NON-CODING RNA EXPRESSION PATTERNS OF TWO DIFFERENT TELEOST GONAD MATURATION STAGES

Maria Papadaki[1,2], Elisavet Kaitetzidou[1], Constantinos C. Mylonas[1], Elena Sarropoulou[1*]

[1]Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, P.O. Box 2214, Iraklion, Crete 71003, Greece
[2] University of Crete, Department of Biology, P.O. Box 2208, Heraklion 71409, Crete, Greece

*Correspondence: sarris@hcmr.gr

Abstract: (max 250 words.)

Non-coding RNAs (ncRNAs) are involved in several different regulatory pathways including reproduction. In teleost fish, efficacious reproduction is heavily dependent on the completion of the reproductive cycle. The presence of ncRNA however, as well as their expression dynamics and putative regulatory role in mature and immature gonads have not yet been extensively explored. Therefore, the abundance of ncRNAs in mature and immature female sharpsnout seabream (*Diplodus puntazzo*) was investigated. The sharpsnout seabream is a rudimentary hermaphrodite which, in captivity, displays dysfunctions in the gonad maturation process. Our analyses revealed a gonad specific read length distribution with two main peaks representing miRNAs (21nt-26nt) and piwiRNA (27nt-34nt). Besides, distinct expression patterns for several ncRNA biotypes including microRNAs (miRNAs), piwi RNAs (piRNAs), and ribosomal RNAs (rRNAs) were detected. Identified miRNA accounted to 938, corresponding to ~13% of obtained transcripts. Among the differential expressed ncRNAs, 10 (~7%) were annotated as miRNA, out of which 2 were found in higher abundance in immature gonads (miR-125c and miR-24) and 8 (miR-451, miR-7a, miR-122-1, miR190a, miR129, ENSGACT00000029608, ENSGACT00000029489, and ENSGACT00000029667) were found to be higher expressed in mature gonads. Putative miRNA targets, including lncRNAs and genes are proposed. Target genes are involved in several processes of fish oocyte development, such as steroidogenesis, proteolysis, and apoptosis, and may explain hormone regulation. This study demonstrates a gonad maturation biased ncRNA profile which in turn may support the role of ncRNAs in ovarian physiology and reproductive performance of fish, stressing the specific function of each RNA biotype in oocyte development.

Keywords: non-coding RNA, gonad maturation, teleost, differential expression, epigenetics

Introduction

Major parts of the genome undergo transcription but are not further translated into proteins. It has been shown that these non-coding RNA (ncRNA) transcripts encompass key cellular functions and, thus, the main focus has been given on their capability to modulate gene expression. Since ncRNAs change gene expression without DNA sequence alteration, they are considered as epigenetic modifiers, as are DNA methylation and histone modification. Epigenetic modifications are known to play a significant role in embryogenesis, gametogenesis, cell differentiation, and adaptation. Besides, they have been frequently linked to a broad range of diseases. In teleost fish, early environmental influences have been shown to exert a significant epigenetic effect on different traits later in life, such as sex differentiation, metabolism, growth, egg size, lifespan and migration tendency (Jonsson and Jonsson 2014). Moreover, recent studies have investigated the role of epigenetics in fish gametes and early developmental stages (Kaitetzidou et al. 2015; Labbé et al. 2017; Anastasiadi et al. 2018). In contrast to DNA methylation and histone modifications, which belong to the well-known and classical epigenetic mechanisms, ncRNAs are the most recently discovered class of epigenetic effectors, regulating gene expression at the post-transcriptional level.

The main types of ncRNAs with regulative functions are micro RNAs (miRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), piwi interacting RNAs (piRNAs) but also long non-coding RNAs (lncRNAs) (Robles et al. 2019). The latter two classes, piRNAs and lncRNAs are a less well-studied type of transcripts and consequently they are not well annotated (Lipovich et al. 2010; Liao et al. 2011; Bhartiya et al. 2013). On the one hand, piRNAs have been shown to control mainly transposable elements (TE) in mammalian germlines, and on the other hand, it has also been reported that piRNAs may be associated to multifunctional regulatory molecules (Larriba and del Mazo 2018). Concerning lncRNAs, in mammals, it has been shown that they are relatively abundant and that they are involved in several precise cellular functions, among which the regulation of transcription (Khaitovich et al. 2006). In teleosts, some studies have been carried out showing tissue-specific expression (Kaushik et al. 2013), as well as the implication of lncRNAs in immune regulation (Bennett et al. 1976). It has also been shown

that lncRNAs have distinct roles regulating the expression of messenger RNA linked to sex-related differences (Zhang et al. 2019). Among this diverse group of ncRNAs that alters gene expression post-transcriptionally, the most widely studied are miRNAs. They usually inhibit gene expression when binding to target mRNA, either by repressing translation, or by causing mRNA degradation. MiRNAs are highly conserved and their role in different physiological processes has been the subject of a number of recent investigations in different animals, including teleosts (Bizuayehu and Babiak 2014; Kaitetzidou et al. 2015; Liu et al. 2015; Best et al. 2018). In male and female fish gonads, differentially expressed miRNAs have been identified and attempts have been made to link them to sex differentiation mechanisms (Luo et al. 2012; Jing et al. 2014; Mi et al. 2014; Presslauer et al. 2017; Wang et al. 2017, 2018; Qiu et al. 2018). Nevertheless, investigations to unravel the regulatory roles of miRNAs in female gonads during oogenesis and maturation are scarce.

The sharpsnout seabream is a rudimentary hermaphrodite sparid with possible partial protandry (Papadaki et al., 2008), that exhibits asynchronous ovarian development (Pajuelo et al. 2008). Wild-caught sharpsnout seabream mature and spawn spontaneously in captivity (Papadaki et al. 2008). On the contrary hatchery-reared broodstocks often fail to spawn even if both males and females complete gametogenesis (Mylonas et al. 2011; Papadaki et al. 2018), while at times gametogenesis does not take place at all. The successful integration of a new species in aquaculture is heavily dependent on the completion of the reproductive cycle and spontaneous spawning under captivity conditions. During adaptation fish have to adjust to new environmental conditions and, as a result, they may exhibit various reproductive dysfunctions, from difficulties in mating to the absence of oocyte development and ovulation in females and sperm release in males (Mylonas and Zohar 2009; Mylonas et al. 2010).

To gain insights into the functional and molecular regulatory process of teleost fish gonad maturation we conducted a comprehensive assessment of ncRNAs in mature and immature female sharpsnout seabream based on single-end Illumina small RNA sequencing. Our results highlight the distinct expression profiles of miRNAs but also other types of ncRNAs such as lncRNAs, rRNAs, and piRNAs important for successful female gonad maturation.

Materials and Methods

Ethics

Experiments were conducted at the aquaculture facilities of the Hellenic Centre for Marine Research (HCMR, EL91-BIOexp-04). The experimental protocol was approved by the National Veterinary Services (AP 255356). All procedures involving animals were conducted in accordance with the "Guidelines for the treatment of animals in behavioral research and teaching" (Anonymous 1998), the Ethical justification for the use and treatment of teleost fishes in research: an update (Metcalfe and Craig 2011), the Directive 2010/63/EU of the European parliament and the council of 22 September 2010 (EU 2010), as well as the Greek Presidential Decree 56/2013 on "the protection of animals used for scientific purposes".

Broodstock Maintenance, Sampling, and Histological Processing

Fish used in the present study belonged to two different broodstocks maintained at the facilities of HCMR. Five-year-old sharpsnout seabream born by wild-caught breeders spawning annually at HCMR (Papadaki et al. 2008) comprise the "immature" group (individuals IM-1, IM-2 and IM-3) and wild-caught individuals fished near Agathonisi, East Aegean Sea, Greece and kept in our facilities for more than 3 years comprise the "mature" group (individuals M-1, M-2 and M-3). Immature individuals were five-year-old fish that had entered vitellogenesis but failed to spawn during the two previous spawning seasons. In the current spawning season their ovaries were arrested at the primary oocyte (immature) reproductive stage, with no evidence of any gametogenesis (vitellogenesis) or oocyte maturation. On the other hand, mature fish were wild-caught breeders, able to mature and spawn naturally under captivity. Both broodstocks were placed in 5-m^3 tanks, and consisted of 23 and 13 fish of a mean weight ± standard deviation (SD) of 0.94 ± 0.22 kg and 1.26 ± 0.25 kg, respectively. Fish were kept under a constant temperature of 18-20°C throughout the year and were fed with industrial feed (6 mm, Irida SA, Greece).

On October 29, at the peak of the spawning season of the species (Papadaki et al., 2008) a sampling was conducted, in order to investigate the different ncRNA profiles between immature and mature individuals.

Three female fish from each group were killed with an overdose of anaesthetic, the gonads were excised, weighed and one piece of the gonad was fixed for histological evaluation in a solution of 4% formaldehyde:1% glutaraldehyde (McDowell and Trump 1976); another piece was preserved in RNAlater (Sigma-Aldrich, Germany) at -80°C until RNA extraction.

For histological processing, gonads were dehydrated in a 70–95% ethanol series and embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer, Germany). Serial sections of 3–5 µm thickness were obtained on a microtome (Leica RM2245, Germany) using disposable blades. After drying, the slides were stained with methylene blue/azure II/basic fuchsin (Bennett et al. 1976), examined under a light microscope (50i Eclipse, Nikon, Japan) and photographed using a digital camera (Progres, Jenoptik AG, Germany).

RNA Extraction and Preparation of miRNA Libraries

For total RNA extraction, gonad tissues (20-30 mg) were disrupted in liquid nitrogen with mortar and pestle and sample homogenization was achieved by passing the lysate five times through a 23-gauge (0.64 mm) needle. Isolation of total RNA comprising small RNAs was conducted with the use of the Nucleospin miRNA kit (Macherey-Nagel, Duren, Germany) following the manufacturer's instructions for small and large RNA extraction in one fraction. Putative genomic DNA was digested on the column by RNase-free rDNase as indicated in the manufacturer’s instructions. Total RNA quantity was estimated with Nano-Drop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the quality was further evaluated by agarose (1%) gel electrophoresis as well as by RNA Pico Bioanalysis chip (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, California, USA). The RNA integrity number (RIN) has been shown not to be applicable for female gonads in a number of different fish species (Rojo-Bartolomé et al. 2016; Shen et al. 2017), including the sharpsnout seabream (Manousaki et al. 2014) due to the accumulation of 5S RNA in developing gonochoristic and hermaphroditic gonads.

On the other hand, the 5S/18S index is closely linked to the gonad maturation stage (Rojo-Bartolomé et al. 2017b). The gonad maturation index was calculated as follows: 5S/18S index=log2 (time corrected area of the 5S peak/time-corrected area of the 18S peak).

Subsequently, miRNA libraries were generated applying NEBnext multiplex Small RNA Library Preparation Set 1 for Illumina sequencing (New England Biolabs, Ipswich, MA, USA). Size fraction was carried out as recommended by the manufacturer by running a (6%) polyacrylamide gel (Lonza, Basel, Switzerland) at 4 °C for 1 h. Each sample was tagged with different multiplex identifier tag provided by NEB. The generated miRNA libraries were evaluated by DNA high sensitivity Chip (BioAnalyzer, Agilent) prior to sequencing. Quantification of the libraries was carried out with Qubit (Life Technologies, Carlsbad, CA, USA) and 4 nM was single strand sequenced over 4 lanes on the Illumina NextSeq sequencing platform at the Microchemistry laboratory of Forth, Crete, Greece.

Quality control of all reads was carried out applying the freely available Fastqc v0.10.0 software. (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Sequencing reads were quality and adapter trimmed using Trimmomatic software 0.30 (Bolger et al. 2014) and imported into CLC Workbench (v10.1). The putative ncRNAs were further extracted and all reads were counted accordingly. The minimum sampling count (the number of copies of the raw ncRNAs reads included in the resulting count table) was set to 5.

Annotation

Annotation of obtained ncRNAs to identify putative miRNAs was performed against *Gasterosteus_aculeatus*.BROADS1.ncrna as well as against available miRNA annotations of teleosts, (*Cyprinus carpio, Danio rerio, Fugu rubripes, Ictalurus punctatus, Oryzias latipes, Petromyzon marinus, Salmo salar, Tetraodon nigroviridis, Gallus gallus, Hippoglossus hippoglossus, Paralichthys olivaceus*) humans, (*Homo sapiens*), and mice (*Mus musculus*) in miRBase release 21.1 (Griffiths-Jones et al. 2008). Subsequently, merging of variants of the same miRNAs were carried out resulting in a "sampled grouped" list with the corresponding read counts. All annotated reads were normalized by transcripts per

million (TPM). For all remaining unannotated reads the minimum read count number for each sample was set to 20 and subsequently TPM normalized. In order to generate the "RNA sampled grouped" lists for the unannotated reads the cluster software program cd-hit-est (cd-hit-v4.6.1-2012-08-27) with a sequence identity cut-off 0.9 was run. For further characterization, differential expressed novel ncRNAs transcripts were mapped onto the available genomes of the evolutionary nearest species, namely the gilthead sea bream (*Sparus aurata*), the European seabass (*Dicentrarchus labrax*), the greater amberjack (*Seriola dumerili*), Astatotilapia (*Astatotilapia burtoni*) as well as the three-spined stickleback (*Gasterosteus aculeatus*) and submitted to MirDeep2 (version0.1.2, (Friedländer et al. 2012)). In the present study, only those transcripts fulfilling the basic criteria for miRNA identification (Ambros et al. 2003) which are differential expression pattern, typical hairpin structure as well as identification of putative target sites would have been considered as novel miRNAs. Unannotated differential expressed transcripts by mirBase were additionally submitted to the Ensemble, RNAcentral as well as to the NCBI database applying blastn search with the loosest Blast algorithm available, which is "somewhat similar sequences".

Differential Expression Analysis

Differential expression (DE) analysis was carried out by submitting the obtained count matrix from the merged annotated reads as well as the read counts from the clustered unannotated small RNA to SarTools vs 1.2.0 (Varet et al. 2016) with default parameters. Transcripts with p-value < 0.005 and fold change (FC) >|1.8| were considered as differentially expressed. Heatmap as well as PCA analysis was carried out in R (R Core Team 2017). For further downstream analysis of unannotated transcripts a more stringent threshold was applied, specifically read counts of one condition equals to zero.

Target Search

For target search, open reading frames (ORFs) of the assembled transcriptome (Manousaki et al. 2014) were predicted using Transdecoder (version 5.5.0, (Haas et al. 2013)). Subsequently 3' UTRs were extracted from each transcript. For the characterized differential expressed miRNAs putative targets were

identified applying RNAhybrid (version 2.12, (Krüger and Rehmsmeier 2006)) with default parameters. Energy threshold was set to mfe ≤ -30. Putative targets were re-annotated by applying blastn against the GeneBank nr database of NCBI. For investigating the potential targets with blast match to lncRNA the mfe value was set to ≤-25.

Enrichment Analysis

For each set of identified miRNA targets, enrichment analysis was carried out using the Blast2Go 5 basic software program with FDR value <0.05 and a minimum sequence number of 2. Enriched Biological Process GO categories representing a minimum of 2% of the corresponding 'test set' were visualized in the form of a balloon plot in R (R Core Team 2017). The re-annotated transcriptome assembly, provided by Manousaki et al. (Manousaki et al. 2014) served as "reference set".

Data Access

The raw data has been submitted to the NCBI SRA database under the BioProject accession number PRJNA612198.

Results

Gonad Maturation Failure in Sharpsnout Seabream

Ovaries of immature fish were exclusively occupied by primary oocytes. On the other hand, mature individuals were fully vitellogenic, their ovaries containing primary, early vitellogenic, vitellogenic (M-2), as well as early maturing oocytes (M-1 and M-3) (Fig.1A). The increase of oocyte diameter and the acquisition of yolk in vitellogenic oocytes is followed by the subsequent lipid droplet coalescence and the migration of the nucleus to the oocyte periphery in early maturing oocytes (Fig.1A, Supplemental Fig. S1). Histological results were in accordance with the high 5s rRNA peak at about 180 nt (~25s) of the DNAnalyzer profile (Fig. 1B). The correlation of the 5s rRNA peak and the oocyte development stage has previously been shown by Rojo-Bartolomé and colleagues (Rojo-Bartolomé et al. 2017a, b). In accordance with the latter study, the 5S/18S index of hatchery-reared fish of the present study was higher than that of mature fish. Only the mature individual M-2 had a 5S/18S index closer to one of the immature individuals (Fig. 1B).

High Throughput Small RNA Sequencing Revealed Increased Diversity of ncRNAs

The subsequently generated small RNA libraries resulted in two main bands after gel electrophoresis. The band corresponding to putative miRNAs according to the manufacturer were cut for Illumina sequencing. The average percentage observed at a PHRED-score over 30 were 98%. After quality and adaptor trimming, NextSeq Illumina sequencing resulted in an average of 23 million reads per sample (Supplemental table S1). Read length distribution revealed two main peaks, for both mature and immature fish, at 21-26 nt and 27-34 nt (Fig. 2A).

Subsequent annotation of the reads per sample, resulted in an average of 2.2% *Gasterosteus aculeatus* annotated small RNAs and small RNAs annotated with miRbase accounted an average of 1.4%. The majority of the reads of each sample, (~96%), could not be annotated (Supplemental table S2). Clustering of unannotated reads resulted in 5,360 reads (~77% of the total clustered reads). Besides the very high abundance of unannotated RNAs, biotype distribution classified 13.4% as miRNAs, ~2% as rRNAs and

around 8% were identified as other RNA biotypes (snoRNAs, snRNAs, mtRNAs and miscRNAs) (Fig. 2B).

ncRNA are differentially expressed at two different gonad maturation stages

DE analysis resulted in a total of 152 differentially expressed transcripts between mature and immature female fish, out of which 116 (76% of the DE) belonged to the unannotated transcripts, ~15% to rRNA, ~7% to miRNA and ~2% to other biotypes (snoRNAs, snRNAs) (Fig. 2C and Supplemental table S3). Sequence length distribution of differentially expressed transcripts showed a main peak at 27-34 nt comprising mainly unannotated reads (Fig. 2D). Following Principal Component Analysis (PCA, Fig. 3A) of the differentially expressed transcripts resulted in two distinct groups, with the 1st, 2nd, and 3rd principal component explaining 89%, 8% and 1.3% of the total variance, respectively.

In addition, distinct expression patterns of all differentially expressed ncRNAs between mature and immature ovaries were also found after hierarchical clustering and were visualized in form of a heatmap (Fig.3B). The latter shows that most of the obtained transcripts were more expressed in immature animals. Concerning miRNAs, only two miRNAs (ENSGACT00000028206.1 and miR-125c) were higher expressed in immature fish, while the rest were higher expressed in mature fish. Higher abundance in the mature fish has also been found for the class of rRNA representing about 11% of the differential expressed ncRNAs. On the other hand, more unannotated small RNAs were found to be higher expressed in immature (57%) than in mature fish (19%) (Fig. 3B). Applying mirDeep2 mapper.pl function allowing one mismatch at the seed region successfully mapped ~38% of the unannotated ncRNA to the gilthead sea bream (*Sparus aurata*, Sparidae), ~12% to the European seabass (Moronidae), ~8% to the greater amberjack (Carangidae), ~5% to three-spined stickleback (*Gasterosteus aculeatus*, Gasterosteidae) and ~7% to tilapia (*Astatotilapia niloticus*, Cichlidae). Subsequent hairpin and similarity search did not result in the identification of putative new miRNAs. Further annotation of unannotated differentially expressed transcripts by blastn search against the NCBI nr database revealed 10 transcripts classified as long non-coding (lnc) RNAs (~9%), 12 as rRNAs (~10 %), 20 as mRNAs (~17%) and 73 remained unannotated

(~63%). For the needs of the present work, unknown differential expressed transcripts were also mapped onto a collection containing putative piRNA from the zebrafish as well as all identified piRNAs available at RNAcentral resulting in 13 matches with an e-value <0.05 to putative piRNAs being differentially expressed (Supplemental table S4).

For the differential expressed miRNAs further downstream analysis comprising target search was carried out.

Micro RNAs Target Genes are Involved in Distinct Biological Processes

Micro RNA target genes identified for the differentially expressed miRNAs showed that miR-122-1 and miR-125c are involved in a plethora of different biological processes (BP) including e.g. proteolysis, while miR-129 and miR-24 are involved in only two and three GO-BP categories respectively. Among the four miRNAs the number of targets varied. Two of them, miR-122-1 and miR-125c were found to target most of the available annotated transcripts while the other two miRs, miR-129 and miR-24, targeted genes classified only in two or three categories respectively (Fig. 4). For the miRNAs miR-7a, miR-190, miR-451 not enough target genes were identified to carry out enrichment analysis (22, 1 and 21 respectively) (Supplemental file 5).

Distinct expression pattern of ncRNAs

Eight out the 10 via NCBI blast hit identified lncRNA are found in higher abundance in the immature fish while 8 out of the 10 miRNAs differentially expressed are found in higher abundance in the mature fish (Fig. 5A). This may be an indication for putative interaction between lncRNA and miRNA. The putative lncRNA-miRNA hybridizations of five miRNAs (miR-122-1, miR-129, miR-125c, miR-7a and ENSGACT00000282061) with mfe ≤ -25 are illustrated in figure 5B.

Discussion

The peak of the sharpsnout seabream reproductive season in the Mediterranean occurs from October until December (Papadaki et al. 2008). At this time, hatchery-produced sharpsnout seabream of the present study were immature and their ovaries contained exclusively primary oocytes, whereas wild-caught individuals were mature and their ovaries contained both vitellogenic oocytes and oocytes at the early stages of maturation. This could be reflected also in the total RNA electropherograms of gonads. In immature ovaries the 5S rRNA peak was very high and masked the other two peaks. In the more advanced stages of oocyte development, of the mature fish ovaries, the peaks of 18S and 28S rRNA were more pronounced (Fig.1B). Oocyte development specific rRNA profiles have been shown in a number of fish species (Diaz De Cerio et al. 2012). It has further been proposed that the 5S/18S index can be used to distinguish the oogenesis stage in female fish (Diaz De Cerio et al. 2012; Rojo-Bartolomé et al. 2016, 2017b). In sharpsnout seabream, the 5S/18S ratios, with the exception of individual M-2, were in line with their developmental stage, separating the mature from the immature fish samples. The individual M-2 exhibited a 5S/18S index similar to the one of immature individuals; this is explained by the fact that, although this fish had already entered vitellogenesis, its ovary still contained a large number of primary oocytes, thus explaining its high 5S/18S index (Fig. 1A and Supplemental Fig. S1). Still, ncRNA expression data clustered this individual clearly to the mature individuals (Fig. 3A and B). The accumulation of 5S rRNA in the immature stage, is associated with the preparation of the oocytes for fertilization and the storage of rRNA for later use in protein synthesis during embryogenesis (Diaz De Cerio et al. 2012; Ortiz-Zarragoitia et al. 2014; Rojo-Bartolomé et al. 2016). Later, at vitellogenesis, 18S and 28S rRNAs are produced. Those dynamics of rRNA production during oocyte development are also reflected in the present study with the high percentages of rRNA transcripts (~15%) differentially expressed between immature and mature individuals compared to the percentage of rRNA of all obtained transcripts (~2%) (Fig. 2B and C). Consequently, this result supports the conclusion that rRNA, other than energy reserve before embryogenesis, also serves for protein synthesis during vitellogenesis (Shen et al. 2017). Besides, a notably high number of unannotated transcripts being differentially expressed were

reported in the present study. Similar results have also been reported in zebrafish (*Danio rerio*) gonad ncRNA studies (Presslauer et al. 2017) as well as in gonads of other fish species, such as the Atlantic halibut (*Hippoglossus hippoglossus*) (Bizuayehu et al. 2012), the marine medaka (*Oryzias melastigma*) (Li et al. 2016), and the rainbow trout (*Oncorhynchus mykiss*) (Farlora et al. 2015). The high number of unannotated transcripts pinpoints the need to generate powerful annotation pipelines and further downstream analysis for teleost ncRNA characterization. For miRNAs it has been suggested that their annotation comprises three major steps: i) differential expression ii) conservation among species, and iii) the presence of the typical hairpin structure (Ambros et al. 2003). Concerning the first step, the 116 unannotated transcripts in the present study were differentially expressed. Corresponding analysis for step ii) and iii) preconditioned first the mapping of unannotated transcripts to available genomes and revealed that mapping success was highly dependent on the taxonomic position of the respective species. Conservation among species is one of the main characteristics of miRNAs; therefore, the fact that no miRNAs were identified by carrying out additional mirdeep2 analysis was expected. Among the differentially expressed but unannotated transcripts significant matches to lncRNAs and mRNAs were obtained. While lncRNA represent the largest group of ncRNAs their characterization remains difficult, although today several databases are available and specific lncRNA identification properties have been set. Then again, the presence of a functional open-reading frame (ORF) within a transcript may also comprise a non-coding function. Examples important in reproduction are the sex-determining region Y (SRY) as well as oskar RNAs (Taylor et al. 2015). Consequently, those transcripts annotated as mRNA and being differentially expressed, may also be of importance but were outside the scope of the present study. On the other hand, read length distribution of the generated small RNA libraries in the present study may indicate the presence of piRNAs since a second dominant peak was obtained for the gonad libraries (Figs.2A and D) between 27 and 34 nt. Small RNA libraries were generated following the same protocol as for European sea bass embryonic development stages, which resulted only in one main peak (Sarropoulou et al. 2019). Different read length distributions out of gonad small RNA libraries have also been observed in other fish studies (Presslauer et al. 2017; Wang et al. 2017). piRNAs are dominant in

germ cells and are involved in gonad function and development (Bizuayehu et al. 2012; Gu et al. 2014; Jing et al. 2014; Juanchich et al. 2016; Presslauer et al. 2017; Wang et al. 2017, 2018). Furthermore, in a study on the common carp (*Cyprinus carpio*), it was also found that piRNAs are more abundant than miRNAs in the mature ovary compared to earlier stages (Wang et al. 2017). However, annotation for piRNAs in teleosts is still in its infancy and efforts are needed to establish a reliable teleost piRNA database.

Concerning miRNAs, the percentage of successfully identified miRNAs was relatively low (13.4 %) compared to miRNAs found for example during European sea bass development (Sarropoulou et al. 2019), where more than 50% of the transcripts were annotated as miRNAs. The exploration of the target genes of each of the differentially expressed miRNAs revealed that they belong to specific GO terms. For example, only the miR-129 target genes mapped to the GO term "cellular response to peptide hormone stimulus", while only the target genes of miR-122-1 mapped to the GO term "cellular response to corticosterone stimulus" .Both GO terms respectively were not detected for the other miRNA target genes (Fig.4). Previous studies in miR-122-1 have shown that miR 122-1 is less abundant (a) in livers of vitellogenic female zebrafish, compared to non-vitellogenic ones and (b) in livers of E2-treated male zebrafish compared to untreated controls, suggesting a role for this hepatic miRNA in vitellogenesis (Cohen and Smith 2014). Vitellogenesis includes the synthesis of vitellogenins in the liver, their transportation to the ovary and the formation of yolk proteins (Reading et al. 2016). A possible scenario could be that miR-122 is not or only little present in the liver and in high abundance in the gonads during vitellogenesis and oocyte development. The expression of miR-122 has also been correlated with sex steroid hormone biosynthesis, both in the Nile tilapia (*Oreochromis niloticus*) (Wang et al. 2016) and in humans (Sirotkin et al. 2009). Also the other differentially expressed miRNAs of the present study, have been reported to be involved in oocyte development and ovarian function, both in mammals and in teleosts (Baley and Li 2012; Bouchareb et al. 2017; Reza et al. 2019). miR-7a for example, which was more abundant in mature females with advanced ovarian stages is involved in mammalian ovulation, as its loss can lead to infertility in mice, in both males and females (Ahmed et al. 2017). In fish, miR-7a has

been found in primary growth and previtellogenic oocytes of zebrafish (Wong et al. 2018). Mir-129, which was found in higher copy number in the mature individuals of the present study, was also found in higher abundance in mature ovaries of the Nile tilapia (Xiao et al. 2014) and in mature testes of the rainbow trout (Farlora et al. 2015). In humans, miR-129 has also been related to steroid biosynthesis (Sirotkin et al. 2009). Oocyte development has been linked to the higher abundance of another miRNA, miR-451, which was detected in higher copy numbers in mature ovaries in the present study and was also highly abundant in large compared to small, healthy follicles of bovine (Sontakke et al. 2014). The role of this miRNA, together with a set of other miRNAs, was linked to the development of the dominant follicle in bovine (Sontakke et al. 2014). In Atlantic halibut, it has been shown that the expression of miR-451 is regulated by sex steroid hormones (Bizuayehu et al. 2012).

In contrast, only two miRNAs were found in higher copy numbers in immature sharpshout seabream; ENSGACT00000028206.1, which has been annotated by mirDB as miR-24, and miR-125c. The first miRNA, miR-24, has been shown to be involved in steroidogenesis in human ovaries, enhancing progesterone and inhibiting estradiol (E2) and testosterone (T) release (Sirotkin et al. 2009). This increase of progesterone and inhibition of E2 and T release could lead to the lack of maturation of immature fish. Progesterone is known to suppress gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) release (Skinner et al. 1998), whereas E2 and T are related with vitellogenesis and oocyte maturation, respectively (Nagahama et al. 1994). Thus, an increase in progesterone and a decrease in E2 and T levels are all possible ways of hindering oocyte maturation.

The second small RNA that was found in higher abundance in immature females in the present study, miR-125c, has also been detected in immature zebrafish ovaries [6 weeks post fertilization (wpf)], whereas in testes it is expressed after 6, 9 and 12 wpf (Presslauer et al. 2017). Enrichment analysis of putative miR-125c targets were mapped to numerous GO terms (Fig. 4). In mammals, miR-125c is absent, however, the seed region of miR-125a,b and c is fully conserved (He et al. 2017) and members of the miR-125 family have been associated with a broad range of physiological processes which may explain the finding of numerous GO categories in the present study. Among the top ten GO terms found

there were “negative regulation of cell death” as well as “proteolysis”. Through proteolysis, vitellogenin is transformed into smaller-sized yolk proteins in the maturing oocyte (Selman et al. 1993). In species spawning pelagic eggs, these yolk proteins are further proteolyzed to form small peptides and free amino acids, causing subsequent water intake, oocyte hydration and buoyancy of the fertilized eggs (Cerdá et al. 2007; Finn 2007). Yolk proteins are of course necessary for embryo development, since they provide the nutrients for embryonic and early larval development and metabolism (Patiño and Sullivan 2002; Reading et al. 2018). Assuming that miR-125c inhibits gene expression, proteolysis of vitellogenins and yolk proteins is suppressed, inhibiting maturation of the immature females, which show higher abundance of mir-125c in their gonads. Besides the gene ontology term “proteolysis” it has also been found that miR-125c targets the gene follicle stimulating hormone receptor (fshr) (Fig. 6). First approaches showed that in the absence of fshr, primary ovarian follicle growth was retarded (Zhang et al. 2015). In fish, the initiation of vitellogenesis is triggered by a rise in follicle stimulating hormone (FSH), which is responsible for the subsequent E2 synthesis in the ovary, followed by vitellogenin synthesis in the liver (Nagahama and Yamashita 2008b). The fact that miR-125c was found in higher abundance in immature fish in the present study may indicate the repression of fshr, and thus of E2 synthesis. This may explain the vitellogenesis failure in immature individuals. In the present study, it was also found that miR-125c may interact with one potential lncRNA (Fig. 5), which is in higher abundance in immature fish. Besides the broad range of functions as mentioned before it has been shown that lncRNAs may also act as enhancers of miRNAs (Fernandes et al. 2019).

Noteworthy, fshr has also been shown to be involved in the process of sex reversal (Huang et al. 2019) and in spite of the teleost specific whole genome duplication, it has been accepted that teleosts comprise a single fshr gene (Maugars et al. 2015). Nevertheless, since several splice variants have been reported, a phylogenetic tree comprising 12 teleost species has been generated using the transcript of sharpsnout seabream as reference. It appears that only for the protandrous hermaphrodite gilthead sea bream (*Sparus aurata*) and the rudimentary hermaphrodite sharpsnout seabream miR-125c reaches the needed minimum

free energy threshold to be accepted as putative fshr regulator (Fig. 6). However further studies are needed to confirm this finding.

In conclusion, the results of the present study suggest that miRNAs may contribute to fish oogenesis and oocyte development, being involved in different aspects of the process, such as steroidogenesis, apoptosis and proteolysis. Other than miRNAs, also piRNAs, rRNAs and putative lncRNAs show differential expression between mature and immature sharpsnout seabream females. Additionally, the present study suggests a possible interaction of miRNA-lncRNA during gonad maturation. However, further studies are needed to focus on the relationship between the different types of small RNAs and investigate the mechanisms by which they act to control the reproductive process in teleost fish.

Author Contributions: Conceptualization, E.S., M.P. and C.C.M.; methodology, M.P. and E.K.; software, E.S.; validation, E.S., M.P. and E.K.; investigation, M.P and E.S.; resources, M.P. and C.C.M ; writing—original draft preparation, M.P. and E.S.; writing—review and editing, E.S and C.C.M; visualization, E.S. and M.P.; supervision, E.S. and C.C.M.; funding acquisition, C.C.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partly funded by the European Union's Horizon 2020 research and innovation programme under grant agreement No 727315 (MedAID). Call: SFS-23-2016 improving the technical performance of the Mediterranean aquaculture. This research was supported in part through computational resources provided by IMBBC (Institute of Marine Biology, Biotechnology, and Aquaculture of the HCMR (Hellenic Centre for Marine Research). Funding for establishing the IMBBC HPC has been received by the MARBIGEN (EU Regpot) project, LifeWatch Greece RI, and the CMBR (Centre for the study and sustainable exploitation of Marine Biological Resources) RI.

Acknowledgements: We would like to thank Irini Stratidaki and Niki Gounalaki from the NextSeq sequencing platform at the Microchemistry laboratory of Forth, Crete, Greece for sequencing support.

Conflicts of Interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

References

Ahmed K, LaPierre MP, Gasser E, et al (2017) Loss of microRNA-7a2 induces hypogonadotropic hypogonadism and infertility. J Clin Invest 127:1061–1074 . doi: 10.1172/JCI90031

Ambros V, Bartel B, Bartel DP, et al (2003) A uniform system for microRNA annotation. 277–279 . doi: 10.1261/rna.2183803.One

Anastasiadi D, Vandeputte M, Sánchez-Baizán N, et al (2018) Dynamic epimarks in sex-related genes predict gonad phenotype in the European sea bass, a fish with mixed genetic and environmental sex determination. Epigenetics 13:988–1011 . doi: 10.1080/15592294.2018.1529504

Anonymous (1998) Guidelines for the treatment of animals in behavioural research and teaching. Anim Behav 55: 251–25:Animal protectionAnimal use

Baley J, Li J (2012) MicroRNAs and ovarian function. J Ovarian Res 5:1–7 . doi: 10.1186/1757-2215-5-8

Bennett HS, Wyrick AD, Lee SW, Mcneil JH (1976) Science and art in preparing tissues embedded in plastic for light microscopy, with special reference to glycol methacrylate, glass knives and simple stains. Stain Technol 51:71–97 . doi: 10.3109/10520297609116677

Best C, Ikert H, Kostyniuk DJ, et al (2018) Epigenetics in teleost fish: From molecular mechanisms to physiological phenotypes. Comp Biochem Physiol Part - B Biochem Mol Biol 224:210–244 . doi: 10.1016/j.cbpb.2018.01.006

Bhartiya D, Pal K, Ghosh S, et al (2013) LncRNome: A comprehensive knowledgebase of human long noncoding RNAs. Database 2013:1–6 . doi: 10.1093/database/bat034

Bizuayehu TT, Babiak J, Norberg B, et al (2012) Sex-biased miRNA expression in atlantic halibut (*Hippoglossus hippoglossus*) brain and gonads. Sex Dev 6:257–266 . doi: 10.1159/000341378

Bizuayehu TT, Babiak I (2014) MicroRNA in teleost fish. Genome Biol Evol 6:1911–1937 . doi: 10.1093/gbe/evu151

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120 . doi: 10.1093/bioinformatics/btu170

Bouchareb A, Le Cam A, Montfort J, et al (2017) Genome-wide identification of novel ovarian-

predominant miRNAs: New insights from the medaka (Oryzias latipes). Sci Rep 7:1–11 . doi: 10.1038/srep40241

Cerdá J, Fabra M, Raldúa D (2007) Physiological and molecular basis of fish oocyte hydration. In: Babin PJ, Cerdá J, Lubzens E (eds) The Fish Oocyte: from Basic Studies to Biotechnological Applications. Kluwer Academic Publishers, Dordrecht, The Netherlands, p FOMhydration

Cohen A, Smith Y (2014) Estrogen Regulation of microRNAs, Target Genes, and microRNA Expression Associated with Vitellogenesis in the Zebrafish. Zebrafish 11:462–478 . doi: 10.1089/zeb.2013.0873

Diaz De Cerio O, Rojo-Bartolomé I, Bizarro C, et al (2012) 5S rRNA and accompanying proteins in gonads: Powerful markers to identify sex and reproductive endocrine disruption in fish. Environ Sci Technol 46:7763–7771 . doi: 10.1021/es301132b

EU (2010) Directive 2010/63/EU of the European parliament and the council of 22 September 2010 on the protection of animals used for scientific purposes. Off J Eur Union L 276/33:Animal protection

Farlora R, Valenzuela-Miranda D, Alarcón-Matus P, Gallardo-Escárate C (2015) Identification of microRNAs associated with sexual maturity in rainbow trout brain and testis through small RNA deep sequencing. Mol Reprod Dev 82:651–662 . doi: 10.1002/mrd.22499

Fernandes JCR, Acuña SM, Aoki JI, et al (2019) Long non-coding RNAs in the regulation of gene expression: Physiology and disease. Non-coding RNA 5: . doi: 10.3390/ncrna5010017

Finn RN (2007) The maturational disassembly and differential proteolysis of paralogous vitellogenins in a marine pelagophil teleost: A conserved mechanism of oocyte hydration. Biol Reprod 76: 936-94:vitellogenesishydrationFOMovulation

Friedländer MR, MacKowiak SD, Li N, et al (2012) MiRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. Nucleic Acids Res. doi: 10.1093/nar/gkr688

Griffiths-Jones S, Saini HK, Van Dongen S, Enright AJ (2008) miRBase: Tools for microRNA genomics. Nucleic Acids Res. doi: 10.1093/nar/gkm952

Gu Y, Zhang L, Chen X (2014) Differential expression analysis of *Paralichthys olivaceus* microRNAs in adult ovary and testis by deep sequencing. Gen Comp Endocrinol 204:181–184 . doi:

10.1016/j.ygcen.2014.05.019

Haas BJ, Papanicolaou A, Yassour M, et al (2013) De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nat Protoc. doi: 10.1038/nprot.2013.084

He Y, Huang C-X, Chen N, et al (2017) The zebrafish miR-125c is induced under hypoxic stress *via* hypoxia-inducible factor 1α and functions in cellular adaptations and embryogenesis. Oncotarget 8: . doi: 10.18632/oncotarget.17994

Huang M, Chen J, Liu Y, et al (2019) New insights into the role of follicle-stimulating hormone in sex differentiation of the protogynous orange-spotted grouper, Epinephelus coioides. Front Endocrinol (Lausanne) 10:1–11 . doi: 10.3389/fendo.2019.00304

Jing J, Wu J, Liu W, et al (2014) Sex-biased miRNAs in gonad and their potential roles for testis development in yellow catfish. PLoS One. doi: 10.1371/journal.pone.0107946

Jonsson B, Jonsson N (2014) Early environment influences later performance in fishes. J Fish Biol 85:151–188 . doi: 10.1111/jfb.12432

Juanchich A, Bardou P, Rué O, et al (2016) Characterization of an extensive rainbow trout miRNA transcriptome by next generation sequencing. BMC Genomics 17:1–12 . doi: 10.1186/s12864-016-2505-9

Kaitetzidou E, Xiang J, Antonopoulou E, et al (2015) Dynamics of gene expression patterns during early development of the European seabass (*Dicentrarchus labrax*). Physiol Genomics. doi: 10.1152/physiolgenomics.00001.2015

Kaushik K, Leonard VE, Shamsudheen K V., et al (2013) Dynamic expression of long non-coding RNAs (lncRNAs) in adult zebrafish. PLoS One 8: . doi: 10.1371/journal.pone.0083616

Khaitovich P, Kelso J, Franz H, et al (2006) Functionality of intergenic transcription: An evolutionary comparison. PLoS Genet 2:1590–1598 . doi: 10.1371/journal.pgen.0020171

Krüger J, Rehmsmeier M (2006) RNAhybrid: MicroRNA target prediction easy, fast and flexible. Nucleic Acids Res. doi: 10.1093/nar/gkl243

Labbé C, Robles V, Herraez MP (2017) Epigenetics in fish gametes and early embryo. Aquaculture 472:93–106 . doi: 10.1016/j.aquaculture.2016.07.026

Larriba E, del Mazo J (2018) An integrative piRNA analysis of mouse gametes and zygotes reveals new potential origins and gene regulatory roles. Sci Rep 8:16–18 . doi: 10.1038/s41598-018-31032-1

Li JW, Lin X, Tse A, et al (2016) Discovery and functional characterization of novel miRNAs in the marine medaka Oryzias melastigma. Aquat Toxicol 175:106–116 . doi: 10.1016/j.aquatox.2016.03.013

Liao Q, Liu C, Yuan X, et al (2011) Large-scale prediction of long non-coding RNA functions in a coding-non-coding gene co-expression network. Nucleic Acids Res 39:3864–3878 . doi: 10.1093/nar/gkq1348

Lipovich L, Johnson R, Lin CY (2010) MacroRNA underdogs in a microRNA world: Evolutionary, regulatory, and biomedical significance of mammalian long non-protein-coding RNA. Biochim Biophys Acta - Gene Regul Mech 1799:597–615 . doi: 10.1016/j.bbagrm.2010.10.001

Liu J, Luo M, Sheng Y, et al (2015) Dynamic evolution and biogenesis of small RNAs during sex reversal. Sci Rep 5:1–18 . doi: 10.1038/srep09999

Luo GZ, Hafner M, Shi Z, et al (2012) Genome-wide annotation and analysis of zebra finch microRNA repertoire reveal sex-biased expression. BMC Genomics 13: . doi: 10.1186/1471-2164-13-727

Manousaki T, Tsakogiannis A, Lagnel J, et al (2014) The sex-specific transcriptome of the hermaphrodite sparid sharpsnout seabream (*Diplodus puntazzo*). BMC Genomics. doi: 10.1186/1471-2164-15-655

Maugars G, Dufour S, Vaudry H (2015) Demonstration of the coexistence of duplicated LH receptors in teleosts, and their origin in ancestral actinopterygians. PLoS One 10:1–29 . doi: 10.1371/journal.pone.0135184

McDowell EM, Trump BF (1976) Histologic fixatives suitable for diagnostic light and electron microscopy. Arch Pathol Lab Med 100:405–414

Metcalfe JD, Craig JF (2011) Ethical justification for the use and treatment of fishes in research: an update. J Fish Biol 78:393–394

Mi X, Wei Z, Zhou Z, Liu X (2014) Identification and profiling of sex-biased microRNAs from sea urchin Strongylocentrotus nudus gonad by Solexa deep sequencing. Comp Biochem Physiol - Part D Genomics Proteomics 10:1–8 . doi: 10.1016/j.cbd.2014.01.001

Mylonas CC, Zohar Y (2009) Controlling reproduction in aquaculture. In: Burnell G, Allan G (eds) New Technologies in Aquaculture: Improving Production Efficiency, Quality and Environmental Management. Woodhead Publishing Ltd, Cambridge, UK, pp 109–142

Mylonas CC, Fostier A, Zanuy S (2010) Broodstock management and hormonal manipulations of fish reproduction. Gen Comp Endocrinol 165:516–534 . doi: 10.1016/j.ygcen.2009.03.007

Mylonas CC, Zohar Y, Pankhurst NW, Kagawa H (2011) Reproduction and broodstock management. In: Pavlidis M, Mylonas CC (eds) Sparidae: Biology and Aquaculture of Gilthead Seabream and Related Species. Blackwell Science Publishers, London, pp 95–131

Nagahama Y, Yoshikuni M, Yamashita M, Tanaka M (1994) Regulation of Oocyte Maturation in Fish. Fish Physiol. doi: 10.1016/S1546-5098(08)60074-6

Ortiz-Zarragoitia M, Bizarro C, Rojo-Bartolomé I, et al (2014) Mugilid fish are sentinels of exposure to endocrine disrupting compounds in coastal and estuarine environments. Mar Drugs 12:4756–4782 . doi: 10.3390/md12094756

Pajuelo JG, Lorenzo JM, Domínguez-Seoane R (2008) Gonadal development and spawning cycle in the digynic hermaphrodite sharpsnout seabream *Diplodus puntazzo* (Sparidae) off the Canary Islands, northwest of Africa. J Appl Ichthyol. doi: 10.1111/j.1439-0426.2007.01010.x

Papadaki M, Papadopoulou M, Sigelaki I, et al (2008) Egg and sperm production and quality of sharpsnout sea bream (*Diplodus puntazzo*) in captivity. Aquaculture 276:187–197 . doi: 10.1016/j.aquaculture.2008.01.033

Papadaki M, Mazzella D, Santinelli V, et al (2018) Hermaphroditism and reproductive function of hatchery-produced sharpsnout seabream (*Diplodus puntazzo*) under attenuated annual thermal cycles. Aquaculture. doi: 10.1016/j.aquaculture.2017.09.028

Patiño R, Sullivan C V (2002) Ovarian follicle growth, maturation, and ovulation in teleost fish. Fish

Physiol Biochem 26: 57-70:oogenesisFOMovulation

Presslauer C, Tilahun Bizuayehu T, Kopp M, et al (2017) Dynamics of miRNA transcriptome during gonadal development of zebrafish. Sci Rep. doi: 10.1038/srep43850

Qiu W, Zhu Y, Wu Y, et al (2018) Identification and expression analysis of microRNAs in medaka gonads. Gene. doi: 10.1016/j.gene.2017.12.062

R Core Team (2017) R: A language and environment for statistical computing

Reading BJ, Sullivan CV, Schilling J (2016) Vitellogenesis in Fishes ☆. Elsevier Ltd.

Reading BJ, Andersen LK, Ryu YW, et al (2018) Oogenesis and egg quality in Finfish: Yolk formation and other factors influencing female fertility. Fishes 3:1–28 . doi: 10.3390/fishes3040045

Reza AMMT, Choi YJ, Han SG, et al (2019) Roles of microRNAs in mammalian reproduction: from the commitment of germ cells to peri-implantation embryos. Biol Rev 94:415–438 . doi: 10.1111/brv.12459

Robles V, Valcarce DG, Riesco MF (2019) Non-coding RNA regulation in reproduction: Their potential use as biomarkers. Non-coding RNA Res 4:54–62 . doi: 10.1016/j.ncrna.2019.04.001

Rojo-Bartolomé I, De Cerio OD, Diez G, Cancio I (2016) Identification of sex and female's reproductive stage in commercial fish species through the quantification of ribosomal transcripts in gonads. PLoS One. doi: 10.1371/journal.pone.0149711

Rojo-Bartolomé I, Martínez-Miguel L, Lafont AG, et al (2017a) Molecular markers of oocyte differentiation in European eel during hormonally induced oogenesis. Comp Biochem Physiol -Part A Mol Integr Physiol 211:17–25 . doi: 10.1016/j.cbpa.2017.05.018

Rojo-Bartolomé I, Valencia A, Cancio I (2017b) Transcription of ribogenesis genes in fish gonads: Applications in the identification of stages of oogenesis and in environmental monitoring of intersex condition. Mar Pollut Bull 121:292–301 . doi: 10.1016/j.marpolbul.2017.06.027

Sarropoulou E, Kaitetzidou E, Papandroulaki N, et al (2019) Inventory of European sea bass *(Dicentrarchus labrax*) sncRNAs vital during early teleost development. Front Genet 10:1–11 . doi:

10.3389/fgene.2019.00657

Selman K, Wallace RA, Sarka A, Qi X (1993) Stages of oocyte development in the zebrafish, Brachydanio rerio. J Morphol 218: 203–2:Brachydanio, zebrafish, oogenesis, FOM, histology

Shen ZG, Yao H, Guo L, et al (2017) Ribosome RNA Profiling to Quantify Ovarian Development and Identify Sex in Fish. Sci Rep 7:1–11 . doi: 10.1038/s41598-017-04327-y

Sirotkin A V., Ovcharenko D, Grossmann R, et al (2009) Identification of microRNAs controlling human ovarian cell steroidogenesis via a genome-scale screen. J Cell Physiol 219:415–420 . doi: 10.1002/jcp.21689

Skinner DC, Evans NP, Delaleu B, et al (1998) The negative feedback actions of progesterone on gonadotropin-releasing hormone secretion are transduced by the classical progesterone receptor. Proc Natl Acad Sci U S A. doi: 10.1073/pnas.95.18.10978

Sontakke SD, Mohammed BT, McNeilly AS, Donadeu FX (2014) Characterization of microRNAs differentially expressed during bovine follicle development. Reproduction 148:271–283 . doi: 10.1530/rep-14-0140

Taylor DH, Chu ET, Spektor R, Soloway PD (2015) HHS Public Access. Mol Reprod Dev 82:932–956

Varet H, Brillet-Guéguen L, Coppée JY, Dillies MA (2016) SARTools: A DESeq2- and edgeR-based R pipeline for comprehensive differential analysis of RNA-Seq data. PLoS One 11:1–8 . doi: 10.1371/journal.pone.0157022

Wang F, Jia Y, Wang P, et al (2017) Identification and profiling of *Cyprinus carpio* microRNAs during ovary differentiation by deep sequencing. BMC Genomics 18:1–17 . doi: 10.1186/s12864-017-3701-y

Wang P, Wang L, Yang J, et al (2018) Sex-biased miRNAs of yellow catfish (*Pelteobagrus fulvidraco*) and their potential role in reproductive development. Aquaculture 485:73–80 . doi: 10.1016/j.aquaculture.2017.11.020

Wang W, Liu W, Liu Q, et al (2016) Coordinated microRNA and messenger RNA expression profiles for understanding sexual dimorphism of gonads and the potential roles of microRNA in the

steroidogenesis pathway in Nile tilapia (*Oreochromis niloticus*). Theriogenology 85:970–978 . doi: 10.1016/j.theriogenology.2015.11.006

Wong QWL, Sun MA, Lau SW, et al (2018) Identification and characterization of a specific 13-miRNA expression signature during follicle activation in the zebrafish ovary. Biol Reprod 98:42–53 . doi: 10.1093/biolre/iox160

Xiao J, Zhong H, Zhou Y, et al (2014) Identification and characterization of MicroRNAs in ovary and testis of Nile tilapia (*Oreochromis niloticus*) by using Solexa sequencing technology. PLoS One 9: . doi: 10.1371/journal.pone.0086821

Zhang S, Wang Y, Jia L, et al (2019) Profiling the long noncoding RNA interaction network in the regulatory elements of target genes by chromatin in situ reverse transcription sequencing. Genome Res 29:1521–1532 . doi: 10.1101/gr.244996.118

Zhang Z, Lau S-W, Zhang L, Ge W (2015) Disruption of Zebrafish Follicle-Stimulating Hormone Receptor (fshr) But Not Luteinizing Hormone Receptor (lhcgr) Gene by TALEN Leads to Failed Follicle Activation in Females Followed by Sexual Reversal to Males. Endocrinology 156:3747–3762 . doi: doi:10.1210/en.2015-1039

Figure legends

Figure 1. A. Histology ovarian photos of three immature (on the left: IM-1, IM-2 and IM-3) and three mature (on the right: M-1, M-2 and M-3) sharpsnout seabream. Immature fish ovaries were filled with primary oocytes (po), whereas mature fish gonads contain early vitellogenic (eVg), vitellogenic (Vg) and early maturing oocytes (eOM). Scale bars indicate 500 μm. **B.** Electropherograms of total RNA of immature and mature sharpsnout seabream gonads. The number shown in red on each chart represents the 5S/18S index.

Figure 2. A. Read length distribution of small RNA sequences of mature and immature sharpsnout seabream ovaries. Red colour indicates immature fish and blue colour mature fish. **B.** Pie chart of all isolated small non-coding RNA biotypes found in libraries of mature and immature sharpnout seabream ovaries. **C.** Pie chart of statistically significant differentially expressed ($P<0.05$) small non-coding RNA percentages found in libraries of mature and immature sharpsnout seabream ovaries. **D.** Read length distribution of different biotypes of small non-coding RNAs. miRNA: micro RNA, snoRNA: small nucleolar RNA, snRNA: small nuclear RNA, rRNA: ribosomal RNA, mt-tRNA: mitochondrial transfer RNA, miscRNA: miscellaneous RNA.

Figure 3. A. Principal component analysis of differentially expressed small RNA transcripts of immature (IM-1, IM-2 and IM-3) and mature (M-1, M-2, M-3) sharpsnout seabream ovaries. X, Y and Z axis show principal components 1, 2 and 3 that explain 89%, 8% and 1,3 % of the proportion of variance, respectively. N = 6 data points. **B.** Heatmap visualization of differentially expressed non-coding RNAs of immature and mature sharpsnout seabream gonads (padj<0.05). Individual fish are indicated at the bottom of each column (IM-1, IM-2 and IM-3 are immature and M-1, M-2 and M-3 are mature fish). Each row represents the expression of one non-coding RNA, and purple and light purple represent high and low abundance, respectively.

Figure 4. Balloon plot displaying the miRNA target genes identified for the differentially expressed miRNAs. The colour indicates the number of targets, whereas the size of the circle indicates the percentage of target genes of each GO term identified for each miRNA.

Figure 5. A. Heatmap visualization of differentially expressed long non-coding RNAs and miRNAs of immature and mature sharpsnout seabream gonads (padj<0.05). Individual fish are indicated at the bottom of each column (IM-1, IM-2 and IM-3 are immature and M-1, M-2 and M-3 are mature fish). Each row represents the expression of one non-coding RNA, and purple and light purple represent high and low abundance, respectively. **B.** Illustration of putative miRNA –lncRNA hybridization applying RNAhybrid analysis with minimum freedom energy (mfe) <-25. LncRNAs were found after blast search of unannotated *D.puntazzo* transcritps against the NCBI nr database and were used as targets for the miRNAs.

Figure 6. Molecular phylogenetic analysis. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 14 nucleotide sequences. Graphical illustration of the hybridization to the 3'UTR with miR-125c applying RNAhybrid is shown next to the respective species. Asterisks indicates a mfe values of < -25. 3'UTRs were not available for the three-spined stickleback, the yellowtail amberjack and the spotted gar. For Human and mouse hybridization the miR homolog miR125-b was used.

Additional files

Supplemental figure S1. Histology ovarian photos of three immature (on the left: IM-1, IM-2 and IM-3) and three mature (on the right: M-1, M-2 and M-3) sharpsnout seabream at two resolutions, 4X and 10X. Immature fish ovaries were filled with primary oocytes (po), whereas mature fish gonads contain early vitellogenic (eVg), vitellogenic (Vg) and early maturing oocytes (eOM). Scale bars indicate 500 μm and 200 μm respectively.

Supplemental table S1. Number of NextSeq Illumina sequencing reads, after quality and adaptor trimming, of small RNA libraries of sharpsnout seabream gonads. Gonadal samples belonged to immature (IM-1, IM-2, IM-3) and mature (M-1, M-2, M-3) individuals.

Supplemental table S2. Number of annotated small RNA reads (total, with *Gasterosteus aculeatus* and with miRbase), number of unannotated small RNA reads and percentage of unannotated small RNA reads in libraries of sharpsnout seabream gonads. Gonadal samples belonged to immature (IM-1, IM-2, IM-3) and mature (M-1, M-2, M-3) individuals.

Supplemental table S3. Differentially expressed small RNAs ($P<0.005$) in immature (IM-1, IM-2, IM-3) and mature (M-1, M-2, M-3) sharpsnout seabream gonads. Differential expression (DE) analysis was carried out by submitting the obtained count matrix comprising TPM normalized count data from the merged annotated reads as well as the read counts from the clustered unannotated small RNA to SarTools vs 1.2.0 (Varet et al. 2016) with default parameters. Transcripts with p-value < 0.005 and fold change (FC) >|1.8| were considered as differentially expressed.

Supplemental table S4. Unannotated and annotated transcripts of non-coding RNAs by blastn search against the NCBI nr database. Gonadal samples used for the construction of RNA libraries belonged to immature (IM-1, IM-2, IM-3) and mature (M-1, M-2, M-3) individuals.

Supplemental table S5. Target genes of differentially expressed miRNAs found in immature and mature sharpsnout seabream gonads.

Figure 1

Click here to access/download;Figure;Figure 1.jpg

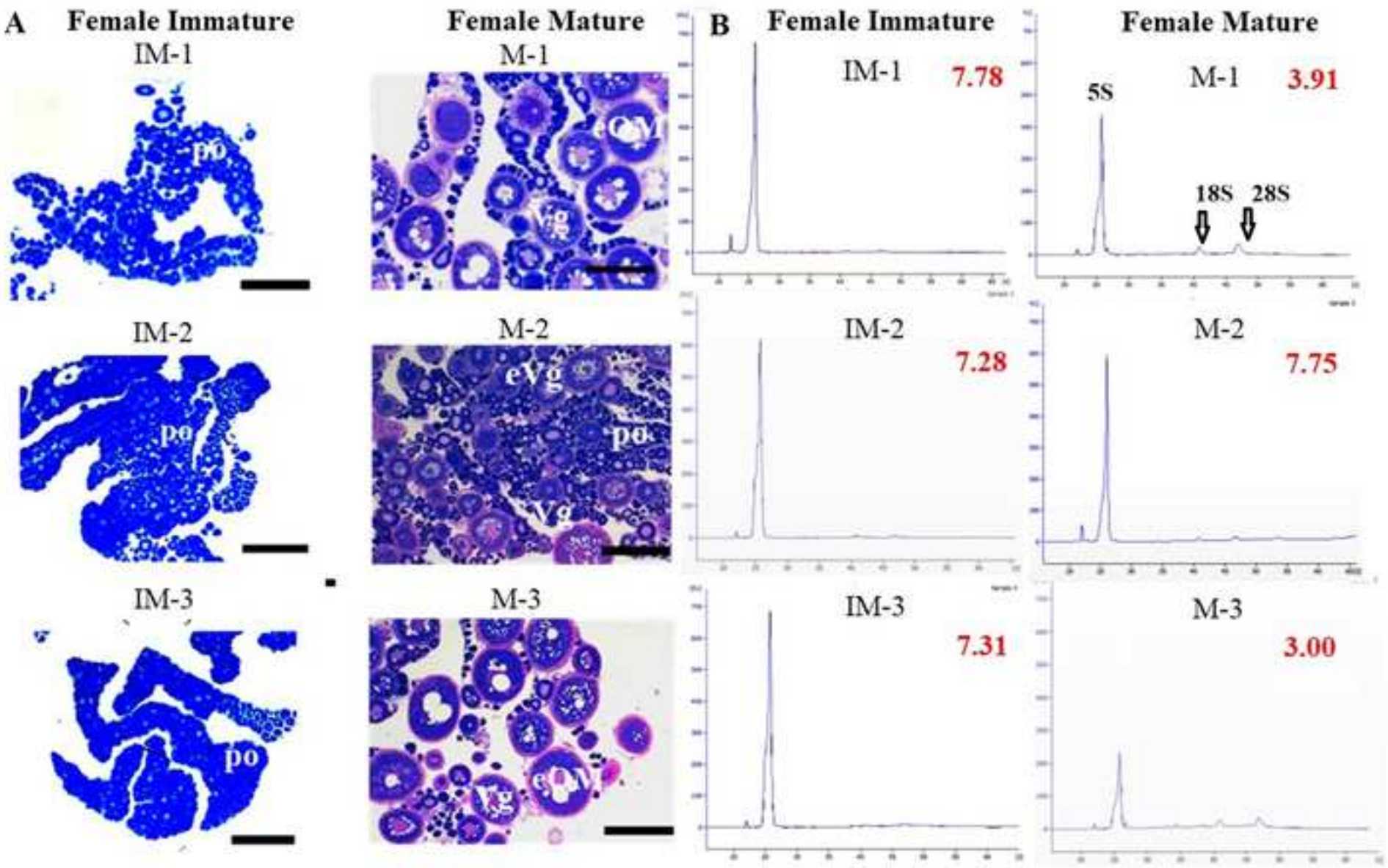


Figure 2

Click here to access/download;Figure;Figure 2.jpg

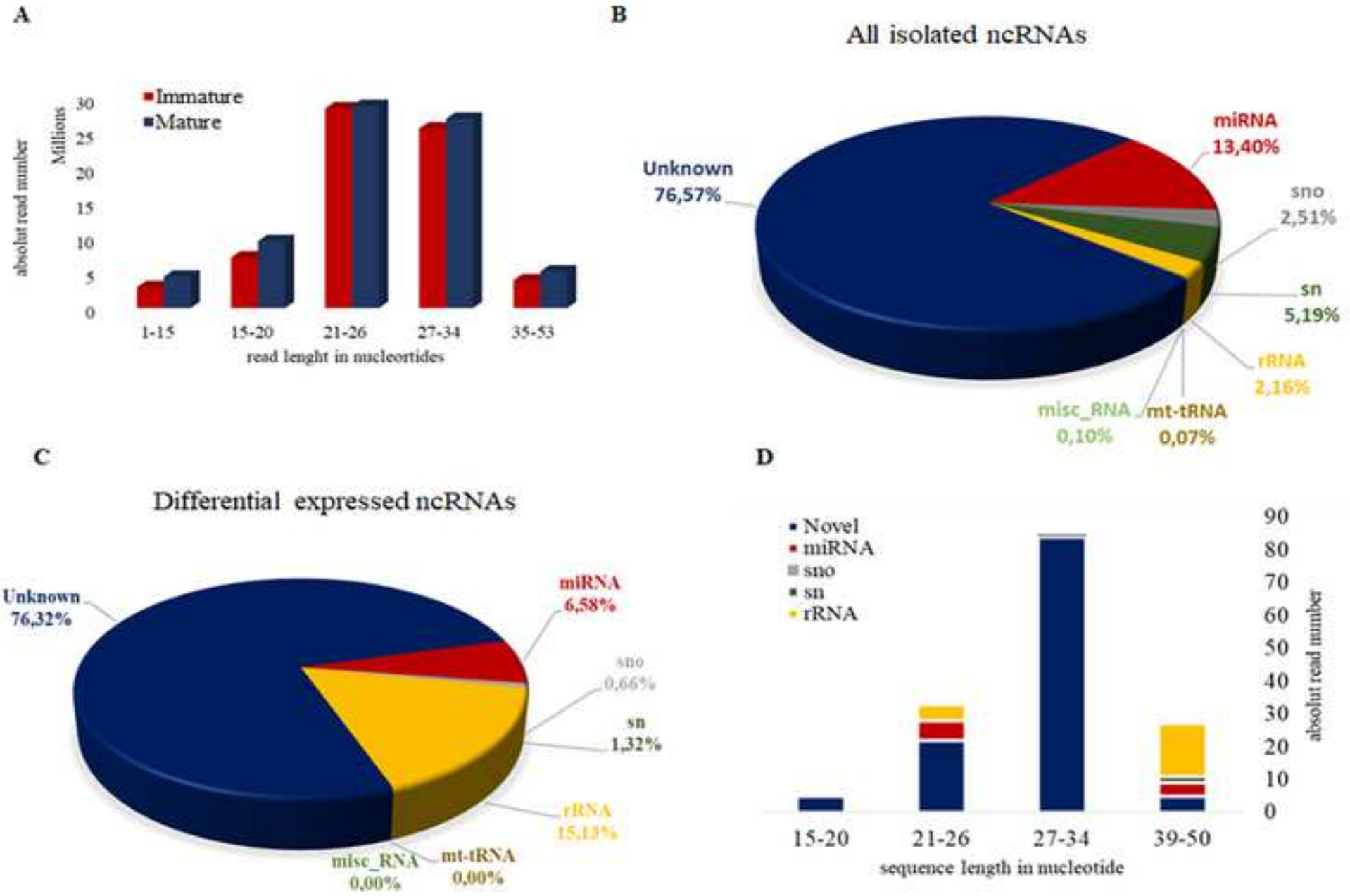


Figure 3

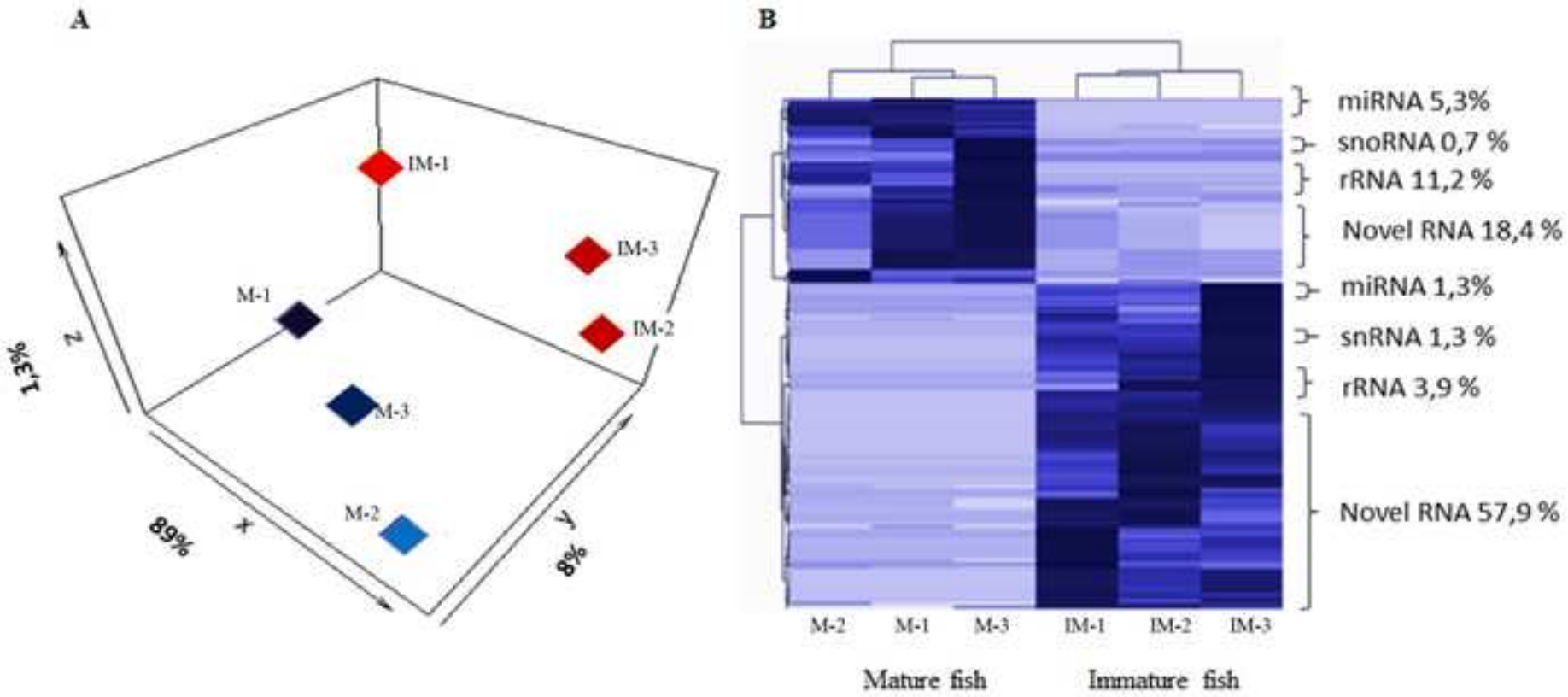


Figure 4

Click here to access/download;Figure;Figure 4.jpg

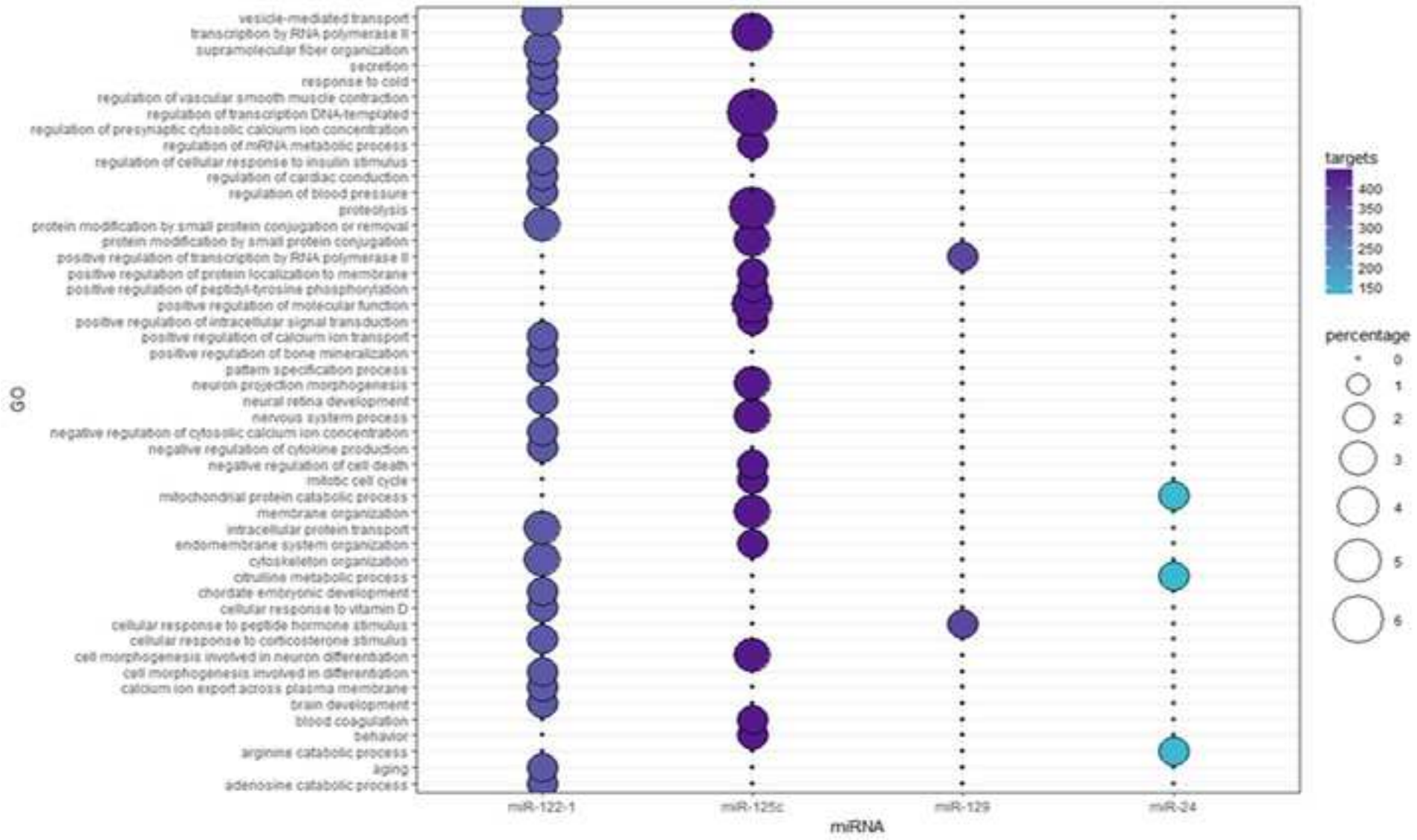


Figure 5

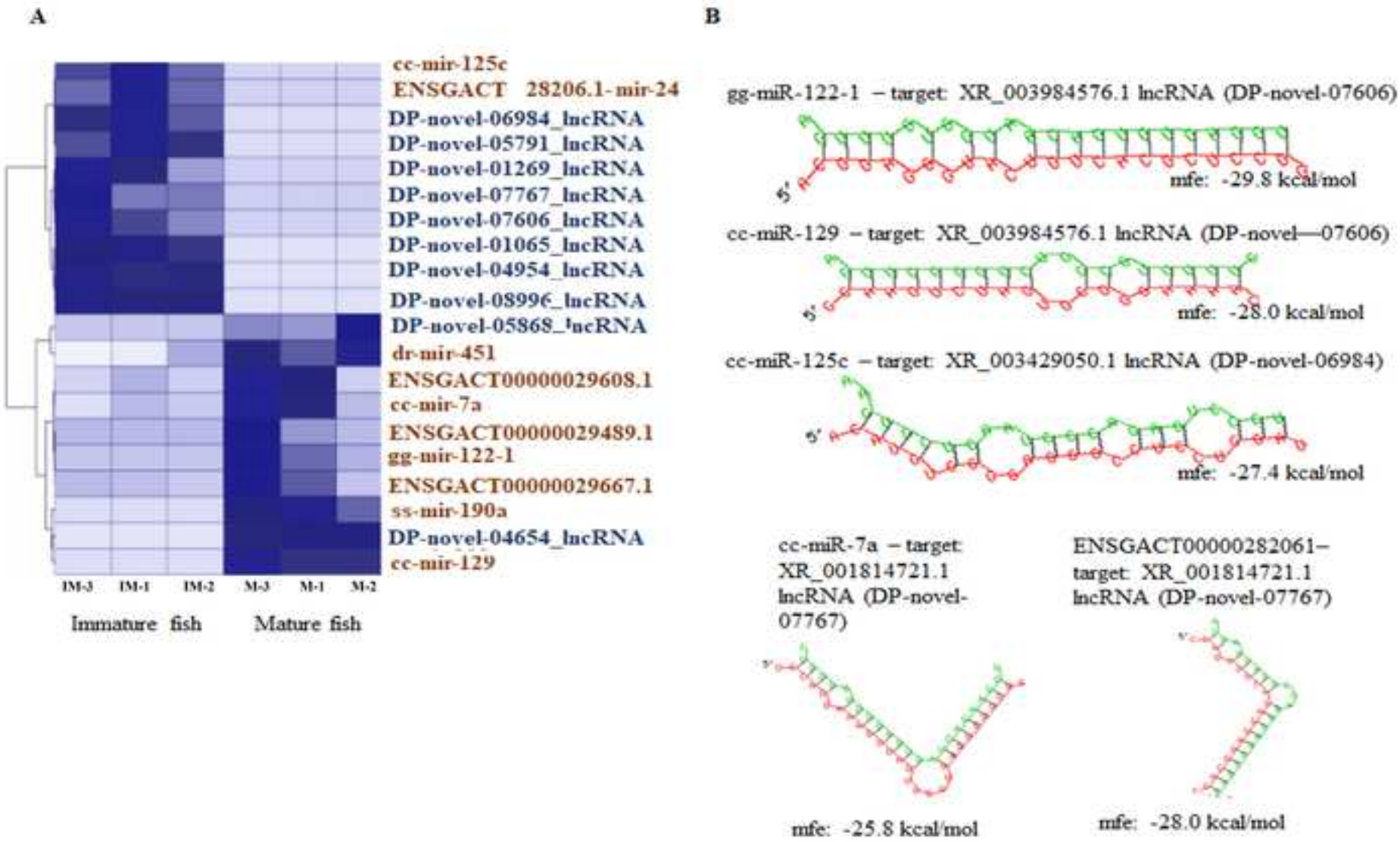
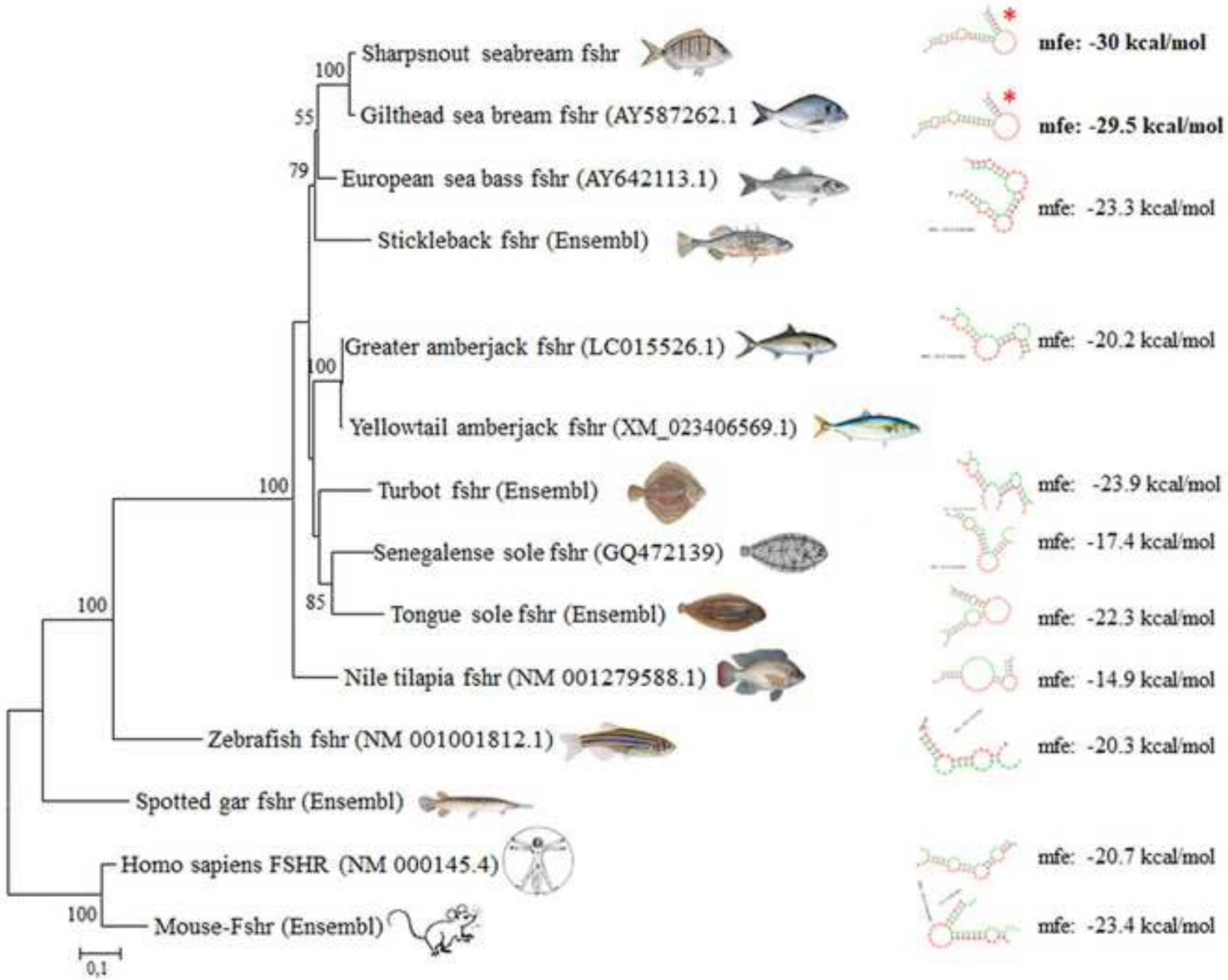


Figure 6

Click here to access/download;Figure;Figure 6.jpg



Supplemental figure S1

Supplemental table S2

Supplemental table S3

Click here to access/download

Supplementary Material

Supplemental table S3.xlsx

Supplemental table S4

Click here to access/download

Supplementary Material

Supplemental table S4.xlsx

Supplemental table S5

Click here to access/download

Supplementary Material

Supplemental table S5.xlsx