Non-coding RNAs: Uncovering their Potential Relevance in Fish Nutrition

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Abstract

The optimization of industrial production would only be possible with the discovery, identification and characterization of biological processes in which a nutrient or any other factor acts, as well as when their genes and genetic networks revealed. With the advent of Next Generation-Sequencing technologies, the discovery of non-coding RNAs having a key role on the control of a diverse set of biological functions in multicellular organism will allow a deeper knowledge on genes and genetic networks control such processes in farmed fish species. Here, the basics of non-coding RNAs regarding their features, biogenesis and mode of action will be briefly reviewed, while the research works specifically conducted until now on the identification of non-coding RNAs in different farmed fish species, developmental stages and tissues using high throughput technologies will be described and compared. Several non-coding RNAs have been associated with early developmental events, immune response to pathogen infections, sexual differentiation and maturation, and nutrition. While the research on miRNAs is the most abundant, new efforts on the characterization of long non-coding RNAs and PIWI-interacting RNAs profiles provided new insights on how these non-coding RNAs are also involved in fish nutrition. Finally, the future perspectives and considerations on the potential use of non-coding RNAs (mainly those found in circulation) in relevant cultured fish species as new reliable biomarkers of physiological condition will be pointed out.

Keywords: ncRNAs, miRNAs, nutrigenomics, transcriptomics, NGS.

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1. Beyond the central dogma: the complex world of non-coding RNAs.

Industrial production optimization would only be possible with the discovery, identification and characterization of biological processes in which a nutrient or any other factor acts, as well as when their genes and genetic networks revealed. In eukaryotic genomes only a small fraction of the DNA codes for proteins, but the non-protein coding DNA harbors important genetic elements directing the development and the physiology of the organisms, like promoters, enhancers, insulators, and non-coding RNA genes. For years, the Crick's central dogma – DNA is transcribed to mRNA and mRNA is translated to proteins – was believed (Crick, 1970). While DNA encoding proteins represents the 2% of the mammalian DNA, the remaining DNA is non-coding sequences (the "junk" DNA). More recently, among this 98%, it was discovered that although not transcribed, some DNA sequences harbors basic information to regulate the transcription of protein-coding genes (e.g. promoters, enhancers, insulators, etc) while other regions are actively transcribed in non-coding RNAs that are essential to assist on protein-coding genes translation (e.g. ribosomal RNAs and transfer RNAs). With the advent of the next generation-sequencing (NGS) technologies, the notion of "junk" DNA was rejected and opened a new gate towards the understanding of the complexity of higher multi-cellular organisms that the protein-coding region fails to explain (Lozada-Chávez *et al*. 2011). Nowadays, high-throughput transcriptomic analyses have revealed that eukaryotic genomes transcribe up to 90% of the genomic DNA (The ENCODE Consortium, 2004). The vast majority of this genomic DNA is transcribed as non-coding RNAs (ncRNAs) either as infrastructural ncRNAs, including ribosomal (rRNAs), transfer (tRNAs), small nuclear (snRNAs) and small nucleolar RNAs (snoRNAs); or as regulatory ncRNAs, mainly classified into microRNAs (miRNAs), P-element–induced wimpy testis (PIWI)–interacting RNAs (piRNAs), small interfering RNAs (siRNAs), long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), promoter-associated RNAs (PARs) and enhancer RNAs (eRNAs; Kaikkonen *et al*. 2011).

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2. Non-coding RNAs: families, origins and functions.

Over the last two decades, several evidences were obtained supporting the key role of those regulatory ncRNAs in a diverse set of biological functions in multicellular organism. Although the most of the knowledge came from studies in mammalian species, the widespread and cost-effective use of NGS technologies allowed the identification of these ncRNAs as well as the protein-coding genes involved in their biogenesis and protein-interactions in several taxonomic groups including viruses, bacteria, plants, Cnidaria, Platyhelminthes, insects and non-mammalian vertebrates (fish, birds and reptiles; Rosani *et al*. 2016). Although only few functional studies have been already performed to demonstrate powerful regulatory action of ncRNAs, the appearance of all these ncRNAs along evolution, some of them with regions partially conserved between different taxons, is already a strong evidence of the important role that each type of ncRNAs might have. The most well-known actions of ncRNAs are dealing with the regulation of gene expression, translation and transposon activity. The most studied ncRNAs due to the regulatory action on eukaryotic genes and other genetic elements are the small ncRNAs (sncRNAs): siRNAs, miRNAs and piRNAs (Ghildiyal and Zamore, 2009; Kim *et al.* 2009; Malone and Hannon, 2009), being the last ones the less studied sncRNAs. While siRNAs and miRNAs take part to the same silencing machinery, piRNAs are particularly involved on the silencing of germ-line transposons, among other roles. RNA silencing provides highly specific inhibition of gene expression through complementary recognition of RNA targets. The RNA-induced silencing complex (RISC) forms the core of the RNA silencing machinery, and consists of a protein from the Argonaute (AGO) family and a small RNA that acts to guide RISC to its targets (a ‘guide’ RNA). Once loaded with a sncRNA, AGO proteins inhibit the expression of their targets, either by cleavage using SLICER endonuclease activity, or by attracting additional proteins that can affect translation, RNA stability or chromatin structure. The majority of eukaryotic organisms possess more than one AGO protein, and the functions of individual members of the family are often non-redundant (Siomi *et al.* 2011). On the other hand, long non-coding RNAs (lncRNAs) can participate in physiological and pathological processes through chromosome inactivation and epigenetic modifications, control of mRNA decay and translation, and/or DNA

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sequestration of transcription factors. More recently, circular RNAs (circRNAs) and competing endogenous RNAs (ceRNAs) have been discovered to regulate miRNA function at transcriptional/post-transcriptional level. In addition to their mode of action, those ncRNAs also differed on their biogenesis. The features, biogenesis and mechanisms of action of the most commonly studied classes of regulatory ncRNAs will be briefly described below.

2.1. Small interfering RNAs (siRNAs)

The canonical siRNA is a linear double stranded RNA (dsRNA) perfectly base-paired 21–23 nt in length, containing an mRNA sequence (sense strand) and its complement (antisense active strand; reviewed in Wittrup and Lieberman, 2015). siRNAs are processed by DICER endonuclease (as well as miRNAs) that will silence directly the target when loaded onto RISC. In this sense, siRNAs can mediate silencing of mRNA targets (i) at post-transcriptional level; (ii) at transcriptional level by increasing epigenetic marks (e.g. methylation) of heterochromatin, particularly silencing mRNAs of the same locus from which they are derived; and/or (iii) suppressing retrotransposition (reviewed in Castel and Martienssen, 2013). Those functions of siRNAs lead its use in the treatment of human diseases (Wittrup and Lieberman, 2015), as well as a method for gene knockdown system for functional analysis in model species for developmental biology like zebrafish (*Danio rerio*) (Shinya *et al*. 2013), or as a strategy to control viral diseases in aquaculture (Papic *et al.* 2015).

2.2.MicroRNAs (miRNAs)

The miRNAs are by far the most extensively studied sncRNAs. They are evolutionarily conserved and small single-stranded molecules (20–24 nt) derived from transcripts (pre-miRNAs) and characterized by forming distinctive hairpin structures (reviewed in Ha and Kim, 2014). Pre-miRNAs are sequentially processed into the mature miRNA by DROSHA and DICER, although a DICER independently processing have been recently demonstrated. Mature miRNAs will finally interact with AGO proteins to form RISC. Then, the miRNAs pair with mRNAs, most favorably to the 3′ untranslated region (UTR), although pairing with the 5'UTR or the coding DNA sequence (CDS) has been also demonstrated. In contrast to siRNAs, miRNAs do not perform perfect match with mRNA sequence. Instead,

a not fully complementary pairing with the 2-8 bases (mer) of the 5'UTR of miRNA seed with the specific mRNA region has been shown in order to regulate the post-transcriptional translation of targeted mRNAs (Yartseva *et al.* 2016). Post-transcriptional regulation of targeted mRNAs can be through deadenylation of the mRNA (followed by its degradation), mRNA cleaving, and/or translation repression at initiation or elongation. Furthermore, in addition to these classical roles, miRNAs have also been shown to regulate gene expression at the promoter of the target mRNA through epigenetic mechanisms. In one or another way, miRNAs could target 30-60% of the human transcribed genes (John *et al.* 2004; Sand *et al.* 2012), having strong implications on cell differentiation and cell death processes, stress responses and diseases. A large set of research works have been performed during the last decade to identify and predict the targeted mRNAs (and biological processes) by the miRNAs in different fish species (see below).

2.3.P-element–induced wimpy testis (PIWI)–interacting RNAs (piRNAs)

P-element–induced wimpy testis (PIWI)–interacting RNAs (piRNAs) are known sncRNAs 24-31 nt length, forming complexes with the PIWI proteins from the AGO family, and having a diverse set of functions (reviewed in Siomi et al. (2011), Watanabe and Lin (2014), Iwasaki *et al*. (2015), and Sarkar et al. (2017), among others). The primary role of piRNAs has been shown to silence transposable elements (TEs) in the germline of animals. TEs are genomic parasites that threaten host genomic integrity as they can move to new sites by insertion or transposition and thereby disrupting genes. In animals, endogenous siRNAs also silence TEs, but the piRNA pathway is at on its forefront. By silencing TEs in the germline, piRNAs prevent harmful mutations from being passed to the next generations. Nevertheless, recent research suggested that piRNAs are playing important roles beyond TE silencing, and being reported their expression in tissues others than gonads (reviewed in Sarkar *et al.* 2017). In brief (reviewed in Siomi *et al.* 2011), piRNAs are originated from single-stranded precursors given rise to antisense piRNAs, which then recognize and target the cleavage of transposons by associated PIWI-proteins. This generates additional sense piRNAs arising from the target transposon sequence. Such process is known as the 'ping-pong' cycle, increasing the abundance of piRNAs and transposon silencing. The piRNA mechanism of action is not fully understood but probably involves the arginine methyl-

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transferase PRMT5, tudor domain-containing proteins (TDRDs) and the Maelstrom protein (MAEL; Sokolova *et al.* 2011). Interestingly, a recent study showed as piRNA pathway genes rapidly evolved in the teleost fish compared with mammals, likely to adapt to the higher diversity of transposons in the teleost fish species (Yi *et al*. 2014).

2.4.Long non-coding RNAs (lncRNAs)

LncRNAs are ncRNAs longer than 200 nt, although some of them act as a source of shorter RNAs. Categorization of ncRNAs within the lncRNAs is quite ambiguous/heterogeneous regarding its localization. Some lncRNAs are located in the nucleus while others in the cytoplasm. Moreover, some of them can have or not poly A tail (reviewed in Fatica & Bozzoni, 2013). Nevertheless, all of them tend to show a low level of expression. The degree of conservation of lncRNAs along evolution is quite low, being only the 4-11% of lncRNAs retaining some conserved regions between mammals and fish (Basu *et al.* 2013). The lncRNAs have crucial roles in gene expression control during both developmental and differentiation processes (reviewed by Fatica and Bozzoni, 2013). In brief, at the nucleus, lncRNAs guide chromatin modifiers (DNA methyltransferases and histone modifiers, such as the Polycomb repressive complex PRC2 and different histones) to specific genomic loci in order to induce repressive heterochromatin formation and thus, transcriptional repression. Additionally, they also repress gene transcription by acting as decoys sequestering transcription factors, by allosterically modulating regulatory proteins, and/or by altering nuclear domains and long-range three-dimensional chromosomal structures. In this sense, nuclear lncRNAs can act on the same loci (*cis*-) or on a distant one (*trans*-). Cytoplasmic lncRNAs can modulate mRNA translation through a diverse set of modes: by positive or negative regulation with direct complementary pairing with target mRNA, increasing or decreasing mRNA stability, and/or by binding to and sequestering specific miRNAs. A third type of gene expression regulatory mechanism performed by lncRNAs, and the best-characterized one, is through the dosage compensation and genomic imprinting, relying on the formation of silenced chromatin to produce monoallelic expression of specific genes. The extraordinary complexity of transcriptional regulation performed by lncRNAs is further reflected by a single lncRNA working by different mechanisms depending on the cell type.

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To date, of the tens of thousands of metazoan lncRNAs discovered by high-throughput transcriptome projects, only a handful of lncRNAs have been functionally characterized. Some of them are XIST, HOTAIR, and MALAT1, the last being evolutionary conserved in fish species (Johnsson et al., 2014). Among them, the H19 gene encoding a 2.3-kb ncRNA is highly expressed during embryogenesis but shut off in most tissues after birth, and known to be involved in genomic imprinting. Maternal undernutrition has been shown to regulate the expression of H19 in a sex-specific manner, being maternal low-protein diet the cause of abnormalities in male but not female mice blastocysts (Kaikkonen *et al.* 2011). In fish species, while other specific lncRNAs have been found to induce developmental defects in zebrafish, such as the lncRNAs *cyrano* and *megamind* (Ulitsky *et al.* 2011), modulation of lncRNAs has been associated with the immune response of Atlantic salmon (*Salmo salar*) to pathogenic infections (Boltaña *et al.* 2016).

2.4.1. Circular RNAs (circRNAs) and Competing endogenous RNAs (ceRNAs)

Among the different classes of lncRNAs, two of them act as sponges for miRNAs. Whereas the linear ceRNAs have a short half-life that allows a rapid control of sponge activity, circRNAs have much greater stability and their turnover can be controlled by the presence of a perfectly matched miRNA target site. circRNAs structurally differ from other lncRNAs in that their 3′ and 5′ ends are not free but covalently joined at a site flanked by canonical splice signals in contrast to the regular splicing pattern in which a splice donor is joined to a downstream splice acceptor (reviewed in Ebbsen *et al.* 2016). CircRNAs can be derived from exon of protein-coding genes, from intronic, intergenic, UTR regions, ncRNA loci and from locations antisense to known transcripts; and can comprise one or more exons. Interestingly, since some of these multiexonic circRNAs consisted exclusively of exonic sequences, these circRNAs must be subjected to splicing to remove the introns either before or after circularization, and are being exported afterwards from the nucleus to the cytoplasm through an uncharacterized pathway. The role and biological importance of circRNAs is currently uncharacterized and has been a source of debate. Nevertheless, circRNAs have a great stability since no free extremes are found where exonuclease degradation may act, but also the lacking of a 3′ poly(A) tail, made them resistant to

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deadenylation, decapping and degradation normally observed in mRNAs. Thus, they would be the more efficient sponge for miRNAs as this stability will allow circRNAs to accumulate as well as to maintain the regulatory function for a longer period of time than the ceRNAs. CircRNAs have been predicted and isolated in zebrafish and coelacanth (*Latimeria chalumnae*) (Shen *et al*. 2016) and in large yellow croaker (*Larimichthys crocea*), where GO and KEGG pathway of the genes included in those circRNAs were related with digestive system and metabolism (Xu *et al.* 2017).

2.4.2. Enhancer RNAs (eRNAs)

The eRNAs are another class of lncRNAs. The size of eRNAs range from 0.1 to 9 kb, and show a specific histone methylation signature typical of enhancers (reviewed in Smith and Shilatifard (2014) and Lam *et al.* (2014)). More particularly, eRNAs are produced from DNA regions extremely rich in monomethylation on lysine 4 of histone 3 (H3K4me1) but not so in H3K4 trimethylation (H3K4me3), and are evolutionary conserved. Several evidences indicate that eRNAs function as transcriptional activators, as depleting eRNA led to a gene-specific decrease in mRNA expression. Furthermore, this transcriptional enhancing activity appears to be sequence-specific since substituting eRNA with other open reading frames led to decreased enhancer activity although the transcription start site remained intact (Orom *et al.* 2010). Limited work has been carried out to explore eRNAs in fish species, being only recently and strictly performed on zebrafish eRNAs (Pauli *et al.* 2012; Taminato *et al.* 2016; Trinh *et al.* 2017).

2.5. Overview of the mechanisms of action of ncRNAs

As above-mentioned, each ncRNA can regulate gene expression and translation in a diverse set of ways within each cell. Furthermore, some ncRNAs can also regulate the action of other ncRNAs such as the case of the circRNAs and ceRNAs regarding the action of miRNAs. The different mechanism can be clustered in three types: chromatin remodeling, transcriptional and/or post-transcriptional regulation (reviewed in Kaikkonen et al., 2011).

In one hand, different ncRNAs (lncRNAs and piRNAs) can regulate transcription by recruiting chromatin-remodelling complexes, which in turn mediate epigenetic changes (heritable changes in phenotype and gene expression caused by mechanisms other than

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changes/mutations in DNA sequences). A classic example for this model is X-chromosome inactivation, where polycomb group (PcG) proteins binds to ncRNA XIST expressed on the targeted X-chromosome, and initiating and epigenetic silencing by trimethylation of H3K27. PcG also mediates transcriptional repression through interaction with histone deacetylases and exerts long-lasting silencing by CpG (or CG) islands' methylation through interaction with DNA methyltransferase 3 alpha (van der Vlag & Otte, 1999; Viré *et al*. 2006).

On the other hand, ncRNAs can also repress or activate gene transcription. As an example, DHFR gene contains a major and a minor promoter. The lncRNAs generated from the minor promoter bind both the major promoter (triplex formation) and the general transcription factor IIB leading to the dissociation of preinitiation complex, and thus repressing DHFR gene expression (Martianov *et al.* 2007). On the contrary, ncRNAs can also serve as transcriptional coactivators as illustrated by the 3.8 kb polyadenylated EVF2 ncRNA that form a complex with DLX2 and function as a transcriptional activator of DLX5/6 expression in an enhancer-specific manner (Feng et al., 2006).

Finally, ncRNAs can also act at post-transcription level, modulating mRNA splicing, transport, translation and/or degradation. Although piRNAs can act at post-transcriptional level, the most known ncRNAs performing mRNA post-transcriptional regulation are the siRNAs and miRNAs. The difference between siRNAs and miRNAs is the region of action. While siRNAs silence the locus from which they are derived, miRNAs can act in a wider manner affecting genes others than those from its own locus. Some ncRNAs overlaps with (i) the exon-intron boundary, enabling alternative splicing; (ii) the 5' UTR, CDS and/or 3' UTR regions, blocking translation at initiation or elongation; and (iii) the 3' UTR region inducing deadenylation (followed by decapping and degradation of mRNA) or mRNA cleavage (Wilczynska &Bushell, 2014).

3. Non-coding RNAs in circulation.

The complex developmental program encoded on the DNA and its regulation through the action of the different ncRNAs within each cell in an autocrine fashion is further twisted by the paracrine and possibly in an endocrine manner (Viereck *et al*. 2014) when the whole

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organism is considered. In this sense, the discovery of circulating ncRNAs associated with exosomes and/or lipoproteins opened up the possibility of a holistic and new mediation of gene regulation. A significant and diverse set of ncRNAs have been observed outside the cells, including in different body fluids (reviewed in Allegra *et al.* (2012), Silva& Melo (2015), and Viereck &Thum (2017)). Although the biological consequence of released ncRNAs on distant sites remains to be uncover, the expression patterns of ncRNAs in body fluids are highly correlated with disease states and other physiological conditions in humans, and being recently associated with sex differentiation in the tongue sole (*Cynoglossus semilaevis*) (Sun *et al.* 2017; see below).

The resistance of ncRNAs to RNases and harsh conditions (e.g. boiling, extreme pH, storage at room temperature, or freezing and thawing cycles), but also its presence in body fluids such as blood, serum/plasma, urine, and breast milk, makes circulating ncRNAs suitable for clinical assessment and monitoring patient's pathophysiological state (Viereck and Thum, 2017). In circulation, ncRNAs transport involve encapsulation into membranous vesicles including exosomes (30–100 nm), microvesicles (100-1000 nm), and apoptotic bodies (500-2000 nm); association to RNA-binding proteins, such as nucleophosmin, AGO2 or lipoprotein complexes like low- and high-density lipoproteins. Since the ncRNA content of extracellular vesicles can differ from that of the parental cell, a specific sorting and packing mechanisms might be favored. The ncRNA content of extracellular vesicles can be taken up by recipient cells, enabling cell-to-cell communication, which is potentially mediated via membrane receptors, vesicle fusion with the membrane of target cells, endocytosis, or remain attached to the plasma membrane activating specific signaling pathways (reviewed in Fritz *et al*. 2016).

Among the ncRNAs found in circulation, miRNAs were the most extensively studied (Allegra *et al*. 2012), and have been almost exclusively characterized in mammalian species. One of the major advantages of miRNAs (as well as other ncRNAS) as mediators of cell-cell communication, is that its presence in body fluids and high stability may represent an infinite resource of non-invasive biomarkers, not only in cancer and other diseases, but also in nutrition. Nevertheless, some challenges still limit its use. In general, the potential lack of specificity of one miRNA for a disease or physiological condition as well as the standardization of the analysis of circulating miRNAs (and other ncRNAs)

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regarding the preparation of serum/plasma or the quantification of miRNAs (identification of suitable 'housekeeping' serum miRNA/small RNA). Moreover, extra-caution should be paid on the isolation procedure of circulating ncRNAs as for instance, large part of the circulating miRNAs are originated from blood cells (McDonald *et al*. 2011), and thus hemolysis might mask or provide erroneous ncRNAs biomarkers. The potential use of circulating ncRNAs in relevant aquacultured fish species needs further considerations in order to use them as a new biomarkers of physiological condition. In this sense, most of the aquaculture species do not have its genome sequenced, which difficult the prediction of novel ncRNAs but also investigating the biological processes to which there are associated through their targeted sequences (independently of being DNA and/or mRNA sequences). Although the implementation of NGS technologies on aquaculture research and the decreasing prices of such kind of analysis might promote an increasing knowledge on this issue in the nearest future, nowadays an alternative and acceptable approach could be mapping the isolated ncRNAs with close related species where the genome is already known (e.g. Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*), Japanese puffer (*Takifugu rubripes*), tilapia (*Oreochromis niloticus*), and/or zebrafish). Additionally, the development of more powerful and user-friendly bioinformatics tools will also benefit to the advance in this research topic and eventually, it will allow the implementation of therapeutic strategies based on the modulation of specific ncRNAs to improve immune system, allow monosex production and/or solve sexual maturation problems in captivity. However, to achieve these long-term goals functional studies in model species such as zebrafish or medaka (*Oryzas latipes*), where a diverse set of biotechnological tools are available, is still required.

4. Non-coding RNAs in farmed fish species.

The identification and characterization of different ncRNAs in fish species started from an evolutionary point of view, studying miRNAs following a homology approach on the published genome sequences. Posteriorly, this knowledge was expanded with the sequencing of the genome of different fish species. Some research works have been done on the functional characterization of particular ncRNAs in research model fish species

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(zebrafish and medaka, mainly). Instead, only few research studies were performed on the characterization of particular ncRNAs in specific traits of farmed fish. In this case, the obvious priority to identify the ncRNAs evolutionary conserved (or not) in aquacultured fish species was translated on their discovery applying in silico and/or RNA-sequencing (RNA-Seq) approaches. In this sense, in Table 1, the different studies performed in aquacultured fish species and at distinct developmental stages and/or tissues by high throughput technologies in order to identify ncRNAs are presented.

Research works on ncRNAs were performed in 9 freshwater, 8 marine, 2 catadromous and 1 anadromous farmed fish species, approximately comprising the 51, 32, 6 and 11 % of the studies, respectively. The most abundantly studied fish species are the common carp (*Cyprinus carpio*) (5), the Atlantic salmon (4) and the rainbow trout (4), in line with the fish species from the top-ranking in worldwide production (in Tns and value; FAO, 2016). Surprisingly, an exception to this is the fourth majorly studied fish species, the Atlantic halibut (*Hippoglossus hippoglossus*), with 3 studies, all of them from Dr. Babiak's Lab (University of Nordland, Norway), who is one of the scientific references on ncRNAs research in fish species, mainly regarding the miRNAs.

Among the conducted research, 57 % was on specific tissues (being liver, brain, head kidney and gonads the most frequently studied), 23 % was on different larval developmental stages, and only one work has been conducted in cell lines and another in circulating ncRNAs. Regarding the specific type of ncRNAs studied, the research on miRNAs is the most abundant (comprising almost 83 % of the research studies), being extensively reviewed in Bizuayehu and Babiak (2014), and particularly on those specifically found at the muscle tissue (known as myomiRs; reviewed in Mennigen (2016)). In contrast, only limited, and very recent, research effort has been focus on lncRNAs and piRNAs, representing less than the 12 and 6 %, respectively. Curiously, lncRNAs studies were focus on their association with fish immune response against several pathogens, mostly done by the research group of Dr. Gallardo-Escátare from Chile.

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Table 1. Main research works on non-coding RNAs identification by high throughput technologies conducted in aquaculture fish species.

Fish species	Developmental stage/tissue	ncRNAs	Approach	Analysis platform	Nº of ncRNAs	Associated biological processes	Reference
Oncorhynchus mykiss	Tissues*	miRNAs	Sanger sequencing		54	Several	Salem *et al.* 2010
Paralichthys olivaceus	Larvae	miRNAs	Small RNA sequencing / microarray	Solexa	140	Metamorphosis	Fu *et al.* 2011
Hypophthalmichthys nobilis	Tissues**	miRNAs	Small RNA sequencing		167	Several	Chi *et al*. 2011
Hypophthalmichthys molitrix	Tissues**	miRNAs	Small RNA sequencing		166	Several	Chi *et al*. 2011
Lates calcarifer	Tissues***.	miRNAs	Small RNA sequencing		63	Immune system	Xia *et al.* 2011
Ictalurus punctatus	-	miRNAs	Small RNA sequencing		60	Several	Barozai *et al.* 2012
Oncorhynchus mykiss	Eggs	miRNAs	Small RNA sequencing	Illumina	496	Egg quality	Ma *et al*. 2012
Cyprinus carpio	Larvae	miRNAs	Small RNA sequencing		113	Several	Zhu *et al*. 2012
Hippoglossus hippoglossus	Brain and gonads	miRNAs	Small RNA sequencing	SOLiD	150-168	Sexual differentiation	Bizuayehu *et al.* 2012a
Hippoglossus hippoglossus	Larvae	miRNAs	Small RNA sequencing	SOLiD	201	Several	Bizuayehu *et al.* 2012b
Cyprinus carpio	Muscle	miRNAs	Small RNA sequencing	Solexa	195	Muscle	Yan *et al.* 2012
Ictalurus punctatus	Tissues^	miRNAs	Small RNA sequencing	Solexa	282	Several	Xu *et al.* 2013
Salmo salar	Juveniles	miRNAs	Small RNA sequencing	Illumina HiSeq 2000	888	Several	Bekaert *et al*., 2013

Salmo salar	Tissues^^	miRNAs	Small RNA sequencing	Illumina	193	Several	Andreassen *et al.* 2013
Hippoglossus hippoglossus	Ovaries	miRNAs	Small RNA sequencing	Roche 454	43	Reproduction	Bizuayehu *et al.* 2013
Cyprinus carpio	Skin	miRNAs	Small RNA sequencing	Solexa	73	Pigmentation	Yan *et al*. 2013
Solea senegalensis	Larvae	miRNAs	Small RNA sequencing	SOLiD	320	Thermal plasticity	Campos *et al*. 2014
Megalobrama amblycephala	Liver	miRNAs	Small RNA sequencing	Illumina HiSeq2000	202	Nutrition	Zhang *et al.* 2014a
Gadus morhua	Larvae	miRNAs	Small RNA sequencing	SOLiD	389	Thermal plasticity	Bizuayehu *et al*. 2015
Cyprinus carpio	Epithelial cell line	miRNAs	Small RNA sequencing	Solexa	187	Immune response	Wu *et al.* 2015
Dicentrarchus labrax	Larvae	miRNAs	Small RNA sequencing	Illumina HiSeq2000	1,928	Several	Kaitetzidou *et al*. 2015
Anguilla marmorata	Gills	miRNAs	Small RNA sequencing	Illumina Hiseq2500	647	Osmoregulation	Wang *et al*. 2015
Takifugu rubripes	Tissues[#]	miRNAs	Small RNA sequencing	SOLiD	1420	Several	Wongwarangkana *et al.* 2015
Gadus morhua	Larvae	miRNAs	Small RNA sequencing	SOLiD	348	Several	Bizuayehu *et al.* 2016
Salmo salar	Tissues[##]	lncRNAs	Small RNA sequencing	Illumina MiSeq	5,636	Immune system	Boltaña *et al.* 2016
Larimichthys crocea	Whole organism	miRNAs	Genome prediction	-	199	Several	Huang *et al.* 2016
Oncorhynchus mykiss	Whole organism	lncRNAs	Small RNA sequencing	Illumina HiSeq2000	31,195	Immune system	Paneru *et al.* 2016
Salmo salar	Tissues[###]	lncRNAs	Small RNA sequencing	Illumina MiSeq	918	Immune system	Valenzuela & Gallardo, 2016
Oreochromis niloticus	Ovary and testis	piRNAs	Small RNA sequencing	Illumina Hiseq2000	862,289	Gonad development	Zhou *et al.* 2016
Clarias batrachus	Whole organism	miRNAs	Genome prediction	Illumina HiSeq	210	Several	Agarwal *et al*. 2017

Colossoma macropomum	Liver and skin	miRNAs	Small RNA sequencing	Illumina HiSeq2000	279	Several	Gomes *et al.* 2017
Oncorhynchus mykiss	Intestine	lncRNAs	Small RNA sequencing	Illumina MiSeq	9927	Several	Nuñez-Acuña *et al.* 2017
Oreochromis niloticus	Head kidney	miRNAs	Small RNA sequencing	-	1900	Immune system	Qiang *et al.* 2017
Cynoglossus semilaevis	Exosomes	miRNAs	Small RNA sequencing	Illumina HiSeq2500	723	Sexual differentiation	Sun *et al.* 2017
Cyprinus carpio	Ovaries	piRNAs	Small RNA sequencing	Solexa	8765	Several	Wang *et al.* 2017

*muscle, heart, brain, kidney, liver, spleen, intestine, gill, and skin; ** heart, liver, brain, spleen and kidney; *** Spleen, kidney and liver; ^liver, gill, head kidney, spleen, heart, brain, muscle, stomach, intestines and skin; ^^ liver, spleen, kidney, head kidney, heart, brain, gills, white muscle and intestine; # fast and slow muscles, heart, eye, brain, intestine, liver, ovaries, and testis; ## gills, head kidney, and liver; ### brain, spleen, and head kidney.

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The approach followed in all these studies clearly reflected how recent this research topic is. Only one study used individual Sanger sequencing technology, two were based on the prediction through genomic inference, while the remaining 90 % was performed using high throughput sequencing technologies such as Solexa, SOLiD, Roche 454 and Illumina (HiSeq2000, HiSeq2500 and MiSeq) platforms. The output obtained (number of identified ncRNAs) was really variable, depending on the tissue, organ and developmental stage, the type of ncRNA studied and/or the used sequencing platform. The number of ncRNAs identified normally ranges from 43 to 31,195, with only one exception: the identification of 862,289 piRNAs by Zhou et al. (2016) from the gonads of tilapia, tissues known to abundantly express this kind of sncRNAs. Finally, the expression of all those ncRNAs was associated with different biological processes: flatfish metamorphosis, thermal plasticity, immune system, sexual differentiation, gonad development and reproduction, pigmentation, myogenesis, osmoregulation and/or nutrition (extensively reviewed in the case of miRNAs in Bizuayehu and Babiak (2014)). In this sense, as abovementioned, since different ncRNAs are able to regulate genes at transcriptional and post-transcriptional level and there is an intricately genomic program regulating nutrient absorption (at intestine), metabolism (at liver) and transport; a clear role of those ncRNAs on nutrition might be expected.

5. Non-coding RNAs and nutrition

Despite of the abovementioned limited screening of ncRNAs involved in fish nutrition and metabolism, researchers have taking advantage of the wider and deeper knowledge on this issue from mammalian species. In this sense, how maternal nutrition can alter and shape offspring metabolic profile and/or organ function through the alterations in sncRNAs, such as miRNAs, piRNAs, and tRNAs, has been an intense area of research (reviewed in Loche and Ozane (2016) regarding the particular case of cardiovascular diseases). Furthermore, several evidences suggested that not only the endogenously produced ncRNAs might regulate metabolism, but also those obtained from dietary sources (plant foods and cow milk) might affect it by altering the expression of endogenous miRNA genes in mammalian species (recently reviewed in Cui et al.,

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2017). Encapsulation of miRNAs in exosomes and exosome-like particles confers protection against RNA degradation and creates a pathway for intestinal and vascular endothelial transport by endocytosis, as well as its delivery to peripheral tissues (see above, section 3: Non-coding RNAs in circulation). Thus, food components and dietary preferences may modulate serum miRNA profiles that might finally influence particular biological processes (Cui *et al.* 2017). Taking into account the relevance of human metabolic disorders nowadays in our society, novel pipelines and databases have been developed, including a dietary miRNAs database reporting miRNAs in 15 dietary resources (Cui *et al.*, 2017). These databases might be examples of future needs to study the diverse types of ncRNAs provided to fish from the diverse source of nutrients in aquafeeds, and mainly considering the nowadays driving force in aquaculture sustainability, the replacement of fish oil and meal by different alternative sources (mainly from vegetable origin). Furthermore, and interestingly, Liang *et al*. (2015) demonstrated in mice the efficacy of lncRNA expression profiles in discriminating the types of microbes in the gut (germ-free, conventional and/or gnotobiotic). Since the gut microbiota is known to have pivotal effects on host physiology, metabolism, nutrition and immunity, such work provided an initial resource of gut microbe-associated lncRNAs for the identification of lncRNA biomarkers in host-microbes interactions. Thus, the question for fish farmers and feed developers could be if this might be also the case of farmed fish.

In one hand, several miRNAs have been associated with energy metabolism in fish species though comparative analysis between mammalian and teleost species, despite the limited work conducted with high throughput technologies (RNA-Seq; see Table 1). For a full list of potential miRNAs involved on the regulation of fish metabolisms, readers are highly recommended the recent review of Mennigen (2016). Some relevant examples of different miRNAs and the related metabolic pathways are as follows. While miRNA-33 in lipid metabolism and miRNA-8163 in iron metabolism have been associated due to the presence of those miRNAs on an intron from SREBP and transferrin genes, respectively; other miRNAs were functionally demonstrated to be linked with metabolism. In this sense, inhibition of liver-specific miRNA-122 was shown to decrease postprandial serum triglyceride concentration and conversely cause postprandial hyperglycemia in rainbow trout (Mennigen *et al*. 2014). Also, inhibition of miRNA-17 in rabbit fish (*Siganus canaliculatus*) regulate, at least partially,

docosahexaenoic acid (DHA) biosynthesis from docosapentaeonic acid (DPA) via fatty acid desaturase 2 (FAD2) mediated Δ4 desaturation (Zhang *et al*. 2014b). Furthermore, and although poorly understood, some exogenous metabolic stimuli have been found to regulate metabolic miRNAs. In fact, several miRNAs have been reported to be regulated under post-pandrial and fasting conditions, as well as by the amount of macronutrients (such as lipids) included in the diets. In this sense, rainbow trout miRNA-122, at the same time regulates lipid homeostasis it was found to be post-prandial regulated (Mennigen *et al.* 2012); increasing abundance of let-7d and miRNA-140-5p in zebrafish was reported when fish were under fasting conditions (Craig *et al.* 2014); and the levels of 12 miRNAs were altered in the liver of blunt snout bream (*Megalobrama amblycephala*) when fed with high-fat diet during a prolonged period, and six lipid metabolism-related genes (*fetuin-B*, *Cyp7a1*, *NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 2*, *3-oxoacid CoA transferase 1b*, *stearoyl-CoA desaturase*, and *fatty-acid synthase*) were bioinformatically predicted to be targeted by those differentially expressed miRNAs (Zhang *et al.* 2014a).

Recently, an analysis of the microRNAs expressed at first feeding larvae from a marine fish species (Atlantic cod, *Gadus morhua*) have provided new insights on how these ncRNAs might mediate nutritional effects on growth (Bizuayehu *et al*. 2016). The miRNAs expressed in larvae fed the golden standard diet for marine fish species (zooplankton, mostly copepods), and showing a better growth and development performance, were compared to the ones found in larvae fed the most commonly used live preys in aquaculture: enriched rotifers and *Artemia*. Eight different miRNAs (miR-9, miR-19a, miR-130b, miR-146, miR-181a, miR-192, miR-206 and miR-11240) were found differentially expressed between the two feeding groups in at least one developmental stage of the six compared, and predicted targets of these miRNAs were associated with metabolic, phototransduction and signaling pathways. Furthermore, since miRNAs have also been shown to regulate gene expression through epigenetic mechanisms at the promoter of the target mRNA, in addition to the post-transcriptional regulation (John *et al.* 2004; Sand *et al.* 2012), these results suggest how first feeding might affect fish growth and development later on, at outgrowth phases, through nutritional programing. Moreover, since strong evidences has been provided that effective amounts of exogenous miRNAs (from cow milk) can be absorbed in humans, physiologic concentrations of exogenous miRNAs affect human gene expression *in vivo*

and *in vitro* (reviewed in Cui *et al*. (2017)); effects on growth and development as well as the modulation of miRNAs in Atlantic cod larvae fed golden standard or regular aquaculture diets could be due to those exogenous miRNAs provided by live preys, which miRNAs were also characterized (Bizuayehu *et al.* 2016).

Finally, and similarly to the associated lncRNAs with mice gut microbiota (Liang *et al.* 2015), Nuñez-Acuña *et al.* (2017) demonstrated how the expression profiles of intestinal lncRNAs in rainbow trout differed when fed for 30 days with functional diets based on pre- and probiotics. Thus, ncRNAs modulation through diets and their role on fish metabolisms seems to be evolutionary conserved, open new research challenges to explore the identification, characterization of ncRNAs in farmed fish, as well as its use as reliable and non-invasive (in the case of circulating ncRNAs) biomarkers of fish nutrition, among other relevant biological processes (e.g. resistance to pathogens, sexual differentiation and reproductive performance).

6. Future perspectives and conclusions.

Considering the several evidences of the ncRNAs playing regulatory roles in fish development and in response to different environmental stimuli (*e.g.* rearing temperature, pathogenic infection, water osmolarity and nutritional composition, among others), as well as other factors like sex and genetic background, an interesting goal in fish farming would be to identify and functionally characterize the full spectrum of ncRNAs with respect to normal *versus* abnormal physiological conditions in most importantly produced species. The development of even further powerful and versatile sequencing technologies, and the decreasing price per sequenced sample would clearly benefit the progress on this research topic. In this sense, RNA-Seq has already been applied to a wide range of issues in fish species (reviewed in Li and Li, 2014) and compared to microarray technology, RNA-seq provides nearly unlimited possibilities in modern bioanalysis since it is not limited towards the amount of RNA, the quantification of transcript levels and the previous knowledge on sequences to be detected and/or quantified. RNA-seq analyses, not only allows the expression level of mRNA, but also the detection and quantification of splice variants, and ncRNAs on a genome-wide scale. The recent and rapid advance on NGS technologies has already made DNA sequencing broadly available, allowing the sequencing of important farmed

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fish species, like Atlantic salmon, rainbow trout or turbot (*Scophthalmus maximus*) (Berthelot *et al.* 2014; Lien *et al.* 2016; Figueras *et al*. 2016). Thus, the number of genome sequenced fish species is expected to increase continuously in the coming years. Nevertheless, even in species in which the full genome sequence is still not known, ncRNAs can be identified from the read output from RNA-Seq performing a comparative analysis with the known sequences from other species deposited in relevant ncRNA databases such miRBase (http://www.mirbase.org/; Kozomara and Griffiths-Jones, 2014), piRBase (http://regulatoryrna.org/database/piRNA/; Zhang *et al.* 2014c), and/or lncRNAdb (http://www.lncrnadb.org/; Quek *et al*. 2015) among others. Comparative analysis is a powerful approach to extract functional or evolutionary information from biological sequences (reviewed in O'Brien and Fraser, 2005); however, the low conservation between miRNA-target relationships, especially between teleost fish and mammalian model species, as well as the presence of species specific miRNAs, limit this information gained from this approach in both directions: ncRNAs identification in the species of interest and reliable prediction of the targeted mRNAs or DNA sequences as the conservation of DNA sequences in non-coding regions (*e.g.* gene promoters) is not so high, even when close-related mammalian species are compared (Chiba et al., 2008). Another issue that require attention in such kind of analysis for target mRNA prediction is the existence of different algorithms to rate how the ncRNA-mRNA interaction is most likely to occur. An enormous set of diverse software have been developed in this regard (please, check them at OMICtools; https://omictools.com/; Henry *et al*. 2014), being the most used ones miRanda, TargetScan, PITA and/or RNAhybrid for instance (Riffo-Campos *et al.* 2016).

When quantitative studies are in mind to discover new ncRNAs associated with a particular physiological condition, among other factors, correct normalization procedures are required for getting accurate and reliable results. In this sense, although standard and commercial protocols on isolation, treatment and sequencing procedures are available to decrease intersampling variability, normalization by the addition of known amounts of spike-in ncRNAs or by total reads are the most current and accepted procedures in bioinformatic preprocessing analysis (Tam *et al.* 2015). Particularly problematic is the normalization, standardization and quantification of the analysis of ncRNAs in circulation. Avoiding contamination in plasma preparations and finding a reliable 'housekeeping' ncRNA in the serum are the major goals to be pursued. In any

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case, an additional and final confirmation of the results obtained from RNA-Seq analysis should be performed by RT-qPCR. Researchers have mainly three options to perform an accurate quantification of sncRNAs: stem-loop, locked nucleic acid (LNA) or linear conventional primers; being stem-loop technology the most sensitive and accurate while the LNA the most specific (discriminate between isomiRs; Nolan *et al.* 2013).

Finally, one of the most important investigations to be carried out in the following years will undoubtedly be the development of functional studies. In this sense, experiments *in vivo* (using model species like zebrafish or medaka) and/or *in vitro* where the knocking down or out of the ncRNAs, and/or the particular inhibition, mimicking or overexpression of miRNAs using synthetically produced miRNAs, will be needed to validate the predicted association of these ncRNAs with a particular physiological condition previously explored by RNA-Seq. In this sense, predicted DNA methylation, chromatin-remodeling and/or transcriptional/post-transcriptional mRNA regulation can be empirically demonstrated.

Concluding, new insights on how genes are regulated by ncRNAs at different levels and ways have been recently gained applying NGS technologies in farmed fish species. This basic knowledge open the doors to new strategies to solve old aquaculture problems such as increasing resistance to pathogenic agents, such the use of siRNAs e application to control viral diseases in aquaculture (Papic *et al.* 2015), early nutritional programing by exogenous miRNAs (Cui *et al.* 2017); and/or as reliable biomarkers of fish immunecompetence in the case of lncRNAs (Boltaña *et al.* 2016; Nuñez-Acuña *et al.* 2017) or miRNAs for metabolic and sexual differentiation conditions (miRNAs; Mennigen, 2016; Sun *et al.* 2017). Furthermore, although the biological consequence of circulating ncRNAs on distant sites remains to be uncover, its characterization in body fluids like plasma might be an interesting approach to allow a continuous and less-invasive monitoring method of fish condition in the nearest future.

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