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Epigenetics in teleost fish: from molecular mechanisms to physiological phenotypes

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Key words
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Abstract

While the field of epigenetics is increasingly recognized to contribute to the emergence of phenotypes in mammalian research models across different developmental and generational timescales, the comparative biology of epigenetics in the large and physiologically diverse vertebrate infraclass of teleost fish remains comparatively understudied. The cypriniform zebrafish and the salmoniform rainbow trout and Atlantic salmon represent two especially important teleost orders, because they offer the unique possibility to comparatively investigate the role of epigenetic regulation in 3R and 4R duplicated genomes. In addition to their sequenced genomes, these teleost species are well-characterized model species for development and physiology, and therefore allow for an investigation of the role of epigenetic modifications in the emergence of physiological phenotypes during an organism's lifespan and in subsequent generations. This review aims firstly to describe the evolution of the repertoire of genes involved in key molecular epigenetic pathways including histone modifications, DNA methylation and microRNAs in zebrafish, rainbow trout, and Atlantic salmon, and secondly, to discuss recent advances in research highlighting a role for molecular epigenetics in shaping physiological phenotypes in these and other teleost models. Finally, by discussing themes and current limitations of the emerging field of teleost epigenetics from both theoretical and technical points of view, we will highlight future research needs and discuss how epigenetics will not only help address basic research questions in comparative teleost physiology, but also

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inform translational research including aquaculture, aquatic toxicology, and human disease.

1. Epigenetics and teleost research models

In recent years, the field of epigenetics has received increasing attention, which has resulted in a series of papers aiming to provide historical context for its development (Haig, 2004; Deans and Maggart, 2015) in an effort to define clear working definitions for this dynamic research field (Bird, 2007; Berger et al., 2009; Dupont et al., 2009). Because of the historical utilization of the term epigenetics in different research contexts on the one hand, and the rapid development of molecular epigenetics on the other, agreement on a synthetic, clear-cut definition has proven challenging. While we acknowledge different viewpoints, we will, for the scope of this review, employ an integrative working definition of epigenetics, which firstly emphasizes the link between genes, their products and the temporal emergence of the phenotype. This directly reflects the initial definition of epigenetics by Waddington (1968) as 'the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being'. Since the initial focus on development and phenotype, the investigation of epigenetics has increasingly shifted to molecular mechanisms, which entail factors regulating 'heritable changes in gene expression without changes in the DNA sequence' (Riggs et al., 1996), which we include as a second definition.

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Together, we therefore define epigenetics as the study of factors that heritably regulate the spatio-temporal genome expression that underlies the emergence of physiological phenotypes. Under this definition, epigenetic regulation at the molecular level is mediated by three principal mechanisms, all of which can heritably alter gene expression without a change in the DNA sequence. Two of these mechanisms, histone modifications and DNA methylation, regulate gene expression at the levels of chromatin structure and DNA, while microRNAs (miRNAs), a type of small non-coding RNA, constitute a post-transcriptional mechanism regulating abundance and translation of specific mRNAs. Together, these epigenetic mechanisms can be affected by environmental and/or endogenous signals to regulate genome-wide gene expression, leading to the emergence of physiological phenotypes at higher levels of biological organization (**Figure 1**). These processes can be spatially restricted to specific tissues or cell types and can occur across different temporal scales, which encompass both ontogenesis and the lifespan of an individual organism (termed context-dependent or intragenerational epigenetics) as well as generations (termed germline-dependent or transgenerational epigenetics), as distinguished by Burggren and Crews (2014). Briefly, intragenerational epigenetics encompass the emergence of physiological phenotypes in response to environmental and/or endogenous stimuli directly experienced by an organism during its life. These stimuli can introduce specific epigenetic marks in somatic cells, which may be mitotically transmitted and lead to temporal and spatial changes in expression of the organism's genome, which in turn can introduce physiological plasticity (Burggren, 2014). As the organism is directly exposed to the

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stimulus during its life cycle, this is also termed context-dependent epigenetics. Conversely, transgenerational epigenetics are based on epigenetic changes in the germline ('germline-dependent epigenetics'), which are meiotically stable and hereditary. Such changes may overtly manifest themselves phenotypically in subsequent generations never directly exposed to the initial stimulus or may be masked and emerge only in response to additional context-dependent stimuli experienced by the offspring. Indeed, recent publications reconsidered this phenomenon in the context of the Lamarckian theory of inheritance, which had historically long been neglected in favor of a genocentric view (Burggren, 2014). In this context, it is important to distinguish intergenerational from transgenerational inheritance, which differs conceptually between teleost fish and currently more widely studied mammalian models (**Figure 2**). In pregnant female mammals, subsequent generations are still directly exposed to exogenous or endogenous stimuli as embryo (future F_1 generation) or embryonic germ-cells (contributor to future F_2 generation) within the founder generation (F_0). Therefore, transgenerational effects in female mammals will be evident only from the F_3 onwards, after which they may persist or 'wash-out' gradually over subsequent generations. With regard to male mammals, only direct offspring (F_1) are considered intergenerational because of context-dependent exposure of sperm in F_0 founders. In oviparous teleost fish, irrespective of sex, only the F_1 generation is considered intergenerational, while both F_2 and F_3 generations are considered transgenerational (**Figure 2**). Semantics aside, oviparous teleosts will likely prove advantageous for transgenerational epigenetics, compared to mammals, as the study of

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maternal effects is much more feasible especially when investigating oocyte contributions. This is because unfertilized viable eggs are easily obtained to study molecular mechanisms, can be externally fertilized, and are amenable to genetic and pharmacological manipulation via microinjection, providing an avenue for mechanistic studies investigating maternal-lineage specific epigenetic effects.

In addition to both abiotic and biotic naturogenic environmental stimuli any organism experiences across intra- and multigenerational timescales (**Figure 1**), recent focus has shifted to ever increasing anthropogenic influences. Endocrine disrupting chemicals in particular have been identified as epigenetic determinants involved in the intra-, inter-, and transgenerational emergence of physiological phenotypes in mammals (Crews and Gore, 2012). Compared to mammalian model systems however, the investigation of epigenetic mechanisms in teleost fish has received less attention. This is in spite of the fact that comparative studies of epigenetic mechanisms in the large and physiologically diverse infraclass of teleost fish have the potential to provide important insights in the context of genome evolution, as teleost fish have undergone one, or in the case of salmoniform fish, two additional rounds of genome duplication (Volff, 2005; Betancur-R, 2013). Teleost fish also hold, in addition to their value as research models in comparative physiology, significant importance for translational research, especially in the growing sector of aquaculture (Naylor et al., 2009; Panserat and Kaushik, 2010; Ulloa et al., 2014), aquatic toxicology (Corcoran et al., 2010; Cossins and Crawford, 2005; Overturf et al., 2015; Williams et al., 2014), and human disease (Lieschke and Curry, 2007; Schartl,

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2014). As such, advances in understanding epigenetic determinants of physiological phenotypes in teleost fish are anticipated to translate into applications in these areas.

For the purpose of this review, we will principally focus on three key teleost model species, the cypriniform zebrafish, *Danio rerio*, and the salmoniform rainbow trout, *Oncorhynchus mykiss*, and Atlantic salmon, *Salmo salar*. We focus on these species firstly because annotated genome sequences are available (Howe et al., 2013; Berthelot et al., 2014; Lien et al., 2016), secondly because of their evolutionary position encompassing both teleost- (Ts3R) and salmoniform-specific (Ss4R) genome duplication events (Volff, 2005, Betancur-R, 2013), and thirdly because of their wide use as teleost research models in basic comparative physiology research as well as translational research (Ekker and Akimenko, 2010; Craig, 2013). Consequently, these species are of particular interest in advancing the field of comparative epigenetics in teleost fish. Indeed, while fish genomes and their gene expression have been and continue to be well-studied (**Figure 3A-B**), the study of regulatory molecular epigenetic mechanisms (**Figure 3C**), specifically histone modifications (**Figure 3D**), DNA methylation (**Figure 3E**), and miRNAs (**Figure 3F**), are only beginning to be investigated in teleost fish. In the first section of this review, we use available, NCBI-deposited genome sequence datasets (https://www.ncbi.nlm.nih.gov/genome) to comparatively describe the presence and function of genes representative of three major molecular epigenetic pathways of histone modifications, DNA methylation, and miRNAs in zebrafish (ID 50), rainbow trout (ID 196), Atlantic salmon (ID 369) and the rat, *Rattus norvegicus* (ID 73). By comparing

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deposited sequence synteny and predicted amino acid (AA) sequence similarities of these epigenetic regulator-encoding genes and their products, we aim to identify potential differences and particularities in teleost molecular epigenetic toolboxes compared to a mammalian reference. In the second section of this review, we highlight emerging evidence for the involvement of histone modifications, DNA methylation, and miRNA in intra-, inter- and transgenerational determination of physiological phenotypes in teleost fish in response to environmental stimuli. Since research in comparative epigenetics in teleost fish is still emerging, we extend our review to include additional fish species, in cases where information for our focal teleost species is limited. In the third and final section, we then aim to synthesize the reviewed primary literature to identify emerging themes and current knowledge gaps in the field of teleost epigenetics and to prioritize research needs and critically review available technical approaches to address them. We conclude by highlighting importance of this basic research and its implications for translational research.

2. The molecular epigenetic toolbox in teleost fish

2.1. Histone modifications

The most important histone modifications can broadly be categorized by posttranscriptional modification of specific histone amino acid residues by reversible acetylation/deacetylation, methylation/demethylation and phosphorylation/dephosphorylation (Bannister and Kouzaridis, 2011). Generally speaking, these modifications affect the interactions of basic, positively charged histone proteins with

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negatively charged DNA, resulting in different chromatin states, exemplified by relaxed, accessible heterochromatin and more condensed, closed euchromatin. These chromatin states, in turn, are linked to active and inactive gene expression by regulating the accessibility of DNA to proteins and long non-coding RNAs involved in transcriptional regulation of genes (Allis and Jenuwein, 2016).

Within the major groups of histone modifications, histone acetylation dynamics (**Figure 4A**) that largely affect the ε-amino group of lysine (K) residues at the N-terminal of H3 and H4, are mediated by histone acetylases (HATs) and histone deacetylases (HDACs), respectively. HATs are, based on their location in cytosolic and nuclear cellular compartments, further classified into two major families termed type-A and type B. While type B HATs play a role in initial nuclear deposition of nascent histones, the more diverse type A family is the principal regulator of chromatin states and transcription, and is hence considered in more detail in this review. Type A HATs are, based on sequence homology and conformational structure, which also dictate their site-specificity (Hodawadekar et al., 2007), further divided into GNAT, MYST and CBP/p300 families, examples of which are provided in **Figure 4A**. Conversely, the HDAC-mediated reversal of histone K acetylation are largely non-specific, but HDAC enzymes are, based on sequence homology, nevertheless divided into classes I-IV (Yang and Seto, 2006).

Histone methylation dynamics (**Figure 4B**) largely, but not exclusively, occur on histone K residue side chains. In contrast to histone acetylation, histone methylation occurs in gradual steps of mono-, di-, and tri methylated states, and does not change the overall

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charge of the AA residue (Ng et al., 2009). Histone lysine methylases (HKMTs) are specific with regard to site and degree of K methylation and N-terminal K residues are methylated by enzymes containing a SET-domain (Bannister and Kouzaridis, 2011). Similarly, lysine-specific demethylases (LSDs) are discriminatory with regard to site and degree of methylation (Bannister and Kouzaridis, 2011).

Histone phosphorylation dynamics (**Figure 4C**) are regulated by histone kinases and phosphatases, respectively, which mainly phosphorylate and dephosphorylate N-terminal histone serine (S) and tyrosine (Y) residues. Currently, little is known regarding the recruitment and specificity of these enzymes, but highly dynamic histone phosphorylation is considered to influence histone conformation and DNA interaction by introducing a negative charge (Bannister and Kouzaridis, 2011).

In teleost fish, representative examples of the HAT classes show differential retention patterns in zebrafish and salmoniform genomes compared to the rat, indicating that components of histone acetylation were differentially affected by Ts3R and Ss4R genome duplication events. For example, the GNAT family HAT *atf2,* which specifically acetylates K residues at positions 5, 12 and 15 in H2B, and positions 5, 8 and 16 in H4 to activate transcription (Kawasaki et al., 2000), is present in a single locus across all investigated genomes (**Figure 5A**). Both MYST family HAT *kat5a* (**Figure 5B**), which acetylates the K5 residue of H2A and K14 of H3 and K5, K8, K12 and K16 residues of H4 to activate transcription (Kimura and Horikoshi, 1998), and p300/CBP family HAT *ep300* (**Figure 5C**), which acetylates K residues in all four core histones to activate transcription

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(Ogryzko et al., 1996), are present as paralogues in zebrafish, with additional duplication and differential retention of these paralogues in salmoniformes. Conversely, *hdac3*, *hdac5*, *hdac11* and *sirt1* genes, coding for representative examples of nonspecific HDAC classes I-IV that mediate transcriptional silencing, are present in single loci in rat and zebrafish, but are duplicated in salmoniform genomes (**Figure 6A-D**), with the exception of *hdac11,* the only member of the class IV histone deacetylases.

Similar to the pattern described for genes encoding for histone acetylation dynamics, representative HKMTs involved in histone methylation dynamics are present in zebrafish both as paralogues, as in the case of *kmt5a* and *suv39h1,* (**Figure 7A-B**), or as single loci, as in the case of *ezh2* and *ehmt2* (**Figure 7C-D**). Following Ss4R, these genes are differentially retained in salmoniform genomes. Functionally, Kmt5a mediates transcriptional silencing by monomethylating the K20 residue of H4 (Nishioka et al., 2002), while Suv39h1 exerts the same effect by catalyzing K9 trimethylation on H3 (Rea et al., 2000). Similarly, Ezh2 induces transcriptional silencing via methylation of K27 in H3 (Cao et al., 2002), while Ehmt2 elicits transcriptional repression effect via K9 methylation in in H3 and transcriptional silencing via K27 of the same histone (Tachibana et al., 2001). Genes coding for representative examples of LSDs, specifically *kdm1a*, *kdm6a* and *kdm6b* (**Figure 8A-C**), appear to be present in single loci in rat and zebrafish, but are consistently retained in duplicate loci in salmoniform genomes following Ss4R. Functionally, Kdm1a specifically removes H3K9me1/2, but cannot attack the K9 in a trimethylated state (Shi et al., 2004). The related LSDs Kdm6a and Kdm6b specifically

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demethylate H3K27 (W. Jiang et al., 2013). Collectively these enzymes promote transcription by antagonizing repressive methylation marks previously described.

In the three investigated teleost genomes, genes involved in pathways modulating histone phosphorylation dynamics appear to have been differentially retained in teleost evolution. While representative examples of histone kinase genes, specifically *rps6ka5* and *aurkb* appear to be present in single loci in rat and zebrafish, duplicates are retained in salmoniform genomes following Ss4R (**Figure 9A-C**). Functionally, Rps6ka5 represses transcription via S1 phosphorylation of H2A (Zhang et al., 2004), while promoting mitosis via the phosphorylation of the S10 residue of H3 (Soloaga et al., 2003). The later modification is equally mediated by Aurkp, which functions to reorganize chromatin during mitosis (Hsu et al., 2000). Conversely, the existence of multiple paralogues of representative histone phosphatase genes, such as *ppp2ca*, *ppp2cb*, *ppp6c*, in salmoniformes suggest a diversification of pathways involved in controlling histone phosphorylation status via dephosphorylation (**Figure 10A-C**).

2.2 DNA methylation

2.2.1 Maintenance and *de novo* DNA methylation

DNA methylation enzymes act to methylate cytosines which are mostly, but not exclusively (Ramsahoye et al., 2000), located in the context of genomic CpG dinucleotides. Such DNA methylation can subsequently modify gene transcription as, for instance, methylation of CpG sequences at transcription start sites (TSS) has been associated with long-term silencing (Jones, 2012). However, DNA methylation occurs in a variety of

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genomic contexts outside of promoter regions, and potentially different functional roles of these DNA methylation patterns in regulating the genome are still being explored (Ambrosi et al. 2017). Thus, based on genomic context, DNA methylation marks may have permissive roles in gene expression, or primarily mediate chromatin integrity.

Based on studies using mammalian model organisms, the enzymes catalyzing DNA methylation are termed DNA methyltransferases (DMNTs), and can be divided into DNMT1 and DNMT3 families (**Figure 11**). While DNMT1 is principally associated with maintenance of DNA methylation in replicating cells (Robertson and Jones, 2000), the mammalian DNMT3 family is further divided into DNMT3A, DNMT3B and DNMTL, and is implicated in *de novo* establishment of methylation marks (Cheng, 2014). However, this distinction likely represents an over-simplification, as overlapping molecular functions have been identified for DNMT1 and DNMT3 proteins (Kim et al., 2002; Arand et al., 2012).

In teleost fish, the evolutionary history of *dnmt* genes has been described for zebrafish, where Ts3R has differentially affected *dnmt1* and *dnmt3* loci (**Figure 12**). While *dnmt1* is found as a single copy in zebrafish (**Figure 12A**), two paralogues of *dnmt3a* (**Figure 12B**) and four paralogues of *dnmt3b* (**Figure 12C**) exist, the latter of which have been linked to lineage-specific gene duplication events (Campos et al., 2012). Overall, zebrafish contain a single putative maintenance DNA methyltransferase and six putative *de novo* DNA methyltransferases, which, based on their identified splice variants (Smith et al., 2005) and developmental expression profiles (Smith et al., 2011), are thought to

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correspond to mammalian DNMT1 and DNMT3 families. Functionally, knockdown of zebrafish Dnmt1 and Dnmt3 in has revealed distinct non-complementary functions in specific tissue development (Rai et al., 2010), and differences in developmental *dnmt3* paralogue expression profiles have reinforced the notion of potential subfunctionalization of *dnmt3* paralogues in zebrafish (Campos et al., 2012). In salmoniformes, the current the NCBI deposited rainbow trout and Atlantic salmon genomes allow for the unequivocal identification of two distinct *dnmt1* loci, providing evidence for the retention of an additional gene copy following the salmoniform-specific genome duplication (**Figure 12A**). As for *dnmt3*a, Ss4R has resulted in three and four identified paralogues in rainbow trout and Atlantic salmon, respectively (**Figure 12B**), while *dnmt3b* paralogues appear to have undergone a complex evolution in the salmoniform lineage that includes duplication, differential retention and loss of specific *dnmt3b* paralogues (**Figure 12C**). This is in line with the observed diversification of this gene family in teleost fish compared to mammalian models in general (Goll and Halpern, 2011; Firmino et al., 2017), raising the question of functional relevance. While the specific function of each individual *dnmt3b* paralogue in teleost fish remains unclear, their functional study in teleost fish may prove a fruitful area of investigation. In mammals, DNMT3B plays a crucial role in DNA methylation in stem cells (Baubec et al., 2015), centromeric regions, and germline genes (Walton et al., 2014), and the expansion of the *dnmt3b* family in teleost fish, and salmoniformes in particular, may thus have functional relevance in these processes. A major difference between teleost and mammalian DNA methylation machinery is the absence of DNMT3L (**Figure 12D**),

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whose presence is restricted to genomes of eutherian mammals. In these animals, DNMT3L is involved in genomic imprinting in spite of the lack of a C-terminal catalytic domain (Bourc'his et al., 2001; Chédin, 2011). The functional consequence of its absence in teleost fish is not known, but it has been speculated that parent-of-origin-specific epigenetic marks, which ensure monoallelic maternal or paternal expression, may be a feature restricted to eutherian mammals expressing DNMT3L (Chédin, 2011). However, as the question of whether parental imprinting exists in teleost fish remains debated (Labbé et al., 2016), it is clear that if it does exist, different molecular machinery is involved compared to mammals.

2.2.2 DNA demethylation

DNA demethylation can occur either passively through cell division and absence of maintenance DNA methylation, or actively via enzymatic pathways, which catalyze the removal of DNA methylation (Wu and Zhang 2010; Bhutani et al., 2011). Active DNA demethylation in mammals is largely mediated via 5-methylcytosine (5mC) oxidation catalyzed by members of the ten-eleven translocation (TET) methylcytosine dioxygenase family, yielding 5-hydroxymethylcytosine (5hmC) and subsequently 5-formyl-cytosine and 5-carboxylcytosine (Xu and Wong, 2015, Kamstra et al. 2015b). Of note, 5hmC itself is abundant in the brain of mice (Kriaucionis and Heintz, 2009) and zebrafish (Kamstra et al. 2015b), suggesting a possible epigenetic function of 5hmC beyond being an intermediate of active demethylation. Methylcytosine oxidation is followed by action of

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thymine DNA glycosylase (TDG), which recognizes the higher oxidation products of 5mC and initiates base excision repair, ultimately restoring these as unmethylated cytosines (Kohli and Zhang, 2013). Altogether this demethylation process is known as the TET-TDG pathway (Kohli and Zhang, 2013). An alternative pathway has been uncovered in mammals (Morgan et al., 2004), which involves 5mC conversion to thymine through deamination by activation induced cytosine deaminase (AICDA), as well as the apolipoprotein B mRNA editing enzyme, catalytic polypeptide protein (APOBEC). The resulting thymine can then be restored to unmethylated cytosine by base excision repair (Nabel et al., 2012). However, *in vitro* conversion of 5mC to thymidine occurs at a substantially lower rate than cytosine to thymidine, and the importance of the AID/APOBEC pathway *in vivo* remains controversial (Nabel et al., 2012; Xu and Wong, 2015). As research investigating demethylation mechanisms is still an emerging area of study, we focused on the two principal pathways (TET-TDG and AID/APOBEC-TDG) described to date (**Figure 13**). With regard to the TET pathway, zebrafish, like mammals, encode one copy of *tet1-3* in their genome, while salmoniformes retain duplicates of all *tet* genes following Ss4R (**Figure 14A-C**). In the case of *tet2*, evidence suggests that salmoniform-lineage specific duplications of the *tet2b* gene resulted in an expansion leading to a total of 3 *tet2* paralogues (**Figure 14B**). With regard to the AICDA/APOBEC pathways (**Figures 14D-H**), evidence suggests potential duplication of *aicda* following Ss4R (**Figure 14D**), and differential patterns of *apobec* gene retention between genomes, (**Figures 14E-H**). While *Apobec1* and *Apobec3* are found in the rat, but are absent from teleost genomes, *apobec2* and *apobec4*

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are present as paralogues and individual genes, respectively. The functional significance of the expansion of the *apobec2* gene family in teleost fish is currently unknown. The *tdg* locus, whose gene product catalyzes the final reconversion to cysteine from substrates stemming from both TET and AICDA/APOBEC pathways, appears to have undergone a lineage specific duplication event, leading to the presence of two paralogues in zebrafish and four in salmoniformes (**Figure 14I**). The AICDA/APOBEC pathway alternatively utilizes methyl binding domain protein 4 (MBD4) in the final step to revert methylated cytosine to cytosine (Hashimoto et al., 2012). In the genomes of the rat and the three investigated teleost fish, a single *mbd4* locus is retained following whole genome duplication genome (**Figure 14J**). While little is known regarding the demethylation activity of both pathways in teleost fish in general, evidence from zebrafish thus suggests activity of both the canonical TET-TDG pathway (Almeida et al., 2012; Ge et al., 2014), as well as the AID/APOBEC pathway (Rai et al., 2008; Abdouni et al., 2013).

2.3 miRNAs

2.3.1 miRNA biogenesis

miRNAs are small non-protein-coding RNA molecules, which target specific mRNAs through complementary base pair binding in the 3' untranslated region (3'UTR) to reduce the stability and translation of the target mRNAs (Ha and Kim, 2014). Mature miRNAs are highly conserved in most eukaryotes (Hertel and Stadler, 2015), and are characterized in an increasing amount of teleost fish species (reviewed by Bizuayehu and Babiak, 2014; Mennigen, 2015). As initially demonstrated in mammals and invertebrates,

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teleost microRNAs repress target mRNA abundance or translation (Bazzini et al., 2005). Indeed, the canonical pathway for miRNA biogenesis (**Figure 15**) is highly conserved in animals with the exception of early metazoans (Moran et al., 2017), and is well characterized (Ha and Kim, 2014). Briefly, transcription of miRNA genes yields primary miRNA (pri-miRNA), which is bound and cleaved by the nuclear microprocessor consisting of the endonuclease DROSHA (or ribonuclease 3, RN3), and the DiGeorge syndrome critical region 8 protein (DGCR8). The pre-miRNA is actively translocated out of the nucleus by exportin-5 (XPO5), then processed in the cytoplasm by the endoribonuclease DICER to yield mature miRNA. In some cases, post-transcriptional modification of mature miRNAs yields so-called isomiRs, which differ from the original mature miRNA sequence either by addition or deletion of individual nucleotides at either the 3’ or 5’ end derived from differential processing during biogenesis or posttranscriptional modifications, or by enzymatically edited nucleotides within the miRNA sequence (Morin et al., 2008; Neilsen et al., 2012). While minor, these variations may be functionally relevant, especially when they shift the seed sequence of miRNA molecule (nucleotide position 2-7), which are particularly important in mediating specific target recognition (Neilsen et al., 2012). The mature miRNA duplex is subsequently loaded into the RNA-induced silencing complex (RISC), which contains Argonaute protein 2 (AGO2), the principal mediator of decay or translational repression of target mRNAs. Knockout studies in both zebrafish (Giraldez et al., 2005) and the human cell line HCT116 (Kim et al., 2016), show that Dicer/DICER activity is necessary for miRNA biogenesis via

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the canonical pathway, while knockout of other components such as XPO5 allows miRNA maturation, albeit with changes in the mature miRNA profile (Kim et al., 2016).

Interestingly, zebrafish retain a single gene copy of canonical miRNA biogenesis pathway components (**Figure 16A-E**), suggesting that the Ts3R event, which resulted in the retention of many protein-coding gene paralogues, did not require the retention of duplicates of the miRNA biogenesis pathway genes. Conversely, salmoniform species, which have undergone an additional Ss4R event, possess paralogues of all miRNA biogenesis pathway genes, with the exception of *drosha,* for which only a single copy has been retained in salmoniform genomes (**Figure 16A**). While salmoniformes are the only identified species to date which possess paralogues of several canonical miRNA biogenesis genes, possible functional implications are currently unknown. In light of the fact that individual miRNAs have a high retention rate compared to protein coding genes in salmoniform genomes (an average of three loci per miRNA compared to an average of two loci in zebrafish and a single locus in mammals, as described by Berthelot et al., 2014), this may reflect an increased demand for miRNA processing in the regulation of salmoniform genomes. Conversely, as paralogues, proteins involved in miRNA biogenesis may have undergone subfunctionalization in salmoniformes and thus evolved to differentially regulate gene expression. To provide an initial distinction between these hypotheses for the purpose of this review, we utilized a two-tiered *in silico* approach. Firstly, we reviewed mammalian literature to identify functionally important AA residues (roles in substrate binding, processing, and activity) identified through modeling and

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mutational analysis, and mapped these identified AA onto specific predicted protein sequences from the sequenced teleost genomes in an effort to predict possible functional differences between salmoniform paralogues. Secondly, we utilized a recently developed comparative fish-specific gene expression atlas termed Phylofish (Pasquier et al., 2016) to identify possible differential expression of paralogues between tissues. This approach revealed specific differences in AA critically involved in miRNA binding between salmoniform XPO5 paralogues (**Figure 17A**), which are furthermore differentially expressed in different rainbow trout tissues (**Figure 17B**). While a possible subfunctionalization in this pathway needs experimental validation, this example serves to illustrate the point that functional differences in the miRNA biogenesis pathway may exist between specific teleost species and mammals.

2.3.2 miRNA turnover and degradation

Recent evidence increasingly suggests an important role of miRNA degradation pathways in the maintenance of cellular miRNA abundance (Sanei and Chen, 2015). Such miRNA turnover can be achieved via both mRNA target-dependent and target-independent mechanisms (Zhang et al., 2012; Marzi et al., 2016). Overall, miRNA turnover appears to be complex, as evidenced by miRNA populations with shorter (hours) and longer (days) turnover following ablation of miRNA biogenesis (Gantier et al., 2011; Guo et al., 2015) and pulse-chase labelling experiments (Marzi et al., 2016). Several proteins involved in miRNA turnover have recently been characterized in invertebrate and mammalian models, and are oftentimes highly specific to individual miRNAs and their families, providing a

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possible explanation for the different half-lives observed between specific miRNAs. For the purpose of this review, we focused on the most characterized proteins involved in miRNA turnover to date (**Figure 18**), but note, that while the functional relevance of these gene products in teleost fish is likely, especially given their ancient phylogenetic origin and conserved functionality in both invertebrate and mammalian model systems, their role in teleost fish has been poorly, if at all investigated directly. Briefly, two members of the family of terminal uridyl-transferases (TUT4 and TUT7), which evolved through a gene duplication in the vertebrate lineage (Modepalli and Moran, 2017), cause 3’ uridylation of several miRNAs (Thornton et al., 2014; B. Kim et al., 2015; Gutierrez-Vazquez et al., 2017). This, in turn, destines the modified miRNAs for degradation mediated by general exonucleases, including the 5’-3’ exoribonuclease 2 (XRN2; Miki et al., 2014) and DIS3-like 3'-5' exoribonuclease 2 (DISL3L2; Pirouz et al., 2016). Conversely 3’ adenylation mediated by the poly-A polymerase germline development 2 protein (GLD-2), which is evolutionarily related to the URT4 and URT7 family (Chung et al., 2016), has been linked with stabilization of some miRNAs (Katoh et al., 2009; Gutierrez-Vazquez et al., 2017). Deadenylation of GLD2 targeted miRNAs through the poly(A)-specific ribonuclease (PARN) and CUGBP Elav-like family member 1 (CUGBP1) reverses the stabilizing effect of polyadenylation (Katoh et al., 2015). Similar to the canonical miRNA biogenesis pathway, components of the machinery involved in miRNA turnover have been retained as single copies in the zebrafish genome, while components involved in aspects of miRNA turnover are either equally retained as single copies or as paralogues following Ss4R in

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salmoniform fish (**Figure 19 A-I**). Whether and how differences in the teleost toolbox in miRNA biogenesis and turnover machinery contribute to specific miRNA and isomiR profiles characterized in zebrafish (Presslauer et al., 2017), rainbow trout (Juanchich et al., 2016) and Atlantic salmon (Andreassen et al., 2013), is currently unknown.

3. Comparative dynamics of epigenetic marks in teleost and mammalian life-cycles

In addition to the described differences in the 'molecular toolbox' of epigenetic pathways regulating histone modifications, DNA methylation, and miRNA abundance between teleost fish and mammals, it is clear that this may translate into differences in function and dynamics of these epigenetic marks. This evidence is reviewed in the following section.

3.1 Histone modification dynamics in teleost fish

The dynamics of histone modifications in fish have largely been studied in the context of zebrafish development, where specific histone marks have been described at global genome level using ChIP assays in conjunction with proximal promoter DNA microarrays (Wardle et al., 2006; Lindeman et al., 2009; Lindeman et al., 2010) or DNA-sequencing (Trompouki et al, 2011; Bogdanović, 2013), and at the gene-specific level using ChIP assays in conjunction with real-time RT-PCR (Seiliez et al., 2015; Biga et al, 2017). Through these studies, it has become clear that specific histone marks are highly conserved between zebrafish and mammals (Cunliffe, 2016) and are linked to chromatin states which are bivalent, permissive or inhibitory for gene expression at least in part by

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regulating accessibility to transcription factors (Joseph et al., 2017). However, while both the genome-wide and gene-specific approaches provided evidence for promoters as target of histone modifications, additional target sites outside the promoter area have been described in zebrafish embryos (Perez-Rico et al., 2017), and specific differentiating embryonic tissue (Quillien et al., 2017). While some target sites are partially conserved with mammalian model systems, others, such as enhancers and super-enhancers show a differential genomic distribution pattern compared to mammals, as they are less abundant in gene bodies and introns specifically, but are rather enriched in intergenic regions (Perez-Rico, 2017). In addition to differences in genomic location of histone modification marks, different histone variants have been uncovered in teleost fish, exemplified by the histone H2A variant H2af1o discovered in oocytes of the cypriniform gibel carp, *Carassius gibelio* (Wu et al., 2009). Together with the described genome duplication events, such histone variants further add to differences in the epigenetic repertoire underlying teleost histone modifications compared to mammals, as it reveals a unique structure that functionally results in a destabilization of nucleosomes (Wu et al., 2009). In the same vein, a salmoniform histone cluster identified in rainbow trout and Atlantic salmon that is representative of the vast majority of histone genes in these species are tandemly repeated, suggesting further diversification of the histone repertoire in salmoniformes (Pendás et al., 1994).

While extensive correlative descriptive studies between histone marks and gene expression allowed for the identification and comparative analysis of global and specific

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histone marks in the context of genome regulation in zebrafish, additional studies employing genetic ablation and knockdown of histone modifying enzymes (Lan et al., 2007; Yabe et al., 2009; Zhao et al., 2016), their pharmacological inhibition (Shiomi et al., 2017), and histone-specific transgenic GFP-based reporter lines (Pauls et al., 2001; Zhang and Roy, 2016), subsequently completed our insight into functional roles of the previously described major histone modifications throughout zebrafish development. Specifically, these developmental roles encompass early embryonic development (Andersen et al., 2013; Li et al., 2014), organogenesis (Cayusa Mas et al., 2011), and adult tissue regeneration (Katsuyama and Paro, 2011), in line with the 'epigenetic landscape' of cellular differentiation initially proposed by Waddington (1957).

With regard to early development in zebrafish, the arginine methyltransferase Prmt6 has been shown to be involved in epiboly (Zhao et al., 2016), while the previously described histone kinase Aurkb (**Figure 4**), has been identified as an essential component for furrow formation in early zebrafish embryos (Yabe et al., 2009). At the gastrulation stage, mesodermal competence in ectodermal cells during gastrulation has been shown to be restricted by a H3K27 methylation mark (Shiomi et al., 2017), while the lysine demethylase Kdm6a (**Figure 4**) has been shown to affect hox-dependent patterning and posterior patterning in zebrafish development (Lan et al., 2007).

With regard to organogenesis, largely studied in zebrafish, histone modifications have been shown to play crucial roles in CNS development, especially the brain (Buschbeck et al, 2009, Tsukada et al, 2010; Loponte et al., 2016), specific neuronal

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populations such as serotonergic neurons (Jacob et al., 2014), and sensory organs including the retina (Olsen et al., 2016) and mast cells in the inner ear (He, 2016a) and lateral line (He et al., 2014, He et al., 2016b). Some of these effects are specifically mediated by previously discussed gene products encoding histone modifying enzymes (**Figure 4**). For example, the development of the CNS is mediated by Aurkb (Loponte et al., 2016), while mast cell development has been linked to Kdm1a activity (He et al., 2016). Histone modifications also contribute to the development of the zebrafish cardiovascular system, especially the heart (J. D. Kim et al, 2015; Akerberg 2017) and vascular epithelial cells (Kaluza et al., 2011). In this context, histone modification-dependent zebrafish heart development is, at least partially, mediated by the lysine specific histone demethylase Kdm6b (Akerberg et al., 2017), introduced in **Figure 4**. Zebrafish development of digestive tract organs have equally been linked to histone modifications, including Hat1-dependent exocrine (Zhou et al, 2011) and endocrine (Li et al, 2016) pancreas development, Hdac3-dependent liver development (Farooq et al., 2008), and Suv39a and Ezh2-dependent intestinal development (Rai et al., 2006, Dupret et al, 2017). Finally, the development of the musculoskeletal system has been linked to histone modifications (Tao et al., 2011; Lindgren et al., 2013), including Kdm6a-dependent craniofacial and development and myoblast differentiation (Lindgren et al., 2013). In adult zebrafish, an involvement of histone modifications in tissue regeneration has been demonstrated, exemplified by caudal fin regeneration mediated by Ezh2 (Dupret et al, 2017) and lateral line mast cell regeneration mediated by Kdm1a (Bao et al., 2017).

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All in all, there is overwhelming evidence for developmental roles of all major histone modifications **(Figure 4)** across the zebrafish life cycle. Very few studies exist with regard to developmental histone marks in salmoniform species, but histone marks have been identified to contribute to muscle differentiation in adult rainbow trout *in vitro*, potentially by contributing to differential regulation of *pax7* paralogues (Seiliez et al., 2015). Regardless of the species studied, most studies investigating histone dynamics in teleost have focused on teleost ontogenesis *per se*, rather than their regulation in response to exogenous environmental stimuli.

Historically in rainbow trout, and recently in the zebrafish model, histone marks have also been studied in the context of intergenerational transfer. In rainbow trout, somatic cell histones have been shown to be retained in spermatogenesis where they undergo acetylation, as demonstrated for H2B, H3 and H4 (Candido and Dixon, 1971; Candido and Dixon, 1972; Christensen et al., 1984), methylation, as shown for H3 and H4 (Honda et al, 1975) and phosphorylation, as identified for H2B, H3 and H4 (Sung and Dixon, 1970). As in rainbow trout (Avramova et al., 1983), DNA material in zebrafish sperm is packaged in nucleosomes containing somatic histones and histone variants, which is in contrast to the situation in mammals, where genetic material is packaged by protamines (Wu et al., 2011). Given the presence of histone modifications in teleost sperm, the question of the fate of established marks across generations arises. With regard to genome regulation, the generational barrier is represented by maternal to zygote activation (MZA) at the

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midblastula transition (MBT), at which maternally inherited regulatory transcripts are degraded and give way to zygote-dependent genome regulation (Aanes et al., 2011).

In zebrafish, genome-wide and locus-specific dynamic transition of histone marks have been described between gametes and pre- and post MBT (3 hpf; hours post-fertilization) embryos. Specifically, zebrafish sperm exhibits both complex multivalent chromatin, in which permissive and non-permissive marks exist in close proximity, as well as monovalent permissive histone marks (H3K4me3 and H3K14ac) at genome loci (Lee et al., 2014). These marks were associated with early embryonic gene activation before and after MBT, respectively, suggesting a temporal relevance across MZA. A predictive role for permissive and/or repressive H3 methylation marks in zebrafish sperm for MBT stage gene expression has also been described by Lindeman (2010), however, evidence for *de novo* histone modification following fertilization but prior to MBT has equally been established for other loci in zebrafish (Andersen et al., 2013), suggesting a complex fate of histone marks between generations. Functionally, a previously undescribed genomic localization in 5' and intergenic regions was observed for H3K36me3 marks in zebrafish sperm compared to the typical location at the 3' end of genes identified in late zebrafish embryos and other vertebrates, and these histone marks have been linked to differentially spliced exons that occur before MBT and are believed to play a role in MZA (Aanes, 2013). Due to the low amount of extractable chromatin, histone marks have not been characterized in unfertilized eggs, which would provide complementary information regarding a role for maternal histone-dependent programming of gene expression in the zygote. Together,

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evidence shows that while the intergenerational inheritance of histone modifications, especially via paternal transmission in sperm, are likely functional, their consequences on embryonic development are complex. To our knowledge, no comparison between gamete histone marks between multiple generations has been determined in transgenerational studies in teleost fish, restricting the current evidence for an involvement of histones to intergenerational inheritance (**Figure 2**). Such studies may aid in shedding light into the relative importance of specific histone marks in predetermining gene expression not only across developmental, but also transgenerational trajectories. In salmoniformes, studies investigating the fate of histone marks between generations are lacking, in spite of early research efforts investigating histone marks during spermatogenesis. Given the newly available genome resources (Berthelot et al., 2014; Lien et al., 2016) and the technical feasibility of histone mark profiling in salmoniformes (Seiliez et al, 2015; Marandel et al., 2016), this will be an interesting area of investigation, especially with regard to developmental regulation of paralogous genes.

3.2 DNA methylation dynamics in teleost fish

DNA methylation dynamics are the consequence of the activities of the described DNA methylation and demethylation pathways (**Figure 11** and **Figure 13**), and are considered to be important factors within generations, especially in development and tissue differentiation, and between generations as a molecular effector of transgenerational inheritance (Richardson and Yung, 1999). Studies in teleost fish, and once again in zebrafish in particular (Goll and Halpern, 2011), have revealed functional similarities

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between zebrafish and mammalian model systems in DNA methylation dynamics. Indeed, in zebrafish as in mammals, low genome-wide CpG methylation levels have been described in early ontogenesis, while higher CpG DNA methylation levels are found in differentiated adult tissues (McGaughey et al., 2014; Zhang et al., 2016). Conserved features of the DNA methylome identified in a comparison between zebrafish and mice were the restriction of DNA methylation from promoters and from CpG sites near transcription start sites, and the clustering of methylated CpGs in gene bodies and intragenic regions. In spite of these similarities, both studies equally uncovered some important differences in DNA methylation dynamics between zebrafish and mammals. For example, despite a similar GC content in the genomes of fish and mammals (Varriale and Bernardi, 2006), the zebrafish genome analyzed in liver and brain tissue displays on average a three-fold higher percentage of CpG site specific methylation compared to mice (Zhang et al., 2016). Indeed, based on this study, the authors concluded that species identity was a more important determinant in DNA methylation patterning than adult tissue identity when comparing zebrafish and mouse. The authors attribute this difference between species at least partially to higher abundance of highly methylated transposable elements in zebrafish (Zhang et al., 2016; Chernyavskaya et al., 2017). Another explanation may lie in the fact that teleost fish, including zebrafish, seem to have a different frequency of CpG sites compared to mammals, which suggests that this genomic feature has evolved differently, even between teleost species (Han and Zhao, 2008). Therefore, caution is warranted when using mammalian CpG site identification algorithms to assess the role of DNA methylation status

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in different teleost genomes. This notion is further reinforced by a recent study attempting to link developmental stage-dependent genome-wide DNA methylation patterns with previously characterized gene expression profiles in an effort to gain correlative insight into the functional implications of DNA methylation on gene expression (McGaughey et al., 2014). This study identified that, while methylation of promoter regions is often claimed to be inversely related to gene expression, methylation status of DNA sequences within the genes (especially exons) and of DNA sequences further upstream and downstream of a gene (10 kb) showed even better correlation with gene expression in zebrafish (McGaughey et al., 2014). These findings are in line with other recent work suggesting that DNA methylation patterns in genomic contexts outside the promoter region, such as enhancers, may influence gene expression in zebrafish (Bogdanović et al. 2016, Kaaij et al. 2016). Indeed, enhancer regions may, for example through mutations resulting in differential enhancer methylation, add to the described evolutionary relevance of these elements (Ariza-Cosano et al., 2012).

Together, these studies show that DNA methylation studies that are specific to promoter regions may miss regulatory importance of DNA methylation in zebrafish, and likely in teleost species in general.

With regard to functional involvement of DNA methylation in early development, organogenesis, and adult tissue regeneration, both descriptive and functional studies have linked molecular DNA methylation dynamics to tissue and organism-level phenotypes. Descriptive studies investigated patterns of DNA methylation as previously described or

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expression of enzymes involved in the regulation of DNA methylation dynamics (Mhanni and McGowan, 2002; Takayama et al., 2014; Seritrakul and Gross, 2014), while functional studies used zebrafish lines with mutations or knockouts (Rai et al., 2006; Anderson et al., 2009; Tittle et al., 2011; Seritrakul and Gross, 2017) or pharmacological inhibition (Powell et al., 2013; Dhliwayo et al., 2014; Deveau et al., 2015). For example, knockouts of the maintenance and *de novo* DNA methyltransferases *dnmt1* and *dnmt3bb* (**Figure 12**) revealed a crucial function of DNA methylation on left/right patterning in organogenesis by repressing *lefty2* mRNA abundance (L. Wang et al., 2017). Using a zebrafish *dnmt1* knockout line, Rai and colleagues (2006) have further been shown to regulate terminal differentiation of the intestine, exocrine pancreas, and retina, while being dispensable for liver and endocrine pancreas development. This effect was specifically linked to Dnmt1-mediated DNA methylation, as only introduction of catalytically active Dnmt, but not catalytically inactive Dnmt could rescue the phenotype observed in *dnmt1* knockouts (Rai et al., 2006). Conversely, *dnmt1* mutants reveal time-dependent defects in liver and pancreas organogenesis, but only after 84 hpf (Anderson et al., 2009). Interestingly, the pancreatic effects are specific to acinar cells and spare endocrine cells. Knockout of *dnmt1* as well as Dnmt inhibition has furthermore been shown to regulate zebrafish hematopoiesis (Deveau et al., 2015) and lens development (Tittle, 2011). Overall, compared to the relatively detailed functional investigation of maintenance DNA methyltransferase, the developmental roles of *de novo* methyltransferases have largely been explored indirectly by profiling *dnmt* paralogue expression in different tissues using *in situ* hybridization

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(Takayama et al., 2014). This approach suggested potentially overlapping roles for *dnmt3aa* and *dnmt3ab* in developmental processes of the brain, pharyngeal arches, pectoral fin buds, intestine and swim bladder, while only *dnmt3aa* was expressed in the developing pronephric duct. Conversely, developmental expression of *dnmt3b1* has been observed in the zona limitans intrathalamica, midbrain-hindbrain boundary, ciliary marginal zone, pharyngeal arches, auditory capsule, pectoral fin buds, intestine, pancreas, liver, and hematopoietic cells in the aorta-gonad-mesonephros and in the caudal tissue from 48 to 72 h post-fertilization. The expression and function of the additional teleost lineage-specific *dnmt3b* paralogues (**Figure 12**) remain largely uncharacterized. In addition to DNMTs, the role of demethylation has equally been addressed in zebrafish, specifically by using *tet* knockout lineages. For example, concurrent loss of function of *tet* paralogues (**Figure 14**) either through morpholino-based approaches (Bogdanović et al., 2016) or knockout approaches (Li et al., 2015) results in major developmental defects in embryos surviving to gastrulation stage which include short and blended axes, impaired head structures, small eyes, and reduced pigmentation. In zebrafish *tet2* $^{-/-}$ and *tet3* $^{-/-}$ mutants, retinal neurons, while specified, fail to terminally differentiate as evidenced by a lack of axon formation in retinal ganglion cells (Seritrakul and Gross, 2017).

In adult zebrafish, there is evidence for a role for DNA methylation in tissue regeneration, which may be mediated through both DNA methylation and demethylation pathways. For example, fin regeneration following amputation has been linked to strong *dnmt3aa* and faint *dnmt3ab* and *dnmt4* expression in blastema cells at 72h post-amputation

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(Takayama et al., 2014). Zebrafish *dnmt1* mutants and morpholino-injected larvae revealed increased beta cell regeneration capacity (Anderson et al., 2009). Inhibition of Tet activity that prevented demethylation and generation of Tet-induced cytosine intermediates reestablished the capacity for tissue regeneration in a hyperglycemic zebrafish model for diabetes (Dhliwayo et al., 2014). Finally, retinal injury in zebrafish stimulates Müller glia to undergo a reprogramming event that transitions their identity from quiescent supportive cells to multipotent progenitors capable of repairing the damaged retina, which is associated with a changing DNA methylation landscape (Powell et al., 2013).

The most crucial differences in DNA methylation dynamics between teleost fish, or at least zebrafish and mammalian model systems have been uncovered in an intergenerational context. Initial investigation of temporal DNA methylation dynamics from zebrafish sperm to zygotes at different post-fertilization timepoints provided conflicting results, reporting no changes in methylation on the one hand (Macleod et al., 1999), and demethylation and remethylation events on the other (Mhanni and McGowan, 2004). This difference was attributed to an earlier demethylation window in zebrafish development compared to mammals (Mhanni and McGowan, 2004), a finding recently confirmed by higher-resolution methylation measurements using whole-genome shotgun bisulphite sequencing (L. Jiang et al., 2013; Potok et al., 2013). Of note, both recent studies uncovered not only a temporal difference in DNA methylation dynamics after fertilization between zebrafish and mammals, but also a qualitative difference. In zebrafish, the restored methylation pattern in zygotes at the MBT closely matches the sperm genome methylation

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pattern, which is hypermethylated compared to the hypomethylated oocyte. Thus, the maternally inherited genome undergoes DNA methylation changes to match the paternal methylation pattern at MBT, and the paternally inherited methylation pattern provides a 'blueprint' for early embryogenesis following MBT. Following MBT, increased DNA methylation in CpG sites and LINEs, as well as decreased DNA methylation in SINEs finally lead to a clear differentiation of fully differentiated somatic tissues from embryonic methylation patterns (McGaughey et al., 2014). The parental inheritance of DNA methylation is therefore in stark contrast to findings in mammalian models, where both paternal and maternal genomes have been shown to undergo both rapid active as well as slow passive demethylation after fertilization, the latter of which occurs after conversion to 5hmC (Oswald et al., 2000, Santos et al. 2002; Guo et al., 2014; Amouroux et al., 2016), before *de novo* methylation of the combined genome occurs (Reik et al., 2001). In addition to the nature of parental contribution to DNA methylation patterns in the zygote, a further difference from mammals can be found in the molecular pathways involved, as active demethylation involving 5hmC and TET is not involved in early reprogramming of DNA methylation (L. Jiang et al. 2013; Kamstra et al. 2015b), whereas demethylation involving 5hmC is implicated in paternal genome reprogramming after fertilization in mice (Iqbal et al. 2011). While the link between teleost-specific DNA methylation machinery and the differences in DNA methylation dynamics between (zebra)fish and mammals are currently unknown, the four zebrafish *dnmt3b* paralogues revealed continuous expression leading up to MBT in the zygote (L. Jiang et al., 2013), suggesting that the divergent evolution of

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dnmt3b in teleost genomes (Goll and Halpern, 2011; Firmino et al., 2017) may play a role in establishing paternal-like methylation patterns in the zebrafish zygote. Following initial reprogramming after fertilization, mammalian primordial germ cells (PGCs) undergo an additional de- and remethylation event during gametogenesis (Seisenberger et al., 2012). While the clear-cut differences in the DNA methylation state of mature zebrafish gametes make it very likely that such reprogramming equally occurs in teleost fish, this process has not yet been directly studied in this infraclass (Labbé et al., 2016). Given the widely assumed importance of DNA methylation in germline-dependent transgenerational inheritance of physiological phenotypes in mammals, DNA methylation dynamics in different teleost PGCs need to be comparatively studied in detail. Interestingly, and once again in contrast to mammals, zebrafish primordial germ cell fate begins early in development through maternal deposition of germ plasm components (Yoon et al., 1997; Knaut et al., 2000; Raz, 2003), raising the possibility for a PGC cell-specific DNA methylation dynamics prior to the establishment of a paternal DNA methylation pattern. Early maternally deposited germ plasm has equally been traced in rainbow trout (Yoshizaki et al., 2000) and Atlantic salmon (Nagasawa et al., 2013), and GFP-based cell sorting techniques applied to PGCs in these species (Fan et al., 2008; Kobayashi et al., 2014) will allow the study of specific epigenetic marks in these cells in the future. The study of DNA methylation dynamics in teleost PGCs may also provide important insight into their differential role in sex determination between zebrafish (Slanchev et al., 2005) and Atlantic salmon (Wargelius et al., 2016).

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Finally, at the evolutionary scale, emerging evidence suggests a role for DNA methylation in the regulation of paralogues in duplicated teleost fish genomes (Zhong et al., 2016), confirming reports of a role for differential methylation in duplicated genes in humans (Keller and Yi, 2014). Such epigenetic regulation of duplicated teleost genomes may contribute to the widespread success of teleost fish in occupying diverse ecological niches with a wide range of environmental conditions (Volff, 2005), and future comparative studies involving additional teleost fish species are warranted.

3.3 MicroRNA dynamics in teleost fish

The miRNA repertoires in multiple teleost fish have increasingly been characterized through high-throughput sequencing approaches, which do not require a sequenced genome (Bizuayehu et al., 2014; Mennigen, 2015). Nevertheless, a sequenced genome allows for a better framework for functional studies, as it allows localization of pri-miRNAs in a genomic context to distinguish mature miRNAs from isomiRs and provides preliminary functional context, for example if pri-miRNAs are encoded in host gene introns. Additionally, genomic sequences in conjunction with EST data allow for annotation of protein coding gene 3'UTRs, aiding in the development of species specific miRNA target detection algorithms (Mennigen, 2015). Data stemming from such combined RNA sequencing approaches and genome mapping have highlighted important features of the miRNA and isomiR repertoires of teleost fish. For example, recent profiling and genome annotations of miRNAs in zebrafish (Wienholds et al., 2005; Howe et al., 2013), rainbow trout (Berthelot et al., 2014; Juanchich et al., 2016) and Atlantic salmon

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(Bekaert et al., 2013; Lien et al., 2016) identified novel lineage- and tissue-specific miRNAs. Both zebrafish and salmoniformes appear to preferentially retain increasing amounts of miRNA paralogues compared to protein coding genes over subsequent (Ts3R and Ss4R) rounds of genome duplications (Berthelot et al., 2014), but whether this translates into higher total miRNA content or represents potential for increased differential regulation via divergent promoters is currently unknown. The increased characterization of 3'UTR sequences, for example in zebrafish and rainbow trout (Ulitsky et al., 2012; Mennigen and Zhang, 2016), confirmed a lack of conservation in gene-specific miRNA binding sites in 3'UTR sequence *in silico*, as only ~10% of miRNA–mRNA targets are estimated to be deeply conserved between fish and mammals (Xu et al., 2013). Therefore, miRNA-mRNA networks in teleost fish have experienced extensive evolutionary diversification, and may indeed underlie divergent spatiotemporal gene expression patterns in this large and diverse infraclass including differential spatiotemporal regulation of paralogues. Indeed, studies in cichlids identified 3'UTR polymorphisms as contributors to divergent selection, suggesting an evolutionary importance of rewired miRNA-mRNA relationships in teleost fish (Loh et al., 2011). While the majority of targets may be different even between deeply conserved miRNAs in teleost species and mammals, it is clear that the miRNA mode of action is conserved. For example, miRNA-mediated repression of mRNA translation and/or protein abundance has been identified in developing zebrafish (Bazzini et al., 2005) and adult rainbow trout (Mennigen et al., 2014a).

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With regard to teleost fish development, functional studies have revealed a clear role for miRNAs. While miRNAs are generally not required for patterning and establishment of major cell lineages in early embryogenesis in zebrafish *in vivo* (Giraldez et al., 2005), miRNAs play crucial roles in tissue specific differentiation and maintenance. This is especially evident when investigating tissue-specific miRNAs, including muscle-specific miRNA-1 and miRNA-133, liver-specific miRNA-122, and pancreas-enriched miRNA-375, which have all been shown to play a role in tissue differentiation and/or homeostasis in both zebrafish (Kloostermann, 2007; Mishima et al., 2009; Laudadio et al., 2012) and rainbow trout (Mennigen et al., 2013; Mennigen et al., 2014a; Latimer et al., 2017).

While inter- and possibly transgenerational roles for miRNAs transmitted as parental cargo in sperm or eggs are emerging as determinants of physiological phenotypes in mammals (Rodgers et al., 2015; Smythies et al., 2014; Vilella et al., 2015), their comparative role in teleost fish remains unknown. This is in spite of the fact that miRNAs have been quantified in zebrafish sperm and eggs (Jia et al., 2015; Presslauer et al., 2017) and in rainbow trout eggs (Ma et al., 2012; Ma et al., 2015), underlining the feasibility of investigating potential inter- and/or transgenerational roles for miRNAs in teleost fish. A notable exception to the general lack of knowledge concerning miRNAs as possible mediators of inter- or transgenerational inheritance is the well-established role of miRNA-430, which has been identified as a key regulator of MZA by promoting clearance of

maternally-deposited germ plasm RNAs in somatic but not germ cells (Mishima et al., 2006).

4. A role for (molecular) epigenetics in teleost environmental physiology

Following the description of specific characteristics of the teleost epigenetic toolbox, the spatiotemporal dynamics of epigenetic marks and their mechanistic function in teleost model species, we here address the increasing evidence for environmental and/or endogenous regulation of these epigenetic marks. Specifically, we highlight how molecular epigenetic marks in zebrafish and salmoniform species are altered in response to diverse naturogenic and anthropogenic environmental conditions relevant to aquatic species in intra-, inter- and transgenerational settings (**Figure 1**). Where possible, we aim to describe how this (molecular) epigenetic regulation in response to environmental stimuli integrates into physiological phenotypes. In cases where studies in zebrafish, rainbow trout and Atlantic salmon are currently limited, we include additional teleost species in an attempt to synthesize current information in teleost models.

4.1 Hypoxia

Teleost fish evolved to inhabit diverse niches with varying degrees of oxygen availability (Rogers et al., 2016). While histone-dependent chromatin modifications are involved in the hypoxia response in mammalian models (Melvin and Rocha, 2012, Hancock et al., 2015), the role of histone modifications in context-dependent epigenetic regulation of the genome in teleost fish experiencing hypoxia remains unexplored. Using

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ChIP-assay/DNA-seq and forced expression approaches in zebrafish, detailed, genome-wide maps of hypoxia-responsive elements and hypoxia regulated transcripts have become available (P. Zhang et al., 2014; Greenald et al., 2015), which will greatly facilitate future targeted studies investigating histone-dependent regulation of hypoxia responsive genes in zebrafish. While hypoxic stimuli have been investigated across development in rainbow trout to investigate consequences on metabolic gene expression (Liu et al., 2017a; Liu 2017b), a potential role for histone modifications in the regulation of these genes has not been investigated. In an inter- and transgenerational context, F_0 hypoxia exposure has been linked to increased hypoxia tolerance tolerance in F_1 zebrafish (Ho and Burggren, 2012), and reproductive impairment in F_1 and F_2 male marine medaka, *Oryzias melastigma* (S. Y. Wang et al., 2016). Mechanistically, the hypoxia-induced phenoype in marine medaka, was linked to increased expression of the euchromatic histone-lysine N-methyltransferase 2 (*ehmt2*) in the testes across generations, which, in turn, was linked to hypomethylation of its promoter. The increase in *ehmt2* was linked to an increase in cells positive for its catalyzed histone mark H3K9*me2*, suggesting a concerted role for DNA methylation and histone modifications in this phenotype (S. Y. Wang et al., 2016).

Several studies have investigated hypoxia-induced regulation of miRNA abundance. A hypoxia-inducible factor 1 (*hif1)*-dependent increase in miRNA-462/731 has been described in zebrafish larvae under hypoxic conditions, and miRNA-462/731 was shown to target the genes deadbox helicase 5 (*ddx5)* and Mg^{2+}/Mn^{2+} dependent protein phosphatase (*ppm1da*) to regulate cell survival (Huang et al., 2015). More evidence stems

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from additional studies in the beloniform marine medaka, for which differential expression of miRNAs in several tissues in response to hypoxia has been described (Lau et al., 2014). Specific investigation of these differentially expressed miRNAs under hypoxia have pointed to anti-apoptotic and steroidogenic target genes in the same species, highlighting their role in mediating cell survival on the one hand, but also reproductive impairment observed under hypoxia on the other (Lai et al., 2016; Tse et al. 2015; Tse et al., 2016).

4.2 Temperature

To our knowledge, no studies have comparatively investigated global histone modifications and chromatin states in stenotherm fish species. However, a few studies have investigated the involvement of histone marks in temperature acclimation in eurytherm fish. For example, liver cells of winter-acclimated (8-10°C) common carp, *Cyprinus carpio*, exhibited increased abundance of the heterochromatin marks H3K4me3 and of the histone variant macroH2a compared to summer acclimated (20-22°C) cells. These increased histone marks in cold-adapted carp liver cells correlated with hypermethylated CpG sites in the promoter region of ribosomal protein genes, whose expression was significantly decreased in the winter (Pinto et al. 2005).

With regard to temperature effects on teleost DNA methylation, comparative, ecological-level studies identified global methylation differences across a vast array of teleost fish species as a function of habitat water temperature, with hypermethylated DNA in cold-water species compared to warm-water species (Varriale and Bernardi, 2006;

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Varriale, 2014). In more recent studies, temperature-dependent DNA methylation dynamics have been addressed in muscle tissue of zebrafish (Campos et al., 2012; Han et al., 2016), Atlantic salmon (Burgerhout, 2017) and Senegalese sole, *Solea senegalensis*, (Campos et al., 2013). In zebrafish, *dnmt* paralogues were found to be differentially regulated in response to rearing temperature, with higher temperatures favouring an increase in *dnmt3a* and concomitant decrease in *dnmt3b*, suggesting changes in DNA methylation (Campos et al., 2012). In zebrafish fibroblast cells (ZF4) exposed to a low temperature of 18°C compared to the standard conditions of 28°C, MeDIP sequencing revealed changes in global DNA methylation patterns characterized by an increase after 5d and a decrease after 30d. Overall 21% of DNA methylation peaks were differentially affected by these temperature treatment regimes, of which the majority (92%) did not affect promoter regions. Functionally, differentially methylated gene loci were enriched for functions in folate metabolism, the antioxidant system, the immune system, apoptosis and chromatin modification, suggesting DNA methylation, possibly in crosstalk with histone modifications, plays a role in regulating these pathways involved in cold-acclimation (Han et al., 2016). In Atlantic salmon, embryos selected for fast and slow growth were incubated at 4°C and 8°C prior to seawater transfer, and in the fast-growth group incubated at the warmer temperature, growth performance was correlated with increased larval myogenin expression, which in turn exhibited low DNA methylation levels. This study suggests environment and genome differentially affect epigenetic regulation of myogenin in Atlantic salmon (Burgerhout et al., 2017). In Senegalese sole, *Solea senegalensis*, specific

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analysis of gene promoters using bisulfite sequencing revealed the occurrence of hypermethylated myogenin (*myog*) promoters coinciding with lower growth rates in fish reared at colder temperatures (Campos et al., 2013). An interesting additional context-dependent effect of epigenetic mechanisms in response to temperature but unrelated to the physiology of growth and metabolism has been discovered in seabass, *Dicentrarchus labrax*, a species in which sex is determined by both genetics and temperature (Navarro-Martín et al., 2011). In seabass, temperature-dependent methylation of the *cyp19a* (aromatase) promoter region is an important mediator of sex determination (Navarro-Martín et al., 2011), a mechanism that has subsequently been validated in other fish species and non-teleost species exhibiting temperature-dependent sex determination (Y. Y. Wang et al., 2017; Matsumoto et al., 2013). Potential germline-dependent epigenetic changes in response to temperature in teleost fish have not yet been fully addressed, although Shao et al. (2014) recently demonstrated transmission of temperature effects on sex determination in half-smooth tongue sole, *Cynoglossus semilaevis*, in that some ZW females in the offspring of heat-exposed fish developed as males, even in the absence of elevated temperatures.

Context-dependent regulation of miRNAs in response to developmental or acute changes in water temperature has been relatively widely studied, largely using the zebrafish model (Hung et al., 2016; Johnston et al., 2009; Yang et al., 2011) and several commercially important aquaculture species, including Senegalese sole (Campos et al., 2014) and Atlantic cod, *Gadus morhua* (Bizuayehu et al., 2015). In most cases, muscle

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tissue was targeted for analysis, and transcriptomic screening approaches identified numerous differentially regulated miRNAs between groups developing in different temperature regimes. Some of these temperature regulated miRNAs include deeply conserved and muscle-specific miRNAs termed myomiRs such as miRNA-1/133 and miRNA-206, which have previously been shown to coordinate ontogenic muscle development in zebrafish (Mishima et al., 2009) and Nile tilapia, *Oreochromis niloticus* (Yan et al., 2013). Together, these findings suggest that miRNA control of muscle development can be modulated by temperature. Nevertheless, other than through standard practice *in silico* target prediction (Brennecke et al. 2005), the functional relevance of these temperature-dependent miRNA changes was not probed experimentally. Furthermore, it becomes apparent that a large number of miRNAs regulate temperature-driven processes in a tissue- and species-specific manner, and further investigation is warranted to understand conserved miRNA-mRNA relationships in response to temperature changes at multiple timepoints.

4.3 Salinity and pH

In their diverse aquatic habitats, fish have evolved varied strategies to maintain osmotic homeostasis in environments with different and/or fluctuating salinity and pH (Greenwell et al., 2003). Recent evidence revealed that osmotic challenges result in context-dependent changes in histone modifications, DNA methylation and miRNA abundance in several tissues crucially involved in osmoregulation in cypriniformes, especially gill and kidney. In the gill tissue of the euryhaline teleost *Gillichtys mirabilis*,

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hypoosmotic stress led to an acute and significant decrease in phosphorylated H3 protein abundance, a marker of cell division, while histone H2b was induced at the gene expression level (Evans and Somero, 2008). Conversely, the mRNA abundance of the histone H1 family member was downregulated under hyperosmotic stress (Evans and Somero, 2008). Together, these data show that histone-dependent modification is involved in gill tissue plasticity in response to context-dependent osmotic challenges.

Global DNA methylation changes detected by methylation-sensitive amplified polymorphism (MSAP) were identified between gills of freshwater- and seawater-reared brown trout, *Salmo trutta* (Morán et al., 2013). Similarly, in the same study, DNA methylation changes were also observed following the administration of a salt-enriched diet in freshwater reared brown trout, albeit in the short term only (Morán et al., 2013). Gene specific DNA methylation changes have been described in the half smooth tongue sole, *Cynoglossus semilaevis*, in response to salinity exposure of 0.15% (Li et al., 2017). Specifically, this exposure resulted in retarded growth and was associated with high liver insulin growth factor *(igf)* gene methylation and conversely decreased *igf* mRNA abundance level (Li et al., 2017). No germline-dependent effects of osmotic challenges and underlying DNA methylation patterns have been investigated in teleost fishes to date.

A series of studies in Nile tilapia showed that osmotic stimuli regulate gill and kidney miRNAs and are functionally involved in mediating molecular and organismal level responses to maintain homeostasis when exposed to osmotic stress (Yan et al., 2012a, 2012b; Zhao et al., 2016). In gill tissue, osmotic stress decreases miRNA-429 expression,

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which in turn has been shown to upregulate osmotic stress transcription factor 1 (Ostf1) protein abundance (Yan et al., 2012b). In kidney tissue, miRNA-30c inhibition leads to a loss of osmotic stress tolerance, which, at least in part, may be related to its ability to regulate Hsp70 protein abundance *in vivo* (Yan et al., 2012a). In tissue and cells of Nile tilapia, miR-21 is inhibited under alkaline stress, leading to induction of potentially protective vascular endothelial growth factors *vegfb* and *vegfc* at the mRNA level (Zhao et al., 2016). In zebrafish, studies examining ionocytes in embryos identified members of the miRNA-8 family to bind to the Na^+/H^+ exchanger regulatory factor 1 (*nherf1*), and knockdown of miRNA-8 family members caused increased rates of edema, suggesting a functional role of this pathway in ionocyte-dependent osmoregulation (Flynt et al., 2009). In Atlantic salmon, context-dependent changes in miRNA have been observed: an acidic challenge altered the muscle miRNA profile, although these fish were co-exposed to 80 μg/L aluminum, so the effect of the acidic challenge alone is unclear (Kure et al., 2013). In three-spined stickleback, *Gasterosteus aculeatus*, a model organism to study the molecular mechanisms of freshwater adaptation and speciation, a comparative study of gill tissue of marine and freshwater populations revealed that a total of 10 miRNA genes were localized in so called genomic ‘divergence islands’, which are characterized by a high content of SNPs, suggesting a possible role for differential regulation of these miRNAs in the evolution of freshwater tolerance (Rastorguev et al., 2016). To date no inter- or transgenerational studies on osmoregulation have been conducted in teleost fish.

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4.4 Nutrition

Teleost fish exhibit a wide diversity of feeding strategies, and are represented on practically every trophic level, from herbivores to tertiary predators to decomposers (Gerking, 1994; Braga et al., 2012). As nutrition is of particular interest to aquaculture (Naylor et al., 2009), it is not surprising that most studies investigating nutritional epigenetic regulation in teleost fish stem from a variety of aquacultured species (Panserat and Kaushik, 2010) in addition to zebrafish (Ulloa et al., 2014). Context-dependent nutritional regulation of histone modifications has been described in zebrafish, rainbow trout and blunt snout bream. In muscle of zebrafish, which had been re-fed after a 7d starvation period, the expression of the histone modifying enzymes, *smyd1b* and *ehmt1b* was induced 5-fold, suggesting a role for histone modifications in acute nutritional regulation of the muscle transcriptome (Amaral and Johnston, 2011). Zebrafish myogenic precursor cells starved in a medium devoid of serum and amino acids were shown to induce the expression of several autophagy related genes, which coincided with differential changes of H3K27me3, H3K9me3, and H3K4me3 histone modifications in the promoter region (Biga et al., 2017). In carnivorous and glucose-intolerant rainbow trout, acute feeding of a carbohydrate-rich diet resulted in hyperglycemia as well as global and promoter specific changes of histone marks in the liver (Marandel et al., 2016). Specifically, permissive H3K9ac histone marks were globally increased in rainbow trout re-fed with a protein-rich diet, while H3K36me3 marks displayed greater enrichment around the TSS of specific gluconeogenic gene loci of trout fed a protein-rich diet

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compared to trout fed a carbohydrate-rich diet. Equally in rainbow trout, early life vitamin intake resulted in global H3 acetylation, H3K4 methylation and changes in hepatic DNA methylation (Panserat et al 2017). This is the first evidence to suggest that short-term vitamin stimulus during early life results in gene expression changes driven, at least in part, by methylation and acetylation of histones. In the seabass, *Dicentrarchus labrax*, plant diet supplementation with sodium butyrate, a fermentation by-product, for a period of 8 weeks increased the acetylation level of hepatic H4K8 twofold, while increasing *ehmt2 and hdac11* mRNA abundance in liver (Terova et al., 2016). This coincided with regulation of cytokine-encoding transcripts, however whether both phenomena are linked was not addressed in the study.

Nutritional context-dependent changes in DNA methylation have been reported in both cypriniformes and salmoniformes. In a zebrafish model of diabetes, hyperglycemia results in context-dependent genome-wide hypomethylation of specific CpG sites, which have been shown to be mitotically transmissible to previously unexposed daughter cells during tissue regeneration, providing evidence for their involvement in metabolic memory in impaired wound healing (Olsen et al., 2012). In response to diet-induced hyperglycemia, global and specific DNA methylation changes have been observed in the liver of rainbow trout (Marandel et al., 2016). Specifically, rainbow trout fed a carbohydrate-rich diet exhibited global hypomethylation of hepatic DNA compared to trout fed a protein-rich diet, while hypomethylation of CpG sites in putative promoter sequences of specific *g6pc2* paralogues (glucose-6-phosphatase) correlated with their paradoxical induction in response

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to a high carbohydrate diet (Marandel et al., 2016). Dietary restriction of methionine, a nutritional precursor for the DNA methylation process, resulted in a phenotype of improved glucose tolerance in the same species but did not coincide with global DNA methylation changes in the liver. The liver did however exhibit a tendency for hypomethylation in response to a carbohydrate rich diet in the same study (Craig and Moon, 2013), and analysis of promoter and/or gene-specific DNA methylation changes may provide a more informative link between DNA methylation and physiological phenotype.

Context-dependent nutritional regulation of miRNAs has equally been studied in zebrafish and aquaculture species, largely focusing on nutritional regulation of key metabolic pathways related to macronutrient utilization and tissue differentiation (Mennigen, 2015). In zebrafish, a microarray was used to determine changes in hepatic miRNA and 9 differentially expressed miRNAs were identified, among which *let7-d* and *miR-140-5p* were inversely correlated to predicted target mRNA abundance of the α1 and α2 subunits of the metabolic sensor adenosine monophosphate-activated protein kinase *ampk* (Craig et al., 2014). In rainbow trout liver, postprandial induction of the highly abundant and liver-specific miRNA-122 (Mennigen et al., 2012) was shown to be dependent on insulin and/or macronutrient composition (Mennigen et al., 2014b), and to be functionally involved in the regulation of postprandial glucose and triglyceride homeostasis, likely by regulating fatty acid synthase protein abundance (Mennigen et al., 2014a). In other aquaculture species, macronutrients have also been shown to affect the miRNAs in key metabolic tissues. Studies in the glucose-tolerant blunt snout bream,

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Megalobrama amblycephala, showed that a high starch diet differentially regulated miRNAs in intestine, liver, and brain using next generation sequencing, which were predicted to target genes in insulin signaling, and carbohydrate and lipid metabolic pathways (Miao et al., 2017). In the same species, a high lipid diet equally affected the miRNA profile in the liver and was predicted to target key genes in lipid metabolism, including fatty acid synthase and NADH dehydrogenase (D. Zhang et al., 2014).

Possible intergenerational effects of nutritional programming are beginning to be explored in aquaculture species, for example in rainbow trout adapted to plant-based diets (Lazzarotto et al., 2015; Lazzarotto et al., 2016), however the possible involvement of epigenetic mechanisms in identified transcriptomic changes was not explored in these studies.

4.5 Conspecific and heterospecific social interaction

Among the studies investigating environmental regulation of epigenetic marks in teleost fish, recent evidence suggests involvement of epigenetic mechanisms in response to social interaction in several teleost fish species. For example, a role for histone modifications in response to short, intense interaction with conspecifics comes from a recent study of territorial male three-spined sticklebacks, which were exposed to a brief territorial challenge (Bukhari et al., 2017). In the brain of challenged males, H3K27ac, a marker of chromatin accessibility, changed dynamically and rapidly at several genome loci, as evidenced by 30- to 40-fold changes for individual peaks as early as 0.5 and 2h following the encounter. Differentially expressed genes included those involved in immune function

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and long-term potentiation, suggesting roles for brain histone modifications in recovery, and preparation for future encounters (Bukhari et al., 2017). With regard to context-dependent DNA methylation, a recent manipulative study in the African cichlid fish, *Astatotilapia burtoni*, revealed that pharmacologically induced global hypomethylation changes significantly decreased the likelihood of becoming socially dominant when competing with a conspecific for 30 min (Lenkov et al., 2015). The treatment administered to reduce global methylation, which significantly inhibited social ascension, was specifically linked to increased methylation of a CpG island in the *gnrh* gene, which is known to be rapidly induced in socially ascending males, ostensibly to promote reproductive success (Lenkov et al., 2015). With regard to longer developmental timeframes, different early life social experience (in the form of exposure to parents and sibling conspecifics compared to siblings alone) in the cooperatively breeding cichlid *Neolamprologus pulcher* resulted in lower brain expression of *crf* (corticotropin releasing factor) and *gr1* (glucocorticoid receptor 1; both involved in hypothalamic–pituitary–interrenal (HPI) axis regulation) 1.5 years after the social stimulus (Taborsky et al., 2011), suggesting programming via molecular epigenetic mechanisms. However, specific epigenetic mechanisms were not investigated in this study. Evidence for a role of miRNAs in teleost behaviour includes a study in juvenile zebrafish, which develop hyperactivity following developmental exposure to ethanol (Tal et al., 2012). Specifically, the knockdown of miRNA-9 or miRNA-153, which are aberrantly expressed under ethanol exposure, persistently phenocopy the effect of ethanol to increase swimming, an effect

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linked to changes in brain morphogenesis and craniofacial development (Tal et al., 2012). Whether miRNAs in teleost fish can, in addition to their developmental effects, act as direct mediators of rapid behavioural plasticity as described for histone modifications and DNA methylation is currently unknown. However, recent findings in the fruitfly, *Drosophila melanogaster,* indicate that miRNAs are capable of mediating rapid behavioural responses (Picao-Osorio et al., 2015; Picao-Osorio et al., 2017) and an involvement of miRNAs in diverse acute behavioural responses in teleost fish will provide for an interesting avenue of future investigation.

The involvement of molecular epigenetic mechanisms in possible inter- and transgenerational transmission of social interaction on teleost fish behaviour have not yet been widely investigated in fish, in spite of its direct consequences on organismal fitness. In three-spined sticklebacks, paternal care reduces indices of offspring anxiety, a trait considered to render offspring more vulnerable to predators (McGhee and Bell, 2014). Importantly, reduced anxiety in offspring exposed to paternal care co-related with brain expression of the *de novo* DNA methyltransferase *dnmt3* (McGhee and Bell, 2014), suggesting a context-dependent inter-generational epigenetic effect that is mediated by DNA methylation.

4.6 Pathogens

The environmental regulation of the teleost immune system is widely studied to investigate innate and adaptive immune responses in a basal vertebrate, and to limit disease outbreaks in aquaculture settings (Levraud and Boudinot, 2009; Rubio-Godoy, 2010).

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Similar to other environmental factors, pathogens have been shown to cause histone modifications, DNA methylation changes and alteration of miRNA abundance in teleost immune tissues. For example, microbial priming in zebrafish is facilitated by chromatin modifications in the form of H3K9ac and H3K4me3 marks in promoter regions of the cytokine-encoding genes *il1β*, *il12α*, and *tnfα* following treatments with either trichostatin A, or pargyline, which act to inhibit histone deacetylase and LSD1, a H3K4 demethylase. These histone modifications result in an increased expression of these cytokines in response to DNA from the pathogen *Vibrio anguillarum* prior to the development of adaptive immunity (Galindo-Villegas, 2012). In adult zebrafish with developed adaptive immunity, exposure to a chronic disease inducing strain (E11) of pathogenic mycobacteria caused significant downregulation of several histone family members compared to exposure to an acute disease-inducing strain (Mma20). This suggests an involvement of histone modifications in response to chronic disease inducing strains, which may be related to the coincidental regulation of genes involved in immune system development (Van der Saar et al., 2009). With regard to salmoniformes, histone variants themselves have long been shown to have direct antimicrobial properties, as exemplified by histone variants found in Atlantic salmon and rainbow trout (Richards et al., 2001; Fernandes et al., 2004). However, the role of epigenetic histone modifications in response to pathogens in salmoniform species has not yet been explored.

With regard to pathogen-induced context-dependent DNA methylation changes, a series of studies investigating DNA methylation in grass carp, *Ctenopharyngodon idella*,

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in response to grass-carp reovirus (GCRV) identified differential gene-specific (Shang et al., 2015; Shang et al., 2016) and genome-wide DNA methylation patterns (Shang et al., 2017). Specifically, hypermethylation of individual CpG sites in the flanking and coding regions of *RIG1* and *MDA5* was found to be inversely correlated the encoded mRNA abundance in the spleen and associated with increased disease susceptibility.

Compared to histone modification and DNA methylation, miRNAs have been heavily studied in tissues important in mounting immune responses to viral and bacterial exposures in many teleost fish, and this field of research has also been advanced by studying many aquaculture species of economic interest. In some teleost species, miRNAs have been shown to contribute to host defense by directly targeting viral transcripts. In a greasy grouper spleen cell line (*Epinephelus tauvina*), and a fathead minnow epithelial cell line (*Pimephales promelas*), miR-13 was induced in Singapore grouper iridovirus (SGIV)-infected cells and targeted viral SGIV-MCP (major capsid protein) mRNA, resulting in blockade of MCP translation, and attenuation of viral assembly (Yan et al., 2015). In SSN1 cells of the snakehead, *Channa striata*, infection with snakehead vesiculovirus (SHVV) resulted in down-regulation of miRNA-214, which interferes with viral replication by targeting N and P proteins, suggesting co-evolution of miRNA and pathogen (C. Zhang et al., 2017b). Conversely, in common carp, CyHV3 encoded miRNA MR5057-mir-3p directly regulates gene expression of a CyHV3 encoded dUTPase, while the regulation of possible host targets was not investigated in this study (Donohoe et al., 2015). In a detailed review investigating the regulation of host cell miRNAs by viral and bacterial infections in

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teleost fish, Andreassen and Høyheim (2017) identified distinct (and often teleost-specific) miRNA expression patterns in response to viral infection, bacterial infection, or both, which were postulated to control the time-dependent activation and inhibition of specific host immune responses. Pathogen-dependent regulation of epigenetic marker in the germ-line, and possible consequences on transgenerational phenotypes have not been described in teleost fish to date.

4.7 Anthropogenic effects on teleost epigenetics

In addition to environmental conditions discussed above, anthropogenic activity can influence teleost physiology indirectly, through the modification of naturogenic environmental factors discussed above, or directly in the form of aquatic contaminants. Examples of indirect anthropogenic factors affecting teleost physiology are, for example, hypoxic dead zones (Diaz and Rosenberg, 2008; S. Y. Wang et al., 2016), global warming (Wood and McDonald, 1997; Comte and Olden, 2017), ocean salinity changes (Ojaveer and Kalejs, 2005; Helm, 2010), ocean acidification (Doney et al., 2009; Welch et al., 2014) and algal-bloom derived microcystins (Liu et al., 2014; Brzuzan et al., 2016). As previously described, these environmental factors can elicit molecular epigenetic changes in teleost fish, which contribute to shaping physiological responses across different timescales. In additional instances, anticipated anthropogenic modulation has been linked to intergenerational manifestations of physiological phenotypes. For example, exposure of damselfish, *Acanthochromis polyacanthus*, to acidic environments mimicking ocean acidification levels has been shown to reverse the reaction to a chemical alarm cue within

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the exposed generation as well as in the F_1 generation relocated to control conditions (Welch et al., 2014). Adult zebrafish exposed to microcystin-LR produce offspring which exhibit diminished growth and reduced immune function (Liu et al., 2014). Together, these and other emerging studies show that molecular epigenetic consequences in response to anthropogenic changes in environmental factors warrant further investigation, as these markers may be predictive of physiological disruption or potential for adaptation in the organism or its offspring.

With regard to direct consequences of man-made aquatic pollutants on teleost epigenetics, several classes of contaminants have been linked to histone modifications intragenerationally. Chronic cadmium and copper exposure in a wild population of yellow perch, *Perca flavescens*, for example, revealed a metabolic and immune impairment as determined at the hepatic transcriptome level (Pierron et al., 2011). These gene expression changes may have been driven through histone modifications, which were equally identified as being enriched in the transcriptomic dataset (Pierron et al., 2011). A study conducted in European eels, *Anguilla anguilla*, which investigated environmental contaminants such as cadmium, mercury, polychlorinated bisphenyls (PCBs) and organochloride pesticides in a laboratory setting and compared transcriptomic responses to a wild population may serve as cautionary example, as laboratory housing, rather than contaminants, identified an enrichment of histone modification in the transcriptome (Baillon et al., 2016). This study therefore underlines that care must be applied when

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extrapolating contaminant induced epigenetic regulation between wild and laboratory raised animals.

The plasticizer and endocrine disrupting chemical Bisphenol A (BPA) has recently also been shown to modulate histone marks in ovaries of sexually mature zebrafish, which had been exposed to BPA concentrations consistent with environmental exposures for a period of three weeks (Santangeli et al., 2016). Specifically, BPA exposure decreased the permissive H3K4me3 mark and conversely caused an enrichment of the silencing H3K27me3 mark in the transcription start site (TSS) of the *star* and *fshr* genes, which play roles in steroidogenesis, oocyte growth, and oocyte MATURATION (Santangeli et al., 2016). Since these changes coincided with a BPA induced atresia, zona radiata breakdown, and yolk resorption at the tissue level and complete impairment of fertility after 8 days of treatment at the organismal level, this study implies that underlying histone modifications play a role in BPA-induced reproductive disruption in female zebrafish. To our knowledge, inter- and transgenerational studies of contaminant-induced histone modifications, either in gametes or specific tissues in subsequent generations have not yet been explored in teleost fish.

DNA methylation changes in response to aquatic contaminants have been more widely studied in teleost fish, and have largely focused on determining context-dependent changes in expression profiles of *dnmt* paralogues, Dnmt activity and differential global or specific DNA methylation, the latter of which have been assessed both genome-wide and in promoter regions of specific genes. These studies have almost exclusively been

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conducted in the zebrafish model, testifying to this emerging model's promise to further our understanding of the effects of contaminant exposure on mechanisms regulating DNA methylation dynamics (Kamstra et al., 2015a; Bouwmeester et al., 2016). As such, changes in zebrafish DNA methylation indices have been reported in response to a wide-range of aquatic contaminants, including metals (Gombeau et al., 2016, Zheng et al., 2017; Sanchez et al., 2017; Carvan et al., 2017), industrial products (Laing et al., 2016; Y. Liu et al., 2016; Santangeli et al., 2016; Zhao et al., 2017), pesticides (Wirbisky-Hershberger et al. 2017, Qian et al. 2017), persistent organic pollutants (POPs; Aluru et al. 2014), and polycyclic aromatic hydrocarbons (PAHs; Gao et al. 2017; Kuc et al., 2017). With regard to metals, adult zebrafish exposed to 20 µg/L depleted uranium exhibited both sex- and tissue-specific changes in global DNA methylation between 7-24 days (Gombeau et al., 2016). Exposure of adult zebrafish to 200 µg/L of cadmium following differential rearing at temperatures of 26°C and 34°C for 4 days, revealed a differential effect on methylation of four CpG sites in the promoter and first exon of *hsp70* in the liver (Zheng et al., 2017), providing evidence for intragenerational integration of specific DNA-methylation marks between naturogenic and anthropogenic factors. Zebrafish exposure to 500 ug/L of lead resulted in reduction of *dnmt3* and *dnmt4* expression, which coincided with significant decrease in global methylation levels in the embryo (Sanchez et al., 2017). For industrial products, consequences of exposure to the endocrine disrupting chemical BPA on DNA methylation have been investigated in significant detail in zebrafish, focusing largely on gonadal tissue indices due to observed inhibition of reproductive success. For example, in female

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zebrafish exposed to 5 μg/L BPA, which as previously described exhibited ovarian histone modifications, increases in ovarian *dnmt1* and *dnmt3* and concurrent decreases in *dnmt4, dnmt6* and *dnmt7* transcripts were equally reported, suggesting action of multiple molecular epigenetic mechanisms in contaminant-induced genome regulation (Santangeli et al., 2016). Exposure of breeding pairs to BPA concentrations of 1 mg/L for a period of 15 days resulted in global hypomethylation in ovaries and testes, which coincided with decreased *dnmt1* expression in female ovaries and a decrease in egg fertilization at the organismal level (Laing et al., 2016). Similarly, global DNA methylation was decreased in zebrafish testes following exposure to 15 ug/L BPA for 7 days and in ovaries following exposure to the same concentration of BPA for 35 days (Y. Liu et al., 2016), suggesting that temporal dynamics in contaminant-induced DNA methylation may differ between sexes. In addition to differential regulation of *dnmt* transcript abundances, this study also identified BPA-dependent regulation of *tet* transcripts, pointing to a role for both DNA methylation and demethylation pathways in the effects of BPA on gonadal DNA methylation dynamics (Y. Liu et al., 2016). A gene promoter-specific investigation of BPA effects on DNA methylation in zebrafish was provided by Zhao et al. (2017), who linked the induction of hepatic *esr1* transcripts and of gonadal aromatase *cyp19a1* transcripts in response to a 21 day exposure of 500 and 1500 μg/L BPA to decreased promoter CpG methylation. In addition to these described peripheral effects, BPA exposure in zebrafish also induced long-lasting changes in central regulation of the reproductive axis, as evidenced by the induction of embryonic transcript abundance of *kiss1*, *gnrh3 and fshβ*

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(Qiu et al., 2016). However, epigenetic mechanisms, which may be responsible for longer-term developmental effects on these targets, have not been investigated in this study. Of note, these intra-generational effects of BPA on central components of the endocrine axis of reproduction do not appear to be restricted to zebrafish, as early development BPA exposures equally affected *gnrh3* neuronal developmental trajectories in Japanese medaka (Inagaki et al., 2016). Together, these results suggest possible widespread developmental disruption by BPA, which similar to findings in mammals, is at least partially mediated by context-dependent epigenetic mechanisms (Kundakovic et al., 2011). However, the overall comparability of several studies investigating molecular epigenetic effects in teleost fish are restricted by the choice of specific methods used, which can, as discussed for DNA methylation, affect sensitivity and resolution (Kurdyukov and Bullock, 2016). Global DNA methylation is indirectly assessed by measuring *dnmt* and *tet* mRNA abundance or the activity of their proteins, as well as directly by quantifying global DNA methylation. Global DNA methylation, while easier to obtain, is less meaningful than specific DNA methylation measurements, as it does not allow the linking of specific epigenetic marks to specific gene expression. This can be addressed using higher resolution measurements including genome-wide or gene-specific bisulfite conversion or MeDIP approaches.

Pesticide exposures in the form of atrazine administered at a concentration of 30 μg/L throughout zebrafish embryonic development (1-72 hpf), demonstrated a significant decrease in global methylation, and a concomitant downregulation of *dnmt4* and *dnmt5* transcripts in whole embryos (Wirbisky-Hershberger et al., 2017). Using a MeDIP

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sequencing approach, Qian et al. (2017) identified differentially regulated DNA methylation regions (DMRs) in embryonic DNA from zebrafish that had been exposed to 800 μg/L fipronil between 6-120 hpf. In addition to different DNA methylation patterns that depended on the enantiomer used, DMRs largely affected target genes involved in developmental cell signaling and cellular adhesion processes, which correlated with observed developmental toxicity. Zebrafish exposed to 5 nM of the POP 2,3,7,8-tetrachlorodibenzo-p-dioxin between 4-5 hpf revealed differential regulation of *dnmt* transcripts, with upregulated *dnmt1* and largely downregulated *de novo dnmt3* paralogue transcripts. While these changes did not coincide with global changes in DNA methylation, they did result in CpG methylation changes in specific *ahr* (aryl hydrocarbon receptor) target genes although this did not correspond to changes in *ahr* transcript abundance (Aluru et al., 2015). Zebrafish exposed to the PAH benzo[a]pyrene displayed an upregulation of *dnmt*1 and *dnmt3a* transcripts in embryos immediately after exposure at 96 hpf, which persisted in the adult brain (Gao et al., 2017). Gene promoter specific DMR analysis via MeDIP RNA-seq in conjunction with real-time RT-PCR measurement of target mRNA revealed that *gucy2f* and *drd4* (genes involved in nervous system development) were hypermethylated, and correlated with decreased transcripts in embryo and adult brain (Gao et al., 2017). This suggests long-term programming effects on these genes by DNA methylation in response to embryonic exposure which may contribute to neurodegenerative syndromes in adult zebrafish (Gao et al., 2017).

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The consequences of aquatic contaminants on teleost DNA methylation have also been studied in inter- and transgenerational contexts, albeit to a much lesser extent. F_1 and F_2 generation embryos derived from adult females which had been subjected to 47 day and 32 day exposures of 10 mg/kg MeHg or 20 µg/kg TCDD and mated with unexposed males, revealed no effect on global methylation, but modest changes in specific promoter methylation identified by MeDIP tiling array (Olsvik et al., 2014). However, these marks generally did not correlate to transcript changes as measured by microarray and real-time RT-PCR, suggesting that other mechanisms may be involved in driving the gene expression changes that underlie the observed cardiovascular and neurological effects in the offspring of exposed female zebrafish (Olsvik et al., 2014). Importantly, this study provides evidence for contaminant-induced maternal inheritance of DNA methylation patterns in zebrafish, limiting the prediction that DNA inter- and transgenerational DNA methylation patterns may be largely paternally inherited (L. Jiang et al., 2013; Potok et al., 2013; Kamstra, 2015a). Similarly, a recent study in F_1 and F_2 generation larvae derived from F_0 zebrafish, which had been exposed to 30 µM of mono(2-ethylhexyl) phthalate (MEHP) during early embryonic development (0-6 dpf; days post-fertilization), also challenges the paradigm of exclusive paternal inheritance of DNA methylation patterns in response to contaminant exposure (Kamstra et al., 2017). This is based on the finding that while a few transgenerationally stable DMRs, such as the hypermethylated *cbfa2t2* locus, were identified in 6 dpf old embryos, its methylation status in F_0 sperm was found to be hypomethylated, suggesting non-paternal contributions. Another recent study in zebrafish

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revealed that exposure of F_0 zebrafish to MeHg resulted in hyperactivity and visual deficits in unexposed F_2 descendants compared to control lineage animals, which correlated with DMRs of genes in F_2 zebrafish sperm (Caravan et al., 2017). Because these genes were shown to be involved in in neuroactive ligand receptor interaction and actin-cytoskeleton modulation (Caravan et al., 2017), it was suggested that these 'epimutations' may be markers and mediators of the observed transgenerational physiological effects. However, the developmental fate of these marks within a generation was not addressed in this study and it is unclear whether these sperm 'epimutations' translated into conserved marks in somatic cells (Caravan et al., 2017). Finally, F_0 zebrafish exposed to 5-10 μM Benzo[a]pyrene between 6-120 hpf revealed decreased oxygen consumption and lower heart rates, a physiological phenotype that was equally present in the F_2 offspring. These effects correlated with decreased total embryonic DNA methylation and reduced transcript abundance of several *dnmt* paralogues in the F_0 generation, an effect dependent on Ahr2 receptor recognition of Benzo[a]pyrene (Knecht et al., 2017). However, since epigenetic marks in gametes were not profiled in this study, a possible contribution of molecular epigenetic mechanisms for the transgenerational inheritance of this physiological phenotype warrants future study. Overall, while there is clear evidence for context-dependent DNA methylation in response to aquatic contaminants, the extent to which this contributes to observed phenotypes remains to be elucidated. Similarly, while there is evidence for inter- and transgenerational inheritance of DNA methylation, current evidence contradicts a mere paternal inheritance of paternal DNA methylation patterns altered by

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contaminants. Future studies are clearly needed to investigate the fate of contaminant-induced germ cell DNA methylation as well as the inheritance of contaminant-induced changes in gamete DNA methylation in somatic cells across development. At least at the level of gametes,the experiments described above clearly indicate the feasibility of gamete DNA methylation profiling (Potok et al., 2013; L. Jiang et al., 2013).

Context-dependent regulation of the miRNAome in tissues of a variety of teleost fish exposed to aquatic contaminants has been described for metals (Kure et al., 2013; Qiang et al., 2017), pharmaceuticals (Craig et al., 2014, Cameron et al., 2015), pesticides (Wirbisky et al., 2016) and algal toxins (Brzuzan et al., 2016). These studies generally identify miRNAs at the transcriptome-level (e.g. microarray, or small RNA sequencing) or individual miRNA level (real-time RT-PCR) and correlate these miRNAs to mRNA abundance of *in silico* predicted target genes. For example, the liver of adult zebrafish exposed to the pharmaceutical fluoxetine revealed an upregulation of *dre-let-7d* and *dre-miR-140-5p* correlated with a downregulation of its predicted targets, the α1 and α2 subunits of the energy sensor *ampk* (Craig et al., 2014). However, this study and current studies in general have not mechanistically addressed the functionality of these predicted miRNA-target relationships, and future studies using *in vitro* approaches such as transfection assays with miRNA agonists and antagonists or luciferase assays based on 3'UTR-containing plasmids are clearly warranted in teleost fish in order to substantiate physiologically meaningful miRNA-mRNA interactions in detail. As described, inter- and transgenerational effects of aquatic contaminants are increasingly reported at the

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organismal level in zebrafish whose ancestors had been exposed to aquatic contaminants. Similar to histone modifications, however, and in spite of the technical feasibility to profile microRNAs in fish gametes (Jia et al., 2015; Ma et al., 2015; Presslauer et al., 2017), no studies to date have investigated aquatic contaminant-induced changes in miRNAs in fish gametes. These studies are clearly warranted, given that alterations in the miRNAome in stress-exposed rats have been mechanistically linked to programming the stress-axis in their offspring, providing evidence for miRNA-mediated intergenerational inheritance. (Rodgers et al., 2013; Rodgers et al., 2015).

5. Future basic research directions and possible translational research benefits

Based on the reviewed information, we will here integrate emerging insights to propose future directions for research in the field of teleost fish epigenetics. The first section will identify research areas of priority in teleost epigenetics and critically address the utilization and current limitations of current experimental designs and methodologies in the field. The second section will highlight how the developing area of basic research in teleost epigenetics can inform translational research related primarily to aquaculture and aquatic toxicology, but also human health.

5.1 Basic research priorities for teleost epigenetics and experimental considerations

At the basic research level, epigenetic research in teleost fish is currently largely focused on the zebrafish model, which is considered to be well-suited to link molecular epigenetics to the developmental and transgenerational emergence of physiological

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phenotypes given its sequenced and comparatively well-annotated genome, and available tools for genome-wide or gene-specific profiling and genetic manipulation. Even within the zebrafish model, however, there is a need to increase precision in our understanding of epigenetic pathways at the molecular level in order to link molecular function to physiological relevance. While this can be seen as a historically rooted 'schism' between molecular and developmental or phenotypic understanding of epigenetics, this becomes even more important in zebrafish, where clear differences in the epigenetic toolbox, dynamics and genomic location of epigenetic marks, and their inter- and transgenerational inheritance exist. For example, the roles of teleost-specific *dnmt3b* paralogues and/or teleost-specific miRNAs remain uncharacterized, and should be addressed by CRISPR-Cas9 or morpholino-based approaches in future studies, which have successfully been employed not only in zebrafish, but increasingly in salmoniform species (Wargelius et al., 2016). Additional diversity in the epigenetic toolbox stems from the fact that several components have been retained as paralogues in subsequent rounds of genome duplication, and as suggested by our *in silico* analysis of salmoniform *xpo5,* it is possible that these paralogues have undergone sub-functionalization. This represents another avenue of research that will undoubtedly contribute to better understanding of basic teleost epigenetics.

Outside the immediate toolbox repertoire, epigenetic mark dynamics and functionality reveal both similarities and differences compared to better studied invertebrate or mammalian models. With regard to histone modifications, research largely

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suggests a conservation of histone marks, which, however remain almost exclusively characterized across baseline development in zebrafish and largely omit comparative studies in response to environmental stimuli in this species. In spite of its feasibility, as recently demonstrated in rainbow trout (Marandel et al., 2016), histone modifications in other teleost species remain understudied. In contrast, DNA methylation dynamics in zebrafish are studied in baseline development as well as in response to environmental stimuli, but reveal important functional differences in location and generational transmission. Thus, future genome-wide correlative studies are needed to improve our understanding of DNA methylation loci, as DNA methylation in CpG sites in promoter regions has not always been reported to be the best predictor of gene expression profiles. Emerging studies employing CRISPR-Cas9 based constructs containing catalytic components of Dnmt (Vojta et al., 2016; Lei et al., 2017), or Tet proteins (Morita et al., 2016) have been shown to specifically methylate and demethylate genomic target sequences *in vitro* and *in vivo*, and may allow this question to be addressed functionally by site-specific methylation of promoters, introns, exons, and putative enhancers in zebrafish.

Finally, miRNA networks appear to have undergone extensive rewiring in teleost fish compared to well-studied mammalian models, suggesting possible functional differences at higher levels of biological organization. Currently miRNAs remain the only molecular epigenetic mark that can be directly modulated experimentally with high specificity, for example through antagomiRs, which have been used in an increasing number of teleost fish species *in vitro* and *in vivo* (Mennigen et al., 2015). Conversely,

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functional consequences of methylation and histone modification on individual loci may currently only indirectly be addressed either by global, non-specific pharmacological modulation of epigenetic marks, or by mimicking consequences of specific epigenetic by targeting the regulated gene locus using previously described CRISPR-Cas9 or morpholino-based techniques. In contrast to antagomiR-based miRNA modulation, such modulation will be indirect and less precise, thus limiting our understanding of the functional consequences of specific epigenetic marks at the DNA level. Regardless of the specific epigenetic mechanism studied, previously described genome duplication events have also resulted in the retention of many protein coding genes targeted by epigenetic regulation, adding further complexity to teleost epigenetics. Therefore, as indicated by some pioneering studies, the role of epigenetic mechanisms in the regulation of teleost paralogues warrants further study, as this will improve our understanding of how teleost fish successfully evolved to inhabit diverse ecological niches.

Experimentally, four approaches may prove especially useful to improve our capacity to link epigenetic marks to gene expression and, ultimately, physiological phenotypes. Firstly, epigenetic marks are currently widely studied in isolation, which, in addition to potential differences in the genomic location of regulatory epigenetic marks, may, at least in part, explain the often poor correlations between permissive or repressive epigenetic marks and gene expression. For example, as in mammals, interaction between all major molecular epigenetic mechanisms has been described in teleost fish correlationally as well as mechanistically. For example, correlative evidence for cross-talk

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between histone modifications and DNA methylation stem from studies in zebrafish, where CG-rich promoters have been shown to combine hypomethylation and H3K4 trimethylation marks, indicative of concerted action permissive of gene expression (Andersen et al., 2012). Conversely, a strong antagonism between DNA methylation and the histone mark H3K27me3, has been identified at the genome level across three development stages (dome, 24 hpf, 48 hpf) in early zebrafish development (de la Calle Mustienes et al., 2015). Alongside correlative analysis of epigenetic marks at the genome level, molecular components mediating crosstalk between epigenetic marks are beginning to emerge as well. For example, Dnmt1 knockdown-mediated defects in development of intestine, exocrine pancreas, and retina coincided with reduced H3K9me3 levels, which are established by Suv39h1. Knockdown of Suv39h1 and rescue experiments introducing functional Suv39h1 in Dnmt1 knockout fish clearly revealed that the histone-modification enzyme Suv39h1 mediates Dnmt1 effects on organogenesis (Rai et al., 2006). Equally in zebrafish, it has been determined that the H3 demethylase *kdmb2* recognizes demethylated CpG sites, conferring specificity to its mediated histone modifications (Farcas et al., 2012). Evidence for crosstalk between DNA level epigenetic modifications and microRNAs remains correlational, as seen in studies using zebrafish and rainbow trout. For example, a strong occupancy of pri-miRNA promtors by H3K4me3 has been observed in zebrafish embryogenesis (Nepal, 2016), while in rainbow trout, a recent gene expression study coupled with *in silico* predictions suggested an evolutionary deeply conserved crosstalk between *miRNA-29* and a *dnmt1* paralogue (Kuc et al., 2017). Together, these studies

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clearly point to crosstalk between epigenetic mechanisms in teleost fish. Furthermore, emerging molecular epigenetic mechanisms, such as long non-coding RNAs, which confer specificity to recruitment of the epigenetic machinery at the DNA level (Mercer et al, 2009), are increasingly characterized in teleost models, including zebrafish (Dhiman et al., 2015), rainbow trout (J. Wang et al., 2016; Al-Tobasei et al., 2017) and Atlantic salmon (Boltana et al., 2016). An additional example is the recent description of methylated adenosines (6mA) in zebrafish embryogenesis (J. Liu et al., 2016), which have now been shown to play a role in maternal mRNA clearance (Zhao et al., 2017) and early cell fate determination (C. Zhang et al., 2017a). Both examples testify to the dynamic nature of the field of teleost epigenetics.

A second experimental consideration addresses an important aspect of the epigenetic definition, that of spatiotemporal control. With regard to location, current information in teleosts clearly suggests that epigenetic mark dynamics change in tissue specific development, and future studies are therefore warranted to improve cell population-specific resolutions. The described need to identify the fate of epigenetic marks of PGCs represents an excellent example, and given that GFP-based cell sorting of PGCs has been achieved in teleost fish (Fan et al., 2008; Kobayashi et al., 2014), future studies may utilize this technique to achieve better specificity. With regard to temporal aspects of epigenetic regulation in teleost fish, current studies generally link epigenetic marks and gene expression profiles at a single time point, which may reduce correlations between specific epigenetic marks and gene expression by ignoring the temporal relationships

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between the establishment of epigenetic marks and gene expression. Therefore, similar to a previous suggestion to resolve observed discrepancies between mRNA and protein abundance in teleost fish tissues sampled at single timepoints (Popesku et al., 2010), detailed time-series experiments are therefore warranted for epigenetic marks. Since cost likely represents a limiting factor, the identification of consistently altered mRNA abundance of specific targets in response to experimental conditions may be identified from deposited transcriptomic time-series datasets, allowing pre-screening of specific genes for subsequent determination of epigenetic marks. In addition to developmental stages, this also applies to inter- and transgenerational timescales, for which the characterization of epigenetic marks in gametes and embryos (and specifically their PGCs) before and after MZA are especially warranted. Experimentally, very few studies currently address paternal and maternal contributions in teleost fish, and experimental outcross designs employing experimentally treated males and females mated with wild types of the opposite sex up to the F_2 generation (**Figure 2**) will allow prioritization of the investigation of epigenetic marks in sperm and eggs. Transgenerational experiments will also address specific developmental windows susceptible to context- and germline- dependent epigenetic reprogramming which may alter the emergence of physiological phenotypes across the life-cycle and generations. This will allow, for example, testing of the hypotheses that exposure to a single stimulus translates to longer propagation of altered physiological phneotypes, or that germ-line dependent epigenetics alter physiological responses of future generations to separate environmental stimuli acting by context-

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dependent epigenetics. In this context, it will be increasingly important to clearly separate transgenerational from context-dependent effects, for example by excluding the possibility of maternal transfer of lipophilic hormones (Jeffrey and Gilmour, 2016) or contaminants (Aluru et al., 2010) in oocytes through the use of analytical chemistry methods.

Thirdly, an *a priori* understanding of genetic background and physiological states is equally warranted, as specific teleost strains (Burgerhout et al., 2017) or endogenous factors may factors regulate epigenetic marks in addition to environmental factors (**Figure 1**). The importance of endogenous factors is exemplified by the estrogen-dependent regulation of the abundant and liver specific miRNA-122 in zebrafish (Cohen and Smith, 2014; Cohen et al., 2017), which may introduce sex-specific functional differences in the nutritional regulation and metabolic function of miRNA-122 in fish (Mennigen et al., 2015). Indeed, widespread sexually dimorphic miRNA expression profiles have been identified in developing zebrafish (Vaz et al., 2015), emphasizing that genetic or endogenous endocrine signals contribute to environmental regulation of epigenetic marks in teleost fish.

Finally, because the large and diverse infraclass of teleost fish allows physiological systems to be studied in a variety of environmental and evolutionary contexts, future research should take advantage of the increasingly available teleost genome and transcriptome resources (Mennigen et al., 2015) to fully exploit the potential of this infraclass for comparative epigenetics. The Krogh principle has been historically applied to many fish species, and will undoubtedly lead to better insights into how epigenetics can

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govern physiological systems in extreme environmental conditions across the life cycle, between generations and finally at the evolutionary scale. Indeed, outside the most widely studied zebrafish model, genome-wide and gene-specific approaches to profile histone modification, DNA methylation and non-coding RNA abundance are now used in additional models, such as the rainbow trout (Mennigen et al., 2015; Seiliez et al, 2015; Baerwald et al., 2016; Marandel et al., 2016; Juanchich et al., 2016; Al Tobasei et al., 2016). This has been largely driven by the publication of the rainbow trout genome (Berthelot et al., 2014), showing that genomic resources, rather than technical approaches themselves, represent bottlenecks in expanding teleost epigenetic research to additional species.

5.2 Translational aspects of teleost epigenetics

In addition to the discussed implications for comparative physiology, the emerging field of teleost epigenetics holds promise for three principal areas of applied research, specifically aquaculture, ecotoxicology and human disease. We here briefly explore potential applied benefits of teleost epigenetic research in each of the three areas.

5.2.1 Aquaculture

In the context of aquaculture, the potential for teleost epigenetics has been recognized and for an in-depth discussion, the reader is directed to recent reviews on the subject (Li and Leatherland, 2012; Moghadam et al., 2015; Granada et al., 2017; Gavery and Roberts, 2017). For the purpose of the current review, we will limit our discussion to

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four principal translational benefits. Firstly, the understanding of developmental progression of epigenetic marks in conjunction with ontogenetic development allows for the development of meaningful markers, which given the strong role of epigenetic marks in controlling spatio-temporal gene expression, may represent a way to identify epigenetic biomarkers predictive of future tissue development, growth rates, food conversion, immune competence, reproductive success or other physiological traits relevant to aquaculture operations. Such marks are currently investigated not only at baseline, but also in programming approaches using developmental exposure to environmental factors such as nutrition, temperature, or hypoxia in an effort to modulate specific traits in adult aquaculture species directly. Such research has especially been conducted in the context of nutritional programming of aquaculture species (Geurden et al., 2013; Liu et al., 2017a; Liu et al., 2017b; Panserat et al., 2017) or relevant model species (Ulloa et al., 2014; Rocha et al., 2014, Rocha et al., 2015). Secondly, the identification of epigenetic markers may uncover molecular bottlenecks that may limit specific traits of importance in aquaculture, such as macronutrient-utilization. This is exemplified by glucose intolerance in rainbow trout, which recent evidence has linked to both DNA level epigenetic marks (Marandel et al., 2016) and hepatic microRNAs (Mennigen et al., 2014). Thus, uncovering novel epigenetic targets for nutritional modulation, for example, may lead to specific approaches targeting epigenetic bottlenecks to improve nutrient utilization. Thirdly, the identification of epigenetic markers may provide an additional dimension to complement purely genetic selection for breeding. For example, epigenetic marks are beginning to be characterized in

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eggs (Ma et al., 2012; Ma et al., 2015) and cryopreserved sperm (de Mello et al, 2017) of aquaculture species, and may be used to predict developmental and multigenerational trajectories for specific traits in the future, pending a better understanding of temporal dynamics and functional consequences of these marks. Finally, a recent study revealed that the epigenetic marks, and specifically the methylome in Pacific Atlantic salmon, was significantly affected by the rearing environment in hatchery origin fish released into the wild (Le Luyer et al., 2017), which may contribute to reduced fitness of fish escaping aquaculture operations. Thus, epigenetic marks and their possible transgenerational transfer in offspring between hatchery reared and wild fish populations may address possible environmental consequences of fish released from aquaculture settings.

5.2.2. Aquatic toxicology

In the field of aquatic toxicology, several reviews on the topic already testify to the translational potential of teleost epigenetics (Vandegehuchte and Janssen, 2011; Kamstra et al., 2015a; Aluru et al., 2017). We particularly highlight the large predictive potential of epigenetic marks in the context of aquatic toxicology. The identification of epigenetic signatures in gametes, as well as early embryonic stages, in response to aquatic contaminants may, when coupled with detailed understanding of temporal consequences of specific marks established by basic research, help to better predict physiological consequences and fitness in later developmental stages and offspring. This is true for changes in physiological homeostasis at baseline, but is especially relevant in response to

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additional environmental changes relevant to teleost fishes, which are equally affected through anthropogenic activity. Current studies in teleost fish have, to a large extent, yet to examine integrated consequences of aquatic contaminant exposure in conjunction with additional homeostatic challenges, caused by a change in factors affecting the aquatic environment, for example through global warming or (associated) changes in water chemistry. Importantly, properly conducted experiments can furthermore, as described, help to delineate specific parental contributions, to physiological adaptation or disruption across generations, and have therefore important ramifications towards more targeted intervention in conservation efforts.

5.2.3. Human disease

Because teleost fish exhibit, as the largest vertebrate infraclass, an immense diversity in their physiological adaptations to specific environments, individual teleost species have historically been used as comparative model to study human disease (Schartl et al., 2014). More recently, the well characterized genetic and developmental versatility of the zebrafish model have resulted in an unprecedented utilization of teleost fish in biomedical research, with emerging models for a plethora of human conditions (Lieschke et al., 2007). Especially zebrafish hold promise with regard to research addressing molecular epigenetic determinants of human disease, however, it is important to distinguish translational possibilities and limitations. For example, as discussed in this review, clear differences with regard to molecular epigenetic mechanisms exist between teleost fish and mammalian models, exemplified by largely different miRNA-mRNA networks and

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genome methylation dynamics. Therefore, the specific translation of specific epigenetic signatures to human disease will likely prove challenging. The comparative approach in elucidating differences in epigenetic molecular regulation may however also provide opportunities to increase our understanding of the functionality of human epigenetic marks. For example, the comparative probing of miRNA networks in teleost fish, which are predicted to target only 10% of common mRNA targets compared to mammalian models (Xu et al., 2013), may help to identify the most important, evolutionarily conserved miRNA-mRNA relationships that mediate physiological responses, which often remain unknown in mammals (Mennigen et al., 2015). Along the same vein, comparative analysis of similar miRNA expression patterns in response to specific environmentally stimuli may equally prove of interest.

In addition to a direct translational context of specific epigenetic marks, the zebrafish model allows to screen for global epigenetic activity of specific compounds *in vivo*, as exemplified by the zebraRDM line that allows to visualize DNA methylation dynamics via knockin of an mCherry-fused methyl-CpG binding domain (MBD) probe driven by the *actin2* promoter (R. Zhang et al., 2017). Such tools will likely prove useful not only to screen for environmental factors that might contribute to disease etiology, but also to screen for novel therapeutics affecting global DNA methylation.

At the organismal level, zebrafish represent an excellent model to translate epidemiological evidence indicative of epigenetic disease etiology into testable hypotheses in a vertebrate model. For example, zebrafish are increasingly used as model for metabolic

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disease (Seth et al., 2013; Santoro, 2014; Schlegel and Gut, 2015), as they exhibit largely conserved metabolic pathways involved in regulating energy balance (Forlano and Cone, 2007; Craig and Moon, 2011). While individual hypotheses proposed for metabolic disease, such as the lifestyle hypothesis (Seaman et al., 2013) and the environmental obesogen hypothesis (Baillie-Hamilton, 2002; Grün and Blumberg, 2006) have been tested individually in the zebrafish (Oka et al., 2010; Meguro et al., 2015; Tingaud-Sequeira et al., 2011; Riu et al., 2014), this model allows integration of these hypotheses across developmental and generational timescales in the context of the developmental origin of adult disease hypothesis (Barker, 1990) in a well-characterized vertebrate model. While specific molecular epigenetic marks may differ compared to mammals, such studies conducted in the historical context of epigenetics emphasizing development and phenotypic emergence, rather than specific molecular mechanisms, hold significant promise to improve our understanding of human disease etiology across large timescales, and may allow us to identify specific germ-line dependent paternal or maternal ancestral contribution to metabolic disease, either independently or in addition to an additional context-dependent challenge on energy homeostasis. Indeed, various assays to probe metabolic phenotypes by addressing key contributors of energy balance homeostasis at the organismal level in zebrafish, such as food intake (Hirano et al., 2012; Nishiguchi et al., 2012), metabolic rate (Makky et al., 2008; Stackley, 2011; Dalman, 2013), and locomotory activity (Lange et al., 2013), now allow high throughput analysis, and can be coupled with molecular advantages of the zebrafish model. Thus, in concrete terms, the zebrafish model

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would allow to us to test, for example, the hypothesis that ancestral populations exposed to obesogens may render offspring more susceptible to metabolic disruption in response to dietary challenges.

5.3 Conclusion

Overall, comparative epigenetic research in teleost fish is increasingly gaining traction in the research community. Under the condition that current basic research questions will be addressed in the future, this research field is anticipated to contribute to important translational insights, especially in aquaculture and aquatic toxicology, but also in the context of human disease.

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Figure captions

Figure 1: Conceptual framework of the epigenetic regulation in teleost fish. Environmental (exogenous) and endogenous stimuli are integrated by molecular epigenetic mechanisms to regulate genomic gene expression, which shapes physiological phenotypes at higher levels of biological organization. Such regulation can occur across ontogenesis and result in the emergence of physiological phenotypes within a teleost's lifespan (context-dependent epigenetics) and be transmitted between generations (germline-dependent epigenetics).

Figure 2: Comparative definition of inter- and transgenerational epigenetics between traditional mammalian model systems and oviparous teleost fish. In therian mammals, sex differences exist with regard to transgenerational effects mediated by germ-line dependent epigenetics, since in pregnant females, stimuli act via context-dependent epigenetics on the dam (F_0), as well as on the fetus (F_1) and its primordial germ cells (F_3). Conversely, stimuli acting on sires affect F_0 and the gamete, which will give rise to F_1 offspring. In oviparous teleost fish, fertilization of the egg occurs externally, which limits context-dependent epigenetic regulation to the F_1 generation, while the F_2 generation of either sex is considered transgenerational or affected by germ-line dependent epigenetic regulation.

Figure 3: Historical development of numbers of publications by year (retrieved from NCBI PubMed in November 2017) investigating fish genomes (**A**) and gene expression (**B**) compared to emerging studies focusing on fish epigenetics (**C**) and specifically the principal molecular epigenetic mechanisms involving histones (**D**), DNA methylation (**E**) and microRNA (**F**). In all cases, searches were conducted using the described specific term indicated on the y-axis in conjunction with the term 'fish', 'zebrafish', 'rainbow trout' and 'Atlantic salmon'.

Figure 4: Examples of proteins involved in histone modifications, including histone acetylation (**A**), histone methylation (**B**) and histone phosphorylation (**C**), as determined in mammalian model systems. See text for detailed descriptions.

Figure 5: Inferred evolutionary fate of genes *atf2* (**A**), *kat5a* (**B**), *ep300* (**C**), which represent different HAT classes. Schematics are based on comparative analysis of NCBI deposited genome sequences (https://www.ncbi.nlm.nih.gov/genome) of rat (ID: 73), zebrafish (ID: 50), rainbow trout (ID: 196) and Atlantic salmon (ID: 369). The teleost-specific genome duplication (Ts3R) is indicated by the first red line, while salmoniform species underwent an additional, subsequent lineage-specific genome duplication event (Ss4R). Similarity of paralogues was inferred based on available synteny and predicted protein sequence information.

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Figure 6: Inferred evolutionary fate of genes *hdac3* (**A**), *hdac5* (**B**), *hdac11* (**C**) and *sirt1* (**D**), which represent different HDAC classes. Schematics are based on comparative analysis of NCBI deposited genome sequences (https://www.ncbi.nlm.nih.gov/genome) of rat (ID: 73), zebrafish (ID: 50), rainbow trout (ID: 196) and Atlantic salmon (ID: 369). The teleost-specific genome duplication (Ts3R) is indicated by the first red line, while salmoniform species underwent an additional, subsequent lineage-specific genome duplication event (Ss4R). Similarity of paralogues was established based on available synteny and predicted protein sequence information.

Figure 7: Inferred evolutionary fate of genes *kmt5a* (**A**), *suv39h1* (**B**), *ezh2* (**C**) and *ehmt2* (**D**), which represent different HKMTs. Schematics are based on comparative analysis of NCBI deposited genome sequences (https://www.ncbi.nlm.nih.gov/genome) of rat (ID: 73), zebrafish (ID: 50), rainbow trout (ID: 196) and Atlantic salmon (ID: 369). The teleost-specific genome duplication (Ts3R) is indicated by the first red line, while salmoniform species underwent an additional subsequent, lineage-specific genome duplication event (Ss4R). Similarity of paralogues was established based on available synteny and predicted protein sequence information.

Figure 8: Inferred evolutionary fate of genes *kdm1a* (**A**), *kdm6a* (**B**), *kdm6b* (**C**), which represent different LSDs. Schematics are based on comparative analysis of NCBI deposited genome sequences (https://www.ncbi.nlm.nih.gov/genome) of rat (ID: 73), zebrafish (ID: 50), rainbow trout (ID: 196) and Atlantic salmon (ID: 369). The teleost-specific genome duplication (Ts3R) is indicated by the first red line, while salmoniform species underwent an additional, subsequent lineage-specific genome duplication event (Ss4R). Similarity of paralogues was established based on available synteny and predicted protein sequence information.

Figure 9: Inferred evolutionary fate of genes *rps6ka5* (**A**), *aurkb* (**B**), which represent different histone kinases. Schematics are based on comparative analysis of NCBI deposited genome sequences (https://www.ncbi.nlm.nih.gov/genome) of rat (ID: 73), zebrafish (ID: 50), rainbow trout (ID: 196) and Atlantic salmon (ID: 369). The teleost-specific genome duplication (Ts3R) is indicated by the first red line, while salmoniform species underwent an additional, subsequent lineage-specific genome duplication event (Ss4R). Similarity of paralogues was established based on available synteny and predicted protein sequence information.

Figure 10: Inferred evolutionary fate of genes *ppp2ca* (**A**), *ppp2cb* (**B**), *ppp6c* (**C**), which represent different histone phosphatases Schematics are based on comparative analysis of NCBI deposited genome sequences (https://www.ncbi.nlm.nih.gov/genome) of rat (ID: 73), zebrafish (ID: 50), rainbow trout (ID: 196) and Atlantic salmon (ID: 369). The teleost-specific genome duplication (Ts3R) is indicated by the first red line, while salmoniform species underwent an additional, subsequent lineage-specific genome duplication event

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(Ss4R). Similarity of paralogues was established based on available synteny and predicted protein sequence information.

Figure 11: Key proteins involved in DNA methylation, including DNMT1 linked to the maintenance of DNA methylation marks, and DNMT3A, DNMT3B, DNMT3L linked to the *de novo* establishment of DNA methylation marks as determined in mammalian model systems. See text for detailed descriptions.

Figure 12: Inferred evolutionary fate of genes *dnmt1* (**A**), *dnmt3a* (**B**), *dnmt3b* (**C**) and *Dnmt3l*, which represent different DNMTs. Schematics are based on comparative analysis of NCBI deposited genome sequences (https://www.ncbi.nlm.nih.gov/genome) of rat (ID: 73), zebrafish (ID: 50), rainbow trout (ID: 196) and Atlantic salmon (ID: 369). The teleost-specific genome duplication (Ts3R) is indicated by the first red line, while salmoniform species underwent an additional, subsequent lineage-specific genome duplication event (Ss4R). Similarity of paralogues was established based on available synteny and predicted protein sequence information.

Figure 13: Key proteins involved in two principal active DNA demethylation pathways, which include TET1-3 and TDG as well as AICDA, APOBEC1-4 and MBD4 as determined in mammalian model systems. Dotted lines represent the presence of intermediate cytosine products, which were omitted for simplicity. See text for detailed descriptions.

Figure 14: Inferred evolutionary fate of genes *tet1* (**A**), *tet2* (**B**), *tet3* (**C**) and *aicda* (**D**), *Apobec1* (**E**), *apobec2* (**F**), *Apobec3* (**G**), *apobec4* (**H**), *tdg* (**I**) and *mbd4* (**J**) involved in active DNA demethylation pathways. Schematics are based on comparative analysis of NCBI deposited genome sequences (https://www.ncbi.nlm.nih.gov/genome) of rat (ID: 73), zebrafish (ID: 50), rainbow trout (ID: 196) and Atlantic salmon (ID: 369). The teleost-specific genome duplication (Ts3R) is indicated by the first red line, while salmoniform species underwent an additional, subsequent lineage-specific genome duplication event (Ss4R). Similarity of paralogues was established based on available synteny and predicted protein sequence information.

Figure 15: Key proteins involved in the canonical miRNA biogenesis pathway, as determined in mammalian model systems. See text for explanations.

Figure 16: Inferred evolutionary fate of genes *drosha* (**A**), *dgcr8* (**B**), *xpo5* (**C**) *dicer* (**D**), *ago2* (**E**) involved in the canonical miRNA biogenesis pathway. Schematics are based on comparative analysis of NCBI deposited genome sequences (https://www.ncbi.nlm.nih.gov/genome) of rat (ID: 73), zebrafish (ID: 50), rainbow trout (ID: 196) and Atlantic salmon (ID: 369). The teleost-specific genome duplication (Ts3R) is indicated by the first red line, while salmoniform species underwent an additional,

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subsequent lineage-specific genome duplication event (Ss4R). Similarity of paralogues was established based on available synteny and predicted protein sequence information.

Figure 17: *In silico* evidence supporting sub-functionalization of *xpo5* paralogues in salmoniform genomes. Several specific AA annotated based on identified roles in miRNA recognition and export in mammals (Okada et al., 2009; Wang et al., 2011) are identified as being divergent between salmoniform paralogues (red colour), conserved within fish but different from mammals (green colour) or conserved between fish and mammals (yellow colour) (**A**). *In silico* determination of differential tissue expression levels of *xpo5* paralogues in rainbow trout based on next generation sequencing data from the Phylofish database (Pasquier et al., 2016) (**B**).

Figure 18: Proteins involved in pathways mediating miRNA turnover, as determined in mammalian model systems. See text for explanations.

Figure 19: Inferred evolutionary fate of genes *tut4* (**A**), *tut7* (**B**), *gld2* (**C**) *bcdin3d* (**D**), *cugbp1* (**E**), *henmt1* (**F**) *xrn2* (**G**), *dis3l2* (**H**) and *parn* (**I**), involved in miRNA turnover pathways, as determined in mammalian models. Schematics are based on comparative analysis of NCBI deposited genome sequences (https://www.ncbi.nlm.nih.gov/genome) of rat (ID: 73), zebrafish (ID: 50), rainbow trout (ID: 196) and Atlantic salmon (ID: 369). The teleost-specific genome duplication (Ts3R) is indicated by the first red line, while salmoniform species underwent an additional, subsequent lineage-specific genome duplication event (Ss4R). Similarity of paralogues was established based on available synteny and predicted protein sequence information.

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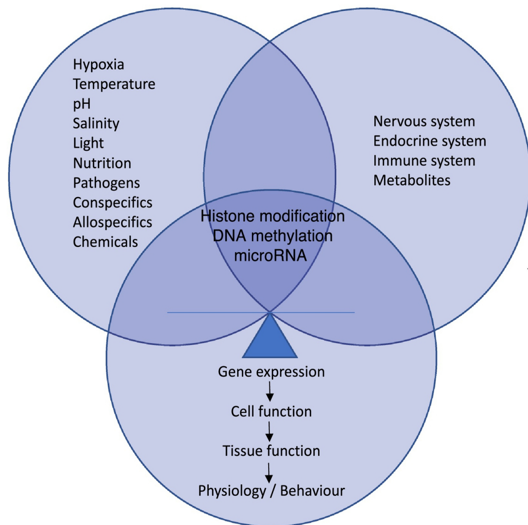

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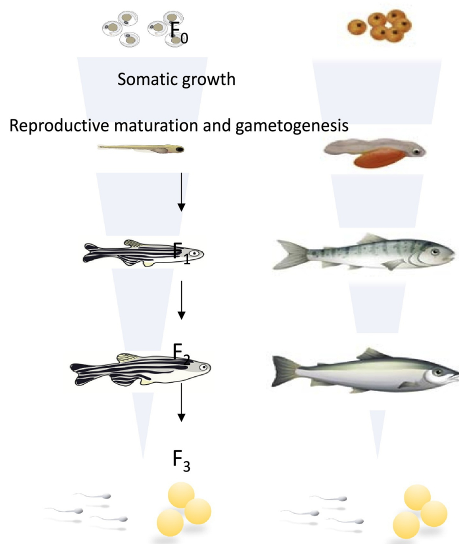
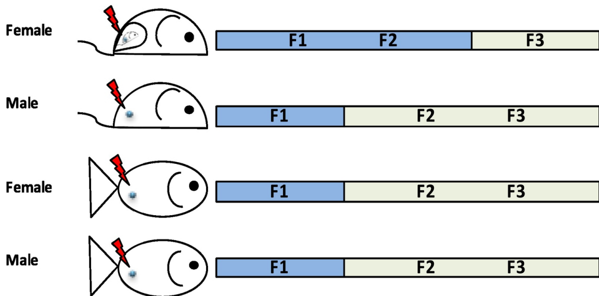


Figure 1



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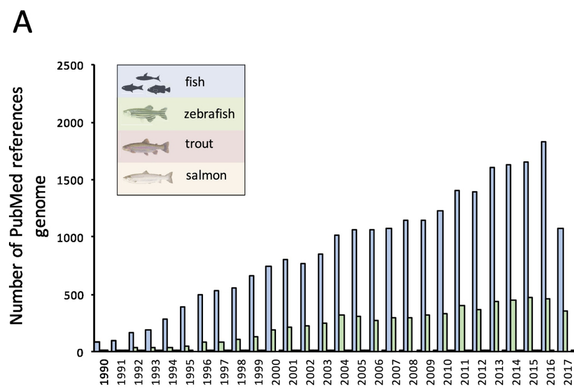

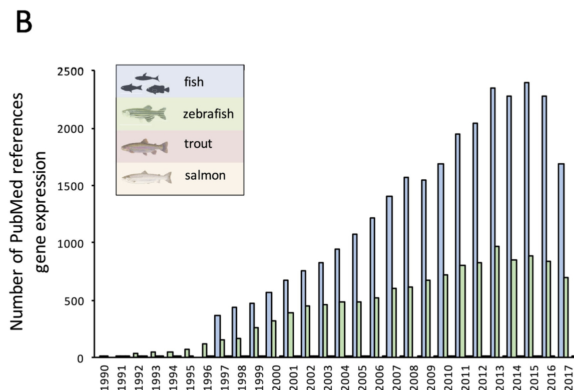

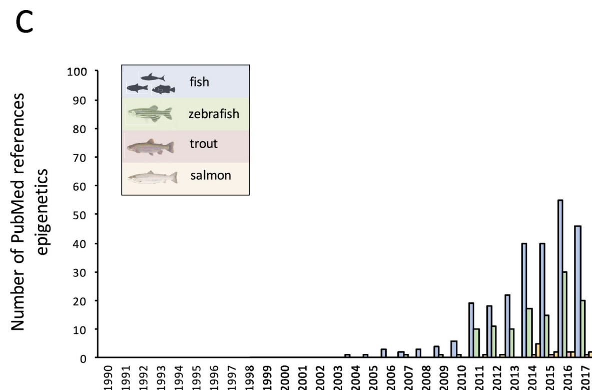

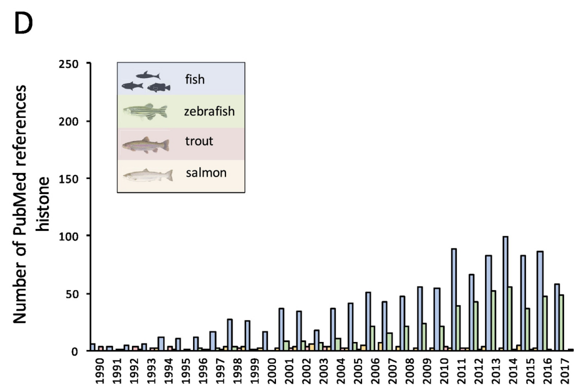

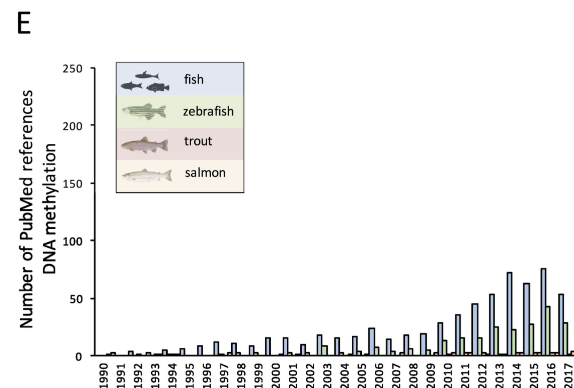

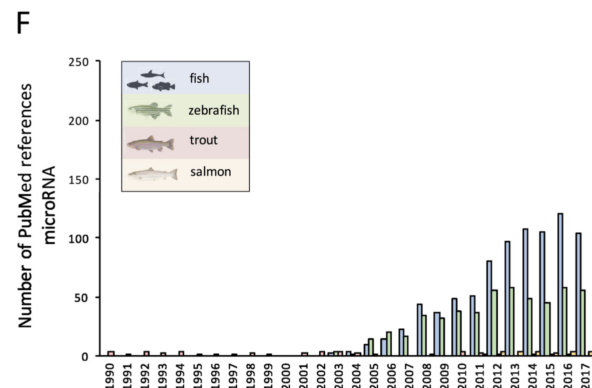

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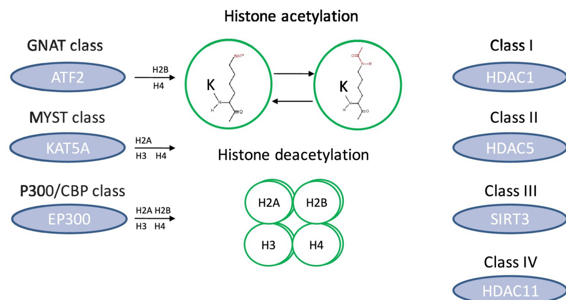

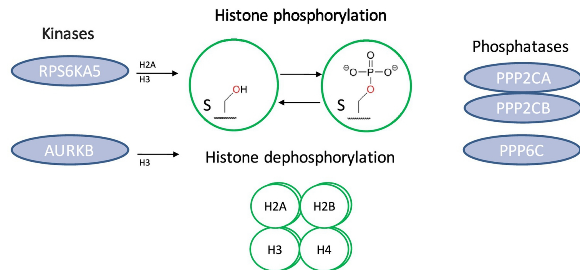

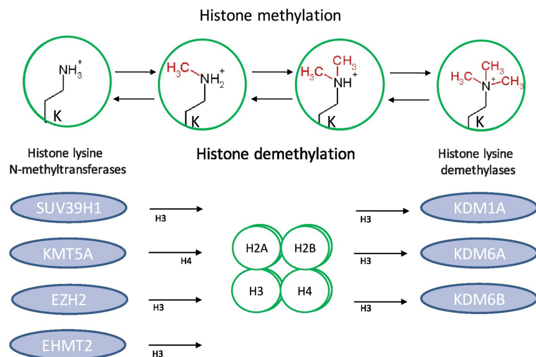

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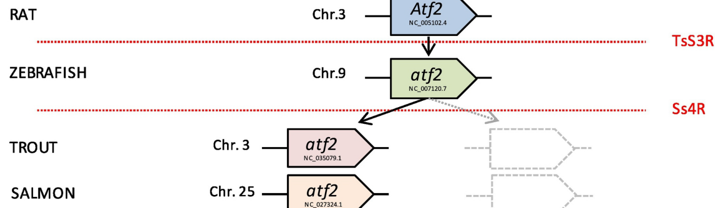

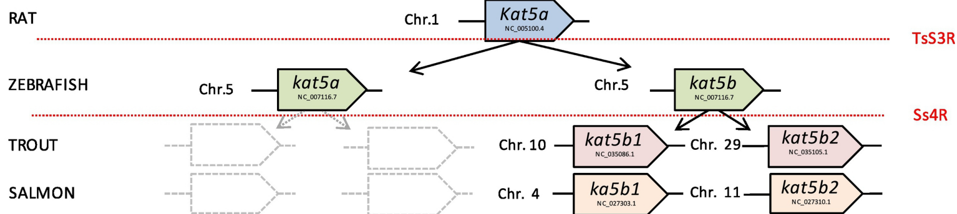

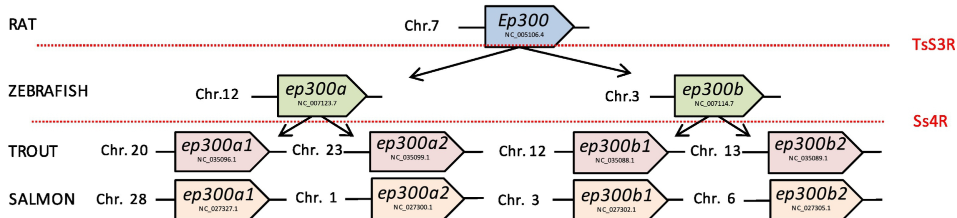

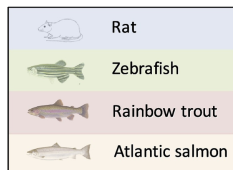

Figure 5

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ZEBRAFISH Chr.14 *hdac3* NC_007125.7

Ss4R

TROUT Chr. 14 *hdac3a* NC_035090.1 Chr. 25 *hdac3b* NC_035101.1

SALMON Chr. 5 *hdac3a* NC_027304.1 Chr. 9 *hdac3b* NC_027308.1

B.

RAT Chr.18 *Hdac5* NC_005109.4

TsS3R

ZEBRAFISH Chr.14 *hdac5* NC_007114.7

Ss4R

TROUT Chr. 12 *hdac5a* NC_035088.1 Chr. 13 *hdac5b* NC_035089.1

SALMON Chr. 3 *hdac5a* NC_027302.1 Chr. 6 *hdac5b* NC_027305.1

C.

RAT Chr.4 *Hdac11* NC_005103.4

TsS3R

ZEBRAFISH Chr.11 *hdac11* NC_007122.7

Ss4R

TROUT Chr. 7 *hdac11* NC_035083.1

SALMON Chr. 22 *hdac11* NC_027321.1

D.

RAT Chr.20 *Sirt1* NC_005119.4

TsS3R

ZEBRAFISH Chr.13 *sirt1* NC_007124.7

Ss4R

TROUT Chr. 1 *sirt1a* NC_035077.1 Chr. 23 *sirt1b* NC_035099.1

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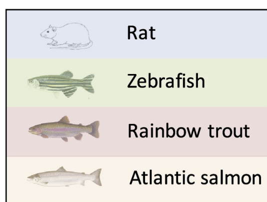

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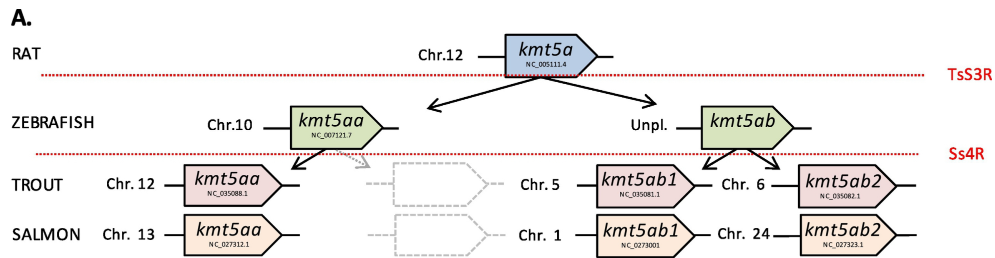

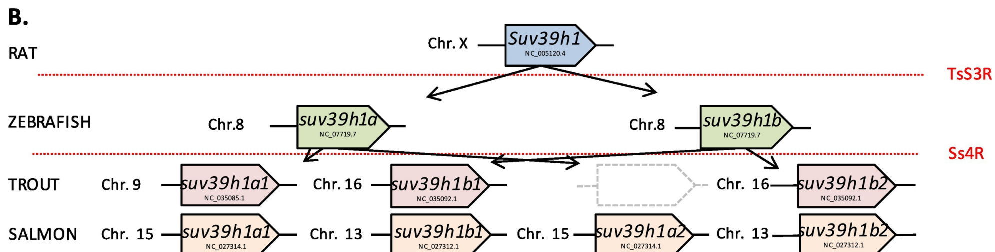

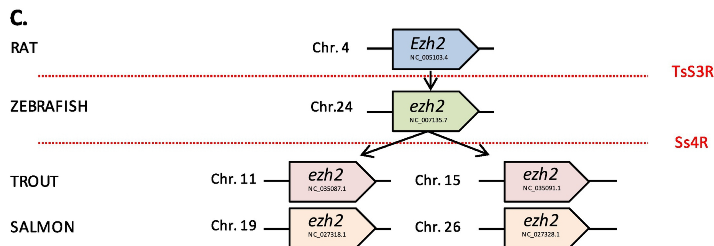

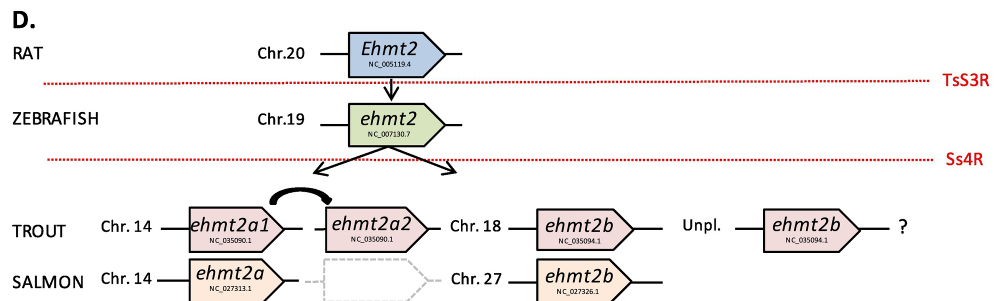

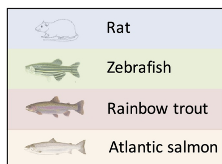

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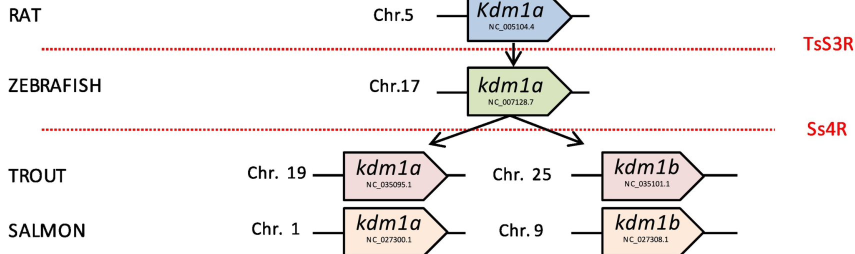

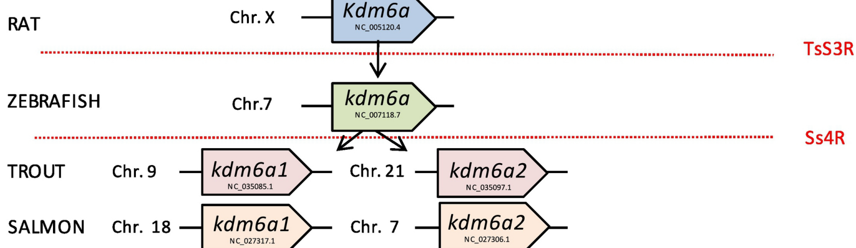

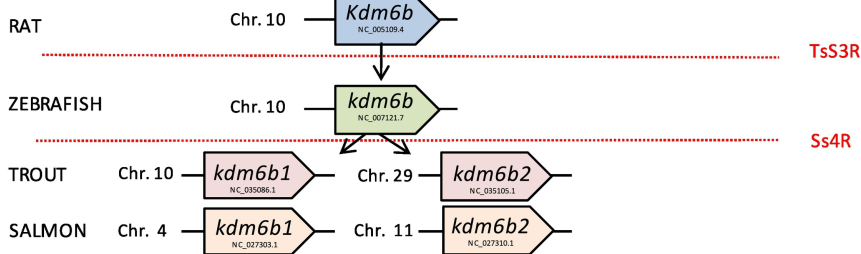

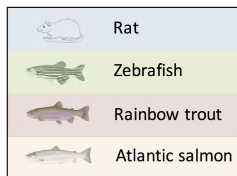

Figure 8

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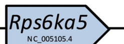

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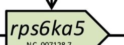
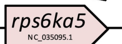
ZEBRAFISH Chr.17

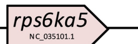


Ss4R

TROUT Chr. 19



Chr. 25



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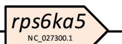
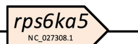
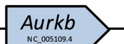


Chr. 9



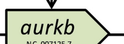
B.

RAT Chr.10



TsS3R

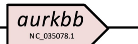
ZEBRAFISH Chr.14



Ss4R

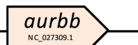
TROUT Chr. 1

Chr. 2



SALMON Chr. 16

Chr. 10



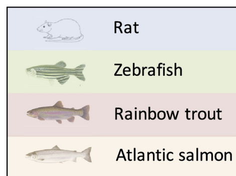
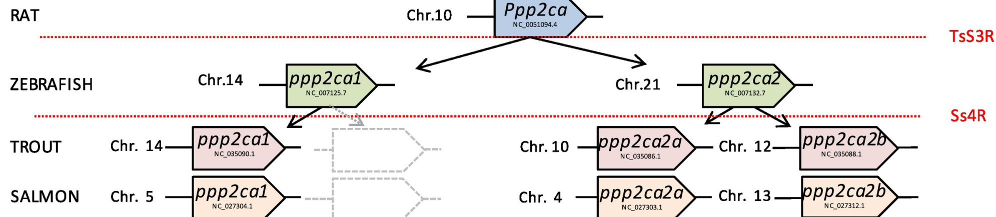
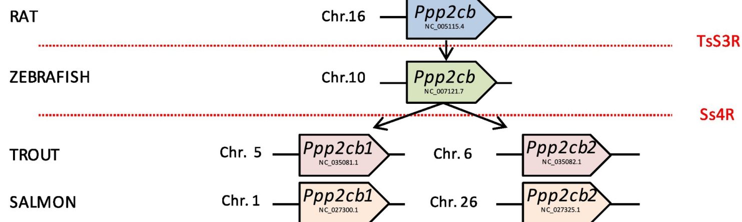
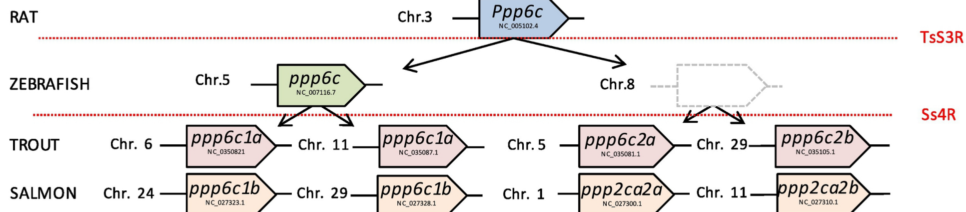


Figure 9







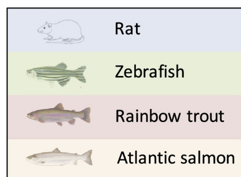


Figure 10

DNMT1

DNMT3A

DNMT3B

DNMT3L

maintenance methylation

de novo methylation

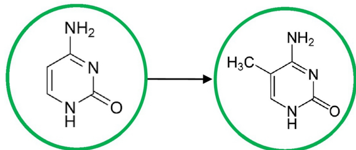


Figure 11

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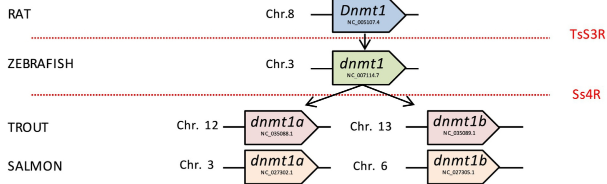

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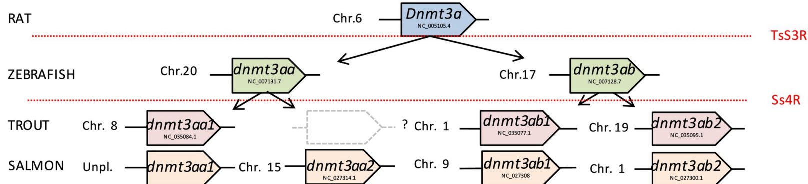

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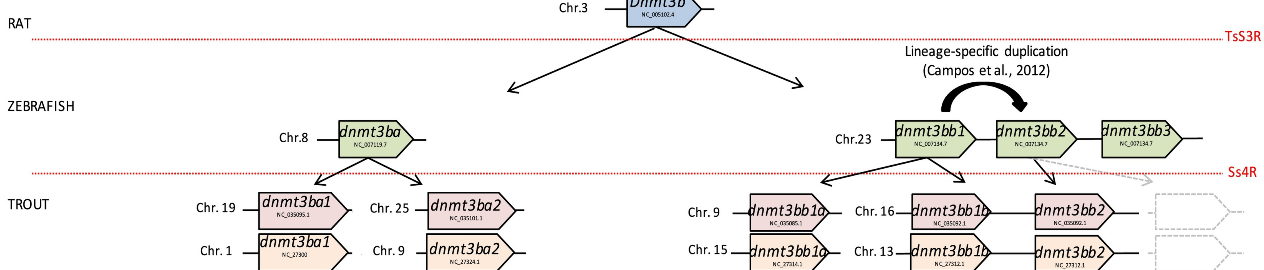
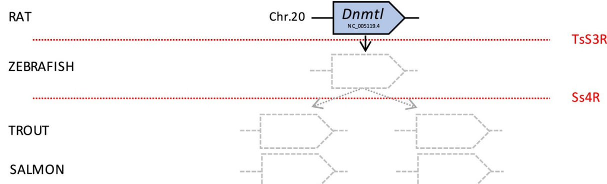
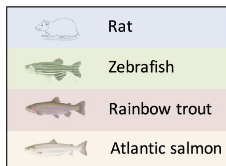


D.





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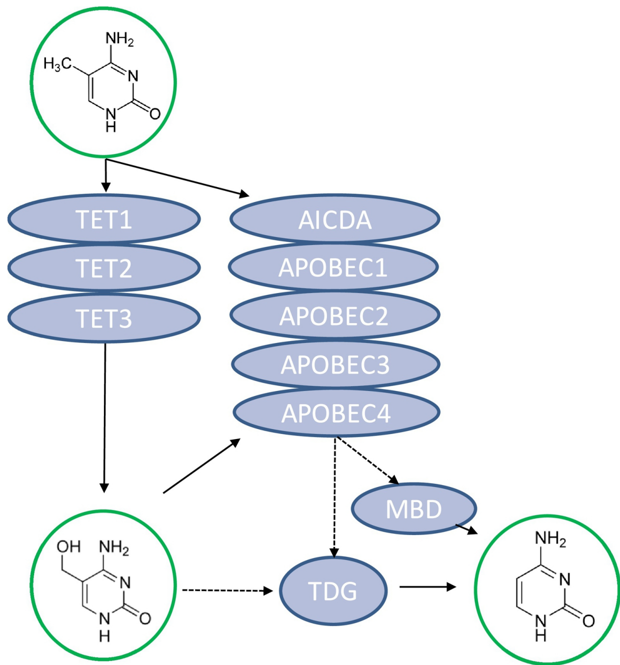
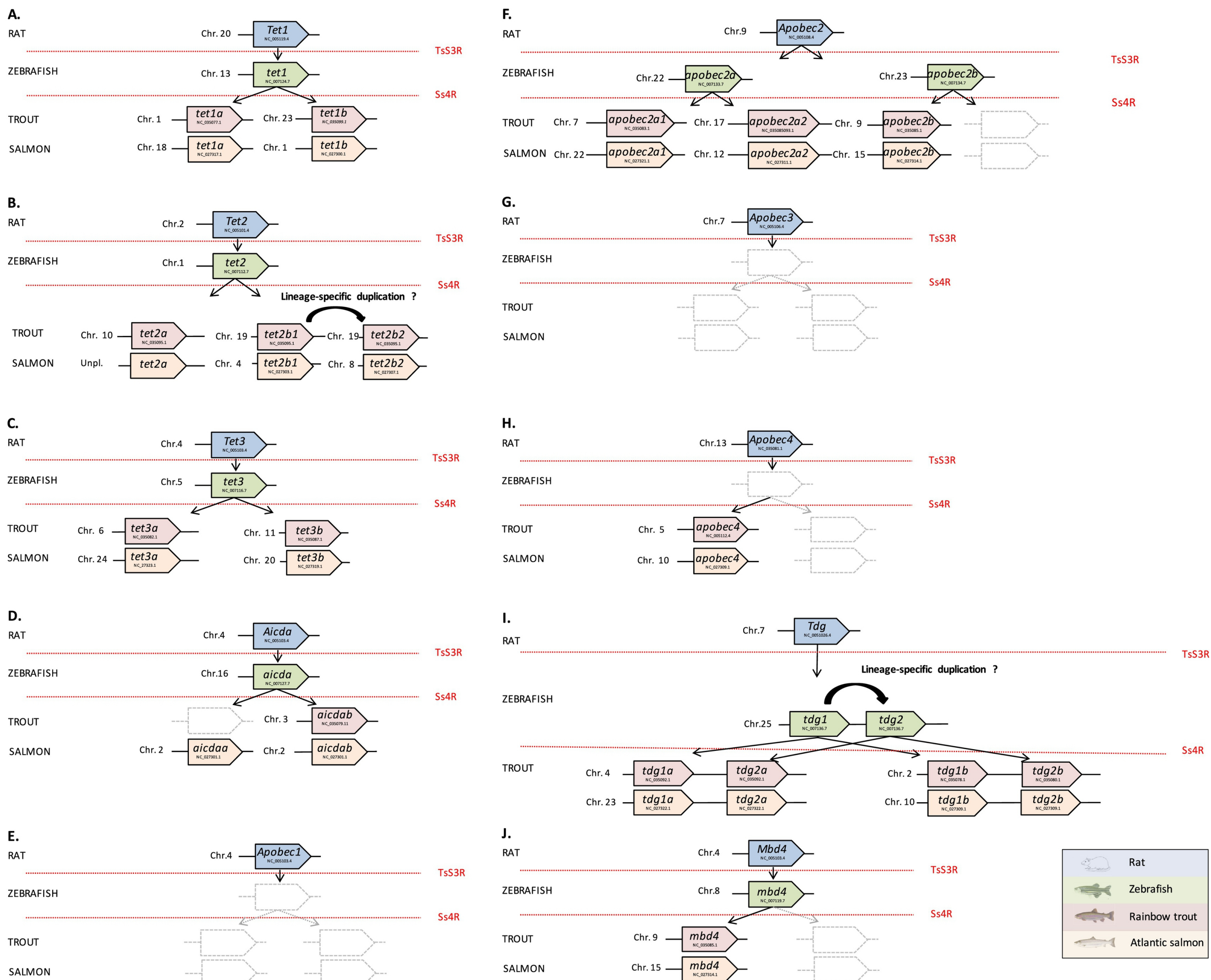


Figure 13



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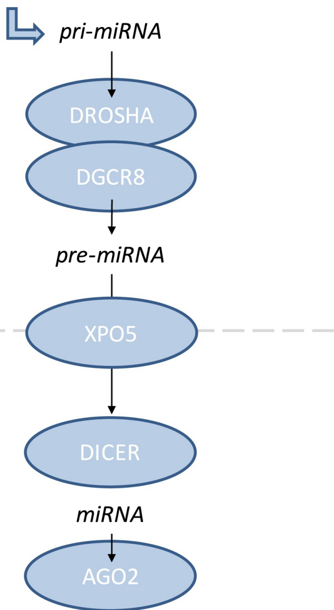
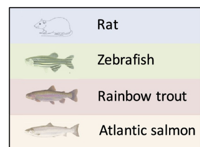


Figure 15



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TsS3R

ZEBRAFISH Chr.12 *drosha* NC_007123.7

Ss4R

TROUT Chr. 11 *drosha* NC_035087.1

SALMON Chr. 19 *drosha* NC_027318.1

B.

RAT Chr.2 *Dgcr8* NC_005101.4

TsS3R

ZEBRAFISH Chr.12 *dgcr8* NC_007123.7

Ss4R

TROUT Chr. 6 *dgcr8a* NC_035082.17 Chr. 11 *dgcr8b* NC_035087.1

SALMON Chr. 24 *dgcr8a* NC_027323.1 Chr. 20 *dgcr8b* NC_027319.1

C.

RAT Chr.9 *Xpo5* NC_005108.4

TsS3R

ZEBRAFISH Chr.13 *xpo5* NC_007124.7

Ss4R

TROUT Chr. 20 *xpo5a* NC_035096.1 Chr. 23 *xpo5b* NC_035099.1

SALMON Chr. 28 *xpo5a* NC_027327.1 Chr. 1 *xpo5b* NC_027300.1

D.

RAT Chr.6 *Dicer* NC_005105.4

TsS3R

ZEBRAFISH Chr.17 *dicer* NC_007128.7

Ss4R

TROUT Chr. 19 *dicera* NC_035095.1 Chr. 25 *dicerb* NC_035101.1

SALMON Chr. 1 *dicera* NC_027300.1 Chr. 9 *dicerb* NC_027308.1

E.

RAT Chr.7 *Ago2* NC_005106.4

TsS3R

ZEBRAFISH Chr.19 *ago2* NC_007130.7

Ss4R

TROUT Chr. 14 *ago2* NC_035090.1 Chr. 19 *ago2* NC_035094.1

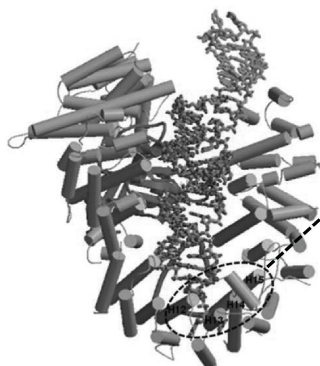
SALMON Chr. 14 *ago2* NC_027313.1

Figure 16

A

```
R.norvegicus_XPO5   SNELLLTQME   QDPCGLNRAR   RATTQLCWPL
D.rerio_XPO5        SDELLLTQLE   DEQMGINRSR   RTAGVLCWTL
O.mykiss_XPO5a      SNEALLTQME   DHTTGINRSR   RTASMVCWTL
S.salar_XPO5a       SNEALLTQME   DYTTGINRSR   RTASMVCWTL
O.mykiss_XPO5a      SSEALLNMLE   EHTTGINRSR   RSASIVCWTL
S.salar_XPO5a       SSEALLNLLE   EHTTSINRSR   RSASIVCWTL
                    *.* **. :*   :    .:**:*  *::   :** *
```

H13	**H14**	**H18**
T641	Q707	R1046
Q642	G715	Q1050
M643	R718	



pre-miRNA
3' overhang
recognition

B

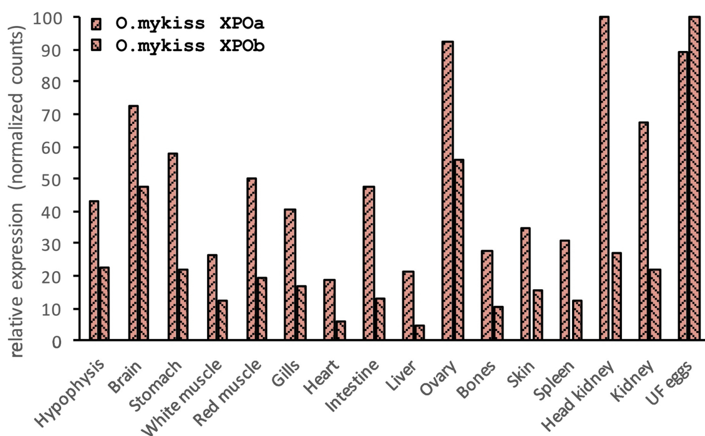


Figure 17

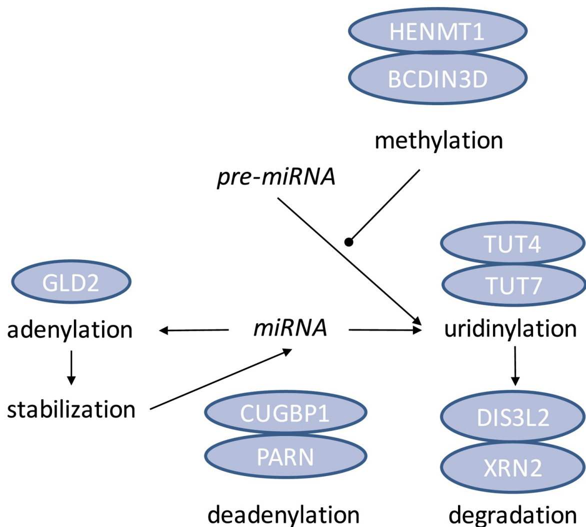


Figure 18

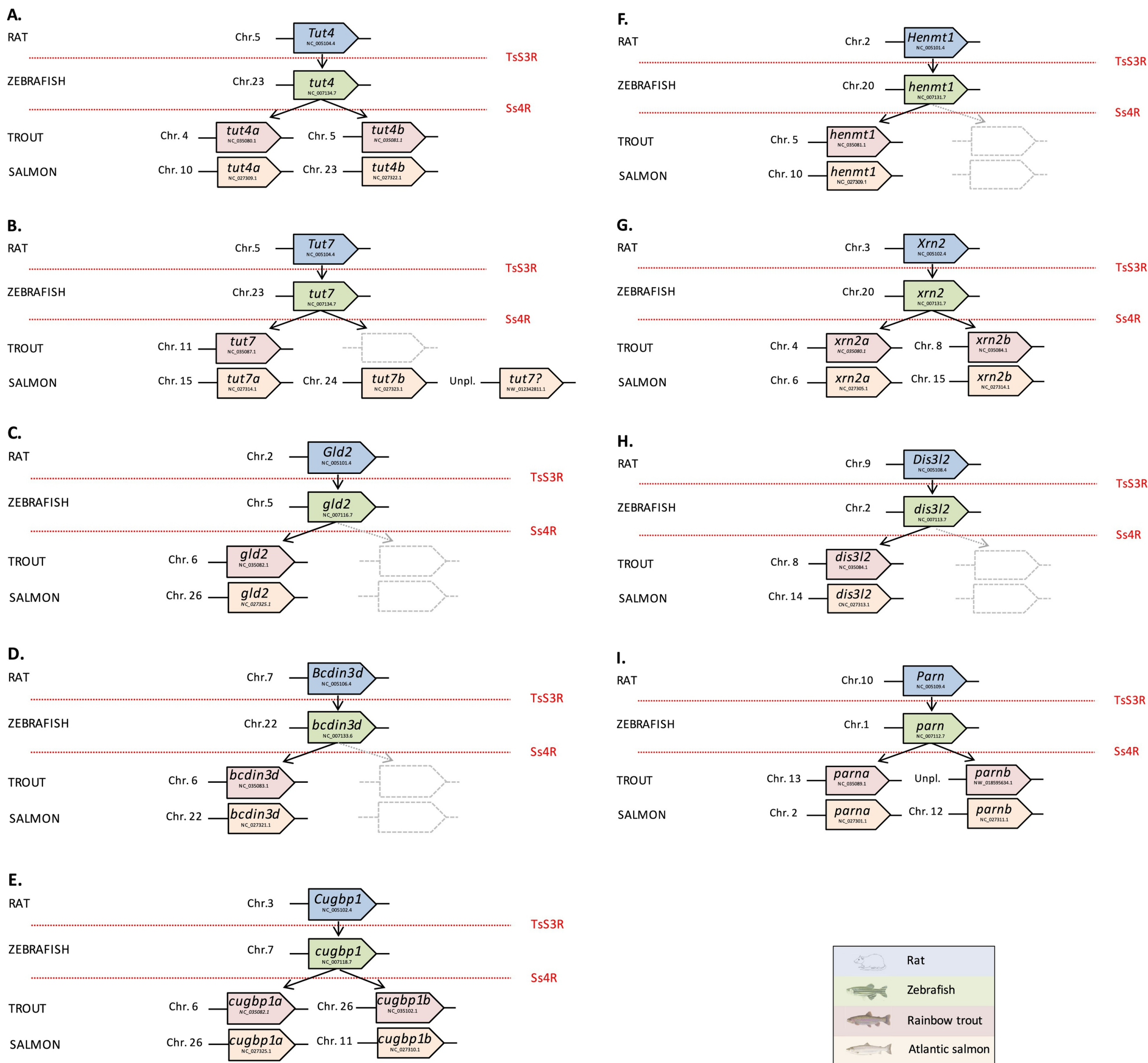


Figure 19