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## Epigenetic inheritance and intergenerational effects in mollusks

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### List of abbreviations

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<b>5mC</b>	5-methylcytosine, a cytosine that has been modified by the addition of a methyl group on its 5th carbon
<b>ADP</b>	Adenosine diphosphate
<b>AFLP</b>	Amplified fragment length polymorphism
<b>BLAST</b>	Basic Local Alignment Search Tool
<b>BS-Seq</b>	Bisulfite sequencing
<b>BS-PCR-Seq</b>	PCr amplification after bisulfite sequencing followed by sequencing
<b>Cd</b>	Cadium

<b>CH3</b>	methyl group
<b>ChIP</b>	Chromatin Immunoprecipitation
<b>COBRA</b>	Combined Bisulfite Restriction Analysis
<b>CpG</b>	A cytosine base followed immediately by a guanine base
<b>CREB2</b>	CAMP response element-binding protein 2, a transcription factor that inhibits long-term memory formation
<b>DNA</b>	Deoxyribonucleic acid
<b>DNMT1</b>	DNA methyltransferase responsible for DNA methylation maintenance
<b>DNMT2</b>	DNA methyltransferases that methylates tRNA
<b>DNMT3</b>	DNA methyltransferases for de novo DNA methylation and maintenance
<b>DNMTs</b>	DNA methyltransferases
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>FISH</b>	Fluorescence in situ hybridization
<b>H2A</b>	one of the 5 types of histones in chromatin
<b>H2AV</b>	a variant of histone H2A
<b>H2A.X</b>	a variant of histone H2A
<b>H2B</b>	one of the 5 types of histones in chromatin
<b>H3</b>	one of the 5 types of histones in chromatin
<b>H3.3</b>	a variant of histone H3
<b>H3K27</b>	the 27th amino acid (lysine) in histone H3
<b>H3K4</b>	the 4th amino acid (lysine) in histone H3
<b>H3K9</b>	the 9th amino acid (lysine) in histone H3
<b>H4</b>	one of the 5 types of histones in chromatin
<b>H4K12</b>	the 12th amino acid (lysine) in histone H4
<b>Jmj</b>	Jumonji (histone demethylase)
<b>MAPK/ERK</b>	Mitogen-activated protein kinase/Extracellular signal-regulated kinase

<b>meDIP-seq</b>	Methylated DNA Immunoprecipitation sequencing
<b>MiRNAs</b>	Micro RNAs
<b>MSAP</b>	Methylation-sensitive amplified polymorphism
<b>MS-PCR</b>	Methylation-specific PCR
<b>pCO<sub>2</sub></b>	partial pressure of CO <sub>2</sub>
<b>PCR</b>	Polymerase chain reaction
<b>PTMs</b>	Post-Translational Modifications
<b>pH</b>	potential of Hydrogen
<b>qPCR</b>	quantitative Polymerase chain reaction
<b>RT-qPCR</b>	quantitative reverse transcription PCR
<b>RNA</b>	Ribonucleic acid
<b>RRBS</b>	Reduced representation bisulfite sequencing
<b>SAM</b>	S-adenosyl methionine
<b>SDS-PAGE</b>	Sodium Dodecil Sulfate PolyAcrylamide Gel Electrophoresis
<b>UV-HPLC</b>	Ultraviolet High performance liquid chromatography

## **Abstract**

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Recent insights in evolutionary biology have shed light on epigenetic variation that interacts with genetic variation to convey heritable information. An important characteristic of epigenetic changes is that they can be produced in response to environmental cues and passed on to later generations, potentially facilitating later genetic adaptation. While our understanding of epigenetic mechanisms in vertebrates is rapidly growing, our knowledge about invertebrates remains lower, or is restricted to model organisms. Mollusks in particular, are a large group of invertebrates, with several species important for ecosystem function, human economy and health. In this review, we attempt to summarize the literature on epigenetic and

intergenerational studies in mollusk species, with potential importance for adaptive evolution. Our review highlights that two molecular bearers of epigenetic information, DNA methylation and histone modifications, are key features for development in mollusk species, and both are sensitive to environmental conditions to which developing individuals are exposed. Further, although studies are still scarce, various environmental factors (e.g. predator cues, chemicals, parasites) can induce intergenerational effects on the phenotype (life-history traits, morphology, behaviour) of several mollusk taxa. More work is needed to better understand whether environmentally-induced changes in DNA methylation and histone modifications have phenotypic impacts, whether they can be inherited through generations and their role in intergenerational effects on phenotype. Such work may bring insight into the potential role of epigenetic in adaptation and evolution in mollusks.

## **Introduction**

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Evolution is a fundamental process at the origin of the biodiversity on Earth. Throughout evolutionary time, biological organisms have changed across generations, leading to contemporary lineages – but evolution and adaptation are still ongoing, and even sometimes boosted by rapid human-driven changes in physical and biotic environments (Chapin et al., 2000; Ceballos et al., 2010; Bellard et al., 2012). Natural selection, the main organizing principle of Evolutionary theory, requires differences in fitness among individuals associated with heritable phenotypic variation. The inheritance system is a key feature for evolution since a phenotype needs to be transmitted across generations to be selected.

During the last couple of years, it has become clear that there are considerable non genetic sources of heritable phenotypes (Jablonka et al., 1992; Jablonka & Lamb, 2008; Danchin et al., 2011).

which led to the notion of a systems concept of inheritance in which genetic, epigenetic, cytoplasmic and microbiome elements interact mutually, and with the environment to code and transmit the phenotype (Cosseau et al., 2017) (see also Jablonka & Noble, 2019). There is a general agreement that, in principle, evolutionary theory should accommodate all forms of inheritance, genetic and epigenetic but also cultural transmission, social learning, niche construction and parental effects – there is however debate over the quantitative importance of

nongenetic inheritance and over whether its recent revival constitutes or not a major turning point in evolutionary theory (Danchin et al., 2011; Laland et al., 2014; Charlesworth et al., 2017).

In this review, we will take the convention to call epigenetic a change in gene function without modifications in DNA sequence. Such changes can be reversible and only some of them are mitotically and/or meiotically heritable – only the latter being relevant for evolutionary change. We focus in particular on changes in gene expression associated with changes in chromatin structure. Chromatin is a complex of DNA and associated proteins whose primary function is to modulate genome packing and organization, as well as its metabolism. Chromatin structural changes can alter the accessibility of DNA by transcription factors and have consequences on gene expression. The main known bearers of epigenetic information are DNA methylation, histone PTMs, nuclear spatial remodelling and non-coding RNA, which complex interplay results in distinct chromatin states, known as chromatin colors (Filion et al., 2011; Van Steensel, 2011). A change in a single or several bearers of epigenetic information at a particular locus may result in changes in chromatin structure at this particular locus, and therefore affect the expression of underlying genes in the affected region. Such a change is called an epimutation. These epimutations may be the results of normal regulation during ontogenesis or cellular metabolism or may be induced by environmental clues.

Most studies in this field have so far focused on model organisms for which several reviews are available (Feng et al., 2010; Zhou et al., 2011; Keung et al., 2015; Schübeler, 2015). These organisms belong to two of the three major clades of bilaterian animals: the deuterostomes (that include vertebrates) and the ecdysozoans (that include arthropods and nematodes). Only a few papers are available for the third major clade (Lophotrochozoans), of which the most diverse phylum is mollusks. In addition, mollusks comprise many species of economic (e.g. edible bivalves such as mussels or oysters) or sanitary (vectors of snail-borne parasitoses such as bilharziasis or fasciolosis) interest. In this review, we first aim at compiling the mechanisms of

epigenetic changes which have been described in mollusks. Then we focus on what is known about intergenerational effects that have been reported in mollusk species. We finally discuss directions for further work, especially to relate molecular aspects of epigenetic information to phenotypes and inheritance patterns – a key aspect to evaluate the importance of epigenetics in adaptive evolution.

## **1- DNA methylation in mollusks**

One of the most studied epigenetic bearers of information is DNA methylation, which involves the biochemical modification of a DNA base. In animals, cytosine methylation most often occurs in a CpG dinucleotide context and is catalyzed by DNA methyltransferases (DNMTs) which add a methyl group (CH<sub>3</sub>) to the fifth carbon of the pyrimidine ring (Bhutani et al., 2011). DNA methylation has been extensively studied in model organisms such as mammals and flowering plants (Law & Jacobsen, 2010) and its role as a heterochromatic feature in gene promoters, repeat elements and telomere elements has been largely validated (Ambrosi et al., 2017). Compared to other bearers of epigenetic information, 5mC appears to be relatively stable and survives common conservation methods for biological material. As a consequence, DNA methylation has been the most studied epigenetic bearer of information in mollusk species (See Table 1 for methods to study DNA methylation).

### **1.1- Mollusks display a conventional invertebrate-like DNA methylation pattern**

Methylated cytosines have been identified in a diverse range of mollusk clades. Indeed, they have been referenced in 8 gastropods species (Fneich et al., 2013, D. V. Nica et al., 2017, Joe, 2013, Müller et al., 2016; Bal et al., 2017; Kong et al., 2017), in 7 bivalves (Sun et al., 2014, Petrović et al., 2009, Ardura et al., 2017, Gavery & Roberts, 2010) and in one cephalopod species (Díaz-Freije et al., 2014) (Table 2). Global cytosine methylation has been shown to vary from 5 to 15% of CpG being methylated based on LC-MS analysis or BSSeq analysis in mollusk

species. This proportion is higher than what is generally found in insects (ranging from 0 to 14%, Bewick et al., 2016) and lower than what is reported in fishes (80% of CpG being methylated, Metzger & Schulte, 2016), plants (30% in CG, CHG and CHH contexts, Law & Jacobsen, 2010) and mammals (70-80% of CpG being methylated, Law & Jacobsen, 2010). DNA methylation features in mollusks are essentially based on data from *Crassostrea gigas* (15 papers concerned *C. gigas* on the 38 founded in the literature concerning DNA methylation in mollusks) and *Biomphalaria glabrata* (3 papers) for which whole methylome characterization has been achieved by BS-Seq or meDIP seq ((Wang et al., 2014; Riviere et al., 2017) (Cosseau personal communication for *B. glabrata*) (Table 2). These two species display the features of DNA methylation patterns encountered generally in invertebrates (Sarda et al., 2012): *C. gigas* and *B. glabrata* have mosaic type DNA methylation (*i.e.* large blocks of fully methylated DNA are separated by large blocks of fully unmethylated DNA) and cytosines are methylated predominantly in the CpG context. The methylation is essentially intragenic (exons and introns) while methylation of repetitive elements and intergenic regions occurs only at moderate levels (Sarda et al., 2012). In *C. gigas*, intragenic DNA methylation level correlates with gene length and gene expression levels (Wang et al., 2014; Olson & Roberts, 2014b; Rondon et al., 2017). Hypermethylated genes are roughly associated with housekeeping functions, and hypomethylated ones are linked to regulated and/or inducible functions (Gavery & Roberts, 2010). Different types of exons have also been associated with different cytosine methylation levels (Song et al., 2017). Of note, for a given gene, correlation between differential gene expression and differential promoter methylation level is barely reported (Tran et al., 2016; Rondon et al., 2017) and seems to be restricted to only a small proportion of genes involved in conserved pathways such as developmental pathways (Rajasethupathy et al. 2012, Saint-Carlier & Riviere, 2015; Wei et al., 2018). Interestingly, positive correlation has been shown between a given promoter methylation and



its associated gene expression (Li et al., 2015) which is in conflict with results on vertebrate model organism where cytosine hypermethylation in promoter regions results in reduction of downstream gene expression. In this sense, the role of invertebrate DNA methylation in mollusk remains an open question and the study of DNA methylation in combination with other techniques such as ATAC-Seq would be necessary to gain insights on this question.

### 1.2- **DNA methylation is a key feature for development in mollusk species**

Cytosine methylation has been shown to be a key feature for developmental process in mollusks (Table 2). A prominent role of DNA methylation for mollusk reproduction success has been suggested based on different studies performed in *B. glabrata* (Geyer et al., 2017) and *C. gigas* (Riviere et al., 2013) for which genes encoding for DNA methylation machinery are over-expressed in gonadal tissue compared to somatic tissue. These studies are supported with analysis using DNA demethylating agent treatments which impact the egg production and embryo development in *B. glabrata* (Geyer et al., 2017, N. Luviano personal communication). DNA methylation variation has also been reported during the early development of mollusks. A global increase in methylation within exons at the expense of other genomic feature occurs in *C. gigas* during larval development (Riviere et al., 2017), for which tissue and development specific expression of gene encoding DNA methylation machinery proteins is observed (Wang et al., 2014). A global demethylation process occurs along the development of *O. vulgaris* (Díaz-Freije et al., 2014; García-Fernández et al., 2017) and age dependent decrease of 5mC levels is also observed in juvenile *P. acuta* snails (Müller et al., 2016a) and in *C. farreri* (Lian et al., 2015). Significant variation in DNA methylation levels is also observed among tissues of *Pinctada fucata* and during early step of development with DNA methylation levels increasing with development (Li et al., 2014). Synaptic plasticity has been extensively studied in *Aplysia* and epigenetic changes have been shown to be key feature for long-lasting changes in the

functional state of neurons (Rajasethupathy et al., 2012). Synaptic memory is facilitated after hypermethylation of conserved CpG island in the promoter of CREB2, a major inhibitor of memory, illustrating again a key role for DNA methylation on developmental process in mollusks.

### **1.3- DNA methylation is responsive to the environment in mollusk species**

Both spatial and temporal variation of abiotic factors have been shown to affect DNA methylation patterns in several mollusk species (Table 2). DNA methylation patterns can vary according to the seasonality (especially of temperature) in the oyster *Isognomon alatus* (Suarez-Ulloa et al., 2019) or according both to temperature and salinity in the Pacific abalone *Haliotis discus hannai* (Kong et al., 2017). DNA methylation differences in response to spatial variation in water current speed (lakes vs. rivers) has been observed in the snail *Potamopyrgus antipodarum* (Thorson et al., 2017). Another example is the invasive populations of *Xenostrobus securis* recently settled in a new area which are less methylated than older ones (Ardura et al., 2017). In addition, chronic environmental exposure to chemical compounds on ecologically relevant species has recently attracted strong attention and a few studies have investigated the impact of environmental stressors on epigenetic bearers of information of terrestrial and aquatic mollusks. Cadmium exposure induces global cytosine (and possibly hydroxycytosine) hypermethylation in *C. aspersus* (Nica et al., 2017(a); Nica et al., 2017(b)). The effect of vinclozolin and prednisolone has also been shown to affect DNA methylation patterns in *P. acuta* (Sánchez-Argüello et al., 2016; Bal et al., 2017). Global level of hydroxymethylcytosine (but not methylcytosine) decreases in *C. gigas* in response to copper exposure (Sussarellu et al., 2018) and a parental effect is reported on DNA methylation patterns in *C. gigas* in response to the diuron herbicide. Indeed in this study, cytosine methylation changes have been observed in specific regions in offspring of oysters which had been exposed to the herbicide during gametogenesis (Rondon et al., 2017). Biotic interactions also often

induce change in DNA methylation. Infection with toxic algae has been associated to a general hypomethylation in different oyster species (Farias et al., 2017; Gonzalez-Romero et al., 2017). Trematode infection in *B. glabrata* affects the expression of genes involved in the response to stress (Ittiprasert et al., 2015) or encoding for DNA methylation machinery proteins and impacts the level of DNA methylation of this mollusk (Geyer et al., 2017), whereas no effect of trematode infection on the level of 5mC is observed in *Zeacumantus subcarinatus* (Joe, 2013). The question of the mechanisms which lead the environmental information to induce epimutations remains however an open question. The cross talk between the nuclear epigenome and mitochondria is an emerging field of interest to investigate this question. Mitochondrial functions provide intermediate metabolites whose derived products (ex: SAM, acetyl coA) are known to modify epigenetic marks in the nucleus (Matilainen et al., 2017; Weinhouse, 2017). Mitochondrion is one of the key target of environmental stress in mollusk species. Mitochondrial toxicity of Cd has been shown in clams (Ji et al., 2019), mitochondrial dysfunctions in mussel hemocytes are induced after an exposure to titanium particles (Wang et al., 2019) and infection of *B. glabrata* with nematodes induces suppression of mitochondrial oxidative metabolism (Tunholi-Alves et al., 2019). In this sense, we believe that investigating the consequences of environmental information on cellular metabolism is of great interest to first investigate the link between the environment and DNA methylation.

## **2- Histone modifications in mollusks**

The primary protein components of chromatin are histone proteins and wrapping of DNA around these histone proteins constitutes the nucleosomes (Kornberg, 1974; Bentley et al., 1984). PTMs of histones result in chromatin structural modifications (Strahl & Allis, 2000) which can alter the accessibility of DNA by transcription factors and have consequences on

gene expression (see: Kouzarides, 2007 for a review). Two types of histones exist: core histones and linker histones. The core histones (H2A, H2B, H3 and H4) are agglomerated with two copies of each to form nucleosomes around which DNA is wrapped (Thomas & Kornberg, 1975). Only one linker histone exists (H1) and it is implicated in linkage between nucleosomes and DNA and therefore plays a critical role in chromatin formation (Kornberg, 1977; see: Fyodorov et al., 2017 for review). Histone N-terminal tail can carry over 60 chemical modifications (Vaquero et al., 2003) such as methylation, acetylation, ubiquitination, phosphorylation, ADP-ribosylation, citrullination and sumoylation (van Holde, 1988; Strahl & Allis, 2000). Post-translationally modified histones interact with each other's and with chromatin modifying enzymes to form complexes which result in the different chromatin colors responsible for the different level of chromatin compaction (Barth & Imhof, 2010). To study histones and histones marks, several techniques are available (Table 3). Among them, techniques based on the use of commercially available antibodies have been shown to be suitable thanks to the high level of evolutionary conservation displayed by these proteins in eukaryotes (See Table 3 for methods to study histone modifications, See Rivera-casas et al., 2017 for accurate description of methods).

Histones and histone variants have been described in many mollusk species (Drabent et al., 1999; Albig et al., 2003; Li et al., 2006; Rivera-Casas et al., 2016). Protamine-like-type, histone H1-type or protamine-type have also been identified in the sperm or testis of diverse mollusks to replace canonical histones (Eirin-Lopez and Ausio 2009, Mennella et al., 2002, Martinez-Soler et al., 2007). Since histones are very conserved proteins along evolution, some studies have been carried on phylogenetic reconstruction and evolution of histones genes in mollusks (Eirin-Lopez et al., 2002, 2004; Armbruster et al., 2005; Li et al., 2006; Bouilly et al., 2010). Other studies have also reported the DNA extracellular track activity of histones showing the potential implication of histone-like proteins in antimicrobial defense in different mollusk

species (Li et al., 2007; De Zoysa et al., 2009; Poirier et al., 2014; Bachère et al., 2015). We will not discuss the details of these analyzes since this review rather focuses on the implication of histone PTMs as an epigenetic carrier of information (Table 2).

### **2.1- Modified histones are key mechanisms for mollusk development**

Tissue and stage specific pattern of the *Jmj* ( expression has been registered and clearly indicate a role for histone demethylase during gametogenesis and embryogenesis in *C. gigas* (Fellous et al., 2014). The expression of these Jumonji histone demethylase gene is also affected in response to temperature changes during *C. gigas* early development (Fellous et al., 2015). These changes correlate with changes in methylation level on H3K4, H3K9, H3K36 and H3K27 and further lead to abnormal development (Fellous et al., 2015, 2019). Consequently, in *C. gigas*, histone methylation patterns are crucial for gametogenesis and early development and environmental cues further interfere with *C. gigas* developmental trajectories possibly via interaction with those methylation patterns. Furthermore, in the cephalopod *Sepia officinalis*, the progressive reorganization of chromatin leading to the formation of mature sperm relies on the mono-acetylation of H4K12 followed by a massive hyperacetylation of histone H4 (Kurtz et al., 2007) therefore suggesting a key role for histone acetylation during gametogenesis in this species.

### **2.2- Histones PTMs are involved in learning and long term memories in mollusks**

Learning and long-term memory formation requires gene expression regulation, and epigenetic chromatin remodeling mechanisms occurs to regulate such processes. Histone modifications have been shown to be key players for regulation of such processes in the two gastropods *Helix lucorum* and *Aplysia sp.*. Stage specific increase of H3 acetylation occurs in response to food aversive learning in *H. Lucorum* in a Mitogen-activated protein kinase (mitogen-activated protein kinase/extracellular signal-regulated kinase: MAPK/ERK dependent manner in the right parietal ganglion (Danilova et al., 2010; Danilova & Grinkevich,

2012). Histone methylation seems to be also involved in this food aversion memory phenotype since the level of methylation on H3K4 trimethylated and H3K9 dimethylated increases after learning (Grinkevich, 2014). In *Aplysia sp.*, the formation of new synapses relies on the activation of key genes whose expression is dependent of chromatin conformation regulated by histone acetylation (Guan et al., 2002; Hart et al., 2011). Moreover, in case of long term facilitation dysfunction, the injection of trichostatin A, an histone deacetylase inhibitor, allows the reestablishment of long term memory (Chen et al., 2014).

### **2.3- Histones PTMs are sensitive to environmental cues in mollusk species**

How the environment may affect histone PTMs is a key question when it comes to study epigenetic-based events. This question has started to be explored in mollusk species through the study of the impact of diverse infectious related stress in various species (Table 2). Changes in phosphor-acetyl histone H3 in the pedal ganglia neurons of *Pomacea canaliculata* after injection of *Escherichia coli* lipopolysaccharide has been reported (Ottaviani et al., 2013). In *B. glabrata*, hemocytes changes in histone H4 expression have been identified in response to *Echinostoma caproni* parasite infection (Bouchut et al., 2006) and genes encoding for histones H2A, H2AV, and H3.3 are up-regulated during infection by the parasite *Schistosoma mansoni* (Adema et al., 2010). Brevetoxin exposure of *C. virginica* also affects the level of serine phosphorylation of histone variant H2A.X. (Gonzalez-Romero et al., 2017). These histone marks have already been identified as a marker of DNA damage and such an increase could have been caused by the genotoxic effect of brevetoxins.

Once again, we underline the importance of studying the consequences of environmental pressure on mitochondrial physiology to investigate a potential link between the environment and histone PTMs. One of the main derived product of mitochondrial metabolism (acetyl coA)

is a major compound involved in histone acetylation (Matilainen et al., 2017; Weinhouse, 2017).

### **3- Intergenerational effects in mollusks**

Environmental factors experienced during the organism's development influence its phenotypes either transiently (e.g. behavior, physiological state) or permanently (e.g. morphology, life-history strategy). The impact of environmental conditions experienced during the development may not be limited to the individuals who experienced them, but may affect generations to follow. Although parental effects have been largely explored (e.g. (Mousseau & Fox, 1998; Crean & Bonduriansky, 2014), the accumulating evidence that past environments can influence the phenotype beyond two consecutive generations (e.g. (Plaistow et al., 2006; Remy, 2010; Shama & Wegner, 2014; Sarker & Peleg-Raibstein, 2019) has renewed the scientific attention for generational carry-over effects, especially because it may enable organisms to cope with fast-changing environment (Gienapp et al., 2008), providing a quicker response than purely genetic evolution. The terminology used to describe such generational carry-over effects is variable without a real consensus within the scientific community. Here, we distinguish the terms multi- and trans-generational effects based on

(Skinner, 2008). 'Multigenerational' indicates that the generational carry-over effects result from a direct exposure to the environmental factor – germ cells/gametes/embryos are directly exposed to the environmental factor when their parents are exposed. For example, in mammals, parental environmental experience (F0) can be transmitted to the F1 directly to the foetus *in utero* and to the F2 via a modification of F1 germ cells that are already present at the foetus stage. On the other hand, 'transgenerational' involves a germ line transmission between generations without direct exposure to the environmental factor. In mollusks, germ cells appear early in the development (Luchtel, 1972; Extavour, 2003; Fabioux, 2004; Fabioux et al., 2004).

Therefore, unless the environmental effect is applied in a narrow window right after fertilisation, it might be necessary to study at least three generations to reach the transgenerational level (i.e. F0: the exposed generation, F1: indirectly-exposed generation via F0 germ cells and F2: non exposed generation) and even through more generations for species with internal hatching (e.g. *Viviparus viviparus*). To simplify the following section, we encompass multi- and transgenerational effects under the terms ‘intergenerational effects’ and we precise when required.

Intergenerational effects are well documented in a great variety of taxa (see for example in plant: Galloway & Etterson, 2007, in Crustacean and plant: Agrawal, 1998, , in fishes: Salinas et al., 2013, in marine polychaetes: Rodriguez-Romero et al., 2015, in mice: Dias & Ressler, 2014, in humans: Veenendaal et al., 2013) and induced by various environmental factors (predation: Walsh et al., 2015, light: Galloway, 2004, temperature: Shama et al., 2014, phenology: Richter-boix et al., 2014, food availability: Schulz, 2010). However, the majority of studies is limited to two or three generations (but see: Plaistow et al., 2006; Remy, 2010; Sarker & Peleg-raibstein, 2019) offering thus a limited understanding of the persistence of such effects and their evolutionary implications. In Mollusks, we found 28 studies investigating intergenerational effects in 8 families and 14 species (cephalopods are not represented; Table 4), which is very limited and may be surprising given their key roles in ecosystem functioning and their health and economic importance. However, although some mollusk species have short generation times (months) and are easily raised in laboratory (e.g.

Planorbidae and Physidae in Gastropods), the majority of species have a long generation time (months to years) or a complex life cycle (e.g. with several pelagic larval stages), for which it is much more difficult to measure the response across multiple generations (Donelan & Trussell, 2015). These studies have been mainly conducted across two consecutive generations (i.e. effect of parental environment on offspring phenotype), except (Parker et al., 2015; Müller



et al., 2016b; Bal et al., 2017; Tariel et al., 2019) who performed a three-generation experiment. They investigated the effects of both abiotic (pCO<sub>2</sub> levels, contaminants, salinity) and biotic (virus, predation cues) environmental factors on various phenotypic traits of offspring (survival, life-history traits, growth/morphology, behavior, shell structure, metabolism, DNA methylation; Table 4). Overall, these studies demonstrated that parental environment can influence the offspring phenotype in mollusks. With so few studies, it still seems difficult to draw general conclusions about the intergenerational effects in mollusks – as already advocated by Ross et al., 2016 (but specifically on the response to changing ocean conditions). However, some points are emerging and deserve to be

highlighted:

1/ The evidence of intergenerational effects may depend on the current environmental conditions experienced by offspring. For example, (Donelan & Trussell, 2015, 2018a, b) examined the influence of parental exposure in *Nucella lapillus* to predator risk cues (crab) on the offspring phenotype (offspring size at emergence, growth, anti-predator behavior). They demonstrated that parental exposure to crab odours influences the offspring phenotype only when offspring are themselves exposed to the same cue. There is no effect of parental environment for offspring in risk-free environment. An opposite result, also illustrating the interaction between parental and offspring environment, was obtained by Luquet & Tariel (2016) in *P. acuta*. The predator-cue parental environment (crayfish) influenced the offspring phenotype (anti-predator behavior, shell thickness and shell size) only when the offspring were raised in control conditions (offspring exposed to predator cues adopted the same defensive phenotype whether or not their parents had been exposed). Zhao et al. (2017, 2018) showed that exposure of the Manila clams (*Ruditapes philippinarum*) to an acidification scenario influenced the offspring phenotype (shell growth, condition index, and metabolism) only when the offspring were themselves exposed to acidic pH. These few examples show that the phenotypic

plasticity of offspring can interact (sometimes in a reinforcing sometimes in an attenuating way) with the intergenerational effects to shape the offspring phenotype. Consequently, it is crucial to design full factorial experiments to investigate the intergenerational effects (Donelson et al., 2018). Such results are largely consistent with studies on other taxa (Salinas et al., 2013; Beaman et al., 2016).

2/ This panel of studies illustrates a large heterogeneity in experimental design, especially regarding the developmental stages at which ancestors are exposed to environmental changes and at which offspring traits are measured. A majority of studies investigated the effect of adult exposure on the postembryonic stage of offspring (Table 4). Some studies highlighted that intergenerational effects can vary according to these timing. For instance, (Kimberly & Salice, 2014) exposed F0 embryos or juveniles of *Physa pomilia* to cadmium and investigated the hatching success of F1 snails. Exposure during the embryonic development of parents decreased the hatching success of offspring while juvenile exposure did not influence it. Reategui-Zirena et al. (2017) explored the consequence of adult exposure to cadmium of *Lymnaea stagnalis* on both the embryonic (hatching success, time to hatch) and the post-embryonic (juvenile survival to cadmium) stages. Intergenerational effects were investigated without cadmium at the embryonic stage but with cadmium at the postembryonic one; however, they seem different between the developmental stages: there were no effects of parental exposure (0 ug/L to 100 ug/L of cadmium) on hatching success while parental exposure increased the cadmium tolerance (survival) of juveniles. Together, these results suggest that the presence or absence of intergenerational effects at a specific life-cycle stage of mollusks does not predict the effects at earlier or later stages and may depend on when the ancestors have been exposed to the environmental factor. Considering the different life-cycle stages is thus important to explore the intergenerational effects in mollusks.

3/ Intergenerational effects may be adaptive or not, and may or not enable organisms to cope with fast-changing environment. The intergenerational effects reported in these studies on mollusks have sometimes positive sometimes negative effects on the performance of offspring depending on the offspring environment (as mentioned above), the species and the environmental factors considered. For example, parental exposure to predation-risk increased the performances and anti-predator defences of offspring (*N. lapillus*: Donelan & Trussell, 2015, 2018a, b; *P. acuta*: Beaty et al., 2016; Luquet & Tariel, 2016; *B. glabrata*: Plautz & Guest, 2013), and similar positive effects are observed in response to other environmental factors (Acidification: Parker et al., 2012; Fitzer et al., 2014; Zhao et al., 2017, 2018; contaminant: (Plautz & Salice, 2013; Reategui-Zirena et al., 2017). In contrast, Fidder et al. (2016) and Bal et al. (2017) showed that parental exposure to fungicide and glucocorticoid influenced negatively the offspring phenotype of *L. stagnalis* and *P. acuta*, respectively (similar negative effects in Kimberly & Salice, 2014 and Griffith & Gobler, 2017). In the *L. stagnalis* study, parental exposure amplified the detrimental effect of the direct exposure of eggs on hatching success and time, likely because it modified adult energetic metabolism, and as a consequence the macronutrient content in eggs they produced changed— a form of toxicity that can affect the early development of the F1 (Fidder et al. (2016)). Similarly, the observation that during continuous exposure to a pollutant over several generations, developmental anomalies and mortality tend to be aggravated at each additional generation (as in *P. acuta* exposed to prednisolone, Bal et al. (2017)) suggests that poor parental condition is transmitted to the next generation through egg quality, resulting in cumulative toxicity over generations. Suski et al. (2012) studied the effects of parental exposure to salinity on hatching success in two gastropod species (*Helisoma trivolvis* and *P. pomila*). They found that parental salinity exposure increased the hatching success of *H. trivolvis* but decreased it for *P. pomilia*. Interestingly, Parker et al. (2017) showed that parental exposure to acidic pH induced faster growth and developmental

rates of *Saccostrea glomerata* larvae considering acidification as a sole stressor. However, considering multiple stressors (temperature, food, salinity), the parental effect decreased the survival of larvae. Consequently, intergenerational effects may sometimes reflect some long-lasting toxicity of an environment rather than the transmission of an acquired tolerance to that environment: case-wise discussion of both possibilities is required before drawing any significant conclusions.

4/ Intergenerational effects may involve a range of genetic and non-genetic processes which are barely addressed in studies reported in mollusk species. Among the non-genetic components, we consider processes such as parental effects, e.g. transmission of nutrients, hormones, proteins (Mousseau & Fox, 1998; Crean & Bonduriansky, 2014), and epigenetic effects (DNA methylation marks, histone protein modifications, non-coding small RNAs (Holeski et al., 2012; Schlichting & Wund, 2014)). These non-genetic components are arousing a high interest in evolutionary ecology. The implication of the epigenetic bearers of information at the origin of intergenerational effects has been established in several model species (Heard & Martienssen, 2014) but very few studies have investigated the mechanisms of intergenerational effects in mollusks. Lafont et al. (2019) demonstrated that *Crassostrea gasar* offspring survived better in the presence of a virus when the mothers had been exposed to the same virus, but they did not find evidence of transcriptomic modification in the offspring, suggesting maternal provisioning of antiviral compounds in the eggs. In *P. acuta*, parental exposure to glucocorticoid pollution did not affect the DNA methylation in the parental snails but induced a significant decrease in global 5mC in the next generation, which may be related to the negative intergenerational effect of toxicant on offspring traits (Bal et al., 2017). In *C. gigas*, Rondon et al. (2017) showed that the parental herbicide exposure had an impact on the DNA methylation pattern of its progeny. However, this change in methylation level correlated with RNA level only in a very small group of genes, suggesting the absence of obvious impact

on molecular phenotype (gene expression and splice variant generation). This absence of obvious link between DNA methylation and gene expression again raises the question concerning the role of intragenic DNA methylation in invertebrate species. DNA methylation patterns may be affected by the conditions to which current or past generations were exposed, and be a manifestation of intergenerational responses just like any other phenotype, without being the vehicle that allows information to cross generations. Consequently, both intergenerational effects and DNA methylation patterns can be induced by environmental conditions in mollusks but the relationship between the two still has to be demonstrated. Moreover, we did not find any study investigating the relationship between histone modifications and intergenerational effects on mollusks.

## **Discussion/Conclusion**

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One of the major issues in modern biology is the role of non-genetic phenotypic variations in organism's adaptation. In particular, epigenetic variation and its capacity to be transmitted to offspring is expected to affect the evolutionary process (Jablonka & Lamb, 1998). In this article, we have narrowed the term epigenetic to chromatin states, supported by bearers of epigenetic information whose states are reversible and inherited through mitosis and possibly meiosis. While research about epigenetics based events and intergenerational effects is booming in model organisms, we have reviewed the current knowledge of both mechanisms in mollusk species. We have clearly highlighted that the intertwined relationships among molecular aspects of epigenetic information, phenotypic variation and inheritance patterns are barely addressed in mollusks and to some extent, in non-model organisms in general. To obtain a better understanding of the evolutionary role of epigenetic processes in the light of intergenerational phenotypic response, three main key research areas are emerging: (i) to investigate how and to what extent the chromatin states are related to the phenotypic variation over generations, (ii) to

estimate the adaptive nature of intergenerational effects and/or chromatin states, and (iii) to track down the chromatin states from somatic to germinal lines over generations in order to determine whether they are the vehicle bearing the heritable information or phenotypic by-products.

- (i) How and to what extent are the chromatin states related to the phenotypic variation over generations

To relate the chromatin states as causal factors of induced phenotypic responses over generations are a challenging task. A fundamental property of chromatin states is not to be permanent, possibly accompanying a progressive reversibility of phenotypic responses over generations (Day & Bonduriansky, 2011). A first step towards understanding how this takes place is a correlative approach. This consists in testing (i) whether an environmental factor induces both specific chromatin states and phenotypes, (ii) whether the reversibility of chromatin states and phenotypes over generations follow the same dynamics (cf. Fig. 1, experimental design 1) and (iii) whether both phenotypes and chromatin states have a similar asymmetry in transmission patterns between the male vs female sex. These objectives require to define the developmental window during which the organism is exposed (early life stages are the most sensitive to environmental variation; (Faulk & Dolinoy, 2011)) (cf. Fig. 1, experimental design 1) and to localize accurately the chromatin states in organs or tissues which are the most suitable to the underlying phenotype. Considering the high level of interplay which might occur between all the bearers of epigenetic information (Eirin-Lopez & Putnam, 2018), the most exhaustive plan is to consider all of them. We focussed our review on DNA methylation and histone modifications but other bearers might be followed. Small RNAs and locus topography in the nucleus are other bearers of epigenetic information which have been shown to be sensitive to environmental stress in mollusks (Biggar et al., 2012, Knight et al., 2010). A whole genome study is the most appropriate to accurately localize the chromatin state.

Such approach could lead to the identification of epimutations which could be considered as a valuable epimarkers induced by the environment and linked to the observed phenotype. The use of epimarker in mollusk has been suggested recently as a mean to set environmental bioindicators (Suarez-Ulloa et al., 2015) and their potential application as a reliable early environmental marker of pollution has been investigated in *C. gigas* (Rondon et al., 2017). Interestingly, tools to address the functional impact of an epimutation on a given phenotype recently became available when considering DNA methylation as the epigenetic bearer of information. The crispr-dcas9 system which contains an inactivated cas9 protein but a fusion with the DNMT3 enzyme (McDonald et al., 2016) allows to methylate a target locus and therefore to induce targeted epimutations whose functional impact may be studied. This tool is promising to investigate the cause / effect relationship of an epimutation (here only considering DNA methylation) for a given phenotype. For now, such an approach is restricted to model organisms (see for example: Liu et al., 2016). Non-model organisms rather use drugs targeting chromatin modifying enzymes which disorganize the chromatin states globally but not specifically (Geyer et al., 2017). Such non-specific methods may have pleiotropic effects which can mask the relationship among environments, chromatin states and traits. Other molecular analysis such as transcriptomics, proteomics might also be of great interest to analyze the molecular phenotypic response. Transposon mobilization, copy number variation and SNPs analysis can complete the overall scheme and extend the study to a level which will allow us analyze the relative contribution of genetic and epigenetic for the observed multigenerational plasticity.

## **ii/ Adaptive nature of intergenerational effects and chromatin states**

To evaluate the evolutionary importance of chromatin states and intergenerational effects in response to environment, it is crucial to determine their effects on the organism's

fitness. The intergenerational nature of the effect does not really matter here; the methods are the same as for classical studies of plasticity. Experiments should evaluate fitness traits of epigenetically induced offspring fitness (e.g. survival, fecundity) and assess them in both types of environments (control and induced) using some form of factorial design. They should also investigate the reliability of the cue perceived by one generation (usually, parents) as a predictor of the conditions of selection in the generation where the response is expressed (usually, offspring) in natural environments. Ideally, the induced phenotypic and chromatin states of the offspring should be measured in parallel (cf. (i)). The timing issue is important here: in order to show that some phenotypic or chromatin-state modification of offspring (in response to, say, parental exposure to a pollutant or virus) are related to fitness effects, these modifications and the associated fitness effects must be present before the offspring themselves are exposed to the pollutant or virus. Irrespective of the new changes that this exposure may induce, if epigenetically modified offspring survive better to it, the modification did prepare them to pollutants or virus, and can be said to be adaptive if in nature, the half-life of pollutant or virus outbreaks makes it likely that offspring environment resembles their parents. The adaptive nature of intergenerational effects and chromatin states is worth to investigate since environmental manipulation could be performed with, the intention to induce an 'epigenetic memory' to produce a desired phenotype. Training the innate immunity has been suggested on fish and could be used in oyster aquaculture since transgenerational immunity and long lasting antiviral innate immune priming has been reported in *C. gigas* (Lafont et al., 2017, 2019).

### **iii/ Chromatin state inheritance across generations**

Globally, there are few studies which indicate that chromatin states can be considered as a full-fledged way of non-genetic heredity (Greer et al., 2015). These studies are strictly limited to model organisms and nothing has been investigated in mollusks to our knowledge. That



chromatin states are the vehicle enabling information to cross generations is yet a fundamental condition to conclude about their evolutionary role. In particular, in order for chromatin states to encode transgenerational response to environment, two major issues must be solved: one is the tissue-specificity of chromatin states, the other is chromatin reprogramming. Let us take the hypothetical example of a pollutant that is detoxicated in the gills and has a transgenerational effect whereby offspring of exposed parents have higher detoxification efficiency. The chromatin state in the gills of the exposed parents may be modified by the pollutant, but this modification may be gill-specific; if the offspring show the same gill-specific modification, the information must have been in some way transmitted to germ cells (and ultimately sperm or eggs) and re-expressed during offspring development but only in gills. It is not likely that the modifications are present already in germ cells and then passed on to gills specifically. In mammals for example, DNA methylation marks and histones modifications undergo two phases of reprogramming (one during germ cells fabrication, the other one after fertilisation) during which these marks are erased and then made anew (Morgan et al., 2005). The occurrence of such a phenomenon in mollusks has been poorly investigated yet, but Li et al., 2018 have shown that DNA methylation level is changing during gametogenesis and embryo development of scallop *Patinopecten yessoensis*. Other works performed on oysters have shown that methylation pattern in *P. fucata* is mainly influenced by oocytes (Li et al., 2015) while paternal inheritance of DNA methylation patterns has been suggested in *C. gigas* (Olson & Roberts, 2014a). The transmission of this information over generations would thus require a transient support saving it and enabling rewriting it later in the development, in a tissue-specific way. An interesting feature of oyster chromatin proteins in germinal cells is the fact that the majority of histones are replaced by different types of SNBPs or protamines-like proteins but some histones persist and could therefore play a role in epigenetic information inheritance (Galindo et al., 1992; Eirín-López & Ausio, 2009). Furthermore, in the context of tracking the epigenetic

effects of environments, it seems crucial to follow the information beyond the observation of epigenetic marks (chromatin states) in one tissue at one stage. In our hypothetical pollutant example, it would be useful to track them both in parental gill, gonad (germ-cell), offspring whole-body larva and offspring adult gill tissue to understand where and when the epigenetic signal is present versus absent versus stored in another form than chromatin states. In some cases, it seems that the erasing is not complete and that some marks escaping from complete reprogramming are transmitted to the next generation (Migicovsky & Kovalchuk, 2011). To understand if these “resistant” bearers have been environmentally-induced and why/how they do not undergo this erasure is a crucial issue.

### **The benefit of knowing more about epigenetics in molluscs**

The questions posed above are quite general and could apply to many under-studied groups, but what benefits can be drawn from more knowledge on epigenetic processes in molluscs specifically? One of the areas where epigenetics may find direct applications is ecotoxicology. A few mollusc species such as *Lymnaea stagnalis* or *Physa acuta* have recently become widely used as sentinels of freshwater pollution. The responses of molecular bearers of epigenetic information to environmental pollutants are rapid, quite easy to assess from wild-caught individuals and, if sufficiently studied and repeatable, may be an interesting alternative to other methods to detect pollution impacts, such as measuring life-history traits or directly dosing pollutants in the animals. This use of epigenetic marks does not require the knowledge of their physiological role, inheritance patterns or adaptive nature. More information on these issues will however be crucial for exploited species such as oysters. Indeed, adaptive epigenetic responses, especially if lasting more than one generation, may help these populations to overcome stresses due to the arrival of new pathogens, or the acidification of seawater – and increase the probability that they have time to adapt genetically. Similarly, more knowledge on epigenetic responses involved in resistance or tolerance to parasites in snail vectors of human

parasites such of *Biomphalaria glabrata* (vector of bilharziasis) and Lymnaeids (vectors of fasciolosis) may be invaluable to improve human health in the forthcoming decades.

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## Figures and tables:

**Figure 1:** Experimental design for gold standard studies on intergenerational effects

Design 1 aims at deciphering the appropriate developmental window which will better integrate the environmental information and to study the persistence (or reversibility) of phenotypic

response and chromatin states. Sampling across generation on different tissues and/or stages from individuals has to be performed. Tissue and stage have to be appropriate and relevant to the environmental effect. Phenotypic traits on individuals (1, 2), chromatin states (3) and genetic based events (4) have to be followed across generation to investigate **how and to what extent are the chromatin states related to the phenotypic variation over generations**. Deciphering the chromatin states through multi-omic approaches may lead to epimarker design which are potential candidates for targeted epimutations (5). This targeted epimutation could allow to **investigate the adaptive nature of intergenerational effect and chromatin states**. Once the appropriate window is determined (Design 1), full factorial experiments where phenotypes are investigated in both control and stress environment across generation have to be designed (Design 2). Studies on a range of phenotypic traits on individuals (1) should allow **investigating the adaptive nature of the observed intergenerational effect and chromatin states**. The epimarkers have to be followed through the chain of transmission from soma to germ cells, from gamete to soma (6) to investigate the **chromatin state across generations**. Continuous arrow indicates sexual reproduction event and meiosis heritability. Dashed arrows indicate development and mitosis heritability.

\*(1) Whether, the observed effect is a multi or a transgenerational effect will rely on the developmental window during which the individuals have been exposed. In the case of our proposed experimental design, we have considered that germ cells are present during exposure and therefore are indirectly exposed to the environment. Consequently, the F1 generation studies are restricted to multigenerational plasticity.

Table 1: Methods to study DNA methylation

Table 2: References about DNA methylation events in mollusks species

Table 3: Methods to study Histones

Table 4: References about intergenerational events in mollusks species

Figure 1: Experimental design for gold standard studies on intergenerational effects

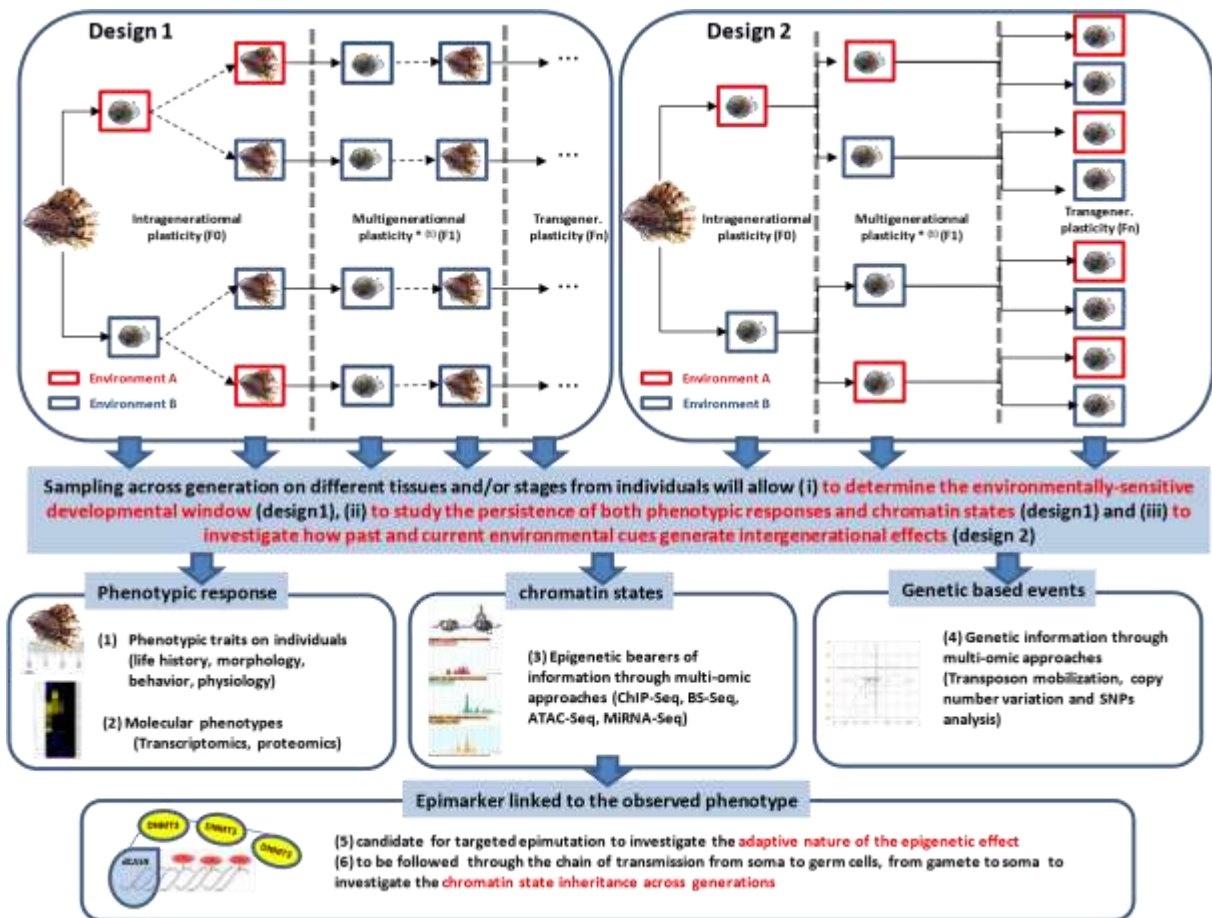




Table 1:

<b>LC-MS/MS</b>	
Mononucleotides generated after DNA digestion with DNA degradase are separated by liquid chromatography and analyzed by mass spectrometry. Allows for determination of the overall cytosine methylation level in a DNA sample. (Fneich et al. 2013)	Low resolution, Sensitive, Quantitative, Medium cost
<b>CpG observed/expected (CpG o/e) ratios</b>	
Higher mutability of methylated CpG introduces a bias in the observed CpG content. Calculating the ratio CpG o/e can infer the 5mC type. On line tools are available to calculate the ratio starting from input fasta files. Allows for prediction of DNA methylation type (Bulla et al. 2018)	Low resolution, fast, low cost, requires cDNA or gDNA sequences
<b>Anti-5mC antibodies techniques</b>	
<i>Anti-5mC antibodies are commercially available and can specifically recognize 5mC in DNA extracted samples</i>	
<b>ELISA</b> based kits are commercially available and allows the detection of 5mC overall variation in DNA samples (Geyer et al. 2017)	Low resolution, not sensitive, quantitative, fast, medium cost
Anti-5mC antibodies coupled with secondary antibody can detect 5mC overall variation in DNA samples spotted on nitrocellulose membrane using a simple <b>Dot Blot assay</b> (Luviano et al. 2018)	Low resolution, not sensitive, quantitative, fast, low cost
Anti-5mC antibodies can be used to immunoprecipitate DNA ( <b>MeDIP</b> ) which can subsequently be analyzed by highthrough put sequencing technology (or microarray). Method to determine the average level of cytosine methylation in a region, at the whole genome scale (Rivière et al. 2017)	High resolution, sensitive, quantitative, high cost
<b>Methyl sensitive restriction assay</b>	
<i>DNA samples are digested with methyl sensitive restriction enzymes</i>	
The methyl sensitive restriction assay can be analyzed by <b>gel agarose separation</b> . This allows to visualize a band shift in the sample digested with the non-sensitive enzyme compared to the sensitive one for which digestion event is more rare and size fragment are bigger (Fneich et al. 2013)	Low resolution, not sensitive, not quantitative, fast, low cost
The methyl sensitive restriction assay can be analyzed by <b>AFLP</b> . The restriction assay is followed by a ligation of adaptors to restriction fragments which can be PCR amplified with primers complementary to adaptor sequence. The amplified fragment can be visualized on gel electrophoresis or via capillary sequencing instrument. Allows for identification of genomic region which display differences of methylation between samples (Jiang et al, 2013)	Medium resolution ,Medium cost, Sensitive, Possible confounding effect with genetic diversity

The methyl sensitive restriction assay can be analyzed by <b>qPCR</b> using primers designed in CpG rich regions. Allows for identification of locus which display differences of methylation between samples (Ittiprasert et al. 2015)	Medium resolution, medium cost, fast, sensitive
<b>Bisulfite treatment of gDNA</b>	
<i>Treatment of DNA with sodium bisulfite converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected</i>	
In the Combined Bisulfite Restriction Analysis ( <b>COBRA</b> ), bisulfite treated DNA is PCR amplified, resulting in cytosine residues at initially methylated positions, and thymine residues at initially unmethylated position. Digestion with appropriate enzymes will allow for detection of methylation dependent retention or loss of CpG-containing restriction enzyme sites. Allows for detection of DNA methylation presence at a specific genomic locus	Medium resolution, sensitive, low cost, fast
PCR amplified fragments from bisulfite converted DNA are PCR amplified and analyzed by sanger sequencing ( <b>BS-PCR-Seq</b> ). Allows for identification of accurate 5mC position within a locus (Fneich et al. 2013, Geyer et al. 2017, Rondon et al. 2017)	Medium resolution, sensitive, quantitative, low cost, fast
Bisulfite converted DNA is analyzed by high throughput sequencing ( <b>BS-Seq</b> ). Allows for identification of accurate 5mC position at the whole genome scale (Rondon et al. 2017)	Very high resolution, sensitive, quantitative, very high cost <sup>*(1)</sup>

<sup>\*(1)</sup> BS-Seq analysis can be combined with other techniques which allow to decrease the sequencing effort and therefore the cost of the technique. Before bisulfite conversion, genomic regions containing 5mC or genomic region of interest can be enriched and therefore less sequencing effort is necessary to reach a good coverage of these regions of interest.

- The immunoprecipitation with antibodies which target Methyl Binding Domain proteins (MBD2/3 in mollusks) or antibodies which target 5mC (MB-Seq = combination of MeDIP and BS-Seq, (Jia et al., 2018) and ensure an enrichment of methylated DNA. This is worth in mollusk species since DNA methylation is organized in a mosaic pattern and long genomic regions are totally devoid of methylated DNA (Gavery & Roberts, 2013; Moroz & Kohn, 2013).

-Using exon capture probe to specifically target intragenic methylation is worth in mollusk species since DNA methylation is essentially intragenic. This can be applied in *C. gigas* in large range of samples (J. Vidal-Dupiol, personal communication).

- Reduced representation bisulfite sequencing (RRBS) uses a restriction enzyme which specifically cuts in CGs and therefore ensure enrichment for CpG regions of the genome. This technique has not been applied in mollusks yet and is not suitable for mosaic type DNA methylation

Table 2: References about DNA methylation and histone modifications events in mollusks species:

<b>DNA methylation</b>				
<i>References about DNA methylation events in mollusks species</i>				
<b>Specie</b>	<b>Taxonomy</b>	<b>Reference</b>	<b>Function</b>	<b>Method</b>
<i>Aplysia</i>	<u><i>Aplysiidae</i></u>	Rajasethupathy et al. 2012	Memory formation	Bisulfite conversion
<i>Aplysia</i>	<u><i>Aplysiidae</i></u>	Moroz and Kohn, 2013	Senescence	DNA methylation assays
<i>Biomphalaria glabrata</i>	<u><i>Planorbidae</i></u>	Fneich et al, 2013	Description	Bisulfite conversion
<i>Biomphalaria glabrata</i>	<u><i>Planorbidae</i></u>	Geyer et al, 2017	Biotic stress	Bisulfite conversion
<i>Biomphalaria glabrata</i>	<u><i>Planorbidae</i></u>	Ittiprasert et al, 2015	Biotic stress	Methylation-sensitive qPCR
<i>Cantareus aspersus</i>	<u><i>Helicidae</i></u>	Nica et al, 2017 (a)	Abiotic stress	ELISA
<i>Cantareus aspersus</i>	<u><i>Helicidae</i></u>	Nica et al, 2017 (b)	Abiotic stress	ELISA
<i>Chlamys farreri</i>	<u><i>Pectinidae</i></u>	Sun et al, 2014	Description	MSAP
<i>Chlamys farreri</i>	<u><i>Pectinidae</i></u>	Lian et al, 2015	Aging	ELISA
<i>Crassostrea gasar</i>	<u><i>Ostreidae</i></u>	Farias et al, 2017	Biotic stress	ELISA
<i>Crassostrea gigas</i>	<u><i>Ostreidae</i></u>	Wei et al, 2018	Description	Pyrosequencing/Sodium bisulfite
<i>Crassostrea gigas</i>	<u><i>Ostreidae</i></u>	Sussarellu et al, 2018	Abiotic stress/Development	UV-HPLC/MEDIP
<i>Crassostrea gigas</i>	<u><i>Ostreidae</i></u>	Rondon et al, 2017	Abiotic stress/ Transgenerational	BS-Seq
<i>Crassostrea gigas</i>	<u><i>Ostreidae</i></u>	Gavery and Roberts, 2010	Description	MS-PCR/BS-PCR
<i>Crassostrea gigas</i>	<u><i>Ostreidae</i></u>	Olson and Roberts, 2014a	Transgenerational	BS-Seq
<i>Crassostrea gigas</i>	<u><i>Ostreidae</i></u>	Olson and Roberts, 2014b	Description	BS-Seq
<i>Crassostrea gigas</i>	<u><i>Ostreidae</i></u>	Jiang et al, 2016	Transgenerational	F-MSAP
<i>Crassostrea gigas</i>	<u><i>Ostreidae</i></u>	Wang et al, 2014	Description	BS-Seq

<i>Crassostrea gigas</i>	<u>Ostreidae</u>	Gavery and Roberts, 2013	Gene expression	BS-Seq
<i>Crassostrea gigas</i>	<u>Ostreidae</u>	Jiang et al, 2013	Description	AFLP/MSAP
<i>Crassostrea gigas</i>	<u>Ostreidae</u>	Saint-Carlier and Riviere, 2015	Gene regulation	BS-Seq
<i>Crassostrea gigas</i>	<u>Ostreidae</u>	Song et al, 2017	Gene expression	BS-Seq
<i>Crassostrea gigas</i>	<u>Ostreidae</u>	Riviere et al, 2017	Development	MeDIP
<i>Crassostrea gigas</i>	<u>Ostreidae</u>	Riviere et al, 2013	Development	MeDIP

<i>Crassostrea gigas</i>	<u>Ostreidae</u>	Gonzalez-Romero et al, 2017	Biotic stress	MSAP
<i>Haliotis discus hannai</i>	<u>Haliotididae</u>	Kong et al, 2017	Abiotic stress	MSAP
<i>Lymnae</i>	<u>Lymnaeidae</u>	Forest et al, 2016	Long term memory, Biotic stress	DNA methylation inhibitor
<i>Octopus vulgaris</i>	<u>Octopodidae</u>	Díaz-Freije et al, 2014	Development	MSAP
<i>Octopus vulgaris</i>	<u>Octopodidae</u>	García-Fernández et al, 2017	Development/Abiotic stress	MSAP/ELISA
<i>Physa acuta</i>	<u>Physidae</u>	Bal et al, 2017	Abiotic stress	Global DNA methylation assay
<i>Physa acuta</i>	<u>Physidae</u>	Muller et al. 2016	Abiotic stress and development	DNA methylation assay
<i>Physa acuta</i>	<u>Physidae</u>	Sánchez-Argüello et al, 2016	Abiotic stress and development	MSAP
<i>Pinctada fucata</i>	<u>Pteriidae</u>	Li et al, 2015	blesure biotic or abiotic?	MSAP
<i>Pinctada fucata</i>	<u>Pteriidae</u>	Li et al, 2014	Development	MSAP
<i>Saccostrea glomerata</i>	<u>Ostreidae</u>	Tran et al, 2016	Oestrogen/Metabolism?	Bisulfite conversion
<i>Xenostrobus securis</i>	<u>Mytilidae</u>	Ardura et al, 2017	Adaptation	MSAP
<i>Zeacumantus subcarinatus</i>	<u>Batillariidae</u>	Joe, 2013	Biotic stress	ELISA
<i>Potamopyrgus antipodarum</i>	<u>Tateidae</u>	Thorson et al, 2017	Adptation to habitat	MeDIP

### Histones modification

#### *References about histones modification events in mollusks species*

<i>Aplysia</i>	<u><i>Aplysiidae</i></u>	Guan et al, 2002	Memory formation	ChIP
<i>Aplysia</i>	<u><i>Aplysiidae</i></u>	Chen et al, 2014	Memory formation	Fluorescence imaging
<i>Aplysia</i>	<u><i>Aplysiidae</i></u>	Hart et al, 2011	Memory formation	ChIP
<i>Biomphalaria glabrata</i>	<u><i>Planorbidae</i></u>	Bouchut et al, 2006	Biotic stress	SDS-Page
<i>Biomphalaria glabrata</i>	<u><i>Planorbidae</i></u>	Adema et al, 2010	Biotic stress	Microarray scanning/qPCR
<i>Chlamys farreri</i>	<u><i>Pectinidae</i></u>	Li et al, 2006	Description/Evolution	Homology cloning
<i>Crassostrea gigas</i>	<u><i>Ostreidae</i></u>	Fellous et al, 2015	Abiotic stress	ELISA
<i>Crassostrea gigas</i>	<u><i>Ostreidae</i></u>	Fellous et al, 2014	Reproduction/Development	RT-qPCR
<i>Crassostrea gigas</i>	<u><i>Ostreidae</i></u>	Bouilly et al, 2010	Description	PCR/FISH
<i>Crassostrea virginica</i>	<u><i>Ostreidae</i></u>	Gonzalez-Romero et al, 2017	Biotic stress	Western blot
<i>Helix lucorum</i>	<u><i>Helicidae</i></u>	Danilova et al, 2010	Memory formation	Western blot
<i>Helix lucorum</i>	<u><i>Helicidae</i></u>	Danilova and Grinkevich, 2012	Memory formation	Western blot
<i>Helix lucorum</i>	<u><i>Helicidae</i></u>	Grinkevich, 2014	Memory formation	Western blot
<i>Lucina pectinata</i>	<u><i>Lucinidae</i></u>	Montes-Rodriguez et al, 2018	Description	DNA-RNA sequencing/BLAST
<i>Several mollusks species</i>		Ausio, 1992	Description	Electrophoresis
<i>Mytilus edulis</i>	<u><i>Mytilidae</i></u>	Albig et al, 2003	Description	Cloning/BLAST
<i>Mytilus edulis</i>	<u><i>Mytilidae</i></u>	Drabent et al, 1999	Description	Cloning/Rotating Field Electrophoresis
<i>Mytilus galloprovincialis</i>	<u><i>Mytilidae</i></u>	Gonzalez-Romero et al, 2012	Description	PAGE

<i>Mytilus spp</i>	<i>Mytilidae</i>	Eirin-Lopez et al, 2002	Description/Evolution	FISH
<i>Mytilus spp</i>	<i>Mytilidae</i>	Eirin-Lopez et al, 2004	Description/Evolution	PCR amplification/ FISH
<i>Mytilus</i>	<i>Mytilidae</i>	Rivera-Casas et al, 2016	Description	BLAST/PCR/qPCR
<i>Octopus vulgaris</i>	<i>Octopodidae</i>	Mennella et al, 2002	Reproduction/Description	RP-HPLC
<i>Pomacea canaliculata</i>	<i>Ampullariidae</i>	Ottaviani et al, 2013	Biotic stress	Western blot
<i>Sepia officinalis</i>	<i>Sepiidae</i>	Kurt et al, 2007	Spermatogenesis	Western blot
<i>Zeacumantus subcarinatus</i>	<i>Batillariidae</i>	Joe, 2013	Biotic stress	SDS-Page

Table 3:

<b>Anti-modified histones antibodies techniques applied on protein extracts</b>	
<i>Anti-histones marks antibodies are commercially available and can specifically recognize histones marks in proteins extracted samples</i>	
<b>SDS-PAGE / Western-Blot</b> : protein extracts are separated by SDS PAGE, the gel is transferred on a membrane and revelation is performed using an antibody which targets a specific histone mark. It allows the detection of modified histone overall variation between samples (Gonzalez-Romero et al., 2017).	Low resolution, not sensitive, quantitative, fast, medium cost
<b>ELISA</b> based kits are commercially available and allows for the detection of modified histones using antibodies targeting specific histone marks in proteins samples extracted from tissues, it allows the detection of modified histone overall variation between samples (Fellous et al., 2015)	Low resolution, not sensitive, quantitative, fast, medium cost, more expensive than Western-Blot.
<b>Anti-modified histones antibodies techniques applied on chromatin extracts – Chromatin Immunoprecipitation assay (ChIP)</b>	
<i>Anti-histones marks antibodies are used to immunoprecipitate chromatin extracted from tissue samples</i>	
Immunoprecipitated DNA is analysed by qPCR( <b>ChIP-qPCR</b> ) (Guan et al., 2002) and allows for comparison of histone enrichment profile at a specific locus between several samples.	Medium resolution, quantitative, medium cost
Immunoprecipitated DNA is analysed by high throughput sequencing techniques (ChIP-Seq) or by microarray (ChIP on chip). Allows for accurate localization of modified histone peaks at the whole genome scale (Never applied in mollusk species), possible quantitative comparison of histone enrichment profile between two samples	High resolution, quantitative, very high cost
Immunoprecipitated DNA is bisulfite treat and analysed by high throughput sequencing techniques ( <b>ChIP-BS-Seq</b> ). Allows to investigate the cross talk between DNA methylation and chromatin features	High resolution, quantitative, very high cost

(Brinkman et al., 2012).	
<b>Studies on histones modifying enzymes</b> <i>Histone modifying enzymes are highly conserved and can be targeted in a wide range of species</i>	
<b>Commercially available</b> inhibitors of histones modifying enzymes can be used and subsequent phenotype can be registered following treatment. (Chen et al., 2014, Geyer et al. 2017)	Low resolution, low cost, fast, correlative analysis
Analysis of expression of histone modifying enzyme by qPCR analysis or commercially available ELISA assay may indicate variation in modified histones between samples (Fellous et al 2015)	Low resolution, medium cost, fast, correlative analysis
<b>Imaging techniques</b> <i>Identification and visualization of histones coding genes and modified histone proteins</i>	
Interphase chromosome can be hybridised with fluorescent DNA probed targeting histones loci ( <b>Fluorescence <i>in situ</i> hybridisation (FISH)</b> ) (Bouilly et al., 2010)	Qualitative, medium cost, time consuming
Nuclei from tissue sections can be targeted by antibodies targeting modified histones and revealed by immunofluorescence ( <b>Immunolocalisation</b> ). Allows for detection of particular histone marks in samples (Odoelman et al. 2009)	Qualitative, medium cost, time consuming
<b>Gene and protein analysis techniques</b> <i>Identification and molecular description of histone</i>	
Proteins like histones can be purified with <b>high-pressure liquid chromatography (HPLC)</b> , this technique coupled with <b>mass spectrometry</b> allows to detect and characterized chemical structure of proteins (Mennella et al., 2002)	Qualitative, medium cost, low resolution
Identification of core histones genes and histones variants are possible via <b>PCR, homology search</b> and <b>sequencing</b> (Li et al., 2006)	Qualitative, low cost, medium resolution

Table 4:

<b>Intergenerational effects</b> <i>References about intergenerational events in mollusks species</i>
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<b>Specie</b>	<b>Taxonomy</b>	<b>Reference</b>	<b>Environmental factor</b>	<b>Timing of exposure</b>	<b>Offspring trait</b>	<b>Timing of phenotypic measurements</b>	<b>Nb of generations</b>	<b>Type of effect</b>
<i>Biomphalaria glabrata</i>	<i>Planorbidae</i>	<i>Plautz et al. 2013</i>	<i>Predation</i>	<i>Post-embryonic to adult</i>	<i>survival challenges to contaminants (cadmium, malathion)</i>	embryonic to postembryonic	2	multi
<i>Chlamys farreri</i>	<i>Pectinidae</i>	<i>Yue et al, 2013</i>	<i>bacterial challenge</i>	<i>Adult</i>	<i>bactericidal effet, increase expression of mRNA, survival</i>	eggs, embryonic and postembryonic	2	multi
<i>Crassostrea gasar</i>	<i>Ostreidae</i>	<i>Green et al. 2016</i>	<i>Ostreid herpesvirus 1</i>	<i>Adult</i>	<i>survival to exposure</i>	post-embryonic	2	multi
<i>Crassostrea gasar</i>	<i>Ostreidae</i>	<i>Lafont et al 2019</i>	<i>Ostreid herpesvirus 1</i>	<i>Adult</i>	<i>survival to exposure</i>	post-embryonic	2	multi
<i>Crassostrea gigas</i>	<i>Ostreidae</i>	<i>Rondon et al, 2017</i>	<i>Herbicide (diuron)</i>	<i>Adult</i>	<i>DNA methylation</i>	embryonic	2	multi
<i>Crassostrea gigas</i>	<i>Ostreidae</i>	<i>Jiang et al, 2016</i>	<i>None</i>	<i>Adult</i>	<i>Transmission of methylated loci</i>	None	2	multi
<i>Helisoma trivolvis</i> <i>Physa pomillia</i>	<i>Planorbidae</i> <i>Physidae</i>	<i>Suski et al. 2012</i>	<i>Salinity</i>	<i>post-embryonic to adult</i>	<i>hatching success, time-tohatch)</i>	embryonic	2	multi
<i>Lymnaea stagnalis</i>	<i>Lymnaeidae</i>	<i>Reategui-Zirena et al 2017</i>	<i>Contaminant (cadmium)</i>	<i>Adult</i>	<i>hatching success, time to hatch, juvenile survival</i>	embryonic to postembryonic	2	multi
<i>Lymnaea stagnalis</i>	<i>Lymnaeidae</i>	<i>Fidder et al. 2016</i>	<i>Fungicide (pyraclostrobin)</i>	<i>Adult</i>	<i>hatching success, time to hatch</i>	embryonic	2	multi
<i>Mercenaria mercenaria</i> <i>Argopecten irradians</i>	<i>Veneridae</i> <i>Pectinidae</i>	<i>Griffith &amp; Gobler 2017</i>	<i>pH/pCO2/Acidification</i>	<i>Adult</i>	<i>survival, development, size</i>	post-embryonic	2	multi



<i>Mytilus edulis</i>	<i>Mytilidae</i>	<i>Fitzer et al. 2014</i>	<i>pH/pCO2/Acidification</i>	<i>Adult</i>	<i>shell composition</i>	post-embryonic	2	multi
<i>Mytilus edulis</i>	<i>Mytilidae</i>	<i>Kjong et al, 2019</i>	<i>Acidification, hypoxia</i>	<i>Adult and embryos</i>	<i>fertilization, deformation, shell length</i>	embryonic to postembryonic	2	multi
<i>Mytilus chilensis</i>	<i>Mytilidae</i>	<i>Diaz et al. 2018</i>	<i>pH/pCO2/Acidification</i>	<i>Adult</i>	<i>shell length, ingestion rate</i>	post-embryonic	2	multi
<i>Nucella lapillus</i>	<i>Muricidae</i>	<i>Donelan &amp; Trussel 2018</i>	<i>Predation</i>	<i>Adult</i>	<i>behaviour, tissue growth, growth efficiency</i>	post-embryonic	2	multi

<i>Nucella lapillus</i>	<i>Muricidae</i>	<i>Donelan &amp; Trussel 2018</i>	<i>Predation</i>	<i>Adult</i>	<i>offspring size at emergence</i>	embryonic	2	multi
<i>Nucella lapillus</i>	<i>Muricidae</i>	<i>Donelan &amp; Trussel 2015</i>	<i>Predation</i>	<i>Adult</i>	<i>behaviour, foraging, mass</i>	post-embryonic	2	multi
<i>Physa acuta</i>	<i>Physidae</i>	<i>Beaty et al 2016</i>	<i>Predation</i>	<i>Post-embryoniand adult</i>	<i>behaviour, shell size, shape, crush-resistance</i>	post-embryonic/adult	2	multi
<i>Physa acuta</i>	<i>Physidae</i>	<i>Luquet &amp; Tariel 2016</i>	<i>Predation</i>	<i>post-embryonic to and adult</i>	<i>behaviour, weight, shell thickness, shell size, shape</i>	Adult	2	multi
<i>Physa acuta</i>	<i>Physidae</i>	<i>Tariel et al. 2019</i>	<i>Predation</i>	<i>post-embryonic to adult</i>	<i>behaviour, weight, shell thickness, shell size, shape</i>	Adult	3	trans
<i>Physa acuta</i>	<i>Physidae</i>	<i>Bal et al, 2017</i>	<i>Synthetic molecule (glucocorticoid)</i>	<i>Adult</i>	<i>survival, hatching success shell length, shell thickness shell structure, shell calcification</i>	embryonic to adult	3	multi
<i>Physa acuta</i>	<i>Physidae</i>	<i>Muller et al, 2016</i>	<i>Endocrine-disruptor (Vinclozolin)</i>	<i>Adult</i>	<i>Size, mortality, deformation</i>		3	multi

<i>Physa pomilia</i>	<i>Physidae</i>	<i>Plautz &amp; Salice 2013</i>	<i>Contaminant (cadmium)</i>	<i>Adult</i>	<i>juvenile survival challenges to contaminants (cadmium)</i>	post-embryonic	2	multi
<i>Physa pomilia</i>	<i>Physidae</i>	<i>Kimberly &amp; Salice 2014</i>	<i>Contaminant (cadmium)</i>	<i>Embryonic and postembryonic</i>	<i>hatching success</i>	embryonic	2	multi
<i>Ruditapes philippinarum</i>	<i>Veneridae</i>	<i>Zhao et al. 2017 Zhao et al. 2018</i>	<i>pH/pCO2/Acidification</i>	<i>Adult</i>	<i>shell growth, Na/Ca ratio shell growth, condition index, standard metabolic rate, delta C13</i>	post-embryonic	2	multi
<i>Saccostrea glomerata</i>	<i>Ostreidae</i>	<i>Parker et al. 2017</i>	<i>pH/pCO2/Acidification</i>	<i>Adult</i>	<i>growth and developmental rates, survival, standard metabolic rate</i>	post-embryonic	2	multi
<i>Saccostrea glomerata</i>	<i>Ostreidae</i>	<i>Parker et al. 2015</i>	<i>pH/pCO2/Acidification</i>	<i>Adult</i>	<i>survival, shell length, developmental rate</i>	post-embryonic	3	multi
<i>Saccostrea glomerata</i>	<i>Ostreidae</i>	<i>Parker et al. 2012</i>	<i>pH/pCO2/Acidification</i>	<i>Adult</i>	<i>survival, shell length, developmental rate</i>	post-embryonic	2	multi

