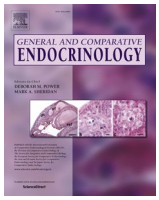
General and Comparative Endocrinology 357 (2024) 114581

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen



Research paper

MicroRNAs are involved in ovarian physiology of greater amberjack (*Seriola dumerili*) under captivity

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ARTICLE INFO

Keywords:
miRNA
Reproduction
Teleost
Greater amberjack
Ovarian dysfunction
Expression
Regulation
Epigenetics

ABSTRACT

Gonad maturation is critical for the reproductive success of any organism, and in fish, captivity can significantly affect their reproductive performance, leading to maturation incompetence and spawning failure. The greater amberjack (*Seriola dumerili*), a fish species recently introduced to aquaculture fails to undergo oocyte maturation, ovulation, and spawning when reared in aquaculture facilities. Since confinement has been shown to influence gonad maturation and completion of the reproductive cycle, investigations into epigenetic mechanisms may shed light on the reasoning behind the reproductive dysfunctions of fish under captivity. Among the known important epigenetic regulators are small non-coding RNAs (sncRNAs), and in particular microRNAs (miRNAs). In this study, immature, maturing (late vitellogenesis), and spent ovaries of captive greater amberjack were collected, and the differential expression of miRNAs in the three different ovarian development stages was examined. Expression patterns of conserved and novel miRNAs were identified, and potential targets of highly differentially expressed miRNAs were detected. Additionally, read length distribution showed two prominent peaks in the three different ovarian maturation stages, corresponding to miRNAs and putative piwi-interacting RNAs (piRNAs), another type of ncRNAs with a germ-cell specific role. Furthermore, miRNA expression patterns and their putative target mRNAs are discussed, in relevance with the different ovarian maturation stages of captive greater amberjack. Overall, this study provides insights into the role of miRNAs in the reproductive dysfunctions observed in fish under captivity and highlights the importance of epigenetic mechanisms in understanding and managing the reproductive performance of economically important fish species.

1. Introduction

Gonad maturation is a complex process that involves the development and differentiation of germ cells and the synthesis of hormones that regulate reproductive function. In males, gonad maturation involves the development of testes and the production of spermatozoa, while in females, it comprises the development of ovaries and the production of mature oocytes. In teleost fish, reproduction is a seasonal process, during which environmental cues, such as temperature and photoperiod, act on the pituitary to induce the production of gonadotropins, follicle-stimulating hormone (Fsh), and luteinizing hormone (Lh), which in turn act on the gonads to induce the production of sex steroids (Yaron and Levavi-Sivan, 2011) and initiate gametogenesis and maturation. In captivity, fish of both sexes may exhibit reproductive dysfunctions (Mylonas and Zohar, 2001). One of the newly introduced species in aquaculture with reproductive dysfunctions is the greater amberjack (*Seriola dumerili*), a popular marine fish species of the Mediterranean region. Recent advances in the control of greater amberjack reproduction under captivity as well as improvements in acclimatization to captivity conditions (Fakriadis et al., 2019; Fakriadis et al., 2020a; Fakriadis et al., 2020b), have led to several achievements in its spawning performance, such as induced spawning with the use of gonadotropin-releasing hormone agonist (GnRHa) implants after cage-to-tank transfer of wild-caught captive individuals (Fakriadis et al., 2020b), and induced spawning of F1 generation fish (Jerez et al., 2018). Yet, despite these advancements in the control of the reproductive cycle of greater amberjack, captive females continue to exhibit incomplete vitellogenesis, failing to mature and ovulate, mainly when reared in tanks. Wild-caught captive females have been shown to exhibit lower estradiol (E2) and testosterone (T) levels, lower gonadosomatic index (GSI), and

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https://doi.org/10.1016/j.ygcen.2024.114581
Received 30 December 2023; Received in revised form 28 June 2024; Accepted 10 July 2024
Available online 14 July 2024

extensive atresia of late vitellogenic oocytes, compared to wild fish (Zupa et al., 2017).

Since a variety of abiotic factors ranging from water quality, temperature, photoperiod, and diet can impact the reproductive success of fish in captivity, investigations into epigenetic mechanisms may shed light on the reasoning behind the observed dysfunctions of fish under captivity. Among important epigenetic regulators are small non-coding RNAs (sncRNAs), and particularly microRNAs (miRNAs), which are non-coding RNA molecules with an average length of 22 nucleotides that affect gene expression at the post-transcriptional level (Bartel, 2004). They are phylogenetically highly conserved, and hundreds of conserved genes encoding miRNAs have been found in various organisms (Ambros, 2004; Dexheimer and Cochella, 2020; Liu et al., 2008). Concerning teleosts, there is an increasing number of scientific articles reviewing the role of miRNAs in a broad range of physiological processes (Bhat et al., 2020; Bizuayehu and Babiak, 2014; Desvignes et al., 2021; Herkenhoff et al., 2018; Rasal et al., 2016; Takacs and Giraldez, 2010), including various aspects of gonadal development and function, such as sex determination, germ cell proliferation and differentiation, and steroidogenesis (Sarropoulou and Fernandez, 2023). One of the most prominent examples, is the gonad-specific miR-202, which has been shown to exhibit higher expression in vitellogenic ovaries of rainbow trout (*Oncorhynchus mykiss*) (Juanchich et al., 2013) and the knockout of its gene has led to reduced fertility in the medaka *Oryzias latipes* (Gay et al., 2018). Another miRNA with an important ovarian function is miR-125b, which has been shown to putatively target the follicle-stimulating hormone (Fsh) receptor gene in the sharpsnout seabream *Diplodus puntazzo* (Papadaki et al., 2020). Differential expression of miRNAs during oocyte development and maturation has been investigated in several species (Juanchich et al., 2013; Lan et al., 2019; Papadaki et al., 2020; Presslauer et al., 2017; Wang et al., 2017). Recently, circulating miRNAs have also been shown to be differentially expressed during the reproductive cycle in rainbow trout (Cardona et al., 2021) and for sex determination in the greater amberjack (Deng et al., 2023). The aim of the present study was to identify putative miRNAs with important roles in the reproductive dysfunctions faced by greater amberjack during captivity and detect putative targets that lead to the prevention of oocyte maturation, ovulation, and spawning. The results of the present study may also be of great relevance to other farmed fish species exhibiting reproductive disorders.

2. Materials and methods

2.1. Ejthics approval

Ethical approval for the study was obtained by the relevant Greek authorities (National Veterinary Services) under license No 255356 (ΑΔΑ: 6Λ4Σ7ΛΚ-ΩΜΥ). All procedures involving animals were conducted following the "Guidelines for the treatment of animals in behavioral research and teaching" (Anonymous, 1998), the Ethical Justification for the use and treatment of fishes in research: An update, and the "Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes" (EU, 2010).

2.2. Fish sampling

Fish used in the present study were sampled according to their histological phenotypes and categorized into: "immature" (Im) fish which were 1-year-old captive individuals sampled in June 2018 in an experiment following the sex differentiation process of a greater amberjack stock produced in a registered fish farm (Papadaki et al., 2021). The second and the third groups were classified as "vitellogenic" (Vg) and "spent" (Sp), respectively, and consisted of captive individuals kept in a registered fish farm and sampled in the middle and at the end of the spawning season of the species in 2015 (Zupa et al., 2017).

During the samplings, fish were captured from the cages, anesthetized with clove oil or phenoxy-ethanol, and sacrificed for gonad dissection. Gonads of Im fish were excised after anesthesia with clove oil, whereas Vg and Sp gonads were excised after anesthetization in phenoxy-ethanol, according to the respective papers (Papadaki et al., 2021; Zupa et al., 2017). Four ovarian samples were collected from each group (Im-1, Im-2, Im-3 and Im-4, Vg-1, Vg-2, Vg-3, Vg-4, Sp-1, Sp-2, Sp-3, and Sp4), one portion of the gonad was kept in 4 % formaldehyde: 1 % glutaraldehyde (McDowell and Trump, 1976) for histological processing, and another one was submerged in RNAlater (Sigma-Aldrich, Germany) and transferred to −80 °C until RNA extraction.

2.3. Histology

For histological processing, ovaries were dehydrated in a 70–95 % ethanol series and embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer, Germany). Serial sections were obtained at 3–5 μm thickness on a microtome (Leica RM2245, Germany). Staining of the sections was performed with methylene blue/azure II/basic fuchsin (Bennett et al., 1976) and stained slides were examined under a light microscope (Nikon Eclipse 50i, Japan).

2.4. RNA extraction and evaluation

Total RNA extraction of all gonad samples was carried out by disrupting 30 mg in liquid nitrogen with mortar and pestle followed by sample homogenization by passing the lysate five times through a 23-gauge (0.64 mm) needle. Homogenized samples were further processed by applying the Nucleospin miRNA kit (MachereyNagel, Duren, Germany) following the manufacturer's instructions. The RNA quantity was estimated with a Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE), and the quality was evaluated by 1 % agarose gel electrophoresis as well as by RNA Pico Bioanalysis chip (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, California, USA) (Supp. Table S1).

2.5. sncRNA libraries construction and sequencing

sncRNA libraries were generated by applying the NEBnext multiplex Small RNA Library Preparation kit for Illumina sequencing (New England Biolabs, Ipswich, MA, USA). The protocol for greater amberjack gonads was optimized according to the manufacturer's instructions by using 1 μg total RNA and 13 PCR cycles. Size fraction was carried out running a polyacrylamide gel (6 % TBE gel, Lonza, Basel, Switzerland) at 4 °C for 1 h. Each sample was tagged with a different multiplex identifier tag provided by NEB. The generated sncRNA libraries were evaluated by DNA high-sensitivity chips (Bioanalyzer, Agilent) and quantified by Qubit (Life Technologies, Carlsbad, CA, USA) as well as by the DNA high-sensitivity chip measurements. Differentially indexed libraries were pooled at a concentration of 4 nM and single-strand sequenced over 4 lanes and 55 cycles on the Illumina NextSeq500 sequencing platform at the Genomics Facility of the Institute of Molecular Biology & Biotechnology, Forth, Crete, Greece.

2.6. Sequencing reads analysis

All reads were submitted to quality control using the open-source Fastqc v0.10.0 software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc). Quality and adapter trimming was carried out using Trimmomatic software 0.30 (Bolger et al., 2014) and imported into the CLC genomics Workbench (v10.1). Putative sncRNAs were further extracted, and all reads were counted accordingly. The minimum sampling count (the number of copies of the raw sncRNAs reads included in the resulting count table) was set to 5. To annotate putative miRNAs, obtained sequencing reads were mapped against the database miRbase (release 21.1) (Griffiths-Jones et al., 2008) available miRNAs of

teleosts and human, gorilla, and mouse. These were prioritized as follows, *Astatotilapia burtoni, Oryzias latipes, Tetraodon nigroviridis, Fugu rubripes, Danio rerio, Cyprinus carpio, Gadus morhua, Hippoglossus hippoglossus, Paralichthys olivaceus, Ictalurus punctatus, Salmo salar, Petromyzon marinus, Gorilla gorilla, Homo sapiens, and Mus musculus* as well as against the three-spined stickleback (*Gasterosteus_aculeatus*) sncRNA database Gasterosteus_aculeatus.BROADS1.ncrna. Subsequently, the merging of variants of the same miRNAs was performed, which resulted in a list of "sampled grouped transcripts with the corresponding read count. Not annotated transcripts were extracted with read counts > 20 in each of the samples. For further downstream analysis, total read counts of all unannotated samples higher than 500 were used. To determine putative novel miRNAs mirDeep2 (version 2.0.1.3) software program was applied with default parameters and the genome sequence of the greater amberjack, (*Seriola_dumerili*.Sdu_1.0,) available at the ENSEMBL database.

2.7. Differential expression analysis

Differential expression analysis as well as the Principal Component Analysis (PCA) of annotated and unannotated transcripts was assessed by DeSeq2 implemented in SarTools vs 1.2.0 (Varet et al., 2016) and carried out in R [R Core Team 2017] with default parameters. Transcripts with padj.<0.05 and log2fold change (log2FC) >|1| were considered as differentially expressed.

2.8. Identification of target mRNAs of the most abundant differentially expressed miRNAs

For the most abundant, differentially expressed, and characterized miRNAs from each gonadal maturation stage, putative targets were identified. Therefore, the greater amberjack 3′UTRs were retrieved from the Ensembl database, and the hybridization dynamics were assessed by applying RNAhybrid, version 2.12 (Kruger and Rehmsmeier, 2006), with the energy threshold set to mfe ≤ − 20. Furthermore, only 3′UTRs with perfect complementary base-pairing to the miRNA seed region (base pairs 2–7) were taken into consideration. Identified targets that are involved in i) ovary formation, ii) ovarian maturation, iii) ovarian steroidogenesis, iv) apoptosis, and v) autophagy according to already published data (Aranyakanont et al., 2020; Bogoch et al., 2022; Dechaud et al., 2021; Fabra and Cerda, 2004; Gao et al., 2022; Ge et al., 2017; Guzman et al., 2014; Liu et al., 2022; Luckenbach et al., 2008; Nyuji et al., 2020; Nzioka et al., 2023; Parajes et al., 2013; Wang et al., 2022; Wu et al., 2018; Yamamoto et al., 2016; Yang et al., 2022; Yokokawa et al., 2023) were further investigated.

2.9. Enrichment analysis

Enrichment analysis was carried out for identified miRNA targets involved in reproduction, steroidogenesis, and apoptosis. Identified miRNA targets were further submitted to AmiGO2 (https://amigo.geneontology.org/) gene ontology database and the GO classes belonging to the category "biological process" were selected (FDR P<0.05). A data matrix was generated by counting the number of miRNAs, assigned to each of the GO classes. For each GO class, the frequency of enriched miRNAs in Im, Vg, and Sp was calculated.

3. Results

3.1. Histology

Histological analysis showed that the ovaries of the group classified as immature (Im) were truly immature containing mainly oogonia and primary oocytes (Fig. 1a). Ovaries of the group classified as vitellogenic (Vg) exhibited, as expected, primary oocytes, cortical alveoli oocytes, vitellogenic oocytes, and atretic oocytes (Fig. 1b). At the end of the spawning season, sampled ovaries were in a regressed state and were classified as Spent (Sp), containing mostly primary and cortical alveoli oocytes and signs of atresia (Fig. 1c).

3.2. sncRNA data generation

After quality and adaptor trimming, each sample had an average of around 11.5, 16, and 12 million reads for Im, Vg, and Sp respectively (Suppl. Table S2). Plotting the percentage of obtained read length showed two main peaks in the three different ovarian maturation stages, one at 19–24 nt, which corresponds to the read length of miRNAs, and one at 25–30 nt, pinpointing to putative piwi-interacting RNAs (piRNAs) (Fig. 2a). Regarding sncRNAs expression patterns PCA analysis separated the three different ovarian maturation stages into three distinct groups with the Vg stage being closer to the Sp than to the Im stage (Fig. 2b). Annotation of obtained sncRNA transcripts revealed six different types of sncRNA [miRNA, small nuclear RNA (snRNA), small nucleolar (snoRNA), ribosomal (rRNA), ribosomal RNA gene, mitochondrial (mtRNA)] as well as 12,328 unique sncRNA which were not annotated named as "unknown" within the present work (Fig. 3a). The read length distribution of the unknown transcripts illustrated in Fig. 3b showed a relatively higher amount of reads between 25 and 30 nt (Fig. 3b).

3.3. Differential expression analysis

Reads annotated as miRNA and with a significant differential expression threshold of padj. < 0.05 and log2FC > |1| revealed a total of 198 comparing Im versus Vg (45 miRNAs depleted and 153 enriched at the immature stage). 196 comparing Im versus Sp (25 miRNAs depleted and 171 enriched at the immature stage), and 39 comparing Vg versus Sp (18 miRNAs depleted and 21 enriched at the vitellogenic stage) (Supp. Table S3). The number of common and stage-specific differentially expressed miRNAs is illustrated by a proportional Venn diagram

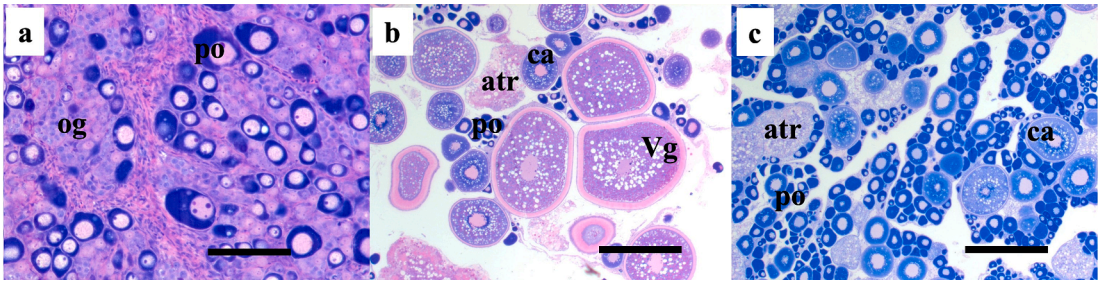

Fig. 1. Histological sections of ovaries from captive greater amberjack. a. Ovary of the Im group, collected from immature 1-year-old fish, showing mostly oogonia (og) and primary oocytes (po). b. Ovary of the Vg group, collected in the middle of the spawning season of captive wild individuals with po, cortical alveoli (ca), vitellogenic (Vg), and some atretic (atr) oocytes. c. Ovary of the Sp group, collected at the end of the spawning season of captive wild individuals, showing po, ca, and atr oocytes. The scale bars indicate 100 μm (A), and 500 μm (B and C).

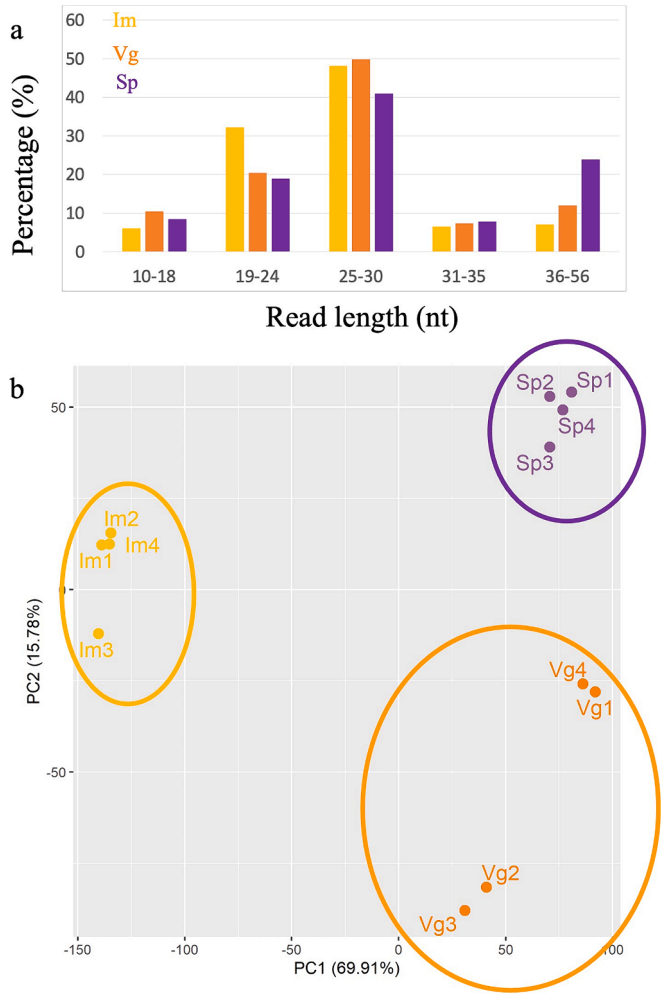

Fig. 2. a. Read length distribution of small RNA sequences of different developmental stage ovaries of greater amberjack. Colors indicate the ovarian development stage; Yellow: immature ovary (Im), orange: vitellogenic ovary (Vg), purple: spent ovary (Sp). b. PCA analysis of sncRNAs of immature (Im-1, Im-2, Im-3 and Im-4), vitellogenic (Vg-1, Vg-2, Vg-3 and Vg-4) and spent (Sp-1, Sp-2, Sp-3 and Sp-4) ovaries of greater amberjack. The first principal component (PC1) is anticipated to separate samples from the different biological conditions. The first two components of the PCA, with percentages of variance associated with each axis are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

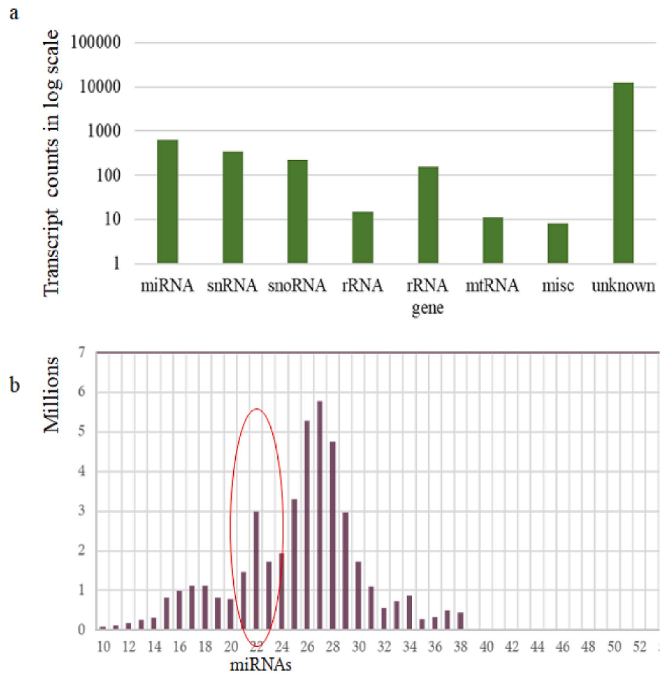

Fig. 3. a. Distribution of obtained small non-coding RNAs according to their transcript types found in immature (dark yellow), vitellogenic (orange), and spent (purple) gonad libraries of greater amberjack. miRNA: microRNA, snRNA: small nuclear RNA, snoRNA: small nucleolar RNA, rRNA: ribosomal RNA, rRNA gene: ribosomal RNA gene, mtRNA: mitochondrial RNA, miscRNA: miscellaneous RNA. b. Read length distribution of unknown transcripts from immature, vitellogenic, and spent ovaries of greater amberjack. The typical read length of miRNAs and is represented by a red circle in the figure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4). Differentially expressed miRNAs found to be common between the comparisons Im vs Sp and Vg vs Sp amounted to 8 miRNAs and between the comparisons Im vs Vg and Im vs Sp amounted to 119 miRNAs including two novel miRNAs (SD_99906 and SD_73346). Among the three comparisons, only one miRNA was found to be differentially expressed, namely miR-100a-1 (*Salmo salar*), and some of its targets related to reproduction have been identified (Supp. Table S4). Three members of the same miRNA family, namely miR-100 (*Cyprinus carpio*), miR-100–1 (*Astatotilapia burtoni*), and miR-100–2 (*Astatotilapia burtoni*) were common in the comparisons Im vs Vg and Im vs Sp (Fig. 5a and b) and two, namely miR-21–1 (*Gadus morhua*) and miR-21–2 (*Gadus morhua*) were the same in the comparisons Im vs Sp and Vg vs Sp (Fig. 5 b and c). After identifying possible miRNA targets involved in reproduction, steroidogenesis, and apoptosis (Supp. Table S5), different GO terms were assigned to the miRNA targets. In all the GO terms examined, Im gonads exhibited the highest number of enriched miRNAs compared to Vg and Sp ovaries (Fig. 6). Differentially expressed miRNAs targeting potential molecular marker genes in apoptosis during follicular atresia identified by Gonzales-Kother et al., 2020 are illustrated in Fig. 7 in form of miRNA-mRNA hybrids.

3.4. Data access

The raw data has been submitted to the NCBI SRA database under the BioProject accession number PRJNA998261.

4. Discussion

Fish may display reproductive dysfunctions in captivity, which range from inconsistent spawning, failure to undergo oocyte maturation and ovulation, and incomplete oogenesis (Mylonas and Zohar, 2001). Therefore, in the present study, the focus was given to three female gonad maturation phenotypes, i.e. immature (Im), maturing (i.e. late vitellogenic) (Vg), and spent (Sp) ovary. The Vg and Sp gonads exhibited advanced atresia and signs of regression, which was recently shown to be characteristic of captive fish along with reduced gonadosomatic index and sex steroid hormone plasma levels (Zupa et al., 2017), while the Im gonad comprised, as expected, oogonia and primary oocytes.

This study aimed to compare the miRNA profiles of three different maturation phenotypes of the greater amberjack. Over the past few decades, research has demonstrated the existence of specific miRNA signatures associated with particular phenotypes. This indicates that miRNA profiles originating from the same phenotype will exhibit a similar set of differentially expressed miRNAs, even when the samples' origin and genetic background differ (Bizuayehu et al., 2015; Bovolenta et al., 2020; Flynt et al., 2009; Goodale et al., 2019; Yan et al., 2012). One of the objectives of the present study was to identify microRNAs (miRNAs) that are uniquely expressed in the immature gonads of fish

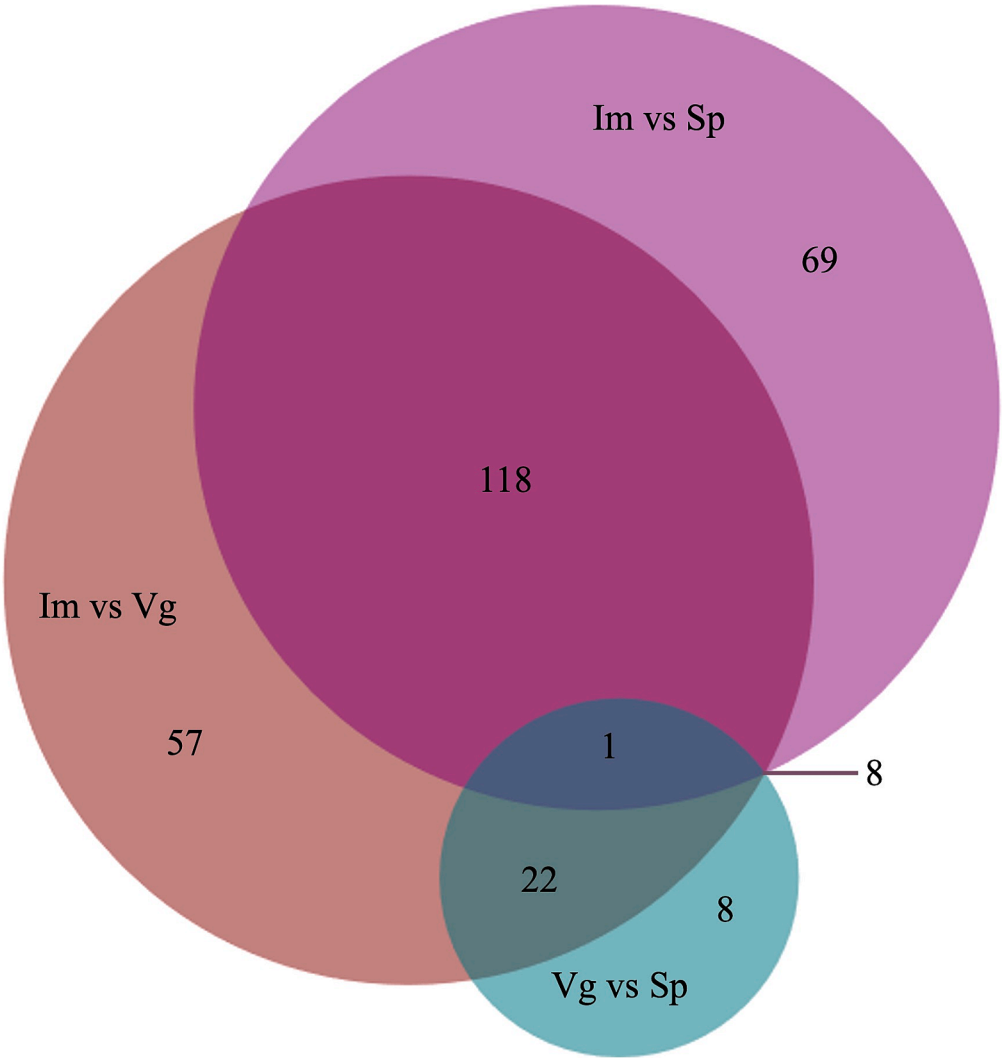

Fig. 4. Venn diagram of differentially expressed miRNAs among immature, vitellogenic, and spent ovaries of greater amberjack. One common miRNA was found to be differentially expressed among the three different reproductive development stages: miR-100a-1 (*Salmo salar*).

species that fail to undergo gonadal maturation. Therefore, the samples of the immature gonads were of different origin than the vitellogenic and spent samples, representing, however, the phenotype of the immature gonad of greater amberjack under captivity. For all samples under study, we achieved to obtain over 10 million reads per sample, which has been reported to be a reliable sequencing depth for differential expression analysis (Liu et al., 2014; Sun et al., 2014). A higher sequencing depth will undoubtedly yield more informative reads, thereby increasing the statistical power to detect differential expression for miRNAs with lower expression levels. Conversely, miRNAs with very low expression levels are unlikely to lead to gene repression and are therefore not a focus of this study. The read length distribution revealed two main peaks, as expected for the three ovarian stages. The first peak corresponded to miRNAs, while the second most probably corresponded to putative piRNAs. PiRNAs are another type of non-coding RNA with a germ-cell-specific role (Houwing et al., 2007). These two peaks have been observed in sncRNAseq data of gonads in several different fish species (Gu et al., 2014; Houwing et al., 2007; Jing et al., 2014; Li and Ge, 2020; Papadaki et al., 2020; Tang et al., 2022; Wei et al., 2022; Xiao et al., 2014). Whereas, in both male and female primordial gonads (i.e. undifferentiated gonads) of the tiger pufferfish *Takifugu rubripes*, only one peak was observed at 22 nt (Yan et al., 2021), in adult gonads of the same species, both peaks were shown (Wongwarangkana et al., 2015). In the common carp (*Cyprinus carpio*), the second peak is apparent in juvenile and adult gonads, but less dominant in primordial gonads (Wang et al., 2017). This suggests a possible role of piRNAs at later ovarian maturation stages. However, this study focused on the detection of miRNAs and putative novel miRNAs in the greater amberjack gonads. PCA analysis of differentially expressed miRNAs distinguished clearly the three ovarian maturation stages with Vg and Sp ovaries being closer located to each other with similar PC1 values, than to Im ovaries. The groups Vg and Sp are representative of the dysfunctional gonad while the Im stage refers to the developing gonad. This difference is also reflected in the number of miRNAs that are higher expressed in the different comparisons between gonadal types of the present study. Besides, more miRNAs were found to be higher expressed in the Im stage compared to the Vg and Sp stages targeting genes involved in apoptosis, autophagy, regulation of steroidogenesis and steroid metabolism, as well as cell division and reproduction. This highlights the high activity of this stage and its importance in ovarian development and maturation. The significance of this stage was similarly emphasised in the case of the Japanese flounder (*Paralichthys olivaceus*) (Qu et al., 2022), where the number of differentially expressed genes was also found to be higher in the primary oocytes, as was the number of higher expressed miRNAs in the present study.

Three highly expressed miRNAs identified within the present study in the greater amberjack gonads were miR-100a-1, miR-202, and miR-21. Of these, miR-100a-1 was found to be enriched in Im compared to

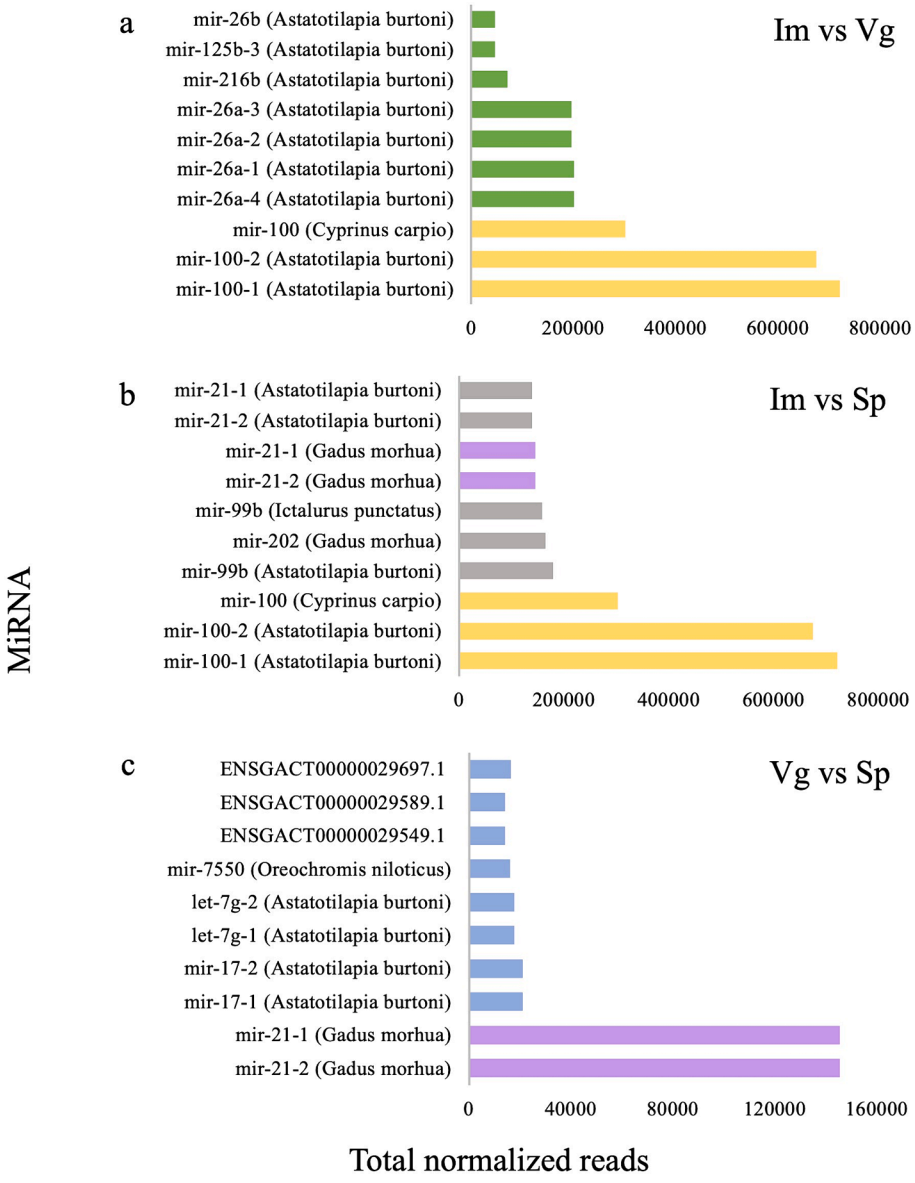

Fig. 5. Total read counts in all the samples of the ten most abundant differentially expressed miRNAs in the three different greater amberjack ovarian development stage comparisons, between immature and vitellogenic ovaries (a), between immature and spent ovaries (b) and between vitellogenic and spent ovaries (c).

both Vg and Sp stages. Notably, miR-100a-1 was the only miRNA differentially expressed in all three comparisons among the different ovarian development stages. MiR-100 is known to be one of the most abundant miRNAs in fish gonads with important roles in apoptosis, steroidogenesis, and oogenesis (Jing et al., 2014; Tao et al., 2016). In the present study, putative miR-100a-1 targets genes were identified involved in apoptosis, steroidogenesis, and oogenesis, i.e. pdcd4b, eva1a, ambra1b, hsd17b4, hsd17b12b, map4k4, map2k5, mapk13, mapk9, cdc25b, sox19b, ctsa, bnip2, il13ra2. The second miRNA, miR-202 has also been detected as a gonad-specific miRNA and is commonly found in high abundance in the ovaries of different fish species (Bouchareb et al., 2017; Ma et al., 2012; Tao et al., 2016; Wongwarangkana et al., 2015; Zayed et al., 2019). In the present study, miR-202 was found to be in higher abundance in Im compared to Sp gonads, which may indicate a leading role in early oogenesis, as it has been also shown in the medaka (Gay et al., 2018). The third highly abundant miRNA, miR-21, has been identified as being particularly enriched in the Im and Vg gonads, in comparison to the Sp stage. Abundant expression of miR-21 has also been reported in primordial gonads of tilapia (*Oreochromis niloticus*) (Tao et al., 2016) and of common carp (Wang et al., 2017). To note, that in mice, miR-21 has been found to block apoptosis in granulosa cells (Carletti et al., 2010). Apoptosis-related genes putatively targeted by miR-21 in the present study involved eva1a, api5, boka, casp9, pdcd4b, klf6, ctsd, cdc25b, and pcna.

Another known apoptosis-related miRNA, let-7g, was found to be enriched in Vg compared to Sp ovaries. In porcine it has been suggested that let-7g represses MAP3K1 and thus induces apoptosis (Cao et al., 2015). In the present study, putative targets of let-7g involve apoptosis-related genes, i.e eva1a, ambra1b, bokb, bnip2, and casp9 and thus let-7g, in this case, would repress apoptosis in the Vg stage like miR-21. Taken together, let-7g, miR-21, and miR-100a-1 could serve as potential apoptosis biomarkers in spent ovaries of the present study. One miRNA that also may be a significant biomarker, but has not yet been detected is in the Ensembl database as miRNA annotated transcript (ENSGACT00000029268.1). The latter has been detected to be highly expressed in Sp and Vg but not in Im. It should be noted, that the impact of miRNAs on gene regulation has been demonstrated to be more complex than previously thought. In addition to the previously identified effects, miRNAs may also show cooperative effects, whereby the

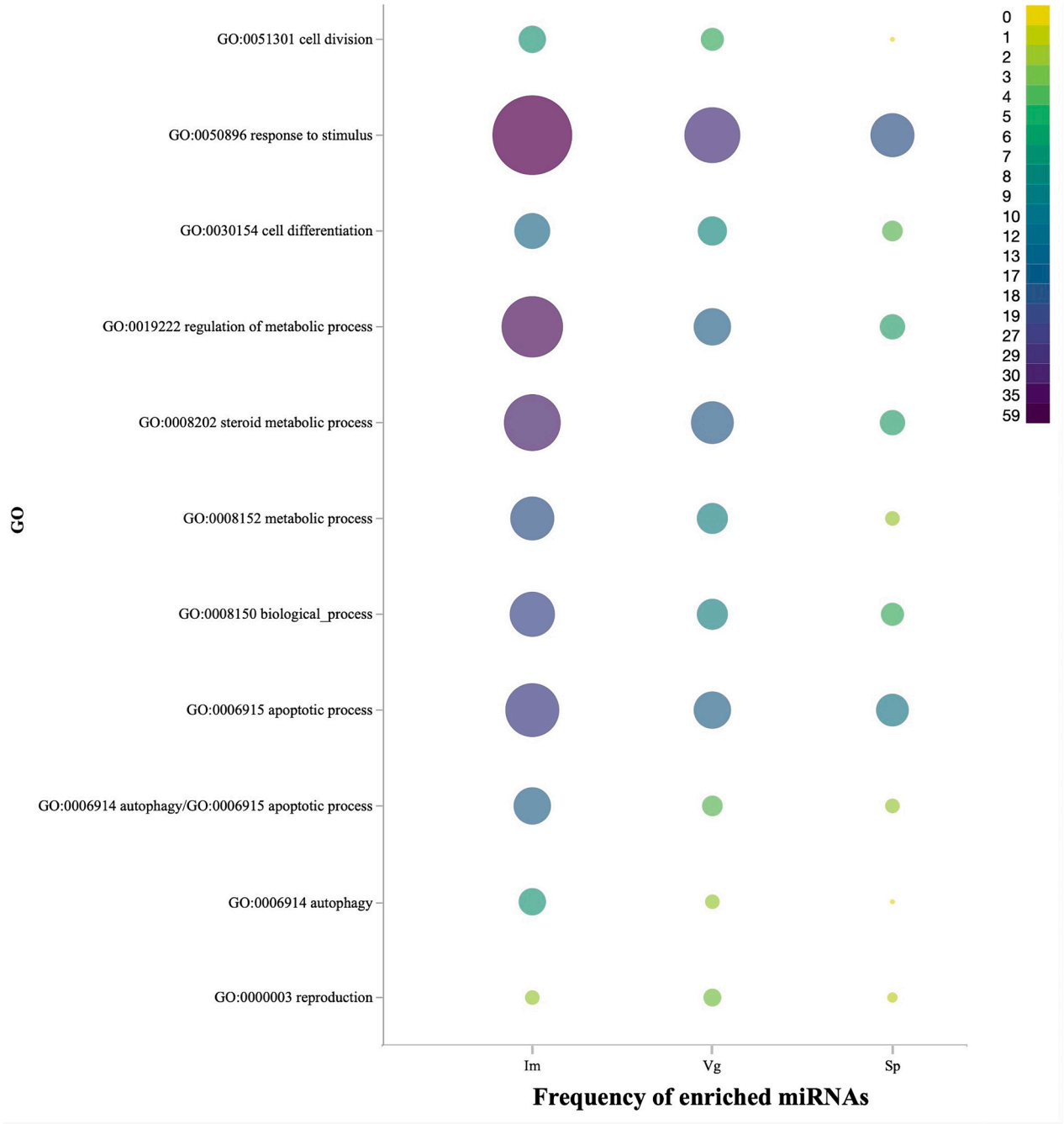

Fig. 6. Bubble plot displaying the enriched GO terms (FDR $P < 0.05$) identified for the targets of the enriched miRNAs of immature (Im), vitellogenic (Vg), and spent (Sp) gonads of greater amberjack. Identified targets involved in i) ovary formation, ii) ovarian maturation, iii) ovarian steroidogenesis, iv) apoptosis, and v) autophagy were used and a GO term was assigned to each one of them. The color and the size of the circle indicate the number and the percentage of enriched miRNAs for each GO term, respectively.

number, distance, and distribution of miRNA binding sites with the 3′UTR may influence the actual gene regulation (Diener et al., 2023). In the present study, four binding sites for the apoptosis and atresia-responsible gene programmed cell death 4a (pcd4a) were identified, as well as four binding sites for the gene Kruppel-like factor 6, including two novel miRNAs (SD_9906 and SD_73346) (Fig. 7). In the case of pcd4a, the novel miRNA is found in higher abundance in the Vg and Sp stages compared to the Im stage. This would indicate the repression of pcd4a. However, the other three miRNAs were found to be enriched in the Im stage, suggesting that they are repressing the pcd4a gene. Concerning the Kruppel-like factor 6 gene, the novel miRNA SD_73346 and miR-2016b are enriched in the Vg and Sp stages, while the miR21-1/-2 and miR-202 are enriched in the Im stage compared to the Sp stage. Further research is required to fully comprehend the underlying mechanisms and their implications for gene regulation in different biological contexts.

Overall, the study offers valuable insights into the role of miRNAs in ovarian development and maturation in the greater amberjack. It also suggests that miRNAs may be a useful tool for understanding the molecular mechanisms underlying reproductive dysfunctions in captive fish. Further research is needed to fully understand the functional roles of these miRNAs and their potential for non-lethal applications in diagnosis and improving the health and well-being of captive fish.

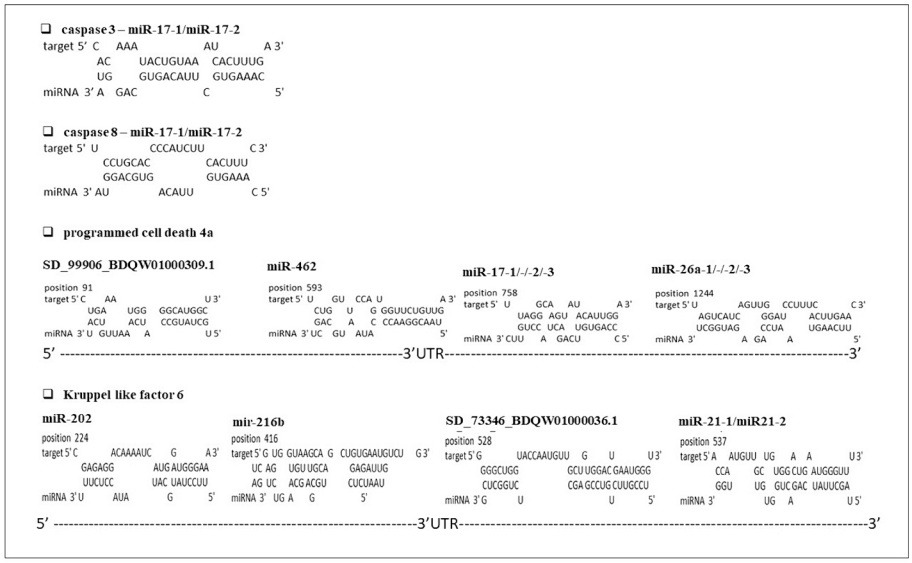

Fig. 7. miRNA-mRNA hybrids of differentially expressed miRNAs targeting potential molecular marker genes in apoptosis during follicular atresia identified by Gonzales-Kother et al., 2020. The 3' untranslated regions (UTRs) of the genes are depicted schematically, from the 5′ to the 3′ end. The specific binding sites are positioned along the 3′ UTRs.

Author contributions

MP, CCM, ES. conceived of the original idea for the manuscript. MP and ES. conceived of the approach to analyses and final framing of the ideas presented in the manuscript. MP, CCM, and ES edited the final 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.

CRediT authorship contribution statement

Maria Papadaki: Writing – original draft, Resources, Methodology, Data curation, Conceptualization. **C.C. Mylonas:** Writing – original draft, Writing – review & editing, Validation, Funding acquisition, Conceptualization. **Elena Sarropoulou:** Writing – original draft, Writing – review & editing, Visualization, Supervision, Resources, Software, Validation, Project administration, Methodology, Formal analysis, Data curation, Conceptualization.

Data availability

All reads generated in the present study from Illumina sequencing were submitted to the Sequence Read Archive (SRA) of NCBI under the BioProject accession number PRJNA998261.

Acknowledgments

Authors would like to acknowledge Aldo Corriero, Rosa Zupa and Valentino Pousis for providing us with the samples of Vg ovaries as well as Elisavet Kaitetzidou for valuable help in the laboratory, the Genomics Facility of the Institute of Molecular Biology & Biotechnology, Forth, Crete, Greece. and the IMBBC IT team and the computational resources and to perform this research which were 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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2024.114581.

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