Identifying sexually dimorphic circulating microRNAs in gonochoristic and hermaphroditic marine fish species.

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Abstract

In many fish species, males and females are hard to distinguish at the juvenile stage and size differences appear during maturation, often favoring females. Molecular tools for sexing live fish would benefit aquaculture, fisheries, and conservation research. Here we aimed to explores to what extent circulating microRNAs (miRNAs) can decipher phenotypic males from phenotypic females in gonochoristic fish species (the European seabass *Dicentrarchus labrax*, the Turbot *Scophthalmus maximus*, the Red drum *Sciaenops ocellatus*, and the Blue Runner *Caranx crysos*) as well as sexual stages in a protandrous hermaphrodite species (the Gilthead seabream *Sparus aurata*). We collected and extracted total RNA from 258 plasma samples, of which 96 samples with satisfactory RNA quality were sequenced using small RNA-seq. Circulating miRNAs detected in the plasma allowed to easily discriminate species, but some miRNAs were significantly correlated to one sex, independently of the species. In immature fishes, 3 miRNAs: miR-21a-3p, miR-18a-3p, and miR-29-1a-5p were overexpressed in females compared to males, while in mature fish, miR-21a-3p exhibited an opposite pattern. In the Gilthead seabream, we detected that both the miR-21a-3p and the miR-125b-2/3-5p were likely involved in the sexual transition from male to female. A complementary analysis on the 3′UTR sequences of all fish species allowed to predict potential mRNA targets of those two miRNAs, some of them being particularly relevant regarding sexual development (*i.e.* wnt4, esrrb, esrrga and hsd17b1). The identification of miRNAs like miR-21a-3p and miR-125b-2/3-5p as potential sex markers could offer a new, poorly-invasive method to monitor sex and developmental stages in fishes.

1. Introduction

In many fish species, it is almost impossible to distinguish males from females based on external phenotypic characteristics such as color or size at the juvenile stage. Sexual dimorphism, when it appears, is often materialized by differences in size that occurs during maturation. For most marine fishes, the sexual size dimorphism is in favor of females, although this general pattern is not the rule (Horne et al., 2020). Having access to molecular tools allowing to sex any alive fish would thus be a great asset for both aquaculture and fisheries sectors, but also for research with conservation purpose. For instance, in commonly produced marine fish species such as the European seabass *Dicentrarchus labrax*, the Gilthead seabream *Sparus aurata*, the Turbot *Scophthalmus maximus* and the Red drum *Sciaenops ocellatus*, the sex of individuals can only be accurately assessed during maturation, when it is possible to collect gametes. This poses a problem for broodstock management. First, late maturation complicates breeding programs by requiring to keep individuals for many years, even though they may potentially not be used. Second, in the above-mentioned fishes, mature females are much bigger than males, and producing predominantly females would be economically relevant for the aquaculture industry. Having reliable information of sex would also benefit research on wild fish movements patterns such as for the Blue Runner *Caranx crysos* (Brown et al., 2010) that are impossible to sex through external characteristics. This will hence participate to increase our knowledge on fish reproduction and other life history traits by working on alive fish, not on fish purposely sacrificed to know their sex (Bakari et al., 2024; Sley et al., 2012). Finally, non-lethal samplings would also participate to the "3Rs rule" which aim at Replacing, Reducing (the number of animals used), and Refining (anesthetize, provide pain relief) the use of animals for scientific purpose in the context of increasing their welfare.

The development of highly reliable alternatives to hormonal measurements that would be easy-to-implement and require low sample volume would therefore be of great interest. Circulating microRNAs (miRNAs) in the plasma as markers of sex in fish perfectly fulfill these roles. MiRNAs, short and conserved non-coding nucleotide sequences typically spanning 20-22 nucleotides, play pivotal roles in regulating numerous biological processes, mainly by post-transcriptionally repressing target mRNAs (Bartel, 2018). These microRNAs function as gene regulators through base-pairing interactions with mRNAs, with a sequence similarity as low as 6 to 8 nucleotides (seed sequence) being adequate to disrupt mRNA

function. Consequently, a single miRNA has the potential to interact with multiple mRNAs, potentially numbering in the hundreds, while individual mRNAs may also be targeted by multiple miRNAs (Bartel, 2009). MiRNAs exhibit high evolutionary conservation, supporting the notion of their fundamental role in metazoan biology (Bartel, 2018). They can also be quantified in various organs but also in extracellular fluids such as serum, blood plasma, urine, and saliva (Mohr and Mott, 2015). These advantageous characteristics of miRNAs make them highly suitable as biomarkers for tracking physiological states. Since their discovery, miRNAs have been extensively employed in human health diagnostics, particularly for tumor detection (Duttagupta et al., 2011; Lu et al., 2005), with growing interest in their application as biomarkers across other species, including fishes (Cardona et al., 2021, 2022). Evidence suggests distinct expression patterns of miRNAs between male and female gonads in diverse fish species (Bhat et al., 2020; Gu et al., 2014; Jing et al., 2014; Tao et al., 2016). However, until now, sex-specific miRNAs have not yet been identified in extracellular fluids such as plasma.

Here, we propose to identify potential circulating miRNAs as sex markers in the plasma of both immature and mature fish belonging to four gonochoristic species: the European seabass, the Turbot, the Blue runner and the Red drum, as well as one protandrous hermaphroditic species, the Gilthead seabream that naturally changes sex from male to female. To do so, we performed a deep small-RNA sequencing on a large number of blood plasma samples of the above-mentioned fishes.

2. Material and methods

Experiments were performed in accordance with relevant guidelines and regulations provided by the ethic committee (no 36) of the French Ministry of Higher Education, Research and Innovation and the experiment received the following agreement number: APAFIS #30612-2021031812193539.

2.1. Sample collection

All samples were collected between 2020 and 2021. Regarding European Seabass, a total of forty immature (mean weight: 44 ± 11 g and length: 15 ± 1 cm) and twenty mature (1387 ± 303 g and 46 ± 4 cm) fish were randomly collected from the experimental aquaculture station of Ifremer (Palavas-les-Flots, France) and Aquanord aquaculture site (Gravelines, France) respectively. Sixty-five Gilthead Seabream at different reproductive stages (see sex assessment session below and Table 2) were randomly collected from Aquanord aquaculture site (Gravelines, France). Thirty-one wild adult Blue Runners (mean weight 303 ± 70 g and length 24 ± 1.7 cm) were collected at the Station Ifremer Le Robert (Martinique, France) as well as forty-two hatchery produced red drum (mean weight 478 ± 102 g and length 34 ± 2.3 cm). Thirty-two immature turbots (mean weight 110 ± 20 g and length 19 ± 1.2 cm) and twenty-eight immature turbots (mean weight 880 ± 240 g and length 35 ± 2.9 cm) were collected at the France Turbot aquaculture site (Tredarzec, France).

All fish were euthanized with a lethal dose benzocaine (150mg/L). For each individual, a blood sample (1 ml) was collected from the caudal vein thanks to EDTA-coated syringes fitted with 23 G needles. Mature gonochoristic fish were visually sexed, while immature fish were histologically sexed. Regarding the protandrous Gilthead Seabream, a picture of the gonad was taken right after blood sampling, and 5 different stages (from juveniles' males to adult female) were assessed depending on the ratio of the female/male gonad (Table 1). After centrifugation of blood samples (3000 x g, 5 min), the plasma was stocked at -80°C. Note that the temperature of centrifugation was equal to the temperature at which the fish was living.

	n	Weight (g)	Length (cm)	Gonad Ratio Female/Male
Immature Male	10	221 ± 45	21 ± 1	
Mature Male	5	854 ± 170	32 ± 2	1.8
Intersex 1	10	840 ± 129	33 ± 1	18
Intersex 2	4	870 ± 179	33 ± 2	48
Mature Female	9	1745 ± 244	41 ± 1	

129 Table 1: Details of the weight, length, gonadal developmental stages and number of
130 Seabream used for Small RNA sequencing.
131
132 **Identification of markers**
133
134 *Histology*
135 Gonads of immature fishes (European seabass, Turbot and Red Drum) were fixed in Bouin's
136 fluid for 6 to 8 h and rinsed in clear water for one hour. Then, they were rinsed in EtOH 70%
137 for several days and placed in a dehydration automate (STP 120, MM, France). Each gonad
138 was embedded in paraffin and cut at 5µm sections. Slides were stained using the Masson's
139 trichrome methods (MYREVA SS30, MM, France).
140
141 *RNA extractions*
142 Thawed plasma of each fish was homogenized in QIAzol lysis reagent (Beverly, MA, USA)
143 following manufacturer's instructions. The total RNA was resuspended in 15 µL of RNAse
144 free water. MiRNAs were quantified using the smallRNA Analysis kit (DNF-470-0275) on
145 a Fragment Analyzer (Agilent). Based on the fragmentation profile (with or without pic
146 between 17 and 25 nucleotides), the estimated quantity (ng/µl) and percentage (%) of
147 miRNAs relative to other small RNAs, a subsample of individuals was selected for libraries'
148 construction.
149
150 *Small RNA Sequencing*
151 Libraries were constructed using the NEXTFLEX Small RNA-seq kit v3 (Perkin Elmer,
152 #NOVA-5132-05). Briefly, a 3' Adenylated adapter was ligated to the 3' end of 0,5 ng of
153 microRNA and purified to remove 3' adapter excess. A 5' adapter was ligated to the 5' end
154 of the 3' ligated microRNA. The resulting construction was purified to remove 5' adapter
155 excess. 5' and 3' ligated microRNAs underwent a reverse transcription using a M-MuLV
156 reverse Transcriptase and a RT primer complementary to the 3' adapter. Resulting cDNAs
157 were used as a matrix in a 25 cycles PCR using a pair of uniquely barcoded primers. The
158 resulting barcoded library was size selected on a Pippin HT (SAGE Science) using 3 %
159 agarose cassette (#HTG3004) and aiming for a size range between 147 bp and 193 bp. Once
160 size selected, libraries were verified on a Fragment Analyzer using the High Sensitivity NGS
161 kit (#DNF-474-0500) and quantified using the KAPA Library quantification kit (Roche, ref.

KK4824). Following this second quality check, we selected 38 samples of Seabream (Table 1), 6 samples of Blue Runners, 13 samples of Red Drum, 26 samples of Turbots and 13 samples of European sea bass (Table 2) for being sequenced.

	Blue runner			Turbot					
	Mature			Immature			Mature		
	n	Weight (g)	Length (cm)	n	Weight (g)	Length (cm)	n	Weight (g)	Length (cm)
Females	3	281 ± 33	24 ± 0.9	8	110 ± 25	19 ± 2	5	935 ± 250	35.5 ± 3
Males	3	320 ± 77	24 ± 2	10	119 ± 15	20 ± 1	3	766 ± 232	34 ± 4
	Red Drum			European Seabass					
	Immature			Immature			Mature		
		Weight (g)	Length (cm)	n	Weight (g)	Length (cm)	n	Weight (g)	Length (cm)
Females	5	508 ± 109	35 ± 2	3	34 ± 2	14 ± 0.1	3	957 ± 161	45 ± 7
Males	8	516 ± 75	35 ± 2	4	45 ± 13	15 ± 1	3	1331 ± 138	47 ± 1

Table 2: Details of the weight, length, maturity stage and number of fish of each species used for Small RNA sequencing.

MiRNA alignment and quantification

Image analyses and base calling were performed using the NovaSeq Control Software and Real-Time Analysis component (Illumina). Demultiplexing was performed using Illumina's conversion software (bcl2fastq 2.20). The quality of the raw data was assessed using FastQC from the Babraham Institute and the Illumina software SAV (Sequencing Analysis Viewer). The raw reads were trimmed using Cutadapt (version 3.5) (Martin, 2011) to remove the sequencing adapter (TGGAATTCTCGGGTGCCAAGG) at the 3′-end. Additionally, 4 bases were also trimmed from the 5′-end and 3′-end of the reads as indicated in the manual of NEXTflex Small RNA-Seq Kit v3 from Bio Scientific. Before counting step, samples with a rRNA degradation profile were filtered out. This resulted in 13 and 16 immature and mature fish, respectively, for the experiment on sex and 21 fish for the experiment on stress. MiRNA analysis was performed with Prost ! v0.7.60 pipeline (Desvignes et al., 2019). In this pipeline, alignment was performed with BBMap (Bushnell, 2014) version 38.90 to genome of interest : the European sea bass *Dicentrarchus labrax* GCA_000689215.1_seabass_V1.0, the one of the Red drum *Sciaenops ocellatus* (GCA_014183145.1_ASM1418314v1), the Gilthead seabream *Sparus aurata* (GCF_900880675.1_fSpaAur1.1_genomic.fna), the Jack C*aranx ignobilis*, phylogenetically closest species of the Blue Runner *Caranx crysos* (GCA_021432705.1) and the Turbot *Scophthalmus maximus* (GCF_013347765.1). Next, sequences with overlapping genomic positions were grouped together. Only sequences with a minimum of 30 reads (when adding up all samples for a given species) were retained, to perform the differential expression

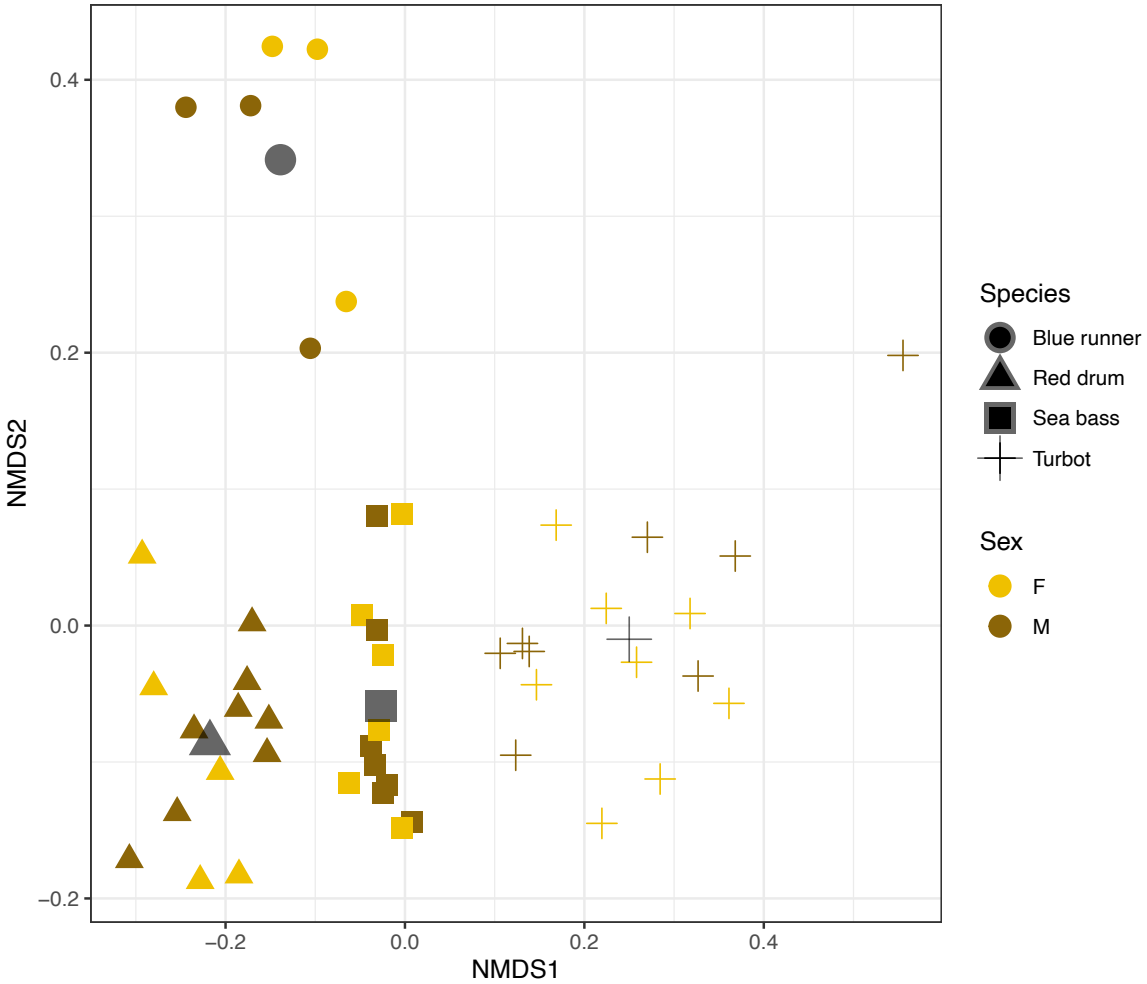
190 analysis. The miRNA annotation and counting were retrieved by Prost! (parameters in Text
191 S1) from a custom annotation provided in FishmiRNA (https://www.fishmirna.org/) using
192 *Gasterosteus aculeatus* miRNA sequences (the phylogenetically closest species).
193
194 *Statistical analysis*
195
196 A Non-metric Multi-dimensional Scaling (NMDS) approach was used to compare species,
197 sex and gonadal maturity stages based on all miRNAs sequenced. A r^2 analysis indicated a
198 significant effect according to the condition tested. The package vegan v2.6-2 (Oksanen et
199 al., 2013) was used and the construction of the dissimilarity matrix was based on the Bray-
200 Curtis methods.
201 Differentially expressed miRNA were identified using one Bioconductor (Gentleman et al.,
202 2004) package: DESeq2 1.32.0 (Love et al., 2014). Data were normalized using the default
203 method for DESeq2 package. MiRNA with adjusted p-value below 5% (according to the
204 FDR method from Benjamini-Hochberg) were considered significantly differentially
205 expressed between sex (males or females) or gonadal stages for the Gilthead seabream. To
206 better depict the miRNAs potentially discriminating males from females we conducted a
207 Principal Component Analysis (PCA) on miRNAs with unadjusted p-value below 5%
208 following the DESeq2 comparing all species together for only immature or mature stages.
209 We also performed a NMDS to discriminate gonadal stages over sex-change of the Gilthead
210 seabream. All statistical analyses were conducted with the R v 4.1.0 (Core Team, 2020).
211
212 **Prediction of target**

213 We retrieved the 3'UTR sequences from the European seabass, the Red drum, the Gilthead
214 seabream and the Turbot (note that annotated 3'UTR sequences were not available for the
215 Blue Runner). To identify miRNA targets, we used the freely available Perl script of
216 TargetScanHuman 8.0. (https://www.targetscan.org/vert_80/) (Agarwal et al., 2015) that we
217 adapted for our fish species. This allowed to obtain a list of potential target genes by
218 searching for the presence of conserved 8mer, 7mer, and 6mer sites that match the seed
219 region of each miRNA of interest. Then all sequences of potential target genes for the 4 fish
220 species were aligned using the MUSCLE Alignment in Geneious Prime 2019.2.3 and the

221 position of the seed was confirmed in order to corroborate that the position of the match is
222 evolutionary conserved between these species, enforcing the credibility of the target.

223 **Results**

224 On the 258 plasma samples collected and for which the RNA was extracted, a total of 96
225 samples for which the quality of the RNA was considered satisfying were sequenced. The
226 Non-metric multidimensional scaling (NMDS) of all miRNAs detected in the plasma of the
227 four gonochoristic fish species allowed to well discriminate the species ($r^2 = 0.82$; p-value <
228 0.001***; Figure 1); and gonadal developmental stages (mature vs immature, $r^2 = 0.07$; p-
229 value < 0.05*) but not sex (females vs males, $r^2 = 0.003$; p-value = 0.87). Therefore,
230 subsequent analyses were performed within each gonadal stage.
231



232
233 Figure 1. Non-metric multidimensional scaling (NMDS) of all miRNAs detected in the
234 plasma of four gonochoristic fishes. On NMDS, each point represents an individual form

235 each of the 4 species, differing by their shape. Yellow points represent females, while brown
236 points represent males.
237
238 **Immature gonochoristic fish**
239
240 From the DESEQ2 analysis, we detected 19 miRNAs for which uncorrected p-value were
241 below 0.05, that allowed to well discriminate males from females in the first axis of the PCA
242 (46 % of variance explained), independently of the species (Figure 2A). Among those 19
243 miRNAs 3 were highly significant: miR-21a-3p, miR-18a-3p and miR-29-1a-5p (Figure 2A,
244 in red, corrected p-value < 0.05), although this pattern was mainly driven by Turbots.
245

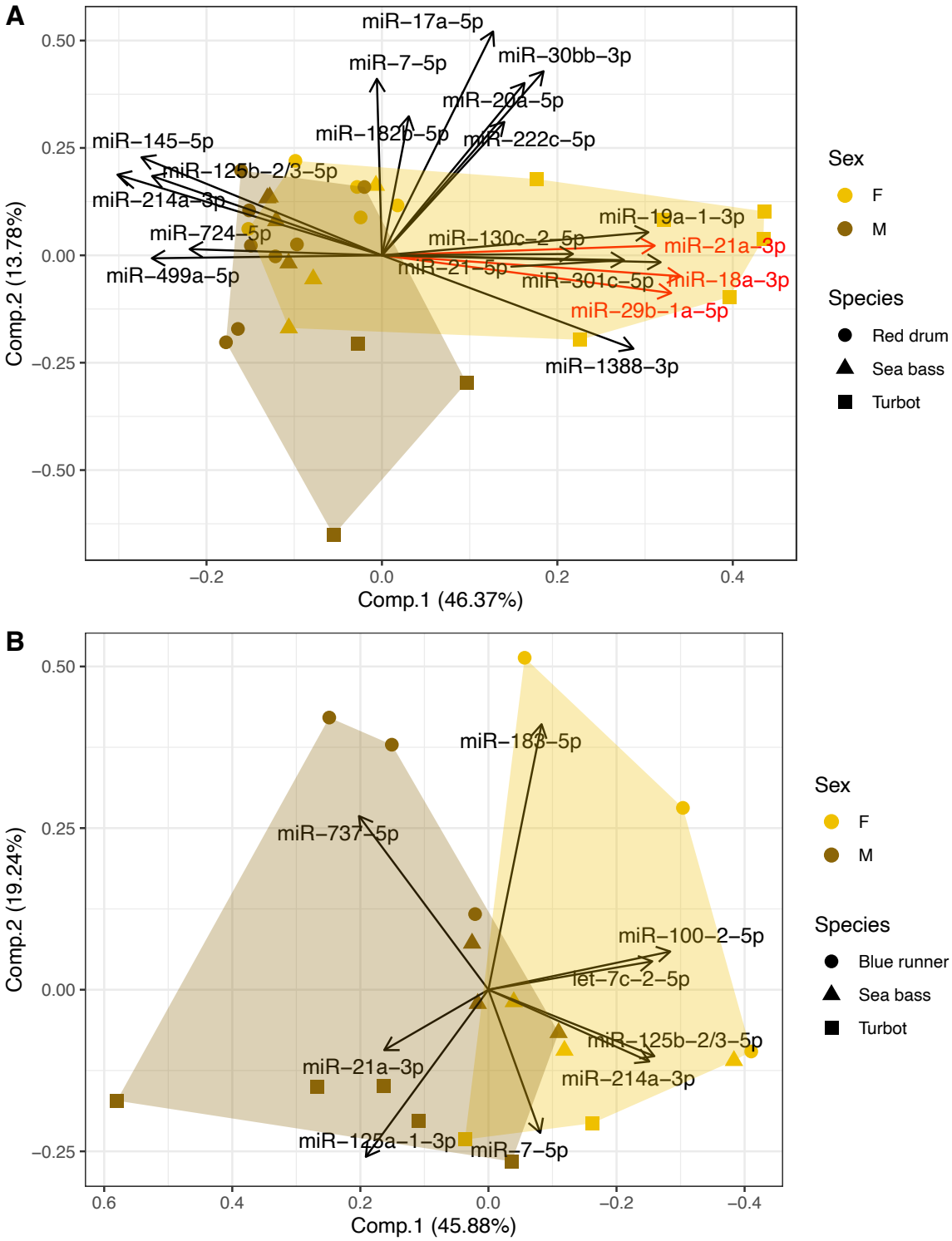


Figure 2. PCA of the most differentially expressed miRNAs in the plasma of A) Immature gonochoristic fish and B) Mature gonochoristic fish. Each symbol represent an individual of a species, and each arrow, a variable : miRNA. MiRNAs represented in red are significant (corrected p-value < 0.05) in the DeSeq2 analysis. Females and males are represented by yellow and brown dots respectively.

252

253 **Mature gonochoristic fish**

254

255 The DESEQ2 analysis yielded 9 miRNAs for which uncorrected p-values were below 0.05,
256 that allowed to well discriminate mature males from mature females in the first axis of the
257 PCA (46 % of variance explained), independently of the species (Figure 2B). None of them
258 were significant when applying the FDR correction method from Benjamini-Hochberg.
259 Overall, 3 miRNAs were common to both analyses. The miR-7-5p presented a similar
260 pattern between mature and immatures samples, with females tending to have more miR-7-
261 5p than males. On the contrary, both and the miR-125b-2/3-5p and miR-21a-3p presented
262 an opposite pattern between males and females according to the maturity stage. When
263 considering each species, we detected significantly more miR-23a-1-5p in males compared
264 to females as well as significantly more miR-206-1-3p and miR-1-3p in females compared
265 to males in Jacks (adjusted p-values < 0.05 for all comparisons). In immature Red drum we
266 could not identify miRNAs differentially expressed between sex. In the European seabass
267 only, we identified 11 differentially expressed miRNAs between males and females
268 (Houdelet et al. 2023), and two of them were in the common analysis of immature fish, the
269 miR-499a-5p and the miR-1388-3p.

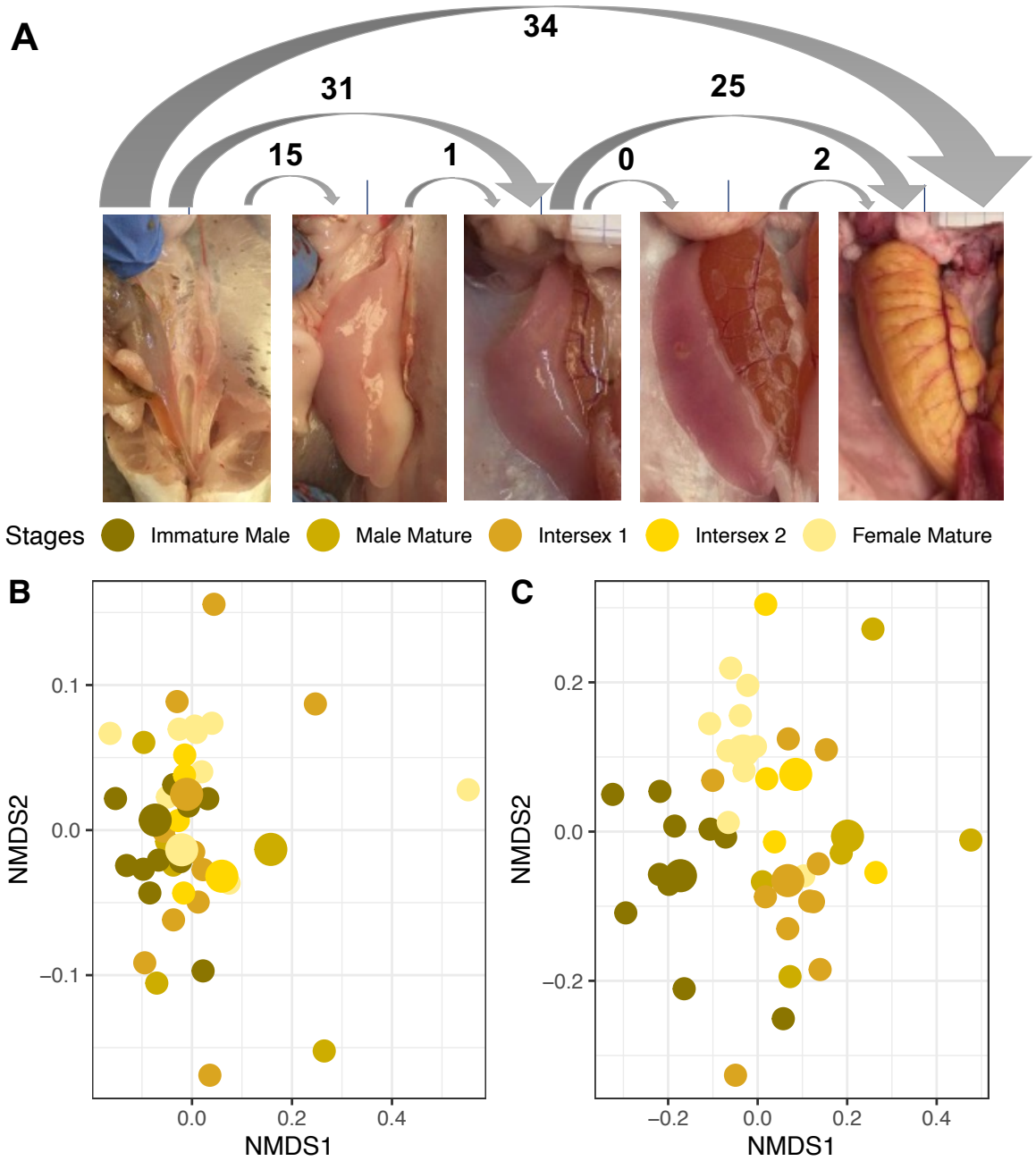
270

271 **In a protandrous hermaphroditic fish: The Gilthead seabream**

272

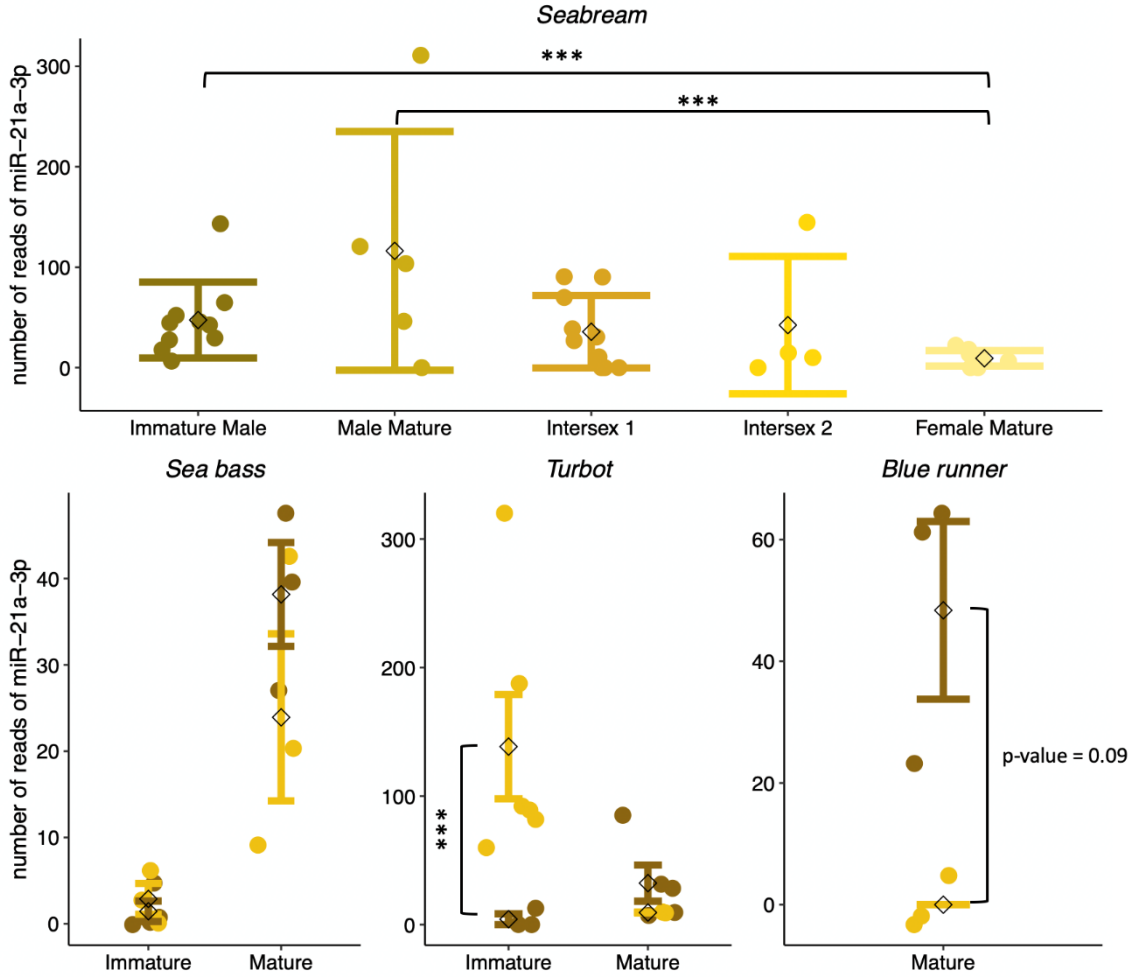
273 The DESEQ2 analyses allowed to detect a total of 76 unique miRNAs differentially
274 expressed between all stages (Supplementary Data 1). Within these 76 miRNAs, 54 miRNAs
275 were at least common to two comparisons (e.g. between immature male and mature male as
276 well as between immature male and intersex 1). The more prominent comparison was
277 between immature males and mature females, with 34 differentially expressed miRNAs
278 (Figure 3A). The NMDS of all miRNAs (stress = 0.16) detected in the plasma allowed to
279 significantly distinguish the five different stages (r^2 = 0.28; p-value = 0.01; Figure 3A, 3B).
280 When focusing on all those discriminating significantly all stages in a pair-wise comparison
281 (1 stage vs 1 Stage, using DESEQ2), the effect of gonadal stage on this new NMDS (stress
282 = 0.17) was more pronounced (r^2 = 0.46; p-value < 0.001, Figure 3C). Since the Gilthead
283 seabream change sex at the same time its grow, it was difficult to decipher sex and age-
284 related miRNAs. Interestingly, we found out that two of our markers detected in immature

285 and mature fish, miR-21a-3p and miR-125b-2/3-5p (Figure 2) were also significantly and
286 differentially expressed between both immature and mature males and mature females in the
287 Gilthead seabream (Figure 4,5). The miR-125b-2/3-5p presence was dependent on the
288 developmental stage, with more reads in mature females compared to mature males and more
289 miR-125b-2/3-5p reads in immature males compared to immature females (Figure 5).
290
291



292
293 **Figure 3** (A) The 5 different gonadal stages, for which plasmatic miRNAs were sequenced.
294 The number bellow each arrow represent the number of miRNAs differentially expressed

295 between stages. (B) Non-metric multidimensional scaling (NMDS) of all miRNAs detected
296 in the plasma of the Gilthead seabream according to their gonadal developmental stages. (C)
297 NMDS of all miRNAs differentially expressed between the different gonadal developmental
298 stages. The different colors distinguish the different developmental stages.
299



300
301 **Figure 4** Copy number of miR-21a-3p in Gilthead Seabream, European Sea bass, Turbots
302 and Blue runner. Females and males are represented by yellow and brown dots respectively.
303 Each bar represents the mean (a black diamond) ± se.
304

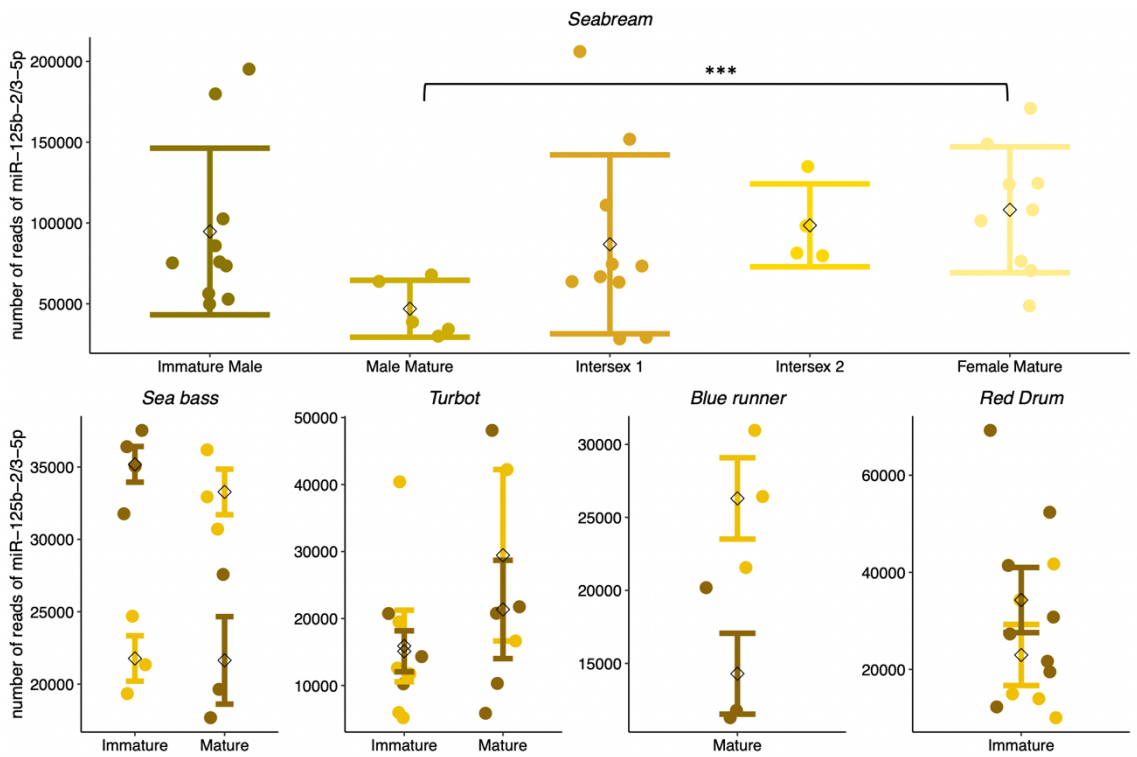


Figure 5 Copy number of miR-125b-2/3-5p in Gilthead Seabream, European Sea bass, Turbots, Blue runner and Red Drum. Females and males are represented by yellow and brown dots respectively. Each bar represents the mean (a black diamond) ± se.

Target prediction of miR-21a-3p and miR-125b-2/3-5p

The seed sequence used for miR-21a-3p was GACAACA and CCCTGAG for miR-125b-2/3-5p. Those seeds were common for all fish species. We detected 23 possible gene targets of miR-21a-3p that were common to 4 species: the Gilthead Seabream, the European Seabass, the Turbot, and the Red Drum; and 184 targets that were common to at least 3 of these 4 species (Supplementary data 2). Among the 184 genes, 6 were of interested regarding hormonal regulation: wingless-type MMTV integration site family, member 4 (wnt4), estrogen-related receptor beta (esrrb), estrogen-related receptor gamma a (esrrga), insulin-like growth factor 2 mRNA binding protein 2a (igf2bp2a), insulin-like growth factor 1 (igf1) and insulin receptor a (insra). To confirm that the seed region of miR-21a-3p could bind to the same position of the 3'UTR sequence for all fish, they were all aligned using Geneious

325 Prime and a consensus sequence was built. For wnt4, all 3'URT aligned completely with the
326 7 bases of the seed region of miR-21a-3p (Supplementary Figure 1). For insra, the 3'UTR
327 sequence of the Turbot was not available, but the seed region of miR-21a-3p aligned at a
328 common 3'UTR sequence region for the Red drum, the European seabass and the Gilthead
329 seabream. For esrrga, the seed region aligned with the 3'UTR of the Red drum, the European
330 seabass and the Turbot. There was only one base difference for the Gilthead seabream (A
331 instead of G; Supplementary Figure 1), which could also be an error of sequencing. For
332 esrrb, the seed region aligned with the 3'UTR of the Gilthead seabream, the European
333 seabass and the Turbot. There was only one base difference for the Red drum (C instead of
334 G; Supplementary Figure 1). Regarding igf1, only 6 bases the seed region of miR-21a-3p
335 aligned with the four 3'UTRs sequences. Regarding igf2bp2a, the seed region aligned with
336 the 3'UTR of the Gilthead seabream, the European seabass and the Red drum at the same
337 location, but not for the Turbot.
338 We detected 23 possible targets genes of miR-125b-2/3-5p that were common to 4 species:
339 the Gilthead Seabream, the European Seabass, the Turbot, and the Red Drum; and 90
340 potential targets that were common to at least 3 of these 4 species (Supplementary data 3).
341 Among the 90 genes, 2 were of interested regarding hormonal regulation: Igf2bp2a, which
342 was also a potential target of miR-125b-2/3-5p, although the seed sequence aligned only
343 with 6 nucleotides (6mer) in the four fish species. Another potentially interesting target of
344 miR-125b-2/3-5p was hsd17b1, but it also aligned with only 6 nucleotides at the same region
345 for 3 species: the Gilthead seabream, the European seabass and the Red drum (the 3'UTR
346 sequence of hsd17b1 was not available for the Turbot).
347
348 **Discussion**
349
350 Our study revealed that circulating miRNAs detected in the plasma are highly species-
351 specific, but some miRNAs were significantly correlated to one sex, independent of the
352 species. This is the case for miR-21a-3p, miR-18a-3p, and miR-29-1a-5p, which are over-
353 expressed in immature females compared to immature males. For mature fishes, 9 miRNAs
354 allowed discrimination between males and females, but when tested individually, none of
355 them was significantly associated with one sex (using DESeq2 analysis). Surprisingly, for
356 the gonochoristic fish species for which both immature and mature fish were available
357 (Turbots, European seabass, and Red drum), libraries were successfully constructed for 57%

358 of the RNAs available in immature fish and only for 30% of the RNAs available in mature
359 fish. For instance, 39 RNA samples were available for mature Red drum, but none of them
360 was of sufficient quality for constructing small RNA-seq libraries. This highlight sharp
361 differences in plasma composition between immature and mature fish and might be a reason
362 why we could not detect miRNAs differentially expressed according to sex in mature
363 individuals. Nonetheless, when considering all miRNAs with an uncorrected p-value < 0.05,
364 two miRNAs, namely miR-21a-3p and miR-125b-2/3-5p, were of particular interest with
365 opposite expression patterns between males and females according to the maturity stage.
366 Interestingly, these two miRNAs were also detected as differentially expressed between
367 individuals with a mature testis and those with a mature ovary in the protandrous Gilthead
368 Seabream.
369
370 Specifically, miR-21a-3p was over-expressed in Gilthead Seabream presenting a mature
371 testis compared to individuals exhibiting a mature ovary, and the same tendency was
372 observed for mature individuals in gonochoristic species: the European Seabass, the Turbot,
373 and the Blue Runner. The opposite pattern was detected in immature European Seabass and
374 Turbot, where immature females (all species together) presented significantly more reads of
375 miR-21a-3p than immature males. Interestingly, this miRNA was found to be abundantly
376 expressed in ovaries of zebrafish and common carp (*Cyprinus carpio*), depending on the
377 developmental stage (F. Wang et al., 2017; Wong et al., 2018; Zayed et al., 2019). Now,
378 when comparing miRNAs between sex, expression levels of miR-21-3p was more than 4-
379 fold in XX ovary as compared to XY testis of immature (1-year old) yellow catfish
380 (*Pelteobagrus fulvidraco*)(Jing et al., 2014). It is worth noting that none of these studies were
381 performed in plasma, where one could expect lower miRNA quantities relative to organs.
382 For instance, in a pre-experiment in which we sequenced gonads and plasma of the same
383 European seabass individuals (n = 10), we detected about 15 times more miR-21a-3p in the
384 gonad than in the plasma (mean number of reads: 473 vs. 32).
385 Yet, most of our knowledge regarding the role of miR-21a-3p in controlling gonadal
386 development comes from mammals, such as mice, sheep, porcine, and bovine (Arefnezhad
387 et al., 2024; Reza et al., 2019). MiR-21 plays a significant role in oocyte maturation, as well
388 as in the development of blastocysts and embryos. It is notably overexpressed during the
389 transition from germinal vesicle to oocytes, which are arrested at the metaphase II
390 (Arefnezhad et al., 2024). In addition, MiR-21-3p is highly expressed in atretic follicles

391 compared to healthy follicles (Donadeu et al., 2017). This would corroborate our findings,
392 where immature females (before the start of vitellogenesis) present more copies of this
393 miRNA than immature males in gonochoristic species, and mature males present more miR-
394 21a-3p than mature females. In addition, those results also well align with what we detected
395 in the protandrous Gilthead seabream that possess an ovotestis, and where ovarian
396 development is blocked in mature males, with potentially high numbers of atretic follicles.
397 The *in silico* analysis revealed that miR-21-3p is a potential regulator of numerous signaling
398 pathways, including meiosis, wingless-type MMTV integration site family, member 4
399 (wnt4), estrogen-related receptors (beta, esrrb and gamma, esrrga), as well as insulin
400 (igf2bp2a, igf1, insra), with conserved target sites among the different fish species
401 investigated. All of these genes play key roles in sex determination and sexual development.
402
403 The miR-125b-2/3-5p also presented an interesting expression pattern that sharply differs
404 according to the developmental stage, but in a direction opposite to that of miR-21a-3p. In
405 all fish investigated, females at a mature stage presented more miR-125b-2/3-5p than males,
406 while immature males tended to have more miR-125b-2/3-5p in the plasma than their female
407 counterparts. When comparing stages within sex, these results are not in line with those of
408 Papadaki et al. (2020) who detected more miR-125c (that share the same seed region that
409 miR-125b) in immature females compared to mature females. The authors proposed the
410 follicle-stimulating hormone receptor (FSHR) as a putative target of miR-125c applying
411 RNAhybrid (version 2.12 (Krüger and Rehmsmeier 2006)) (Papadaki et al., 2020), but we
412 were not able to detect such a target for miR-125b-2/3-5p using TargetScan. One interesting
413 putative target concerning sexual development was hsd17b1, an enzyme that catalyzes the
414 final step of estrogen biosynthesis, by preferentially reducing estrone to yield the potent
415 estrogen 17β-estradiol. In female mice, miR-125b expression was found to be activated by
416 estrogen via Erα, both *in vitro* and *in vivo* (Z.-C. Zhang et al., 2015), while the Luteinizing
417 hormone (LH) inhibited miR-125b-5p expression (X. Zhang et al., 2020). Furthermore,
418 inhibiting miR-125b-5p led to an increase in the expression of genes related to androgen
419 synthesis and stimulated testosterone secretion, while simultaneously downregulating genes
420 related to estrogen synthesis and decreasing estradiol release (X. Zhang et al., 2020). These
421 authors identified Pak3 as a direct target of miR-125b-5p, but such a target was not found in
422 our search of putative targets (X. Zhang et al., 2020). Overall, these results highlight an
423 important crosstalk between miR-125b-5p and oestrogen synthesis-related enzymes, while

much less is known regarding androgens. Yet, in the half smooth tongue sole (*Cynoglossus semilaevis*), high level of miR-125b-5p were detected in the sperm of fish compared to their exosomes in the seminal plasma (Zhao et al., 2023) and it was found to be one of the most abundantly expressed miRNA in both gonads of the yellow catfish (*Pelteobagrus fulvidraco*)(P. Wang et al., 2018).

Our study identified several miRNAs as potential sex markers for marine fish. However, their expression in plasma is closely linked to the animal's sexual developmental stage. This is evidenced by the gradual increase or decrease of miR-125b-2/3-5p and miR-21a-3p, respectively, which characterize the transition from male to female in the Gilthead Seabream. Including both gonochoristic and hermaphroditic species in the study helped to eliminate age as a confounding factor in detecting sex-specific miRNAs in the protandrous Gilthead Seabream. Moreover, detecting these miRNAs in plasma is advantageous for monitoring sexual stage transitions and determining sex in a non-invasive manner. We hope our study will pave the way in developing new miRNA-based sensors that can provide insights into sex and sexual developmental stages in fish.

Data, script, code, and supplementary information availability

All data generated or analyzed during this study are included in this article and in supplementary information. The R script and processed data from the small RNA-seq are available in Zenodo: https://doi.org/10.5281/zenodo.14013730. The sequencing raw data are available through the online ENA (European Nucleotide Archive) platform, under the access number: PRJEB80705.

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Conflict of interest

The authors declare no conflict of interest.

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