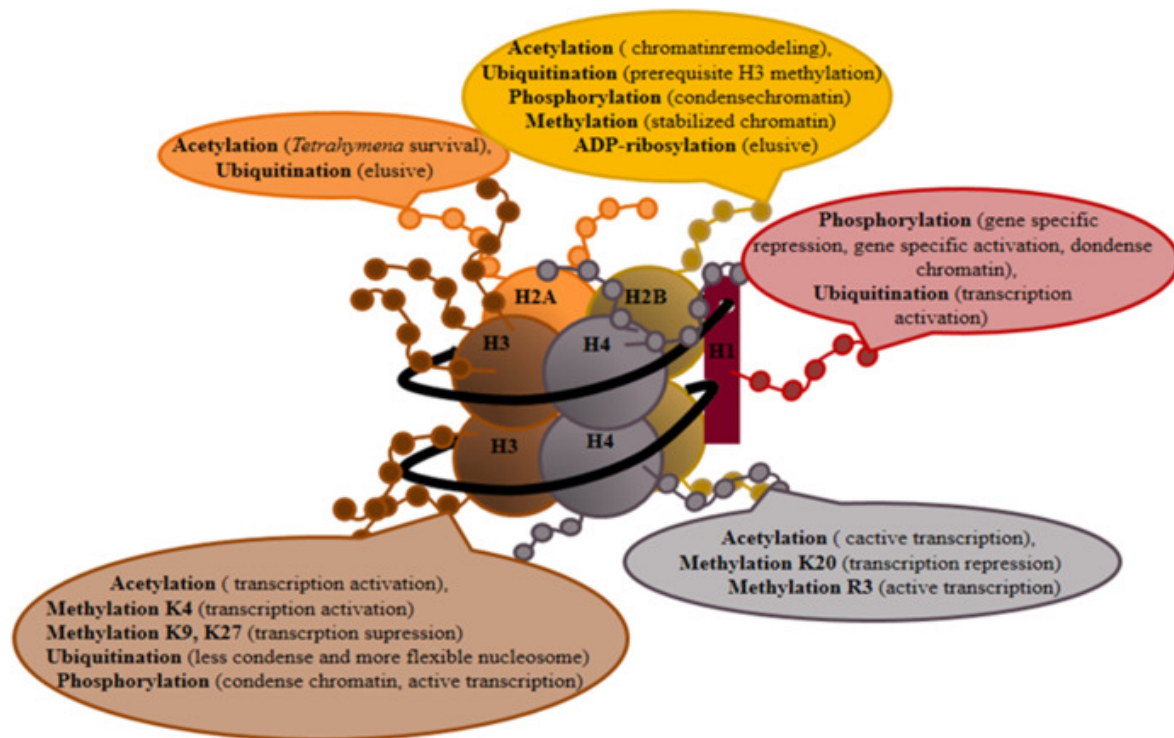


Figure 15 - Histone tails post-translational modifications and their biological roles. These marks can regulate gene expression through forming active regions (euchromatin formation) or inactive regions (heterochromatin formation). From Norouzitallab et al. (2019).

4.2.2.2. Non-coding RNA

Non-coding RNAs (ncRNAs) are RNAs that do not encode a protein. ncRNAs encompass many different types of RNAs such as small interfering RNAs (siRNAs), microRNAs (miRNAs) or transfer RNA (tRNA). These small RNAs (20-30 nucleotides) are generated by a Dicer enzyme from a larger double-stranded or pin-shaped sequence. They associate with Argonaute proteins and form a silencing complex called RISC. This complex targets complementary nucleic acid sequences, which induces inhibition of gene expression (Vandegheuchte and Janssen, 2011). The occurrence and maintenance of ncRNAs probably rise from a protection mechanism against invasive sequences such as transposable elements and viruses (Zilberman and Henikoff, 2005). The majority of mammals' and other complex organisms' genomes are transcribed into ncRNA but their implications in genetic variation is far to be completely understood (Mattick, 2018).

4.2.2.3. DNA methylation

The first epigenetic mark discovered was the cytosine DNA methylation (Riggs, 1975). In eucaryotes, DNA methylation refers to the transfer of a methyl group (-CH₃) from S-adenosyl-L-methionine (SAM) on cytosines at the fifth carbon of the pyrimidine ring (5mC), reaction catalyzed by a group of proteins, the DNA methyltransferases (DNMTs) (Figure 16-A) (Christman, 2002; Holliday and Pugh, 1975; Jang et al., 2017). Highly abundant in the genome, 5mC is commonly referred as the fifth base of DNA. The transfer of a methyl group predominantly happens on cytosine followed by guanine residues (CpG). Three conserved enzymes responsible of this transfer have been described in vertebrates: DNMT1, DNMT3a and DNMT3b (Campos et al., 2012; Goll and Bestor, 2005; Li et al., 1992). DNMT1 is responsible of restoring DNA methylation after replication and DNMT3a and DNMT3b catalyze *de novo* methylation. Another DNMT exist in vertebrates sharing strong sequence homology with the 3 other DNMTs described above, the DNMT2. The role of DNMT2 in DNA methylation appears to be absent. This enzyme is rather specialized in the methylation of cytosine 38 of transfer RNAs (tRNAs) but has been shown to be involved in the methylation of other RNAs favoring their folding and stability, which could provide a protective function (Alexandrov et al., 2006; Goll et al., 2006; Jeltsch et al., 2006; Schaefer et al., 2010).

DNA methylation does not exclusively occur on CpG sequences and has been found on cytosines followed by adenine, thymine and other cytosine. This type of methylation is referred as non-CpG methylation and is catalyzed by the *de novo* methyltransferases, but the exact mechanism is still poorly understood (Figure 16-B) (Jang et al., 2017).

Methylation of high-density CpG regions (CpG Islands) has been extensively described as a mechanism associated with the regulation of gene expression, mostly, with gene expression silencing. In fact, the methyl groups cause either a modification of the DNA surface disrupting its recognition by various enzymes or transcription factors, or allow the binding of methylated DNA binding proteins (MBPs), which recruits histone deacetylase (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). CpG islands are widely distributed across genome; in promoter, gene body and intergenic regions. DNA methylation is a stable and heritable epigenetic state that plays important roles in diverse cellular processes such as genomic imprinting,

retrotransposon silencing, monoallelic X chromosome inactivation in females or the maintenance of epigenetic memory (Bird, 2002; Heard, 2005; Martienssen et al., 2004) (Bird, 2002). It seems that the methylation level of the promoter-associated CpG Island of a gene is closely related to its expression level, with hypo-methylated signatures tending to be expressed in more tissues and to have stronger expression (Du et al., 2012). But the association between hyper-methylated signatures and repression of gene expression is increasingly questioned (Moarii et al., 2015). Hyper-methylated signatures have been found to activate the transcription of some genes by preventing repressors such as CTCFs (chromatin boundary element binding protein) to bind the DNA (Jaenisch and Bird, 2003). CTCFs usually block the interaction between a gene promoter and its enhancer when placed between the two elements and therefore inhibits gene expression (Bell et al., 1999). Stimulation of gene expression through hyper-methylated CpG islands would aim to maintain transcription of genes involved in essential biological mechanisms and involved in the maintenance of basal cell functions, such as housekeeping genes¹ (Foret et al., 2009).

¹ Housekeeping genes are typically **constitutive genes** that are required for the maintenance of basal cellular functions that are essential for the existence of a cell, regardless of its specific role in the tissue or organism. From www.genomics-online.com.

4.2.2.4. Hydroxymethylation

The 5-hydroxymethylation of cytosine (5-hmC) has been newly identified as an epigenetic mark considered as the sixth base in mammalian tissues, which is abundantly found in brain (0.3-0.7 % of all Cs) and embryonic stem cells as well as in many other tissues in smaller proportions. (Pfeifer et al., 2013; Tognini et al., 2015; Zhao et al., 2017). 5-hmC is produced from the oxidation of 5mC by the Ten-Eleven Translocation (TET) family proteins. This mark, thought to be an intermediate of an active demethylation process, is dynamically regulated by life experience and is implicated in many biological processes such as DNA methylation regulation, gene control mechanisms, and several diseases like cancers (Globisch et al., 2010; Guo et al., 2011a, 2011b; Ye and Li, 2014).

4.2.2.5. Topologically associating domains (TADs)

Amongst epigenetic mechanisms, the latest discovered confirms how complex organisms are and how the initial central dogma of molecular biology has evolved. The epigenetic mechanism/mark recently discovered is the topologically associating domain (TAD), which refers to genomic regions with distinct boundaries (Figure 17) (Dixon et al., 2012). Chromatin is organized by regions/TADs within the nucleus. Within a TAD, DNA sequences can physically interact with each other or more frequently than with sequences situated in another TAD, promoting regulatory interactions (enhancer-promoter) (Dowen et al., 2014; Schoenfelder et al., 2015). Genes located in the same TAD seem to be co-regulated and expressed, seem to respond similarly to transcriptional stimuli and share similar epigenetic marks (Dixon et al., 2012). TADs are composed of thousands to millions of DNA bases and are described as the 3D organization of chromosomes. 2 common proteins seem to intervene in TADs' boundaries: the CCCTC-binding factor (CTCF) and cohesins complexes but their exact role in TADs is still unclear (Lazar et al., 2018). Interacting DNA areas generates small loops called sub-TADs. Sub-TADs can interact together to form a bigger DNA loop called TAD. TADs can isolate parts of the genome from each other to avoid any interaction or, on the contrary, allow some parts to get closer from each other. TADs organization inside the nucleus can change from a cell to another after division. The same TADs are present but the geographical organization is different, generating again variability in the regulation of gene expression. Genes situated on one TAD unlikely interact with genomic

region of the next TAD unless a CTCF barrier is removed. The panel of possible interactions generates diversity that can ultimately lead to evolution. TADs have been studied in mouse, monkey and dog and are conserved between these species while sub-TADs are different. This 3D organization balances the constraints of the crowded cell nucleus with the functional dynamics of gene regulation (Rocha et al., 2015).

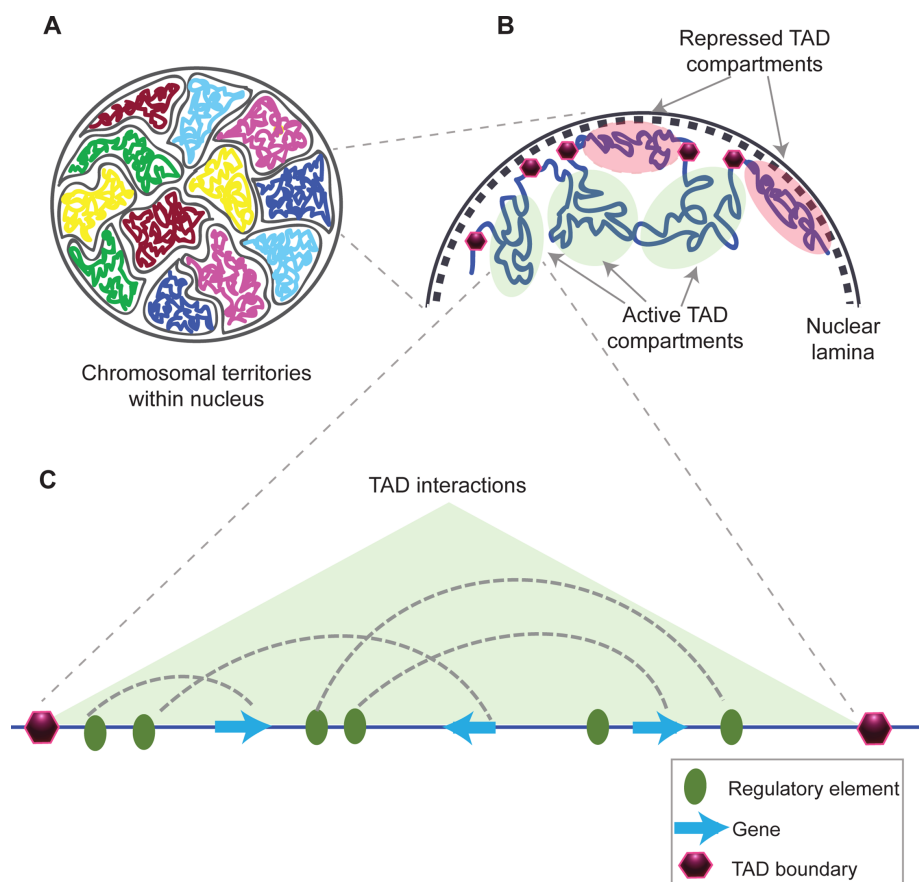


Figure 17 - Structural organization of chromatin. (A) Chromosomes within an interphase diploid eukaryotic nucleus are found to occupy specific nuclear spaces, termed chromosomal territories. (B) Each chromosome is subdivided into topological associated domains (TAD). TADs with repressed transcriptional activity tend to be associated with the nuclear lamina (dashed inner nuclear membrane and its associated structures), while active TADs tend to reside more in the nuclear interior. Each TAD is flanked by regions having low interaction frequencies, called TAD boundaries (purple hexagon). (C) An example of an active TAD with several interactions between distal regulatory elements and genes within it. Adapted from Matharu and Ahituv (2015).

4.2.3. Reprogramming:

Various cell types compose an organism. Each cell type has a function dictated by specific molecular and physiological identity. Amongst cell types, several are highly plastic and can change efficiently from one type to another along with the specific associated functions. This change requires the loss of the initial molecular characteristics to acquire totally new molecular signature without changes in the DNA sequence. This process called epigenetic reprogramming is highly complex because it involves many players cooperating temporally and spatially as well as changes in chromatin state and transcription (Figure 18) (Krishnakumar and Blelloch, 2013).

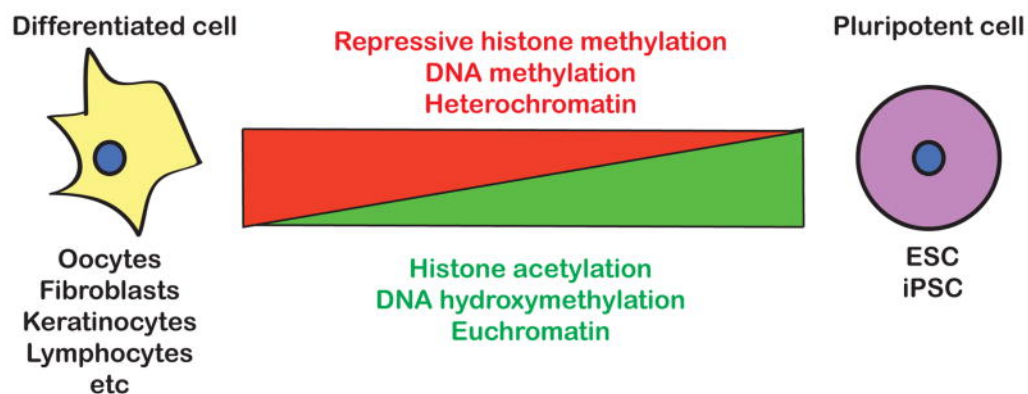


Figure 18 – Key epigenetic changes during transition between differentiated and pluripotent cells. ESC=embryonic stem cells; iPSC=induced pluripotent stem cells. From Krishnakumar and Blelloch (2013).

DNA methylation in vertebrates ¹ establishes during two successive reprogramming events, during gametogenesis and embryogenesis. The epigenome gets globally reprogrammed each time, demethylation followed by remethylation, that makes

¹ Invertebrate and vertebrate genomes express very divergent patterns of DNA methylation. DNA methylation of the genomes of invertebrates mostly occurs on gene bodies while methylation is ubiquitously present on the vertebrate genome (promoter, gene body, intergenic regions) (Keller et al., 2016).

the gonadal cell and zygote particularly sensitive to environmental stimuli (Figure 19) (Hill et al., 2018; Yauk et al., 2008). The erasure of the epigenetic markers required for the proper development of the embryo allows the introduction of new methylation profiles in the early individual. Active and passive demethylation processes can take place in the organism (Figure 16-C). 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). Mutations of TET genes, their reduced expression and the impaired activity of tet proteins have been shown in various type of cancers which reveals their biological importance against cellular transformation (Rasmussen and Helin, 2016). The impaired process of *de novo* methylation simply results in passive demethylation process during DNA synthesis. Therefore, 5mC is progressively diluted after DNA replication preventing the stable inheritance of DNA methylation patterns. The passive DNA demethylation has been shown to promote reprogramming (He et al., 2017).

Little is known about reprogramming in vertebrates except in mammals. In the mouse, DNMT3a and DNMT3b enzymes remethylate the two parental genomes that were actively and passively demethylated until early cleavage (Edwards et al., 2017). In fish, reprogramming have been described in 3 species : the zebrafish (*Danio rerio*) (Fang, et al., 2013; Mhanni and McGowan, 2004), in the mangrove rivulus (*Kryptolebias marmoratus*) (Fellous et al., 2018), and in the medaka (*Oryzias latipes*) (Wang and Bhandari, 2019). It has been emphasized that DNA methylation reprogramming represents a highly critical and sensitive period to environmental stress and its characteristics can vary even among the same taxa such as it was described between these two fish species (Dorts et al., 2016; Fellous et al., 2018).

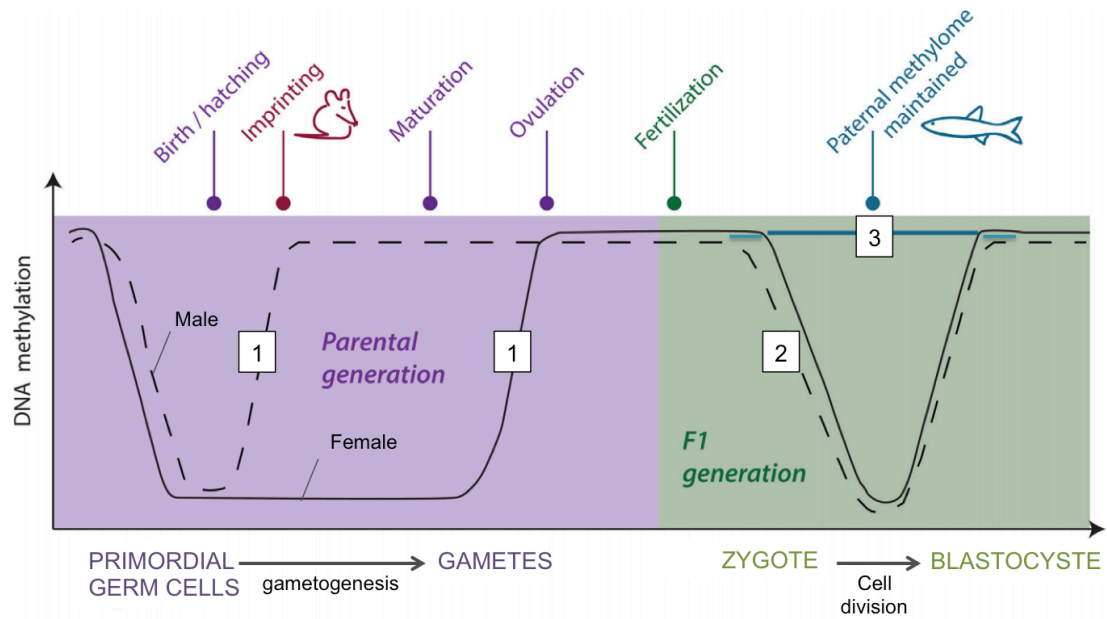


Figure 19 - Dynamics of DNA methylation erasure and reprogramming in primordial germ cells and gametes (prior to fertilization) and early embryonic development of mammals and fish. Two cycles of reprogramming occur; the first in primordial germ cells of the parental (FO) generation (which will eventually become F1), and the second early in embryogenesis of the F1 individual. The methylome is erased in primordial germ cells including on imprinted genes in mammals. (1) The gametes then undergo de novo methylation followed by imprinting in mammals, which is likely absent in fish. Genomic imprinting occurs in most mammals, but it is not yet clear whether or not it occurs in other vertebrates. (2) The second cycle of demethylation/reprogramming occurs after fertilization. In mice, the paternal genome is demethylated before the first replication of DNA, whereas the maternal genome is only demethylated after replication has occurred. (3) Imprinted genes are protected from the second cycle of demethylation and in other species (e.g., zebrafish, some mammals), high levels of methylation of paternal DNA are maintained throughout embryogenesis. Adapted from Brander et al. (2017) and Head (2014).

4.2.4. Epigenetic inheritance

Initially, the biological inheritance through the genetic code was thought to be the exclusive process of traits transmission from one generation to the next. This belief has been shaken by the discovery of trans-generational epigenetic inheritance allowing environmentally induced phenotypes to be transmitted and persist for several generations via epigenetic marks, which influence gene expression (Lacal and Ventura, 2018). Epigenetic inheritance can thus be defined as the non-genetic inheritance of a modified phenotype across generations, without focus on a specific mechanism (Bonduriansky and Day, 2009; Jablonka and Raz, 2009). As epigenetic marks are known to respond faster to environmental influences than the genetic code, epigenetic inheritance and the associated phenotypes would contribute to evolution, but this is still debated (Burggren, 2016; Jablonka, 2017; Lind and Spagopoulou, 2018). The transmission of certain epigenetic marks to offspring can be divided in two concepts: intergenerational and transgenerational epigenetic inheritance. Intergenerational refers to the transmission to one generation to the next (F0 to F1) while the transgenerational epigenetic inheritance refers to the transmission over generations, from F0 to F3 (Burggren, 2016). The information transmission over 2 generations is not sufficient to confirm the transgenerational epigenetic inheritance because the germ cells or the foetus (F1) and its germ cells (F2) could have been exposed to the same condition than the parents (F0) **Erreur ! Source du renvoi introuvable.**(Lacal and Ventura, 2018).

Three types of epigenetic changes over time have been described as consequences of environmental influences or stochastic variability (epimutations): the direct form, the within and across indirect forms. The direct form of epigenetics refers to changes that occur in the lifespan of the individual. When changes occur inside the womb during gestation, we talk about the within indirect epigenetic changes. The last one refers to the true transgenerational inheritance considered authentic exclusively if the acquired phenotypes are still present at F3 generation (Lacal and Ventura, 2018; Norouzitallab et al., 2019).

Epigenetic inheritance can occur through several epigenetic processes but the more extensively studied mechanisms are methylation and non-coding RNA (Pang et al., 2017). The best-understood system of chromatin inheritance is DNA methylation (Lacal and Ventura, 2018). The inheritance of cytosine DNA methylation was partially understood in 1975 (Jablonka and Raz, 2009). During DNA replication, the two strands

separate and the new replicated strand forms, with its mirror image, an hemimethylated duplex. This duplex is recognized by maintenance DNMTs that preferentially add a methyl group on non-methylated C in the new strand (Holliday and Pugh, 1975). This mechanism refers to cellular epigenetic inheritance (Figure 21). The rate of spontaneous gain and losses of methylated sites of an organism (i.e. the epimutation rate) is estimated to be higher than the genetic mutations. As these epimutations can be heritable, this can ultimately lead to adaptation (Jablonka and Raz, 2009).

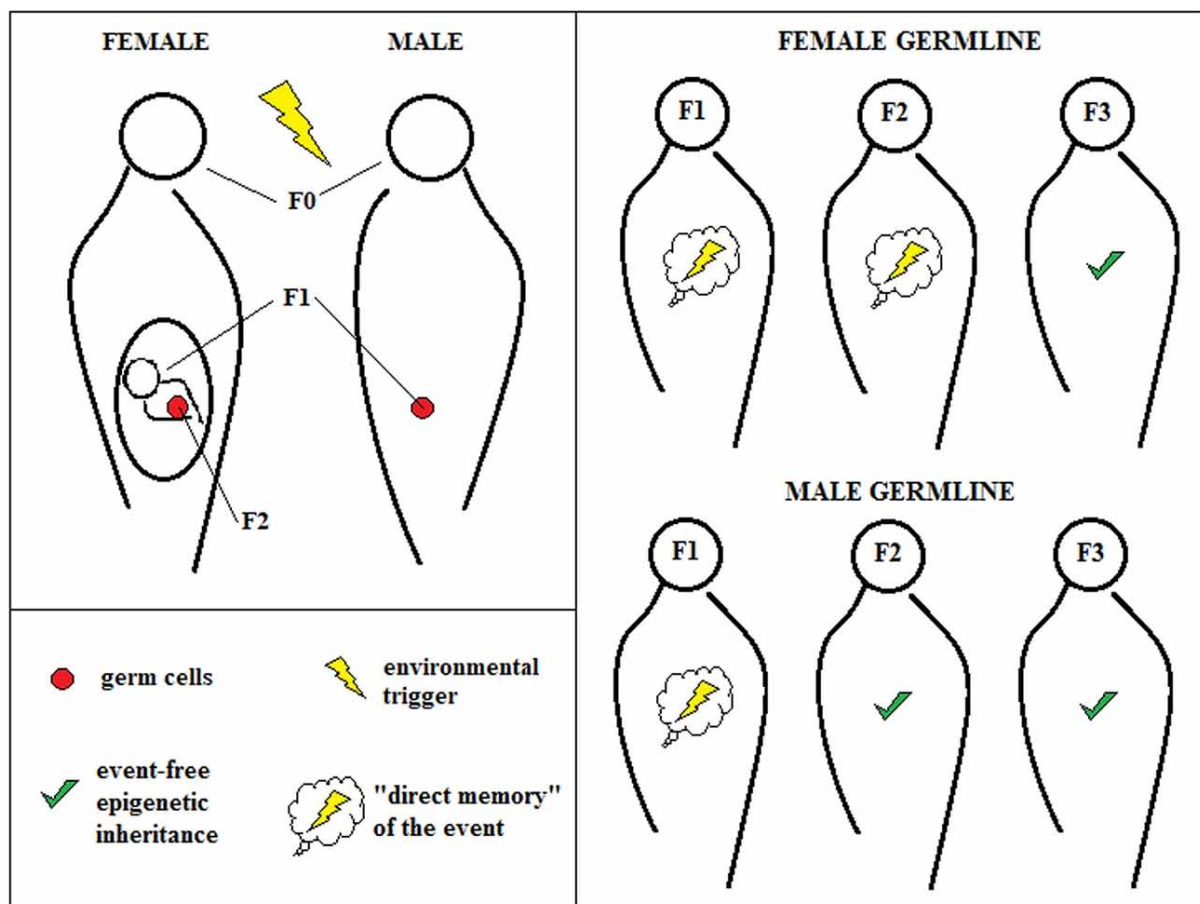


Figure 20 - Transgenerational epigenetic inheritance. According to the classical definition of transgenerational epigenetic inheritance, environmental triggers that hit pregnant female individuals (F0) can affect "directly" not only the first new generation (F1), but also its germ cells that represent the second generation (F2). For this reason, only changes in F3 can be due "purely" to epigenetic inheritance. The male germline, instead, can be affected only for one generation, allowing observing epigenetic inheritance already at F2. From Lacal and Ventura (2018).

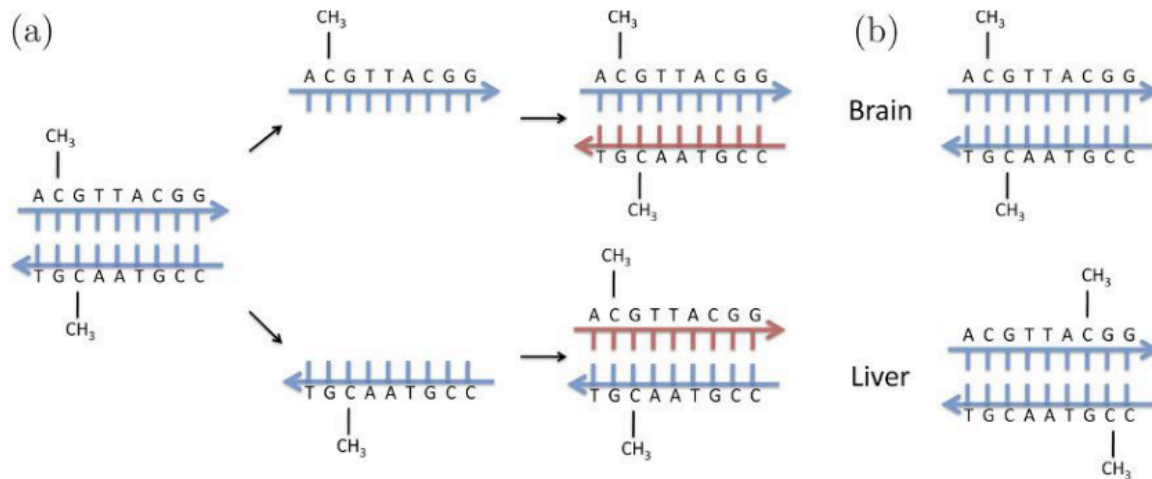


Figure 21 - Drawing illustrating how DNA methylation is inherited in cell division on how it could be involved in tissue differentiation. (a) The fact that the complement of a CpG is also a CpG facilitates the inheritance mechanism. (b) This drawing illustrates how 2 cells can have the same genomic sequence but a different methylation pattern. From (van der Graaf et al., 2015).

4.2.5. Epigenetics in the study of phenotypic plasticity

Living organisms are constantly exposed to diverse environmental stimuli and stressors along their life. To adapt and survive in their environment organisms have to react appropriately by changing rapidly their physiology and/or behaviors, for example. As described in the previous sections, phenotypic plasticity refers to the capacity of one genotype to produce more than one phenotype in response to different environments. The set of phenotypes expressed by an individual from a single genotype across a range of environments represents its reaction norm. Within a population, the adaptive response to environmental stimuli is associated to its genetic diversity, promoting diversity in phenotypes expressed and therefore favoring its adaptive potential. However, such as explained in the previous paragraphs, epigenetic marks and mechanisms are highly sensitive to environmental cues. Through the regulation of gene expression, epigenetic mechanisms have the potential to define and alter phenotypes of organisms and therefore modify their reaction norms (Figure 22). Environment can thus have significant influences on phenotypes. Epigenetics and phenotypic plasticity are therefore inseparably linked together; epigenetic mechanisms are directly influenced by the environment while the plasticity can be influenced by the epigenetic marks. The assimilation of environmental cues across organisms' life through epigenetic marks will

allow the inheritance of acquired phenotypes to subsequent generations via diverse mechanisms of epigenetic inheritance. Through selection, this assimilation can therefore promote evolution (Aguilera et al., 2010; Jablonka, 2017; Norouzitallab et al., 2019, 2016, 2014).

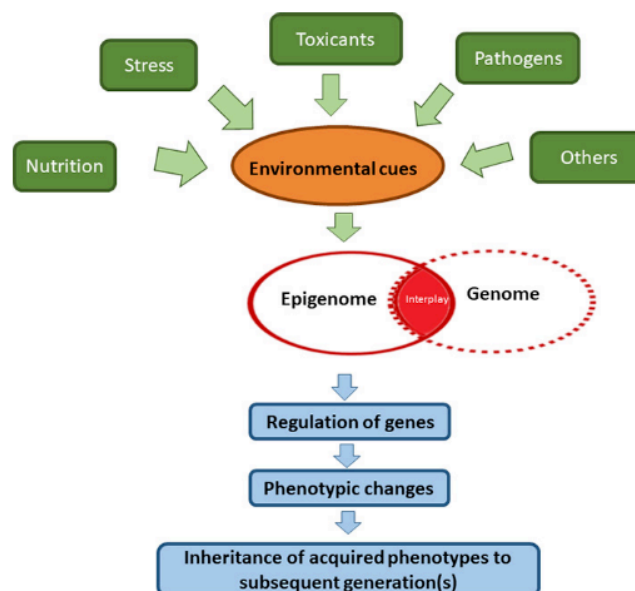


Figure 22 – Illustration of environmental cues integration into organisms' phenotypes through epigenetic changes. From Norouzitallab et al. (2019).

5. Model organism : *Kryptolebias marmoratus*

5.1. Ecology

The mangrove rivulus, *Kryptolebias marmoratus* (Poey, 1880), is an oviparous teleost fish belonging to the rivulidae family (order: Cyprinodontiformes) (Figure 23) that lives in mangrove ecosystems of the Caribbean, Florida, Central and South America (Figure 24-A) (Poey, 1880). More precisely, the mangrove rivulus colonizes a large geographic area including the Florida peninsula and keys, Bahamas islands, Central America and have been seen in some areas of Cuba and Puerto Rico (Avisé and Tatarenkov, 2015; Tatarenkov et al., 2011; Taylor, 2000). This species lives in red mangrove forests (*Rhizophora mangle*) surrounded by brackish water where it occupies a wide range of microhabitats such as crab burrows, ephemeral pools, inside or under leaf litter or damp logs (Figure 24-B) (Avisé and Tatarenkov, 2015; Tatarenkov et al., 2011; D. S. Taylor, 2000). Mangroves physico-chemical conditions are highly variable due to the wet-dry seasonal alternation along with semidiurnal tides with very low tides in spring/summer and very high tides in fall. Oxygen, ammonia, salinity, temperature and water level vary drastically on a seasonal and daily basis (D. S. Taylor, 2000). The ecology of this euryhaline fish species is not well understood but it seems to tolerate from 0 to 68 ppt of salinity as well as from 7 to 38°C (Ellison et al., 2012). Such large range tolerance is suspected to allow fish to survive in seawater during dispersal as well as during dry period in the mangroves (D. S. Taylor, 2000). When environmental conditions become extreme such as elevated concentration of hydrogen sulfide (H₂S) inducing oxygen depletion, the rivulus express a particular behavior consisting of jumping out of the water, behavior referred as emersion (Figure 24-C). Their survival in terrestrial environment up to 2 months is possible through gill and skin remodeling ensuring osmo- and ionoregulation (Costa et al., 2010a; Taylor, 2012). Considerable gill remodeling reduces the effective surface area for gaseous exchange while the skin contains a large population of ionocytes providing Na⁺, Cl⁻ and H₂O exchange across the skin (Wright, 2012). Massive feeding during flooding events would allow rivulus to survive during long emersion and low water conditions. This predator eats a variety of aquatic and terrestrial preys/food such as insects, arachnids, copepods, polychaetes, and fish scales, but can also be cannibalistic and commonly eats its eggs in captivity (Leblanc et al., 2010; Taylor, 1990)(Huehner et al., 1985). Rivulus populations encounter

predation from estuary fish and crustaceans, such as barracudas and blue crabs, mostly during flooding events. In the mangrove forests of Florida and Belize, the water snake *Nerodia clarkii compressicauda* has also been identified as a rivulus' predator (D. S. Taylor, 2000; Taylor, 2012). However they seem to experience a low predatory pressure from birds due to their habitat selection in hidden microhabitats such as fossorial niches (James et al., 2018; Taylor, 1990).

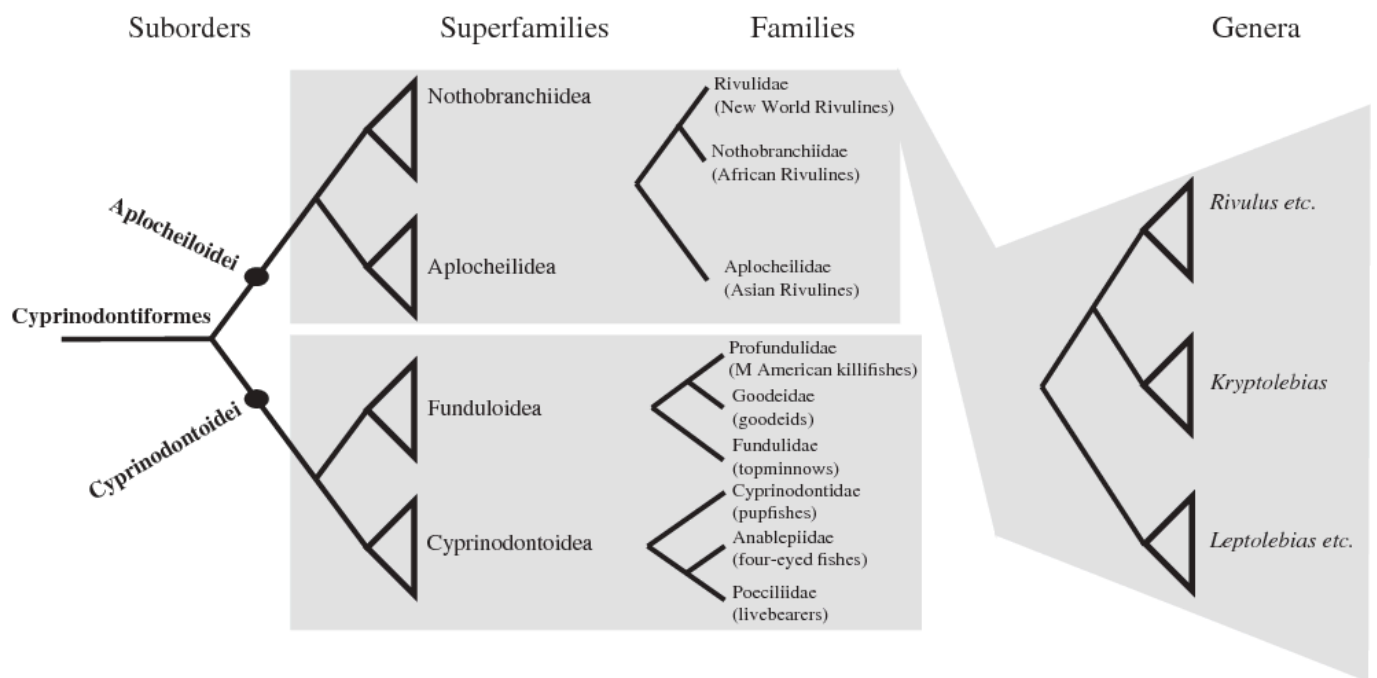


Figure 23 - Macro-phylogeny for cyprinodontiformes. From (Avisé and Tatarenkov, 2015).

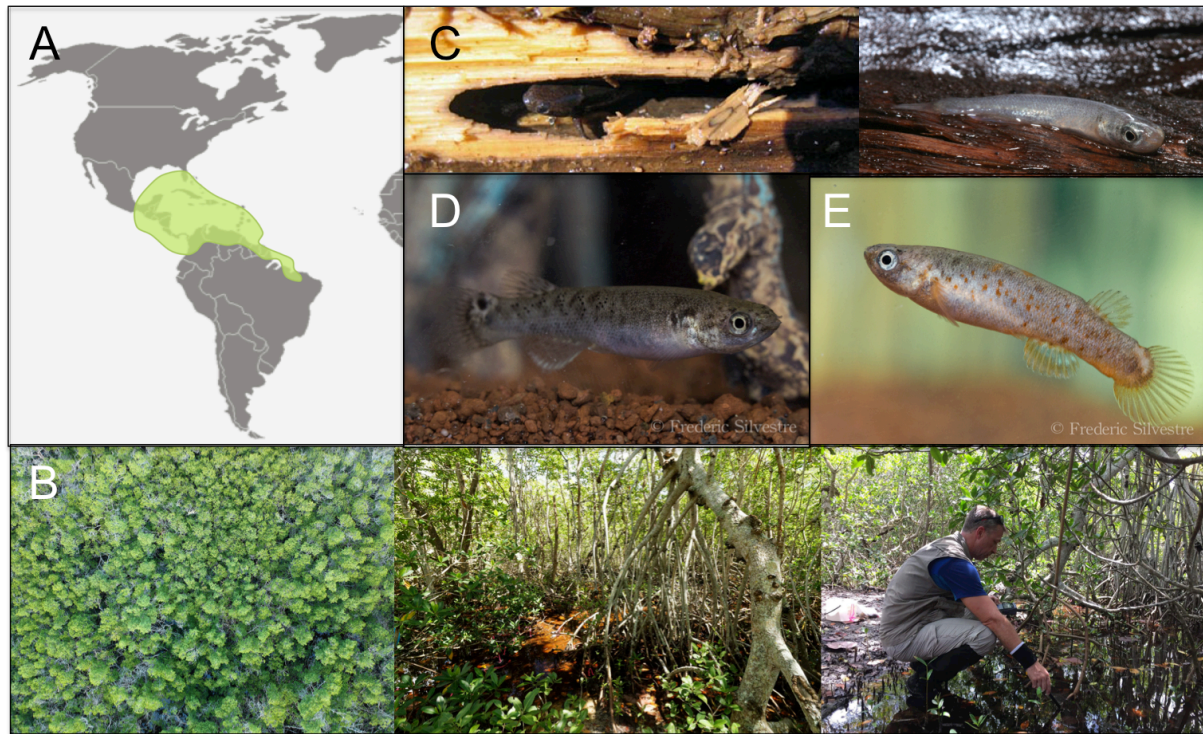


Figure 24 – The mangrove rivulus characteristics: (A) Its living area (in green) located in coastal regions of Caribbean, Florida, central and South America. (B) The red mangrove forest where this killifish lives. (C) Its emersion behavior (photo credits: Ben Chapman and Andy Turko). (D) An hermaphrodite individual. (E) A male. Photo credits: (B-D-E) Frédéric Silvestre, (C-left) Ben Chapman, (C-right) Andy Turko.

5.2. Life cycle and reproduction

Rivulus populations are exclusively composed of hermaphrodites and a small proportion of males (from 2 to 25%) (Figure 24-D and E). This reproductive strategy deprived of females is called androdioecy (Taylor, 2012, 1999). The hermaphrodites are qualified as sequential. The anterior part of the bilobed structure of gonads is made of ovarian tissues that develop during the first days post hatching. Then, testicular tissues develop in the posterior part of gonads and constitute less than 10% of the gonads (Figure 25) (Sakakura et al., 2006; Soto et al., 1992). Sexual maturity is generally achieved between 2 to 3 months (80 to 100 days post hatching). This species expresses 2 modes of fertilization. First, hermaphrodites perform an internal self-fertilization that would have possibly evolved to face low probability to meet a male in their complex environment (Sakakura et al., 2006; Soto et al., 1992)(Cole and Noakes, 1997; Kanamori et al., 2006). More rarely, external cross-fertilization events occur when male individuals drop sperm near unfertilized eggs produced by hermaphrodites. This 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). It appears that predominant selfing shifted to outcrossing in populations with elevated male rate (Mackiewicz et al., 2006b, 2006a). Genetic tests highlighted that cross-fertilization between two hermaphrodites does not exist (Turner et al., 2006).

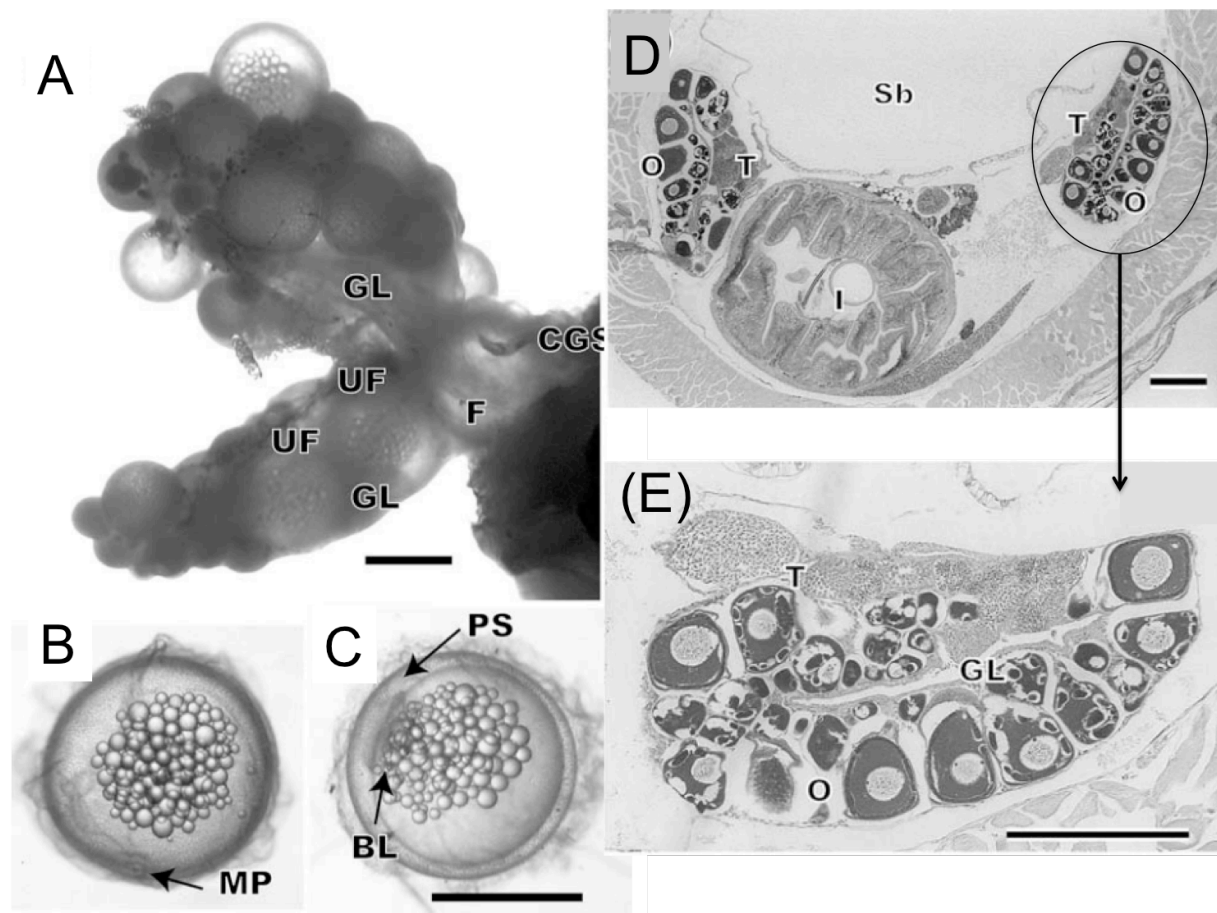


Figure 25 - (A) Dorsal view of the gonads, (B) eggs found in the gonadal lumen (GL), (C) fertilized egg, (D) cross sections of the anterior part of the gonad and (E) zoom on this anterior part of the gonad of *Kryptolebias marmoratus*. CGS: common genital sinus; F: fertilized egg; GL: gonadal lumen; UF: unfertilized egg. Arrows indicate blastodisc (BL), micropyle (MP), and perivitelline space (PS); Sb: swim bladder; I: intestine; O: ovarian region; T: testicular region. Bars (A), (B) and (C)=1 mm; (d), and (e) = 200 μ m. Adapted from Sakakura et al., (2006).

The androdioecy strategy of rivulus presents two kind of males whose appearance depends mostly of the temperature. Primary males develop when their incubation temperature does not exceed 20°C. This males exclusively present testicular tissues in their gonads and produce sperm throughout their entire life (Furness et al., 2015). 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 (Harrington, 1971).

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 24-E and D). Recently, cryptic males were discovered that subtly vary from hermaphrodite phenotypes with absent orange colors (Marson et al., 2019).

Due to difficulties of observing rivulus in its natural environment, information about oviposition is still sparse. However, by combining natural and laboratory observations it appears that the fertilized egg is laid in the terrestrial environment or at the water-air interface. This aerial spawning confers multiple advantages such as increasing oxygen availability and providing protection against aquatic predators. Embryos can enter diapause when they are fully developed and hatch as autonomous larvae after flooding event (Scarsella et al., 2018; Soto and Noakes, 1994).

5.3. Behavior

Living in the mud, stagnant pools, crab burrows, emerged logs or under leaf litter makes the mangrove rivulus behavior difficult to observe in its natural environment (Taylor, 2012). However, some studies have described rivulus behavior such as emersion, habitat preference, and escape behavior (D. S. Taylor, 2000). To focus on more complex behaviors such as personality traits, the mangrove rivulus is a good laboratory model due to its self-reproduction generating isogenic lineages (Abel et al., 1987; Huehner et al., 1985; D. S. Taylor, 2000; Taylor, 1990). The rivulus is subjected to develop social interactions, mostly during low water events. 26 individuals have been

reported at the same time in a single crab burrow in 1990 (Earley et al., 2012b; Edenbrow and Croft, 2012b, 2012c, 2012a, 2011b). Also, hermaphrodites can lay more than 20 eggs in a single clutch all located in the same fossorial niche. Its reproductive biology and the environmental conditions in which it thrives make rivulus highly subjected to develop social relations during early life. However, *K. marmoratus* is considered as a non-gregarious species that preferentially associates with members of their own genotype (kin) (Edenbrow and Croft, 2012b). Commonly known as an aggressive species in the field and in laboratory, the rivulus is able to modulate its aggressive behavior to minimize potential injuries (Hsu and Wolf, 1999, 2001; Huang et al., 2011). Adult fish decrease the intensity of aggression behavior in contact with kin and familiar individuals compared to non-kin and unfamiliar individuals (Edenbrow and Croft, 2012b; Taylor, 1990). Aggressiveness is modulated as well by winning and losing experience in *K. marmoratus* (Huehner et al., 1985; Taylor, 1990).

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 performed by Chang et al., (2012) on boldness and exploration revealed that the intensity of these traits increases during ontogeny and then stabilized at sexual maturity suggesting a developmental flexibility of these behavioral traits in this species. 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 and Croft, 2011). Moreover, some studies suggest that behaviors forming a behavioral syndrome could maintain flexibility at different degrees to respond to environmental variations (Edenbrow and Croft, 2011b).

5.4. Genetic and epigenetic characteristics

Even if self-fertilization is common in plants and invertebrates, *K. marmoratus* and *K. hermaphroditus* are the only known self-fertilizing hermaphroditic vertebrates (Chang et al., 2012). *K. hermaphroditus* reproduces almost exclusively by selfing (97-100%) while in *K. marmoratus* selfing rate varies with geographical localizations (39-99%) (Tatarenkov et al., 2007). Hermaphrodites produce highly homozygous and isogenic individuals only after a few cycles of exclusive self-fertilization (Avise and Tatarenkov, 2015; Costa et al., 2010a; Mackiewicz et al., 2006a; Orlando, 2012;

Tatarenkov et al., 2017). Although this reproduction mode is advantageous in the complex mangrove ecosystem where chances to find a male are low, selfing is commonly associated with inbreeding depression, the reduced survival and fertility of offspring of related individuals (Costa et al., 2010a; Orlando, 2012). But no significant limitations of inbreeding depression has been demonstrated in this species so far (Kelley et al., 2016). Outcrossing generates heterozygous individuals, providing allelic diversity within populations. The genetic characteristics of rivulus populations such as the level of homozygosity and heterozygosity, indicate the portion of male presence in the environment. For example, 25% of the rivulus population living in Belize mangroves is constituted of males, and this number has been recently reported to reach 42% (laboratory personal data), leading to a high rate of outcrossing and therefore of heterozygous individuals as well (Stephen C. Weeks et al., 2006).

The unique selfing characteristics of *K. marmoratus* have motivated many research teams from various fields of biology (behavior, population genetics, ecology, ecotoxicology,...) to use it as animal model (Nakamura et al., 2008). Method initially used to assess homozygosity level in diverse genetic lineages has recently evolve from microsatellite to whole genome sequencing coupled with single nucleotide polymorphisms (SNPs) analysis (Chang et al., 2012; Edenbrow and Croft, 2012c; Kelley et al., 2016; Mourabit et al., 2011; Tatarenkov et al., 2010). The recent technique made it possible to discover some 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 (Lins et al., 2017; Tatarenkov et al., 2010). Hopefully for researchers that justified the use of rivulus on its natural production of clones, most of the SNPs found were located in intergenic regions with only 3.5% in coding regions (10% of the genome)(Lins et al., 2017; Tatarenkov et al., 2010).

Deccribed by Lins et al., (2017), the *K. marmoratus* haploid genome has a total length of 680 Mb, encoded on 24 chromosomes with a CG content equal to 37.76 %. 20954 genes and 634 tRNAs have been predicted. This genome assembly was used for bioinformatics analyses for the proteomic and methylation analyses described in Chapter 1 and 2.

Although *K. marmoratus* are characterized by low genetic variability between individuals of a same isogenic population, it has been noticed that they can express

phenotypic plasticity. As described above, even isogenic individuals raised in the same environment can express variation in their behaviors. They are able to survive in extreme environmental conditions in mangroves as well. In addition to slight genetic variation within population, epigenetic mechanisms such as DNA methylation could be responsible of phenotypic diversity. They are therefore of high interest to disentangle what generates phenotypic plasticity when the contribution of both genetic and environment is considerably reduced (Fellous et al., 2018).

Information about epigenetic mechanisms in the mangrove rivulus is accumulating. Rivulus DNA methylation reprogramming process was described and compared to zebrafish, *Danio rerio*. It appeared that, at the same embryonic stage, this reprogramming is later, deeper and longer than in the zebrafish (Figure 26). A global DNA demethylation occurs directly after fertilization and then is remethylated at specific loci and stages. Although different, this reprogramming event is consistent with the ones occurring in mouse (*Mus musculus*) and zebrafish genomes and seems particularly important in vertebrate development (Fellous et al., 2018).

The time course expression of enzymes involved in epigenetic mechanisms has recently been described in the mangrove rivulus during embryonic development and in adult tissues: DNMTs, TETs, MeCP2 (Methyl CpG Binding Protein 2), Kdm (Histone Lysine Demethylases), Kmt (Histone Lysine Methyltransferases), Kat (Lysine Acetyltransferases), and HDAC (Histone Deacetylases) (Fellous et al., 2019a, 2019b, 2018). Among the multiple results obtained, the involvement of the histone methylation machinery in the development, gametogenesis and neurogenesis in *K. marmoratus* was discovered (Fellous et al., 2019a).

The sex ratio of *K. marmoratus* has been discovered to be modulated by DNA methylation with the identification of several genes that were differentially methylated between males and hermaphrodites. Temperature have been shown to interact with the methylation patterns inducing modulation of the sexual identity in two rivulus lineages (Ellison et al., 2015). Another example of environmental influence on rivulus DNA methylation was recently published by Berbel - Filho and colleagues (2019). They found that the parasite load can modulate DNA methylation, but differentially according to isogenic lineages revealing an interaction between the parasites and the genotype of the lineage on the methylation level. DNA methylation therefore appears to be one of the mechanisms allowing the long-term persistence of self-fertilization in *K. marmoratus*,

which counterbalances the low genetic variations within lineages. By influencing the sex ratio as well, the selfing rate could be modulated by the environmental conditions and therefore increase out-crossing events promoting the genetic diversity.

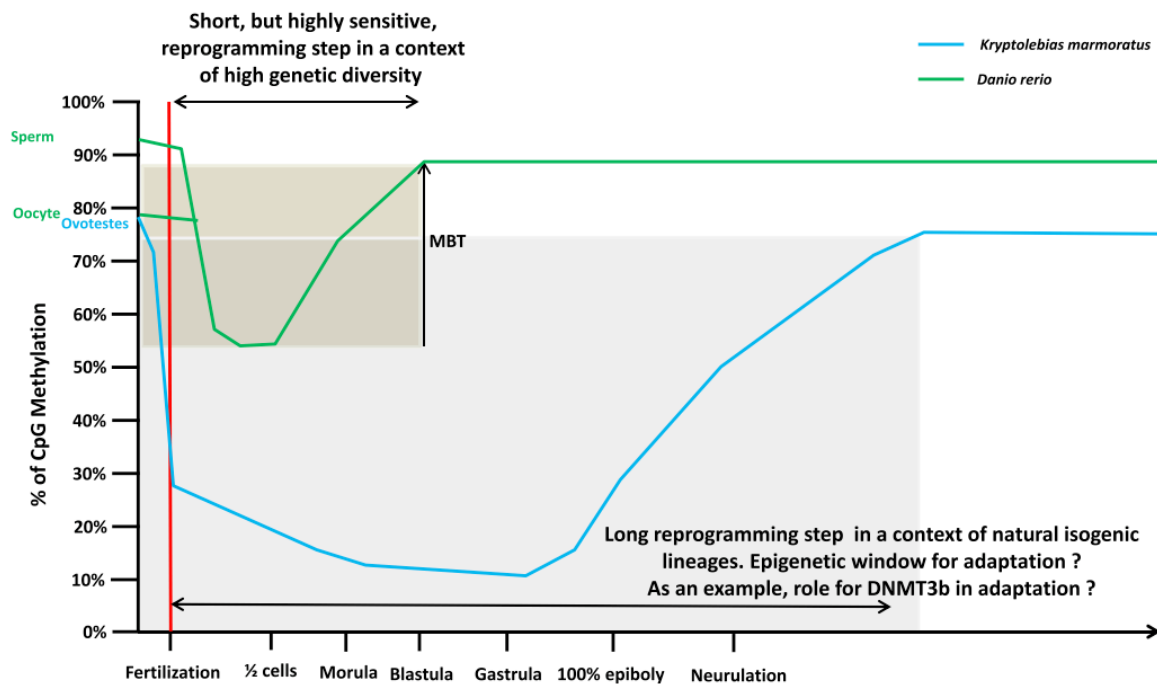


Figure 26 – CpG DNA methylation levels during early zebra fish and mangrove rivulus development. (MBT, Mid Blastula Transition). The developmental stages are indicated under the x- axis. Adapted from Fellous et al., (2018).

5.5. *K. marmoratus* as a model species ?

Unlike the well-known and widely used zebrafish, *Danio rerio*, *K. marmoratus* presents several disadvantageous characteristics, all related to egg production. The spawning cannot be induced due to the absence of egg production synchronization. Moreover, hermaphrodites produce low and inconstant amount of eggs (maximum 10 eggs per week) despite the maintenance of steady environmental parameters such as temperature, salinity and food availability. The internal fecundation leads the embryos to be laid at various developmental stages. Finally, the embryos produced can undergo a diapause limiting the use of elevated number of biological replicates and making difficult to synchronize *in vivo* studies. However, the mangrove rivulus presents advantages to investigate many questions of biology from diverse field research thanks to its unique

reproductive biology among vertebrates. Selfing allows rivulus to express very low genetic variability within lineages (Fellous et al., 2018; Mhanni and McGowan, 2004; Razin and Shemer, 1995). This allows working with natural highly homozygous and isogenic lineages and therefore, reducing genetic noise within lineages in experiments (Fuller et al., 2010). Besides its low genetic diversity within lineage allowing to exclusively focusing on the study of the true reaction norm (Stearns, 1989), rivulus is a useful model to investigate environmental influence on developmental plasticity because it is suggested to quickly acclimate to highly changing environmental conditions directly after hatching. By comparing the numerous existing and described rivulus isogenic lineages, scientists can focus on the genetic bases of phenotypes. Many other advantages related to the use this new valuable model vertebrate species exist and the following list is probably not exhaustive: its sequenced genome, its described DNA methylation reprogramming, its short generation time (\approx 80 to 100 days), its simple rearing environment, the transparency of its embryos, its small size, its robustness...

OBJECTIVES

Animal behavior is the result of a series of physiological and molecular mechanisms that can be modulated by the environment, the genotype of the organism, and both the interaction between genes and the environmental conditions to which the organism is exposed thorough his life. Behavior mediates the interaction between an organism and its environment, and constitutes the ultimate phenotype influencing ecological and evolutionary processes. 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 genetic and environmental variances, as well as their interactions, influence phenotypic variance is crucial to understand individual traits, suits of covarying traits, and animals' ability to acclimate to new environmental conditions during development and adulthood. We hypothesize that epigenetic mechanisms can, at least partly, explain behavioral plasticity and delayed behavioral response in the adults. Consistent individual behavioral differences across time and contexts are particular behaviors known as personality traits that highly influence organism essential behaviors such as social relationship, food access, reproductive success and ultimately organism's fitness. The present study thus aims to investigate the developmental plasticity of behavioral traits and the sequence of key molecular events leading to behavioral modifications that permit the organisms to cope with new environmental conditions. We exclusively worked with self-fertilizing hermaphrodites of mangrove rivulus fish (*Kryptolebias marmoratus*), providing after a few cycles of self-fertilization, natural highly homozygous and isogenic individuals within lineages. This unique vertebrate species with exceptional reproductive strategy thus expresses very low genetic variability within lineages and therefore permits the precise identification of the genetic and environmental sources of phenotypic plasticity.

To complete the general objectives, several specific objectives have been set up. Increasing studies have discovered behavioral variations (individuality) among genetically identical individuals reared in the same environmental conditions. The first specific objective aims to characterize personality traits (boldness and aggressiveness) of an isogenic lineage of the mangrove rivulus, how they co-vary as well as the non-genetic mechanisms generating between-individual behavioral variability in the absence of environmental variability (Chapter 1). To fulfill this purpose, behaviors of isogenic

adult of mangrove rivulus have been tested using respectively the shelter and model tests. Protein expression profiles as well as DNA methylation landscapes have been investigated in their brains. The second objective consists to understand the developmental plasticity of personality traits in the rivulus submitted to social interactions and low salinity during their development and their impacts on life history traits and protein expression profiles in the adult brain (Chapter 2). Those stimuli are environmentally relevant considering rivulus natural habitat. The mangroves are submitted to drastic variations of their physico-chemical conditions on daily and seasonal bases, and the rivulus reproductive biology as well as the environmental conditions in which it thrives make it highly subjected to develop social relations during early-life. The last specific objective is the determination of this developmental plasticity facing a neurotoxin exposure (BMAA), naturally present in rivulus living areas, well known to disrupt proper brain functions leading to neurodegenerative diseases such as Alzheimer, Parkinson or amyotrophic lateral sclerosis diseases in humans (Chapter 3).

Chapter 3: Developmental plasticity of personality traits: effects of a neurotoxin

In the previous chapters, protein expression profiles and DNA methylation landscape were compared between bold and shy as well as between aggressive and non-aggressive individuals. We identified the brain differentially expressed proteins and differentially methylated fragments correlated to between-individual variability in boldness and aggressiveness in isogenic individuals of mangrove rivulus reared in identical environmental conditions. Also, the developmental plasticity of these personality traits were investigated following the exposure to social interactions and low salinity as well as the effects on life history traits and brain protein expression profiles. It appeared that the applied stimuli differently affected fish growth and reproduction as well as the expression of some proteins in fish brain, although the exposure stopped a long time after the brain sampling. However, no effects of stimuli were detected on fish behaviors. The camouflage of stimuli effects on behavior by the elevated behavioral variability between individuals, the low environmental influence in the determinism of behavior or the choice of stimuli not eliciting strong enough effects were amongst the hypotheses raised in the discussion of the chapter 2 and potentially explaining the absence of stimuli effects on fish behaviors.

In this third chapter, we tested the effects of another stress on fish behavior: a neurotoxin, the β -N-methylamino-L-alanine (BMAA). This neurotoxin naturally produced by cyanobacteria, diatoms and dinoflagellates is of increasing interest related to its toxic effects and environmental risk. Intensive farming put pressure on aquatic ecosystems and leads to frequent bloom events due to water eutrophication. BMAA has been reported in marine and freshwater ecosystems around the globe such as several water bodies, including urban waters in the Netherlands, freshwater lakes and brackish water body in Britain, South African freshwater impoundments, Florida bay, Baltic sea, Mediterranean sea, Black sea, Adriatic sea, etc. (Brand et al., 2010a; Lage et al., 2015). Presence and accumulation of BMAA has been detected in organisms ranging from zooplankton, mollusks and fish. In fish, BMAA seems to particularly accumulate in brain tissues. Concentrations recorded in the Baltic sea can reach up to 200 times higher

concentrations of BMAA than did the cyanobacteria (from approximately 0.006 BMAA $\mu\text{g/g}$ dry weight in cyanobacteria of coastal regions of the Baltic sea, 0.033 BMAA $\mu\text{g/g}$ dry weight in zooplankton, and 0.775 BMAA $\mu\text{g/g}$ dry weight in turbot fish brain) (Jonasson et al., 2010).

In the first part of this chapter, the immediate effects of BMAA on locomotion and prey capture behaviors in the rivulus larvae were investigated after one-week exposure to 2 sublethal doses (20 $\mu\text{g/L}$ and 15 mg/L) (Part 1). BMAA is highly suspected to induce neurotoxicity and behavioral impairment as reported in humans. Neurotoxicants are known to be involved in various human and animal diseases (Rey et al., 2013). Environmental exposures to neurotoxic compounds can interfere with normal development during multiple windows of vulnerability that can result in a wide range of detrimental effects for the organism, with impacts on individual fitness and population viability (Cox et al., 2018; Scott and Downing, 2017). This part paves the ground towards a better understanding of the BMAA consequences on simplest behaviors, such as the feeding behavior. Then, the second part of this chapter was dedicated to investigate the developmental plasticity of behavioral traits after a two-weeks BMAA exposure to sublethal doses on newly hatched larvae. Boldness and aggressiveness were recorded in adults at 120 dph, more than 3 months after the exposure stopped. The expression of 7 genes were then measured in fish brain, genes chosen for their implication in neurotransmission, personality traits or known to be BMAA targets.

To fully understand the long-term influence of neurotoxicant exposure on organisms, it is necessary to determine the lasting and delayed effects observed during later life, after exposure has ceased (Carion et al., 2018; Mason et al., 2014). Such long-term effects can be linked to the development of neurodegenerative diseases (ND) in human and support the Developmental Origin of Health and Diseases hypothesis (DOHaD) developed by Baker in the 2000s (Chin-Chan et al., 2015). It is assumed that adult disease states can be triggered by environmental exposure to neurotoxicants earlier in life, or even during embryogenesis (Barker, 2004; Heindel et al., 2017). BMAA is one neurotoxicant hypothesized to cause NDs. It is known that the main mode of action for BMAA is via excitotoxicity, a process that induces an overstimulation of glutamatergic neurons, generating prolonged membrane depolarization, production of reactive oxygen species (ROS) and activation of apoptotic mechanisms (Barouki et al., 2018). However, little is known on its potential long term effects, beside the fact that

BMAA is considered as a slow neurotoxin due to its association with proteins, storage and slow excretion in its free form, causing a long latency between exposure and the observation of initial symptoms (Chiu et al., 2012; Onselen et al., 2018), and has been reported to induce developmental neurotoxicity in animal models (Banack et al., 2007; Faassen, 2014; Murch et al., 2004).

The following paragraph coupled with the schematic illustration (Figure 45) provide a complement of information about how BMAA can interfere at the synaptic level (glutamatergic pathway) in the optic to better understand the choice of the 7 selected genes (in chapter 3-part 2) and the associated mechanisms.

In healthy neurons, extracellular glutamate activates ionotropic receptors (NMDA, AMPA and GluR) and glutamate transporters (Xc-) that are mainly present on astrocytes and postsynaptic neurons ensuring transport of 80 to 90 % of glutamate. Activation of GluR/AMPA and NMDA receptors on postsynaptic membrane respectively induces Na^+ and Ca^{2+} entry into cell and a decrease of K^+ content that creates action potential allowing signal transduction. Glutamate is then transported and recycled in astrocytes to glutamine by glutamine synthetase, then transported outside the cell. This amino acid is ultimately captured by a neural receptor and converted to glutamate to act as a neurotransmitter precursor (Lobner et al., 2007; Platt, 2007). At the same time, extracellular glutamate binds to the antiport cysteine/glutamate receptors to allow entry of cysteine into the intracellular medium. This cysteine helps to form glutathione (GSH), important cell antioxidant (Aniksztejn et al., 2005).

BMAA can interfere with glutamatergic pathway generating various detrimental consequences such as excitotoxicity. *In vivo*, BMAA can bind to a carbonate ion to form β -carbamate. With a similar molecular structure as glutamate, β -carbamate can thus compete for glutamatergic receptors (Figure). Activation of glutamatergic receptor by β -carbamate blocks the entry of glutamate but does not modify the ion exchange. Extracellular glutamate and intracellular Ca^{2+} concentrations increase and cause prolonged cell depolarization (Aniksztejn et al., 2005; Forman et al., 2009). β -carbamate can also compete with glutamate and bind on the Xc- system inducing a dysfunction of the transporter. That prevents cysteine assimilation and induces an excessive exit of glutamate from cell. The absence of cysteine into cell leads to depletion of glutathione that may induce oxidative stress (Bridges et al., 2012). In addition, β -carbamate presence causes a continuous rise of extracellular concentration of glutamate that can

link to ionotropic receptor bringing more and more Ca^{2+} into cell. At higher concentrations ($> 100 \text{ nM}$) Ca^{2+} is toxic (Bridges et al., 2012) leading to mitochondrial dysfunctions and reactive oxygen species (ROS) release into cytoplasm that increases cell oxidative stress. In reaction to oxidative stress mitochondria releases cytochrome c causing cell apoptosis (Swulius and Waxham, 2008). This process caused by hyper stimulation of neural cells through glutamate inducing cell death is called excitotoxicity (Chiu et al., 2011).

Several thousand NCs are present in the environment but their risk assessment is mainly focused on human populations although it constitutes great risk for all living organisms. Neurotoxicity assessment with an ecotoxicological view is therefore needed and is described in the review of Legradi et al., (2018) co-written by our laboratory whose abstract is given below.

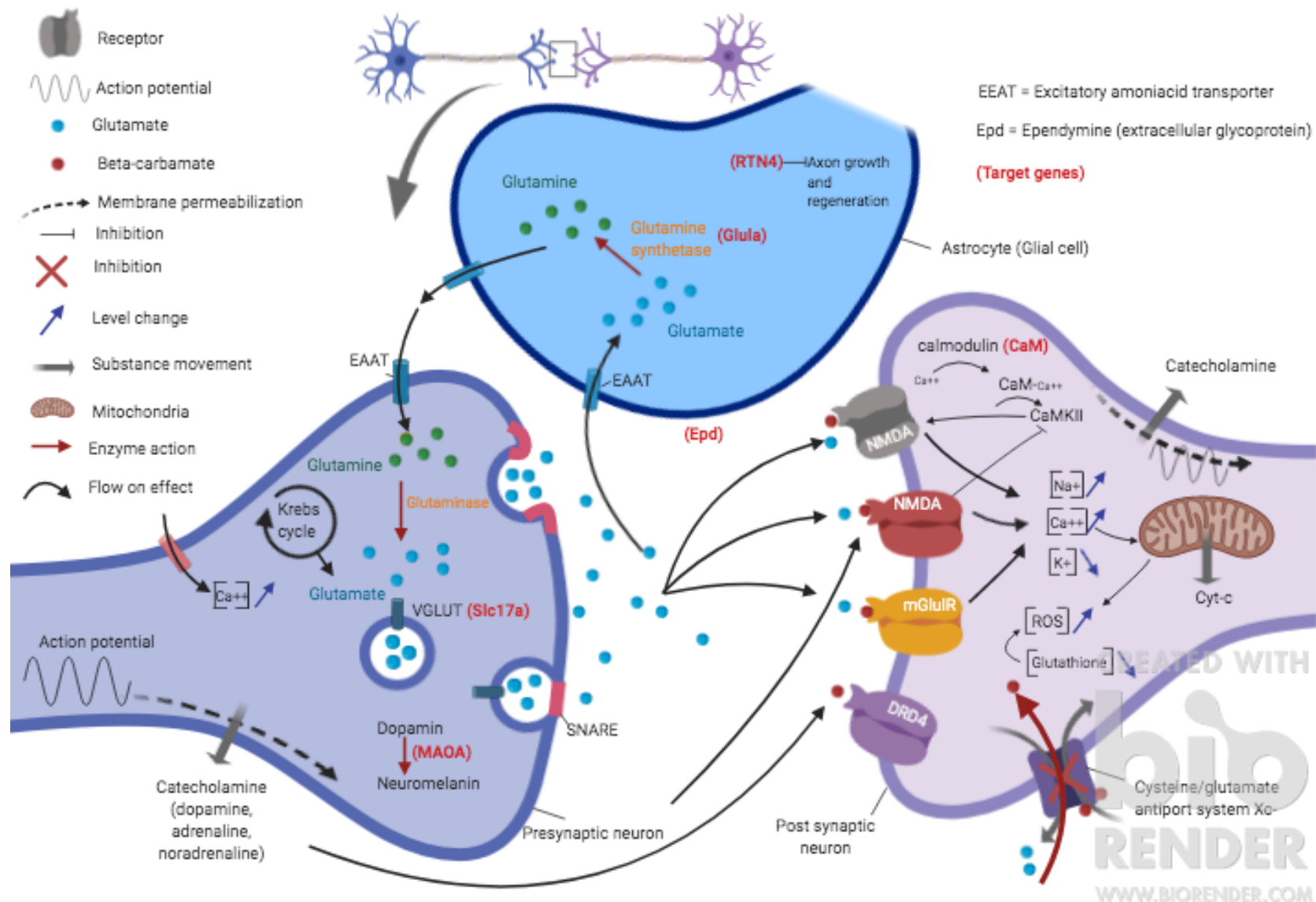


Figure 45 - Illustrative summary of the action of BMAA on neurons and the involvement of the 7 target gene selected in our experiment to measure their relative expression in the adult mangrove rivulus brain exposed to 2 sublethal concentrations of BMAA during early-life.

An ecotoxicological view on neurotoxicity assessment

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ABSTRACT

The numbers of potential neurotoxicants in the environment are raising and pose a great risk for humans and the environment. Currently neurotoxicity assessment is mostly performed to predict and prevent harm to human populations. Despite all the efforts invested in the last years in developing novel *in vitro* or *in silico* test systems, *in vivo* tests with rodents are still the only accepted test for neurotoxicity risk assessment in Europe. Despite an increasing number of reports of species showing altered behaviour, neurotoxicity assessment for species in the environment is not required and therefore mostly not performed. Considering the increasing numbers of environmental contaminants with potential neurotoxic potential, eco-neurotoxicity should be also considered in risk assessment. In order to do so novel test systems are needed that can cope with species differences within ecosystems. In the field, online-biomonitoring systems using behavioural information could be used to detect neurotoxic effects and effect-directed analyses could be applied to identify the neurotoxicants causing the effect. Additionally, toxic pressure calculations in combination with mixture modelling could use environmental chemical monitoring data to predict adverse effects and prioritize pollutants for laboratory testing. Cheminformatics based on computational toxicological data from *in vitro* and *in vivo* studies could help to identify potential neurotoxicants. An array of *in vitro* assays covering different modes of action could be applied to screen compounds for neurotoxicity. The selection of *in vitro* assays could be guided by AOPs relevant for eco-neurotoxicity. In order to be able to perform risk assessment for eco-neurotoxicity, methods need to focus on the most sensitive species in an ecosystem. A test battery using species from different trophic levels might be the best approach. To implement eco-neurotoxicity assessment into European risk assessment, cheminformatics and *in vitro* screening tests could be used as first approach to identify eco-neurotoxic pollutants. In a second step, a small species test battery could be applied to assess the risks of ecosystems.

Part I: Behavioral effects of the neurotoxin β -N-methylamino-L-alanine on the mangrove rivulus (*Kryptolebias marmoratus*) larvae

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Key words

Mangrove rivulus – DOHaD – neurotoxin– behavior – BMAA

Research highlights

BMAA negatively impacts rivulus' capacity of prey hunting.

Needs to test low concentrations of toxicants to assess environmental risk.

The mangrove rivulus is a new valuable model species to test long-term effects of pollutants.

ABSTRACT

Mangrove rivulus, *Kryptolebias marmoratus*, is a hermaphrodite fish capable of self-fertilization. This particularity allows to naturally produce highly homozygous and isogenic individuals. Despite the low genetic diversity, rivulus can live in extremely variable environments and adjust its phenotype accordingly. This species represents a unique opportunity to clearly distinguish the genetic and non-genetic factors implicated in adaptation and evolution, such as epigenetic mechanisms. It is thus a great model in aquatic ecotoxicology to investigate the effects of xenobiotics on the epigenome, and their potential long-term impacts. In the present study, we used the mangrove rivulus to investigate the effects of the neurotoxin β -N-methylamino-L-alanine (BMAA) on larvae behaviors after 7 days exposure to two sub-lethal concentrations. Results show that BMAA can affect the maximal speed and prey capture (trials and failures), suggesting potential impacts on the organism's fitness.

1. Introduction

Pollution involving neurotoxic compounds (NCs) is one of the emerging issues for human health but also for wild species and ecosystems. In wild organisms, the adverse outcome of exposure to NCs is a change in behavior, which can affect an individual's fitness and potentially lead to population decline (Hellou, 2011). Moreover, it is well established that embryonic development and early life stages (ELS) are periods during which the organism is particularly sensitive to environmental stress, and consequently to NCs exposure. In addition to immediate effects on ELS, developmental exposure at relatively low dose can lead to adverse outcomes later in life, at the adult stage, an idea accepted under the concept of Developmental Origin of Health and Disease (DOHaD) (Heindel et al., 2017). However, even if experimental and epidemiological studies support this concept, the mechanisms explaining long-term delayed effects of early life NCs exposure remain largely unclear (Barouki et al., 2018).

Nowadays, an increasing number of people suffer from neurological disorders such as Alzheimer's, Parkinson's or Amyotrophic lateral sclerosis (ALS) diseases, which could be partly related to environmental influences during ELS. Such diseases are triggered by a complex interplay between genes, ageing, and environmental conditions plus a random component (Riancho et al., 2018). In this context, the need for understanding the role played by exposure to NCs has been growing in importance (Chin-Chan et al., 2015). Of particular interest is β -N-methylamino-L-alanine (BMAA), a non-protein amino acid NC produced by the extremely ubiquitous cyanobacteria, dinoflagellates and diatoms (Faassen, 2014). Although the first link between BMAA and neurodegenerative diseases was established more than 50 years ago, evidence of BMAA effects on human brain is still inconclusive and remains controversial (Nunn, 2017). On the other hand, considering that BMAA can be found in aquatic food webs at high concentrations (Brand et al., 2010a), further studies about BMAA risk assessment on aquatic organisms are urgently required.

With this aim in mind, the present work has been developed using a strategic model organism; the mangrove rivulus fish. Together with its sister species, the mangrove rivulus (*Kryptolebias marmoratus*) is the only known vertebrate that naturally reproduce by self-fertilization (Costa et al., 2010a). In nature, this Cyprinodontiform oviparous fish is characterized by an androdioecious mixed-mating

reproductive system, in which hermaphrodites coexist with a low proportion of males (between 5 and 25%). While outcrossing with males is known to happen, it is much less frequent than self-fertilization of hermaphrodites. Consistent self-fertilization is an extreme form of inbreeding and it consequently naturally produces isogenic lineages after a few generations (Tatarenkov et al., 2012). Additionally, despite its low genetic variability, the rivulus displays a high level of phenotypic plasticity and it is capable to efficiently acclimate to the highly variable environment of mangrove forests. These features have recently put the mangrove rivulus in the spotlight of scientific research, as it constitutes an innovative and valuable model for the identification of true cause-effect relationships between the environment, the phenotype and the epigenome.

Impacts of NCs on the brain epigenome are one of the most often cited mechanisms potentially explaining neuronal disorders and DOHaD (Barouki et al., 2018). Effects on the epigenome do not involve changes in the DNA sequence but can modify gene expression patterns in a heritable manner through mechanisms such as DNA methylation (DNAm), histone modifications, and non-coding RNAs. Together, the emerging fields of neuroepigenetics and environmental epigenetics show that exposure to environmental NCs can affect the brain epigenome and consequently lead to impacts on behavior and/or cognitive faculties (del Blanco and Barco, 2018). In this context, the rivulus constitutes an optimal model choice to investigate the role of epigenetic mechanisms in DOHaD and in transgenerational inheritance via sexual reproduction, while minimizing the confounding effects of genetic variation (Fellous et al., 2018; Voisin et al., 2016).

The main objective of this study was to determine the immediate effects of BMAA exposure during ELS on locomotion and prey capture behaviors in the rivulus. Moreover, this preliminary work paves the ground towards a better understanding of the conspicuous consequences that NCs may have for populations of aquatic organisms and their link with molecular mechanisms, such mechanisms including modifications of the expression level of some key genes involved in behavior and in nervous cells metabolism as well as potential variations in epigenetic mechanisms.

2. Materials and methods

Rivulus larvae were exposed to 2 doses of BMAA directly after hatching for 7 days: 20 µg/L and 15 mg/L, plus a control unexposed group (n = 24). Larvae were fed *ad libitum* with *Artemia salina* every day. The BMAA working solution was made from L-BMAA Hydrochloride powder (Sigma-Aldrich®) mixed with 25 ppt (\pm 1) water. About 2/3 of water was renewed every day. During exposure period, mortality was assessed and larvae were individually measured at 1, 3, 5 and 7 days post-hatching (dph) using a Nikon Digital Camera USB3 1/2.5 15 IM/SEC mounted on a Nikon SMZ1270 stereomicroscope and the NIS-Elements® program. Larvae locomotion and thigmotaxis (test adapted from Norton, 2012) were video recorded after 7 days of exposure (10 min of acclimation followed by 5 min of test) in 6-wells microplate. Larvae behaviors were analyzed using Ethovision® software. Afterwards, 10 nauplii of *Artemia* were provided in each well to measure larvae prey capture ability during 5 min. Results are expressed as the percentage of success and failure, calculated as the proportion between successful capture or failed capture trials over the total number of trials.

Agostino and Pearson tests were performed to confirm data normality. Accordingly, one-way ANOVA or non-parametric Kruskal-Wallis tests were applied followed by Dunn's multiple comparisons test in order to evaluate an effect of BMAA on larvae locomotion, thigmotaxis or capability to capture prey. Percentages of success and failure were arcsine square root transformed. Statistical analyses were performed using GraphPad Prism 7 Software. Significance level was set at $p < 0.05$.

3. Results and Discussion

No effect of BMAA exposure was observed on larvae growth, body shape and mortality. Locomotion test showed a significant effect of 20 µg/L BMAA exposure on larvae maximum velocity with a 61 % increase compared to group-controls (Table 16 and Figure 46-a), while no effect has been observed at the highest concentration. The prey capture assay revealed significant effects of BMAA exposure on the percentage of failure in prey capture and on the total trials expressed by larvae to capture prey (Figure 46-b and c). The 20 µg/L and 15 mg/L groups respectively expressed a 14 % and 17 % increases in total trials compared to group-controls. Moreover, these two exposure

concentrations respectively expressed a 13 % and 15 % increases in percentage of prey capture failure compared to controls.

Table 16 Summary table including the number of biological replicates per treatment and results of locomotion and prey capture tests. Mean \pm SD.

Treatments	Number of replicates	Locomotion test			Prey capture test			
		Distance moved (cm)	Thigmotaxis	Maximum velocity (cm/s)	Total trials	Prey capture success (%)	Prey capture failure (%)	Maximum velocity (cm/s)
Ctl	24	39.33 \pm 36.14	0.83 \pm 0.48	2.06 \pm 1.46	10.08 \pm 2.12	94 \pm 17	7 \pm 10	3.09 \pm 5.11
20 μ g/L	23	55.92 \pm 39.13	0.92 \pm 0.34	3.32 \pm 1.90	11.52 \pm 2.27	89 \pm 26	20 \pm 19	5.47 \pm 9.41
15 mg/L	24	49.45 \pm 25.30	0.91 \pm 0.22	2.72 \pm 1.36	11.83 \pm 2.37	97 \pm 22	22 \pm 22	4.31 \pm 6.34

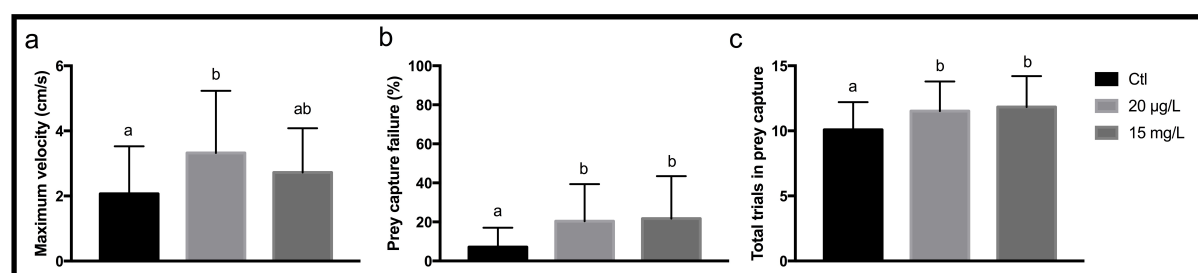


Figure 46 - Immediate effects of 7 days BMAA exposure on newly hatched rivulus larvae behaviors. (a) A significant effect at 20 μ g/L BMAA was observed on larvae maximum velocity (p -value = 0.035). (b) Larvae exposed to 20 μ g/L and 15 mg/L BMAA expressed significant higher rate of failure during prey capture assay (p -value = 0.016 and 0.004, respectively) and (c) significant higher trials to capture prey (p -value = 0.04 and 0.027, respectively) compared to controls. Results are expressed by mean \pm SD. Different letters (a-b) mean significant differences (i.e., p -value < 0.05) between conditions.

According to these results, BMAA exposure during development impacts larvae locomotion and capabilities to behave appropriately to efficiently hunt their prey. BMAA appears to mainly disrupt brain functions. Larvae maximum velocity was impacted by BMAA exposure at lowest concentration (20 μ g/L) while no effect was evident at higher concentration (15 mg/L). This suggests that an environmentally relevant concentration of BMAA applied during fish development can elicit changes in maximum velocity possibly due to neuro-muscular effects and/or impairments in fish perception of its

environment (Purdie et al., 2009a). However, these effects were not observed at a higher concentration suggesting a possible activation of repair mechanisms limiting brain damages (Zupanc, 2009). Interestingly, the total number of prey capture for larvae exposed to BMAA did not differ from control group while larvae showed a higher rate of failure since they tried more to catch a prey. This indicates that larvae exposed to BMAA need more trials to achieve the same success in prey capture with probably more energy expenditure. These observations support the assumptions of movement impairments induced by BMAA exposure and are consistent with literature in behalf of assumptions that BMAA can disrupt synaptic signaling (Frøyset et al., 2016). Effects observed on larvae serve as a baseline to assess possible consequences later in life as movement impairments can lead to lower food intake and consequently impact fish growth, reproduction and therefore organism fitness. Epigenetics can explain long-term latent or transgenerational effects. These hypotheses will be tested in future experiments.

4. Conclusions and perspectives

Overall, 7 days exposure of sub-lethal concentrations of BMAA on newly hatched larvae of mangrove rivulus revealed significant effects on fish behavior. BMAA showed non-monotonic effects, as 20 µg/L exposure increased maximum larvae speed, while 15 mg/L exposure had no effect. This emphasizes the need to test low concentrations when assessing the environmental risk. Moreover, both concentrations under study increased the number of trials necessary for the rivulus to catch the same amount of prey, suggesting impairment in energy expenditure and possible impacts on animal's fitness. Further molecular analyses such as gene-specific DNAm and gene expression in brain will provide new insights into the modes of action of BMAA. Further experiments on the delayed effects in adults will afford a better understanding of long-term consequences of BMAA presence in the environment and the putative roles of epigenetic mechanisms in neurotoxicity. These preliminary findings stress the importance of ecotoxicological studies about BMAA and other NCs on wild organisms, such as the mangrove rivulus, in addition to studies on human neurodegenerative diseases, as its presence in the environment could have consequences on populations' survival even at environmental concentrations, jeopardizing whole communities interconnected in trophic networks.

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General discussion and perspectives

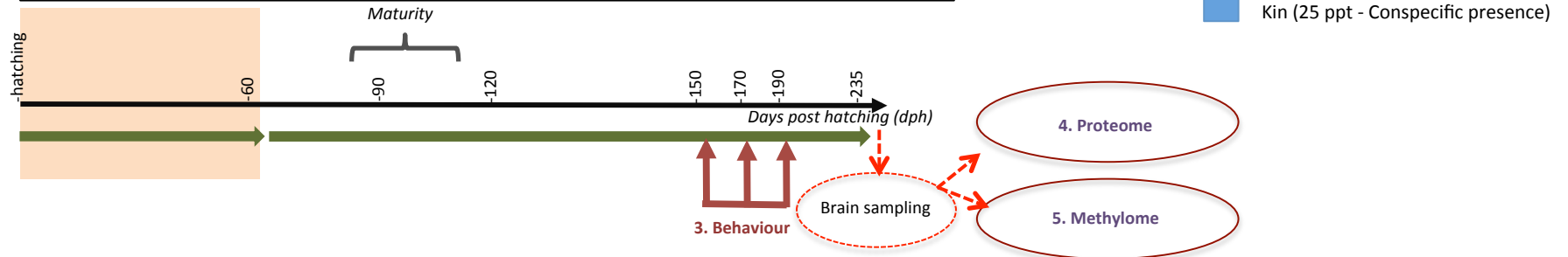
Experimental designs performed, observed effects and related potential perspectives raised at each chapter of the present thesis are illustrated in the following layouts and will be discussed hereafter.

The way an organism behaves in its environment influences numerous parameters and characteristics such as its physiology, reproduction, survival and ultimately its fitness. Behaviors are thus extremely interconnected with ecological dynamics and population evolution (Sih et al., 2010). The widespread existence of personalities across the animal kingdom suggests an evolutionary importance. Until recently, no particular reason was attributed to the presence of personalities in animals, with individual differences in behavior considered to be random variation around an optimal mean response. Many questions about animal personalities exist (Briffa and Weiss, 2010). Why some individuals are more aggressive than others no matter the time or the situation? Why is it not more optimal for an individual to show unlimited behavioral plasticity? The exact answers are still unclear although increasing works in behavioral genetics support that there is a heritable genetic contribution to personality (Beekman and Jordan, 2017; Class and Brommer, 2015; Oers and Mueller, 2010) such as for the exploratory behavior in Great tits (*Parus major*) (h^2 estimates = 0.22-0.41) (Dingemanse et al., 2002).

The potential genetic determinism in animal personality raises questions about the presence of behavioral individuality between isogenic individuals expressing therefore very low to no genetic diversity. A recent study observed behavioral individuality in exploration and activity in a clonal fish reproducing via parthenogenesis, the Amazon molly (*Poecilia formosa*), despite near-identical rearing conditions (Bierbach et al., 2017). The first part of our work (Chapter 1) was dedicated to answer the questions related to this topic: “Can behavioral individuality in personality traits emerge from a natural isogenic population of the mangrove rivulus?” “What are the sources of this individuality?”.

CHAPTER 1

1. Existence of **boldness and aggressiveness individuality** in isogenic lineage of *K. marmoratus* in controlled conditions?
2. Characterization of the **behavioral traits molecular bases**.



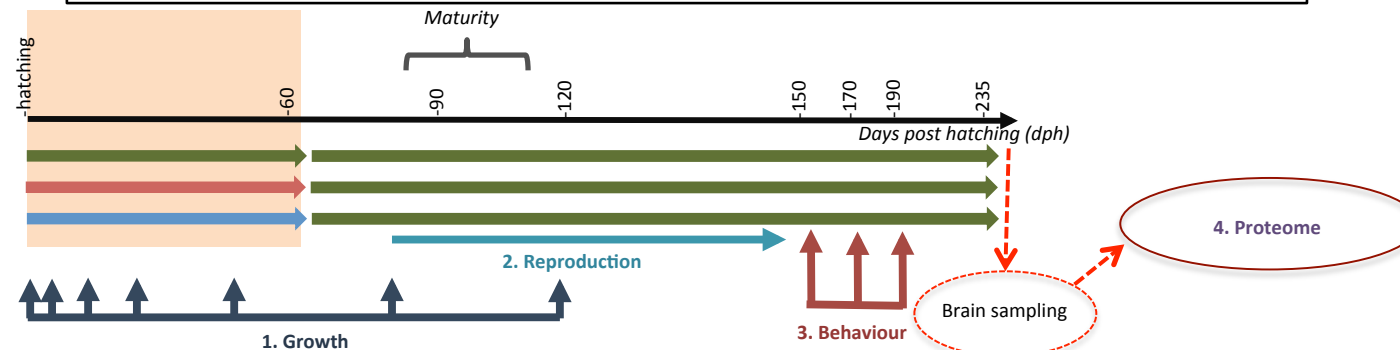
Behaviour
<ul style="list-style-type: none"> - Presence of significant behavioral differences between individuals $V_p = V_G + V_E + (V_{G \times E}) \text{ with } V_G \text{ and } V_E \approx 0$

Proteome (actual state of the molecular phenotype)
<ul style="list-style-type: none"> - Bold/Shy → 23 differentially abundant proteins (DAPs) - Agg/N-Agg → 31 DAPs - No common DAP between behavioral traits - Same final role → NS development maintenance and plasticity - Parvalbumin β → settlement of agg. behavior?

Methylome
<ul style="list-style-type: none"> - Bold/Shy → 31 differentially methylated fragments (DMFs) (Neural development, synaptic plasticity, neurotransmission, brain homeostasis) - Agg/N-Agg → 35 DMFs (Immune response, cytoskeleton and structural proteins) - No common DMF between behavioral traits - No similarities between DMFs and DAPs - Toll-interacting protein → immune response → Agg behavior?

CHAPTER 2

1. **Developmental plasticity of personality traits** within an isogenic lineage of *K. marmoratus*
2. Characterization of brain **molecular phenotype associated** to developmental exposure to stimuli
3. Characterization of stimuli **effects on life history traits**



Growth
<ul style="list-style-type: none"> - Salinity: no effect - Kin: larger fish → mutualistic benefits?

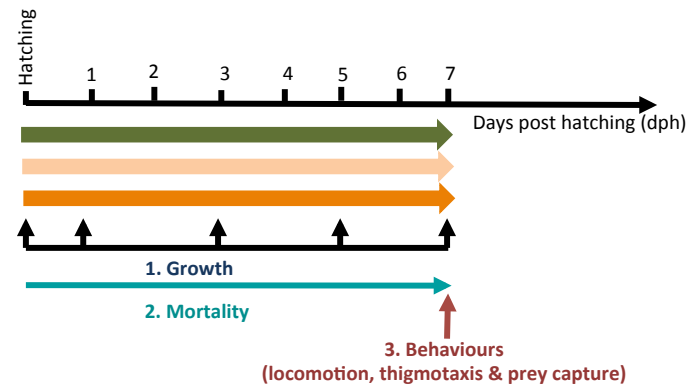
Reproduction
<ul style="list-style-type: none"> - Salinity: earlier reproduction → osmotic stimulus - Kin: reduced egg-laying rate → trade-off with growth

Behaviour
<ul style="list-style-type: none"> - Salinity: no effect on behavioral traits - Kin: no effect on behavioral traits → low environmental determinism of behavioral traits? → immediate effects rather than delayed? → high behavioral variability masks stimuli effects?

Proteome
<ul style="list-style-type: none"> - Salinity: 15 DAPs (energy metabolism, immune response) - Kin: 43 DAPs (neuroendocrine cell secretion, neuronal plasticity, learning process) - No common DAP between stimuli

CHAPTER 3 Part I

1. Determination of **the immediate effects of the neurotoxin BMAA** applied during early life stages of an isogenic lineage of *K. marmoratus*



Growth – body shape
No effect of BMAA

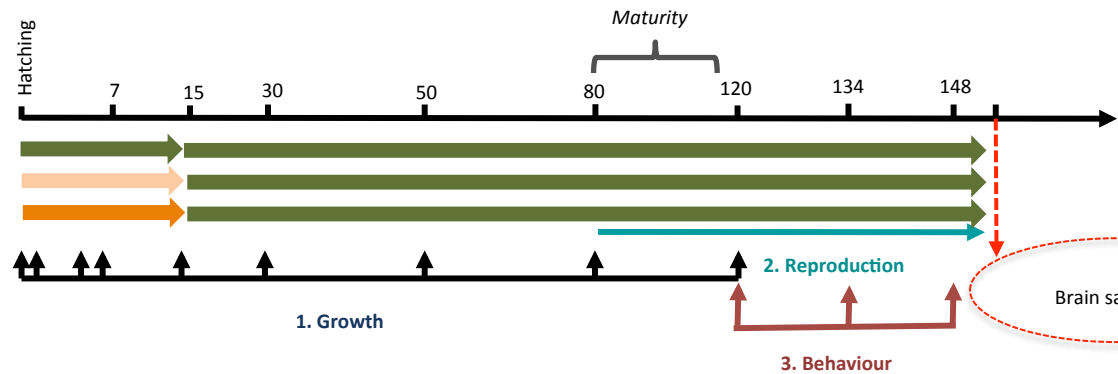
Mortality
No effect of BMAA

Locomotion – thigmotaxis – prey capture
20 µg/L BMAA <ul style="list-style-type: none"> - 61% increase of maximum velocity - 14% increase total trials in prey capture - 13% increase of prey capture failure 15 mg/L BMAA <ul style="list-style-type: none"> - 17% increase total trials of prey capture - 15% increase of prey capture failure <p>More trials needed to achieve the same success in prey capture → Movement and synaptic signaling impairments</p>

- Control
- Low BMAA (20 µg/L)
- High BMAA (15 mg/L)

CHAPTER 3 Part II

1. Determination of **the delayed effects of the neurotoxin BMAA** applied during early life stages of an isogenic lineage of *K. marmoratus* on their **personality traits** and **brain gene expression**



Growth
No effect of BMAA

Reproduction
No effect of BMAA

Behaviour
No effect of BMAA → low environmental determinism → long-lasting effects with aging?

Gene expression
20 µg/L BMAA <ul style="list-style-type: none"> - CaM and MAOA increase >< control group - DRD4, MAOA and CaM decrease >< 15 mg/L BMAA → disruption of glutamate turnover, intracellular dopamine depletion and activation of astrocyte protective mechanisms → those genes do not rule boldness and aggressiveness (polygenic effects?)

Despite the use of isogenic individuals of *K. marmoratus*, behavioral differences in boldness and aggressiveness were observed across the two experiments performed among fish exposed to the same controlled environmental conditions. To distinguish the presence of behavioral individuality/personality traits from random behavioral variation, repeatability estimates (R) were calculated. Repeatability refers to the extent to which individual differences in trait scores are maintained over time and therefore constitutes a statistical parameter to assess the presence of a personality trait (Biro and Stamps, 2015). It is commonly accepted by the scientific community that a R around 0.3 and over reveals behavioral individuality (Bierbach et al., 2017; Dingemanse et al., 2002; Schuster et al., 2017). The repeatability estimates calculated for boldness and aggressiveness in the mangrove rivulus were respectively equaled to 0.21 and 0.11. Dingemanse et al. (2002) considered the presence of behavioral individuality in the exploratory behavior of the great tits with a R = 0.27. The lower repeatabilities observed in our experiments are caused by the drop of fish response with time suspected to come from a habituation phenomenon. The between-individual variability was decreasing with behavioral test replicates over time. This response change with time was stronger with the model test, during which fish were exposed to a dummy. Considering that the lower R observed are caused by the suspected rivulus' learning and memory capacities and that the 0.3 cutoff is a reference for non-isogenic individuals, we consider behavioral individuality in boldness and aggressiveness are highly suspected to be present in the mangrove rivulus. In this section, we will discuss about the potential sources of behavioral individuality in the mangrove rivulus and how to improve behavioral tests performed as well as the R estimates calculation to obtain stronger conclusions.

1. Identifying the sources of behavioral variability leading to individuality of *K. marmoratus* in the absence of genetic diversity within a population

It is established that a third source of random variation, along with genes and the environment, can shape the phenotype variation and is referred as the biological noise (Richard and Yvert, 2014). If this stochastic variation occurs during organism's development at the cellular and molecular levels and generates differences in phenotypic outcomes when genotype and the environment are fixed, it is called the

developmental noise (Kiskowski et al., 2019; Vogt, 2015). The study of phenotypic variation of a population is therefore extremely complex due to the interaction between the three sources of variation: genotype, environment and stochasticity.

Unexpected genetic variations

The origin of the observed behavioral individuality could eventually come from **unexpected genetic variations** between rivulus individuals due to an actual lower level of isogenicity. **Sporadic genetic events** could be responsible of unexpected genetic variation in the tested *K. marmoratus* lineage leading to phenotypic variations, which could potentially explain the behavioral individuality observed for boldness and aggressiveness. *De novo* mutations, transposable elements, variation of tandem repeats could generate genetic variability between individuals (Barrick and Lenski, 2013; Freund et al., 2013).

The spontaneous single mutation rate is generally very low but varies between organisms (Barrick and Lenski, 2013). In bacteria and single-cell eukaryotes, the rate is on the order of 10^{-10} to 10^{-9} per base pair per replication, leading to one mutation every few hundred to several thousand cell divisions regarding the genome sizes of such organisms (from 10^6 to 10^7 bp) (Sung et al., 2012). In more complex eukaryotes, this rate varies from 0.05 to 1.0 per generation across the whole protein-coding portion of genomes (Barrick and Lenski, 2013; Lynch, 2007). Short tandem repeats (STRs) of DNA sequences (also known as microsatellites) are considered as dynamic sequences of the genome mainly present in intergenic regions. But when occurring inside the coding sequence, some can lead to diseases and behavioral impairments such as the fragile X mental retardation in humans due to the expansion of intragenic triplet (CGG) repeats next to FMR1 promoter region on the x chromosome (Jin et al., 2004). Intragenic tandem repeats generate variability, such as in pathogens, which favor their rapid adaptation to their environment and escape from their host immune system by rapidly changing their cell surface antigens (Verstrepen et al., 2005). As STRs are affiliated to the largest contributors of *de novo* mutations in humans and regarding the low *de novo* mutation rate reported in complex eukaryotes, we can consider that *de novo* mutations contribution to behavioral individuality observed in our experiments should be low (Gymrek et al., 2017).

K. marmoratus species is capable to reproduce by selfing. While it can be advantageous not to have to find a mate in a complex muddy environment, it reduces heterozygosity as well as genetic variability. The rate of out-crossing, likely driven by male-hermaphrodite mating, has been shown to vary among populations of *K. marmoratus* (Mackiewicz et al., 2006d). Outcrossing events are reported to be rare in most populations but it can considerably vary by sites such as in Florida populations where the selfing rate is reported from 80 to 90% while in Twin Cayes population (Belize), it reaches 40% with the sex ratio differences suspected to lead to these outcrossing rate variations among populations (Mackiewicz et al., 2006b; Tatarenkov et al., 2015; Turner et al., 2006). Low rate of heretozygosity within population has been studied between various populations of *K. marmoratus* using 32 microsatellite loci (Tatarenkov et al., 2010). Even if this study identified some variations within lineages probably caused by *de novo* mutations, high level of homozygosity within lineages was observed. A recent study used single nucleotide polymorphisms (SNPs) from whole genome sequencing data to determine heterozygosity level in various lineages of rivulus previously referenced by Tatarenkov et al. (2010) as completely homozygous at microsatellite loci (Lins et al., 2017). The assessment of the level of intra-individual heterozygosity in lineages maintained from 1 to 11 generations in the laboratory revealed more variation than previously, mostly due to the higher resolution of the technique used. However, this study confirmed that this variation is rare with most of the SNPs found as singletons mainly in intergenic regions of the genome (Lins et al., 2017). The high level of homozygosity among the DC4 lineage used in the present study, as it was reported by the study of 32 microsatellites (with 31 homozygous, 1 heterozygous), seems thus reliable and low level of genetic diversity between individuals is expected.

The non-genetic sources of behavioral individuality

In humans, it has been established that 40% to 50% of variation in all traits, including personality traits, come from genetic differences (Kandler et al., 2017). This was discovered from a meta-analysis of the genetic contribution to individual differences in 17 804 human traits based on studies of twins (Polderman et al., 2015). Working with humans, even with twins, can't provide the complete isolation of the

environmental influence in the settlement of individual variations. Some genetic determinism has been discovered with the expression variation of a few genes related to boldness and aggressiveness personality traits in diverse species (cfr Introduction). But the genetic variants that influence personality are only beginning to be identified regarding pleiotropic and polygenic effects (Sanchez-Roige et al., 2018). Recent findings in human research have revealed that the genetic basis of personalities, as well as neuropsychiatric diseases, is highly polygenic (Sanchez-Roige et al., 2018). Although unexpected genetic changes such as *de novo* mutations could potentially create variability, it is quite unlikely that a small rate of mutations could lead to behavioral individuality in boldness and aggressiveness in the isogenic individuals of mangrove rivulus as mentioned above. Other mechanisms that do not directly imply DNA coding sequence would therefore create individual differences by impacting gene expression. Our first chapter was dedicated to confirm this working hypothesis. RRBS analyses performed revealed various DNA fragments that were differentially methylated in the brain of individual expressing extreme behaviors in the bold/shy and aggressive/non-aggressive continuums. Through those analyses, the investigation of **potential epigenetic sources of behavioral individuality** and identification of target genes correlated to a particular behavioral trait within a personality continuum were possible. The observed methylation differences occurred at various locations of the genome with most differentially methylated fragments (DMFs) located within intra- and intergenic regions. No DMFs were similar to both behavioral continuums. For the bold/shy comparisons, significant methylation level changes impacting the zinc finger protein 276, the ceramide kinase protein coding genes (both located in the promoter region) as well as the ski oncogene and the NF-X1-type zinc finger protein coding gene (both located in gene bodies) were discussed in the chapter 1. The observed changes potentially revealed that differences in the production of sphingolipids, differences in neurogenesis as well as in various cellular processes partially regulated by zinc finger proteins would be responsible of individuality in the shy/bold continuum. The methylation level of those genes can thus be correlated to the bold level determinism in the mangrove rivulus. For the aggressive/non-aggressive comparisons, significant changes of the methylation level of the promoter region of the Toll-interacting protein and the platelet glycoprotein IX coding genes, both located in the promoter region, as

well as the dihydrofolate reductase coding gene (with intragenic situation) were discussed in the chapter 1. According to the literature, we concluded that the aggression level seems to be modulated by the immune response, such as discovered in humans. The study of gene expression would be further needed to support those results.

Results of the proteomic analyses, aiming to assess the actual state of the molecular phenotype associated to individuality in boldness and aggressiveness personality traits, revealed no significant protein common to both continuums. This suggests the implication of completely different molecular mechanisms. The bold/shy comparison mainly impacted structural proteins whereas the aggressive/non-aggressive comparison seemed to have more effects on proteins directly implicated in the nervous system development, maintenance and synaptic plasticity. However, the structural proteins described in the chapter 1 for the shy/bold axis turned out to be implicated in the modulation of neuron migration, nervous system development, neurotransmission and synaptic plasticity. Although the protein coding genes implicated in individuality in the bold and aggressive personality traits are different, they seem to be involved in the same final goal, the maintenance of nervous system homeostasis.

Interpreting omics results and bringing out strong conclusions from the processing of data coming from such proteome and DNA methylation analyses techniques is difficult due to the numerous and various cellular and molecular mechanisms impacted (Misra et al., 2018). Nevertheless, the identification of potential target genes related to the establishment of behavioral individuality was possible thanks to the use of omics techniques.

Past and actual environmental influences on behavioral individuality

Individual variation can affect intra- and interspecific competition, the structure and dynamics of ecological network, and the degree of phenotypic variation can set the direction and outcome of natural selection (Sih et al., 2012; Wolf and Weissing, 2012). The reasons why consistent individual differences in behavior exist and persist in face of selection are far to be solved. Behavior influences the environment choice through dispersal tendency and migration, mating strategy, social interactions, predator avoidance, feeding strategy, habitat choice and movement patterns. Therefore, individuals can occupy different local environments submitted to various level of

selection pressure due to differences in parasites, predators, competitors and resource densities (Biro and Stamps, 2008; D. Réale et al., 2010; Wolf et al., 2007; Wolf and McNamara, 2012). This **variation in selection pressure** can maintain variability in behaviors between individuals of a same population.

The variability expressed by the mangrove rivulus in their bold and aggressive behaviors can originate from their **highly variable natural environment**. The environmental conditions of mangroves may force them to colonize various habitats (emerged logs, leaf litter, crab burrows, stagnant pools,...). The selection pressure could thus vary between these microhabitats leading to the maintenance of diversity in behavior. This selection pressure would preferentially act on epigenetic mechanisms due to the low genetic diversity between individuals of a same population. Consistent individual differences in energy metabolism is also known to promote consistent individual differences in behavior (Biro and Stamps, 2010). Local variations in food supply may promote the development of differences in energy metabolism and behavioral individuality as well. It was reported that populations expressing differences in personality traits are expected to be less vulnerable to environmental changes (Bolnick et al., 2011; McCann, 2000). 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.

Consistent individual behavioral differences seem often selected as part of a “pace-of-life syndrome” which specifies that closely related populations or species submitted to different ecological conditions should differ in a suite of physiological traits (metabolic, hormonal and immunity) that have coevolved with the life-history particularities of each populations or species concerned. Personalities can thus covary with life-history and physiological differences within-populations as well as between populations and species (D. Réale et al., 2010). For example, variation in the aggressive and activity level in North American red squirrel (*Tamiasciurus hudsonicus*) females showed contrasting reproductive success, the magnitude and direction of the effect changed with the age and possibly food supply (Boon et al., 2007). However, this pace-of-life syndrome can't be generalized to all populations and species. Behavioral traits are not always correlated to another trait forming a syndrome and the correlation with life history traits is not always present (Bell, 2005; Dingemanse et al., 2007). Our results did

not show any behavioral syndrome between boldness and aggressiveness as well as no correlation between behaviors and life-history traits. According to the environmental conditions, the presence of a syndrome can impact individual fitness, can be adaptive or maladaptive with consequences on species distribution and ultimately influencing ecology and evolution of the concerned species (Conrad et al., 2011; Sih et al., 2004; Wolf and Weissing, 2012). The absence of syndrome observed in the mangrove rivulus would not limit the plasticity of each trait independently, which can be advantageous when organisms have to face highly variable environments such as the mangrove habitats. But syndromes such as behavioral syndrome can vary across time/experiments performed, between sex, according to the environmental influence and therefore are function of ecology (Bell and Stamps, 2004; D'Amore et al., 2015; Sih et al., 2015). The expression of a syndrome by the rivulus could thus be different across time, context, and sex or vary with their developmental environmental conditions.

Environmental enrichments in the housing conditions of laboratory animals have been proved to increase behavioral variability. This assessment was recently investigated using five isogenic lineages of *Drosophila melanogaster* raised in various enriched laboratory conditions. This study revealed that enrichment of fruit flies environment increased the behavioral variability compared to non-enriched environment, which was highly dependent on genotype and the interaction with the particular applied enrichment (Akhund-Zade et al., 2019). The fish used in experiments along this thesis are laboratory animals that didn't experience any differences of their environmental conditions across several generations. In the context of differences in local environment shaping individual behavioral variation, maintaining fish in steady laboratory conditions across generations could have smoothed the variability between individuals along generations. However, we still observed variability in boldness and aggressiveness behaviors, with shy and bolder fish as well as aggressive and non-aggressive fish within tested groups. Variability in behavior seems thus to be maintained even in the absence of environmental variation. Some mechanisms could intervene in the transmission of individual behavioral variation from one generation to another with the potential low intervention of the genetic variability. Calculating heritability of these behavioral traits would help to understand how behavioral variability is maintained across generations. The non-genetic individuality (also referred as the intragenotypic

variability (Akhund-Zade et al., 2019; Kain et al., 2015)) in behavior has been observed in many different organisms, even after experimentally homogenizing genotype and environment, such as in mice (Freund et al., 2013; Kurikawa et al., 2018), crayfish (Vogt et al., 2008), fish (Bierbach et al., 2017), nematodes (Stern et al., 2017), pea aphid (Schuett et al., 2011) and fruit flies (Kain et al., 2015). It can not be neglected that non-genetic behavioral variability can rise from very small environmental changes occurring during organism development as well. In laboratory conditions, these **stochastic microenvironmental effects** can occur between individuals, even more considering the rearing conditions of the mangrove rivulus (individually raised in small tanks) (Willmore et al., 2007). Even if salinity as well as temperature and water level of each rivulus tank were steadily maintained, it is quite difficult to monitor small changes occurring between fish. The differentially methylated fragments discovered between fish expressing extreme behaviors in the bold/shy and aggressive/non-aggressive continuums could thus have been generated by local differences of their environmental conditions in mangroves or even by stochastic microenvironmental differences in the lab and transmitted from generations to generations maintaining behavioral variability in the actual laboratory stocks. Mitchell and colleagues (2016) focused on mechanisms underlying the propagation of symptoms of behavioral disorders across generation even in the absence of plausible genetic factors. They discovered parallel non-genetic mechanisms responsible of propagating individual non-genetic traits of a complex psychiatric disease-like phenotype across multiple generations in mice. Somatic mechanism appeared to be responsible of the transmission of “anxiety” and “hypoactivity” traits across generations, whereas gametic mechanism intervened in the transmission of “increased stress-reactivity “ trait, traits found in depression and comorbid generalized anxiety disorders in humans. The transmission of anxiety-like behavior were accompanied by DNA methylation changes converging on the functional network of lipid signaling as well as neurotransmission. The gametic transmission occurring till the F2 generation revealed that the gametically programmed information survived the DNA methylation reprogramming process and persisted till adulthood. But this transmission can't be considered as transgenerational because it was erased in the F3 germline (Mitchell et al., 2016).

Bet-hedging strategy

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. This concept is known as the **bet-hedging strategy**, an evolutionary strategy in which a single genotype produces a distribution 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 (Honegger and de Bivort, 2018). Empirical evidence for bet-hedging is scarce whereas ample theoretical models exist (Honegger and de Bivort, 2018; Kain et al., 2015). Behavioral individuality was recently empirically reported to possibly reflect bet-hedging strategy (Carter et al., 2017; Kain et al., 2015). Kain and colleagues (2015) observed interindividual behavioral diversity (in light and temperature preference-dependent behaviors) in fruit flies (*Drosophila melanogaster*), which was not heritable, that generated interindividual differences in survival and reproduction and reflected a bet-hedging strategy. Vampire bats (*Desmodus rotundus*) have been shown to exhibit social bet-hedging in food-sharing meaning that investing in quantity of social relationship at the expense of relationship quality can reduce the risk posed by unpredictable social environment. They observed that bats invested in cooperative partnership with nonkin individuals by regurgitating food even if a kin relationship was available (Carter et al., 2017). The presence of inter-individual variation in boldness and aggressiveness levels in the mangrove rivulus may thus reflect a bet-hedging strategy in order to reduce risks and ensure the survival of some individuals of the population in case of rapid change of their environment. The red mangrove forests are highly variable environments on a daily and seasonal bases (D. S. Taylor, 2000). With the wet-dry seasonal alternation and the semidiurnal tides, rivulus are submitted to drastic variations of the oxygen, ammonia, salinity, temperature and water level. Knowing the characteristics of the red mangrove environment, it is highly possible that the bet-hedging strategy was selected to optimize survival of rivulus populations where the ratio of males can be very low, therefore limiting outcrossing and genetic diversity.

2. Assessment of the environmental influence in the determinism of personality traits

We observed behavioral variability between isogenic individuals raised in the same environmental conditions leading to the hypothesis that rivulus behavioral individuality comes from a bet-hedging strategy affecting epigenetic mechanisms (Cfr discussion above). However, it is commonly assumed that an organism is the result of a unique interaction between its genes and the environmental conditions to which it is exposed during its life (Crispo, 2007). To assess this environmental influence on the behavioral phenotype we investigated the developmental plasticity of boldness and aggressiveness personality traits to various stimuli/stressors (Chapter 2 and 3). We tested the effects of social interactions, low salinity and the exposure to a neurotoxin applied during the development of the fish, known to be a sensitive window in the organism's life.

Social interaction was chosen as a stimulus because the mangrove rivulus has been shown to differentially modulate its aggressive behavior with kin and nonkin individuals (Edenbrow and Croft, 2012d). We initially individually exposed rivulus to a kin or non-kin individual but the experiment failed with the non-kin exposure. Fish were placed in the same tank than a kin or a non-kin individual separated by a flow-through plastic divider allowing exclusively olfactory and visual contacts. However, it appeared that fish in presence of a non-kin individual were highly motivated to jump on the other part of the tank in order to change compartment and attack. We therefore observed high mortality and could not pursue the experiment with the non-kin exposure. Although this data was not used, this statement permits to confirm that the mangrove rivulus is quite aggressive, can modulate its aggressive behavior according to kin and non-kin individuals and is thus better raised individually per tank than in group, at least for the laboratory stock. It should be interesting to perform this nonkin experiment again by improving partition of the tank dedicated to this exposure.

Low salinity exposure was chosen as a stimulus because rivulus are naturally exposed to drastic variations of salinity on a daily basis in the mangrove habitat. Rivulus exhibit numerous adaptation to live and survive in such habitat (Ellison et al., 2012). Social interactions and low salinity exposure are thus both stimuli that the mangrove rivulus can encounter in its natural environment. The last stimulus chosen is a

neurotoxin, BMAA, that rivulus can encounter as well in its natural environment. However, evidence supports the excitotoxicity of this molecule naturally produced by cyanobacteria, diatoms and dinoflagellates (Berntzon et al., 2015; Lage et al., 2018). This stimulus can thus be seen much as a stressor and was expected to disrupt proper brain development leading to behavioral impairments. Added to the assessment of developmental plasticity of behavioral traits, our study aimed to characterize the brain molecular phenotype as well as some gene expression variation in the brain of fish due to the exposure to those 3 stimuli/stressors.

The chapter 2 was dedicated to characterize the developmental plasticity of mangrove rivulus from the DC4 lineage to social interaction as well as low salinity. This study revealed no effect of either stimuli on boldness and aggressiveness but had distinct impacts on life history traits. Although both stimuli impacted some of the brain protein expression implicated in neuroendocrine cell secretion, neuronal plasticity, learning processes, energy metabolism and the immune response, **the absence of observable effects on fish behavior suggests various hypotheses.**

First, the consequent behavioral variability between individuals may have masked the stimuli effects on behavior. Boldness and aggressiveness in various species have been shown to be influenced by diverse stimuli. To cite a few examples, in the rainbow trouts (*Onchorhynchus mykiss*) the level of stress influences aggression with low stressed individuals being more aggressive and dominant; losing a fight decreases boldness in this species as well (Frost et al., 2007). In the mangrove rivulus, opponent familiarity and contest experience influence contest decisions (Li et al., 2014). In hermit crabs (*P. bernardus*), the contest outcome influences the boldness level with winning enhances shyness (Courtene-Jones and Briffa, 2014). In the sea anemone (*Actinia equina*), the same tendency was observed with losing a contest reducing boldness (Rudin and Briffa, 2012). Improving the power of the analyses by increasing the number of individuals tested would therefore be a perspective to test on the rivulus in future behavioral experiments (Dingemanse and Dochtermann, 2013).

Second, both stimuli could have immediate effects rather than delayed ones on behaviors. It was demonstrated that rivulus preferentially associate with and exhibit less intense aggression towards members of their own genotype, tendency observed in our lab as well (Edenbrow and Croft, 2012d). Experience of a predatory attack can

modulate rivulus secondary males aggressive behavior (Edenbrow and Croft, 2012a). Experiments focusing on salinity influence revealed the modulation of aggressive behavior in the mangrove rivulus and some other fish species respectively (Edenbrow and Croft, 2012d; Lopez et al., 2018; Lorenz et al., 2016). Measuring boldness and aggressiveness right after the exposure and with a delay would help to better assess the influence of the environment on those traits.

Third, it is plausible that the environmental determinism of these two behavioral traits is very low in the mangrove rivulus, which could explain the absence of effects observed in adults. Personality traits are possibly less plastic to stimuli occurring during their development due to their influence on organism fitness. For example, it is generally assumed that elevated temporal variance in life-history traits decreases individual fitness and population growth (Doak et al., 2005). Melbinger and Vergassola, (2015) investigated the impacts of environmental fluctuations on evolutionary fitness functions. They noticed that reduced sensitivities to environmental changes substantially increased organisms' fitness. They concluded that it appeared evolutionary successful to minimize the sensitivity to the environment rather than optimizing the reproduction speed. We could therefore extrapolate the findings of Melbinger and Vergassola, (2015) to behavioral traits, highly influencing organisms fitness through reproduction, mating or food research. The hypothetic low environmental determinism of boldness and aggressiveness would avoid trait homogeneity within population to ensure its acclimation and adaptation to new environmental conditions that may occur in adults. This hypothesis supports as well the potential bet-hedging strategy related to personality traits that has been developed earlier.

The chapter 3 of this project focused on immediate effect of BMAA exposure on rivulus larvae as well as delayed effects on adult boldness and aggressiveness. It can help to support one of the hypotheses settled above. BMAA is highly suspected to impair fish behavior due to the induction of excitotoxicity of this molecule that has been reported many times (Onselen et al., 2018). Although BMAA impaired locomotor behavior in larvae right after the exposure, fish did not experience any delayed effects on their bold and aggressive levels neither on growth and reproduction. These observations therefore tend to support the **low environmental determinism** of behavioral traits. BMAA has been shown to disrupt some genes

expression implicated in key steps on neurotransmission or correlated to personality traits a long time after the exposure period stopped. These observations suggest potential detrimental effects of BMAA at the lower dose (20 µg/L) and repair/protective mechanisms activation at higher BMAA dose (15 mg/L). BMAA can thus be detrimental for the mangrove rivulus but this fish species seems particularly resistant compared to other fish species with potential brain protection mechanisms against BMAA adverse effects that lead to no influence in the adult behavior. We could thus conclude, according to the results obtained along this project that the mangrove rivulus behavioral traits seem to be submitted to low environmental determinism even faced to neurotoxic compounds. However, this hypothesis need to be further confirmed with other stimuli/stressors due to the long retention time of BMAA. The use of a variety of stressors would also provide a better evaluation of the surprising elevated resistance of the mangrove rivulus and the mechanisms associated.

The experimental designs of chapter 2 and chapter 3 aimed to detect environmental effects on rivulus personality traits as well as the molecular proteomic signatures and some genes expression changes of an early larvae exposure on adults. These early-life exposure experimental designs measured the effects respectively with a 90 days and 106 days delay on behavior and with 175 days and 156 days delay on proteomic and gene expression signatures. Proteomic results revealed subtle changes in the protein expression associated to no behavioral changes. Proteomic analyses are most often associated to a large number of significant proteins (Kültz et al., 2013, 2015). The reasons that can explain the small number of significant proteins observed could come from the long delay associated to low environmental stress exposure (kin, low salinity). However, it is also possible that some processes could have created variability in the molecular response assessed in the adults and therefore interferes with the power of statistical analyses. The accumulation of epimutations in the epigenome (epigenetic drift) could have been occurred stochastically or due to environmental influences generating variability in transcription and protein abundance with age (Jones et al., 2015). Phenotypic flexibility could also explain the effect reversibility and therefore low significant protein detection.

3. Adaptive effects of early experience

Two main models describe the adaptive effects of early experience. First, the **inoculation model** considers an upside-down U-shaped relationship between the level of stress during early life and the latter resilience. This means that, on the one hand, a low early stress could leave the organism unprepared for future stressful conditions, and on the other hand a high stress could overwhelm the organism letting him enable to cope with it (Edge et al., 2009). Second, the **adaptive tuning hypothesis** considers that the early environment is adaptive and successfully prepares the organism for its latter environment. However, this model does not fit when the early environment is catastrophically stressful (Bateson et al., 2004; Beery and Francis, 2011; Nettle and Bateson, 2015; Sachser et al., 2011). These adaptive plasticity models can generate two different outcomes, reversible and irreversible plasticity meaning that the phenotype acquired is respectively plastic or stuck in adult. Three main factors can influence the adaptive degree of plasticity. The ability of the organism to detect the changing conditions will modulate the plasticity level. If the individual is not able to properly detect variation of the environment, the phenotype adopted will not be optimal. The accuracy of early cues is also highly influencing the degree of plasticity. If environmental clues are not accurate they can thus not precisely predict future situations. The time between environmental changes is one of the most important factors (De Jong and Gavrillets, 2000; Sih, 2011). If the time between environmental changes is short, it might not be adaptive to be plastic, depending on costs and benefits of the plastic response. The study of real behavioral adaptive responses to stimuli or stressors applied during the organism's development, besides model productions, could provide a better understanding of variation in the response to rapid environmental changes that are and will be increasingly human-induced (Sih, 2011). Varying the type and intensity of stressors applied during rivulus development could therefore help to better assess the effects on their behaviors and to which extend they can vary and ultimately better understand behavioral responses to stress and associated ecological consequences.

4. Limitations of the molecular techniques used to assess behavioral individuality

Although the chapter 1 is quite innovative measuring personality traits in naturally isogenic individuals raised in the same controlled environmental conditions that allow to find out what intervenes in the behavioral variability observed that do not involve genetic and environmental influences, some limitations were encountered. We did not observe any similarities between proteomic and RRBS results limiting the possible connections and strong conclusions. The shotgun proteomic technique used, despite among the best, can't screen the extremely large dynamic range of the proteome. As most of the proteomic workflow, it can detect the most abundant proteins over 4 to 6 orders of magnitude, while in biological samples such as plasma, the range of protein concentration can span 12 orders of magnitude (Surinova et al., 2011; Yates, 2013; Zubarev, 2013). Added to this large dynamic range, sometimes, the most abundant proteins account for a large part of the proteome such as in plasma where the most abundant proteins represent about 90% of the total protein content. The LC-MS/MS shotgun proteomic technique used to assess the brain proteome detected a dynamic range of 6 orders of magnitude with the 25 most abundant proteins accounted for 23% of the total proteins detected. Actual technical limitations can lead to the impossibility to detect low-abundant proteins and therefore missing potential differences in expression of low abundant proteins between conditions.

The RRBS technique used to assess DNA methylation landscape related to boldness and aggressiveness personality traits also presents some limitations. With this technique, DNA samples are treated with sodium bisulfite that converts unmethylated cytosines to uracils, while methylated cytosines stay unchanged. Then, after PCR amplification of libraries, all sites containing methylated cytosine remain cytosine while unmethylated cytosines are displayed as thymines. The assessment of DNA methylation level of a CpG site via high-throughput sequencing requires the comparison of the relative number of reads containing a cytosine (meaning that it was initially methylated) to the number of reads containing thymine (initially unmethylated). Contrary to the "gold standard" of bisulfite sequencing- the whole genome bisulfite sequencing (WGBS) approach - which sequences and maps the whole genome and evaluates methylation status of every cytosine of the genome, RRBS only focuses on CpG-rich sequences due to

the use of a restriction enzyme (MspI) cutting at CCGG sites (Lea et al., 2017; Olova et al., 2018). The libraries generated are therefore enriched in CpG-rich sequences after a step of size-selection of the resulting fragments. The measurement of DNA methylation is limited to regions in proximity to the enzyme recognition sites, focuses therefore on a smaller portion of the genome compared to results obtained with the WGBS technique and miss the methylation of CpGs that are not situated in CpG-rich regions (CpG islands). RRBS is reported to capture about 85% of CpG islands and 60% of promoters which constitutes its biggest disadvantage compare to WGBS (Gu et al., 2011). However, RRBS is a very good compromise between sequencing costs, the coverage of the genome and the information gathered with single-nucleotide resolution. A common challenge to RRBS and WGBS techniques is the interpretation of the results due to the lack of knowledge and consistency about how methylation level influence gene expression according to its genomic location (Moarii et al., 2015). They also have limitations for functional conclusions in species that lack a good reference genome (Paun et al., 2019).

It is not surprising to observe no similar results with both proteomic and epigenetic techniques used in regard of their respective limitations. The association of proteomic and DNA methylation landscape analyses are rarely encountered in the literature, with any related to personality traits. The first chapter of this manuscript is innovative and among the first to use this approach to assess molecular bases of personality traits. Future technical developments added to RNA sequencing techniques will help to bridge the gap between proteome and methylome correspondences and therefore provide new insights about other potential epigenetic mechanisms that are responsible of behavioral individuality emergence. The Crispr-Cas9 approach would be appropriate to confirm the results obtained. Crispr-Cas9 is described as the Swiss knife of genetics for its capacity to modify DNA, and recently DNA methylation, of a wide range of species. Cas9 protein is associated to specific RNA fragments. RNA recruits and guides the Cas9 protein to its DNA homologue sequence. The endonuclease Cas9 cuts the DNA sequence and deactivates the gene associated. The same method with a deactivated Cas9 (dCas9) can be used for the locus-specific targeted manipulation of DNA methylation by fusionning a DNA methylation modulating protein such as DNMT3A (implicated in *de novo* methylation) to the Crisp-dCas9 complex (Urbano et al., 2019). Crispr-Cas9 seems to be a promising method to confirm our results and identify genes

responsible of particular behavioral traits as well as better assessing the role of their DNA methylation in the establishment of behavioral individuality.

5. Retrospective pros and cons about the use of the mangrove rivulus

The mangrove rivulus was used in our study for its unique reproductive strategy. Contrary to the widely used clonal lineages of zebrafish (*Danio rerio*), the capacity of rivulus hermaphrodites to self-fertilize naturally produces highly homozygous and isogenic lineages. It also limits the substantial inbreeding depression of artificial clonal fish strains. The isogenic lineages have naturally evolved and were not selected by human manipulations. These unique reproductive characteristics provide the opportunity to reduce the genetic influence within lineages in the production of organism phenotype. The identification of non-genetic and non-environmental sources of between-individuals phenotypic diversity in experiments is possible through the control of environmental conditions of this species. Rivulus is thus the perfect and only vertebrate that could have been used to fulfill the first specific objective (chapter 1) of this project, i.e. to characterize the brain proteomic molecular signatures and DNA methylation landscape of personality traits. Using a same isogenic lineage submitted to various environmental conditions provides the material to focus on the study of the true reaction norm, isolating the exclusive environmental influence in the production of phenotypes (chapter 2 and 3). We could also focus on the genetic influences in the production of a phenotype by comparing different isogenic lineages of rivulus raised in a same environment. In the context of personality traits, it would be particularly interesting to compare boldness and aggressiveness across various lineages of rivulus in order to better assess the genetic influence of personality traits.

We previously discussed about the potential effect of genetic mutations in the emergence of behavioral individuality among isogenic individuals. Even if this effect is expected very small due to the general low mutation rate, evaluating its implication would provide a better understanding of key targets generating behavioral individuality. For that purpose, the genotyping of individuals after assessing their personality traits added to molecular analyses would give more information in future experiments about the effects of unexpected genetic variability between individuals in the establishment of behavioral variability. We could also use and analyze previous RRBS data and search for

SNPs, even if this data does not include the totality of the genome and focus on CpG-rich regions, it could provide an estimate of the genetic variability. Understanding the effects of genome sequence variation on DNA methylation is needed to have a comprehensive picture of the implication of DNA methylation in regulating key biological processes. Tools to predict the impacts of non-coding variants on DNA methylation has been developed in the last couple of years (Zeng and Gifford, 2017). The presence of a SNP in a coding region can alter protein function. A SNP in non-coding regions can alter the binding of transcription factors, the allele-specific DNA methylation pattern or the allele-specific gene expression. SNPs constitute a potential cause of inter-individual phenotypic differences and are therefore of high interest (Wang et al., 2019). A bioinformatics tool called CpGenie takes high-throughput DNA methylation sequencing data such as RRBS data as input and furnishes predictions of CpG methylation as output. It also predicts the functional consequence of non-coding sequence variants. The implementation of this tool on rivulus RRBS data could thus be interesting as future research in our laboratory (Zeng and Gifford, 2017).

The small size of rivulus brain was also a disadvantage that we encountered during this project. Proteomic and methylation analyses were performed on different sets of individuals due to the technical limitations of extracting both proteins and DNA of high quality from small organs such as the rivulus brain. The integration of various omics data are mandatory to understand the complexity of biological processes but this step is nowadays still challenging despite the amount of studies publishing large-scale multiomics data (Noor et al., 2019). Despite these difficulties, using data coming from various individuals in integrating and interpreting results of different omics tools constitutes a limitation.

In chapter 3, we assessed the delayed effects of the BMAA neurotoxin on life history traits, behavioral traits and some gene expression in rivulus brain. Despite the significant effects on gene expression, no delayed effect was observed on the bold and aggressive levels of fish. We suspect BMAA to interfere with the aging process regarding impacts on brain gene expression as well as the long retention characteristics of BMAA, which could lead to even more delayed effects on fish behavior. To investigate the delayed effects of BMAA exposure on personality traits it would therefore be judicious to use another species with a shorter lifetime to assess the effects in older individuals.

The lifespan of *K. marmoratus* can reach more than 8 years in captivity (Taylor, 2012). The turquoise killifish (*Nothobranchius furzeri*) has been reported as a new valuable model in behavioral ecotoxicology with its extremely fast maturation (<16 days) and short generation time (<3 months) surpassing relatively slow maturation and long lifespan constraints of other model fish species (Harel et al., 2015; Thoré et al., 2019).

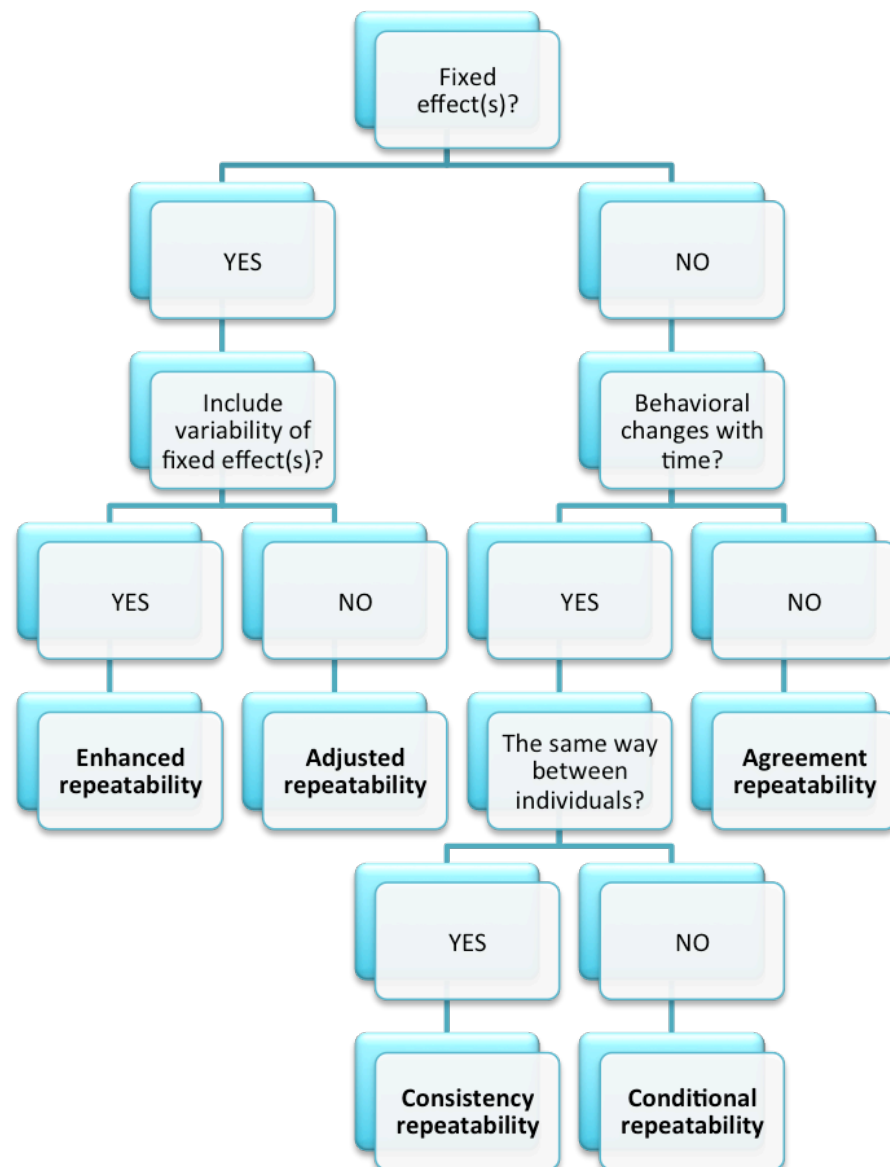


Figure 51 - Decision diagram of best repeatability estimates calculation. Adapted from (Biro, 2012; Biro and Stamps, 2015; Nakagawa and Schielzeth, 2010)

6. Future perspectives to assess personality traits

In the optic of improving our results, a particular attention needs to be provided to the development of most suitable behavioral tests to the mangrove rivulus. Behavioral results have shown a general drop or increase of fish response to both behavioral tests performed. This made difficult the good estimation of the repeatability, which refers to the extent to which individual differences in trait scores are maintained over time and therefore constitutes a statistical parameter to assess the presence of a personality trait (Biro and Stamps, 2015). Repeatability furnishes an estimation of the proportion of total phenotypic variability that can be attributed to the between-individual phenotypic variability. Fish became bolder and less aggressive with time (across behavioral test replicates). However, it appeared that individual response across time did not evolve the same way between individuals. According to the experimental design as well as the responses of individuals to behavioral tests, various type of repeatability can be calculated (Figure 51).

The **agreement repeatability** (R_A), refers to the proportion of the total variability attributed to between-individual variability assuming that individual differences in trait scores are maintained over time (Biro and Stamps, 2015). The **consistency repeatability** (R_C) assumes that individual responses change identically over time while the **conditional repeatability** (R_{time}) expects that individual responses change differently over time. Both R_C and R_{time} refer to the narrow sense repeatability (Biro and Stamps, 2015). However, added to these assumptions of behavioral variation over time, an **enhanced repeatability** (R_E) can be calculated including the variability of a fixed effect in the R calculation, such as a treatment effect for example. According to our behavioral results (with fish response changed differently over time), we should have calculated the enhanced conditional repeatability. However, to have such an estimation, we should have tested each individual more that 3 times over time in order to have a good estimation of the model slope (Bell and Peeke, 2012; Biro, 2012). To avoid the constraint of repeating behavioral tests multiple times and to properly estimate the presence of a personality trait, we should apply different context to the behavioral test replicates, for example by changing the color of the test arena or adding a new object between technical replicates. We did not expect the rivulus to

express such increased/decreased behavioral response over time with the 20 days delay between technical replicates.

The way an individual behave in a specific environment can change due to acclimation, fatigue, sensory adaptation or habituation. Unlike the other, habituation is an active basic learning process that helps animals to focus on important information (Bell and Peeke, 2012; Raderschall et al., 2011). This critical adaptive behavior is a relatively permanent decrease response/behavioral output as a result of repeated stimulation (Park et al., 2018; Rankin et al., 2009; Thorpe, 1956). Habituation is thus a process that optimizes energy expenditure enabling organisms to evolve in dynamic environment (Bell and Peeke, 2012). Habituation can also be submitted to variation between individuals and some studies suggest that individuality in habituation exists – individuals do not learn in the same way – rates of habituation varies between individuals (Bell and Peeke, 2012; Ellenberg et al., 2009).

The change of rivulus behavioral response with time indicates a potential habituation phenomenon, the simplest form of learning, revealing that the mangrove rivulus seems capable to learn from behavioral test and remember it over a long period of time, from few hours to more than 10 weeks (laboratory personal data). Future work will be dedicated to identify the presence of various trajectories of learning, potentially indicating that, even among isogenic individuals, variability/individuality exists in their learning performance as well.

GENERAL CONCLUSIONS

Along this thesis, multiple questions have been addressed around animal personalities: can personality traits appear in genetically identical individuals reared in same environmental conditions? What are the molecular bases of animal personalities? How animal personalities respond to stimuli applied during organisms' development? The interest in the study of animal personality traits rises from their ecological relevance related to their influence on organisms' fitness. Discerning how animal personality traits can develop in the absence of genetic and environmental variability and how they respond to environmental influence is a stake to better assess and understand animal's ability to acclimate and adapt in face of the major current concern of rapid changing environment. To investigate those questions, genetically identical individuals are required. Clones from several animal models exist and are widely used in experiments but most of them were selected by human interventions and therefore did not evolve by the action of natural selection. The mangrove rivulus fish used in this thesis, *Kryptolebias marmoratus*, is a unique vertebrate with a reproductive strategy of androdioecy. Hermaphrodites can self-fertilize and naturally produce, after a few cycles of selfing, highly homozygous and isogenic offsprings. Males exist in a smaller proportion than hermaphrodites whose ratio changes according to geographical areas suspected to influence the out-crossing rate impacting the within-population genetic diversity. The use of natural highly homozygous and isogenic individuals provides a unique sexually reproducing model to isolate and identify genetic and environmental sources of phenotypic plasticity. Working on an isogenic lineage reared in the same environment therefore allowed to investigate molecular mechanisms creating variability such as DNA methylation. The experiments performed during this thesis revealed that between-individual variation in behavior (boldness and aggressiveness) appeared between clones and was correlated to some differences in brain DNA methylation and protein expression despite the absence of environmental differences. Our work furnished new hypotheses about mechanisms underlying personality traits such as the methylation level variation (>40%) of the Toll-interacting protein between aggressive and non-aggressive fish revealing a link between the aggressive level and the fish immune response. Future studies, combined to resolution improvement of molecular

techniques, will confirm our results and may provide deeper insights about the molecular bases of animal personalities. The investigation of the developmental plasticity of rivulus exposed to environmental stimuli (social interactions, low salinity and neurotoxin exposure) during their development have shown various effects on life history traits, adult brain protein expression and gene expression but no delayed effects on fish behavior. These results let consider a low environmental influence on personality traits possibly linked to their considerable impacts on organism's fitness. An evolutionary bet-hedging strategy through epigenetic mechanisms was also envisaged to explain behavioral diversity in the absence of both genetic and environmental variability. The mangrove rivulus was the perfect and unique model to improve our knowledge about the sources of phenotypic variability. Its genetic, epigenetic and ecological characteristics make this incredible little fish an excellent new model species to investigate various biological and evolutionary questions.

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