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1 **Circulating MicroRNAs indicative of sex and stress in the European seabass (*Dicentrarchus labrax*):**
2 **toward the identification of new biomarkers.**

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24

25 **Abstract**

26 MicroRNAs (miRNAs) constitute a new category of biomarkers. Studies on miRNAs in non-mammalian
27 species have drastically increased in the last few years. Here, we explored the use of miRNAs as potential,
28 poorly-invasive markers, to identify sex and characterize acute stress in fish. The European seabass
29 (*Dicentrarchus labrax*) was chosen as model because of its rapid response to stress and its specific sex
30 determination system, devoid of sexual chromosomes. We performed a small RNA-sequencing analysis in
31 the blood plasma of males and females' European seabass (mature and immature) as well as in the blood
32 plasma of juveniles submitted to an acute stress and sampled throughout the recovery period (at 0h, 0.5h,
33 1.5h and 6h). In immature individuals, both miR-1388-3p and miR-7132a-5p were up-regulated in females,
34 while miR-499a-5p was more abundant in males. However, no miRNAs were found to be differentially
35 expressed between sexes in the blood plasma of mature individuals. For the acute stress analysis, five
36 miRNAs (miR-155-5p, miR-200a-3p, miR-205-1-5p, miR-143-3p and miR-223-3p) followed cortisol
37 production over time. All miRNAs identified were tested and validated by RT-qPCR on sequenced samples.
38 A complementary analysis on the 3'UTR sequences of the European seabass allowed to predict potential
39 mRNA targets, some of them being particularly relevant regarding stress regulation, e.g. the glucocorticoid
40 and the mineralocorticoid receptor. The present study provides new avenues and recommendations on the use
41 of miRNAs as biomarkers of sex or stress of the European seabass, with potential application on other fish
42 species.

43

44 **Keywords:** sex differentiation, cortisol, glucocorticoid receptor, fish, blood plasma, miRNAs

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45 **1. Introduction**

46 Identifying new, poorly-invasive, technics to depict clear phenotypes is of major interest in fishery and
47 aquaculture contexts (Brosset et al., 2021; Raposo de Magalhães et al., 2020). For instance, consumers are
48 now highly concerned by the welfare of harvested fish, and accurately monitoring stress is thus becoming
49 crucial for both industries. Managing sex-ratio of farmed population is also primordial for producers, since in
50 many fish species i) there is a strong sexual size dimorphism, and a clear benefit in producing the fastest-
51 growing sex and ii) having a sufficient number of males and females is essential to guarantee successful
52 genetic programs. Sexing wild fish with poorly-invasive tools can also be valuable to fully understand
53 population dynamics in marine animals (e.g., Tunas) in which it is impossible to distinguish males from
54 females based on external phenotypic characteristics.

55 The European seabass (*Dicentrarchus labrax*) is a convenient model to scrutinize all the above-mentioned
56 issues, as it brings together all questioning. First, it is a recognized model of interest in aquaculture and
57 fisheries (Vandeputte et al., 2019). Second, reliable basal levels of cortisol (major indicator of stress) are
58 highly difficult to obtain in this species (Sadoul et al., 2021; Vandeputte et al., 2016) and is considered very
59 high in relation to other species (Samaras et al., 2016). Third, there is currently no practical tools to
60 distinguish males from females in this species that possess a polygenic sex determination system, with effects
61 of the environment (Geffroy et al., 2021; Vandeputte et al., 2007). Indeed, it is only possible to visually and
62 accurately discriminate males from females based on their size when they reach a certain age (i.e., after 3
63 years; Chatain & Chavanne, 2009). Hence, having other, poorly-invasive tools, to monitor sex and stress
64 would be a real asset for this species, but also for many other exhibiting similar characteristics. Circulating
65 microRNAs (miRNAs) could well fulfill these roles.

66 MiRNAs are short and conserved non-coding sequences of nucleotides (20-22 nt) involved in the regulation
67 of multiple biological processes by post-transcriptional repression of target mRNA (Bartel, 2018).
68 MicroRNAs act as gene regulators through a base-pairing interaction with mRNAs, and a sequence similarity
69 of 6 to 8 nucleotides (seed sequence) is sufficient to deregulate a mRNA. Thus, a given miRNA could, in
70 theory, interact with multiple mRNAs (up to several hundreds), and one mRNA could be the target of
71 multiple miRNA (Fabian et al., 2010). MiRNAs present very advantageous characteristics to track
72 physiological states, so that they are considered as efficient biomarkers. First, miRNAs are highly conserved
73 in evolution, which support the prominent hypothesis of a fundamental role in the biology of metazoan

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74 (Bartel, 2009; Desvignes et al., 2019; Wheeler et al., 2009). Second, they can be quantified in different
75 tissues, but also in the extracellular part of various body fluids (e.g., serum, blood plasma, urine, saliva; Mohr
76 and Mott 2015). Since their discovery, they have been widely used for health-associated diagnostics in
77 humans, mainly for tumor detection (Duttagupta et al., 2011; Lu et al., 2005), but there is an increasing
78 interest in using them as biomarkers in other species. MiRNAs were shown to display distinct levels of
79 expression between the gonads of males and females in various fish species (Bhat et al., 2020; Gu et al.,
80 2014; Jing et al., 2014; Tao et al., 2016). However, to the best of our knowledge, sex-specific miRNAs have
81 not yet been described in fluids (like the plasma). A recent study conducted in rainbow trout (*Onchorynchus*
82 *mykiss*) identified a repertoire of circulating miRNAs that reflect the physiological and reproductive state of
83 the species (Cardona et al., 2021). Regarding stress, miRNAs were also shown to transduce different stressful
84 environments (Raza et al., 2022), where the impact of xenobiotics (Burgos-Aceves et al., 2018), handling
85 (Cadonic et al., 2020; Ikert et al., 2021) or thermal stress (Raza et al., 2022) have been pinpointed. Still, we
86 do not yet know how circulating miRNAs correlate to stress-induced cortisol levels.

87 The main objectives of this study were to characterize the circulating miRNAs associated with sex and stress
88 in the European seabass and to evaluate to what extent they can be easily quantified in the plasma by qPCR,
89 with the ultimate goal of using them as biomarkers.

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90 **2. Material and methods**

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

94 **2.1. Sample collection**

95 *2.1.1. Experiment 1: Sex identification*

96 A total of forty immature (mean weight: 44 ± 11 g and length: 15 ± 1 cm) and twenty mature (1387 ± 303 g
97 and 46 ± 4 cm) fish were randomly collected from the experimental aquaculture station of Ifremer (Palavas-
98 les-Flots, France) and Aquanord aquaculture site (Gravelines, France) respectively. A blood sample (1 ml,
99 see details below) was collected from each individual, as well as a piece of gonad for immature fish to
100 histologically sex them (Fig. 1A). For qPCR analyses, supplementary individuals were collected and sexed
101 (validation non-sequenced 1, Table 1) as well as fish from the stress experiment (validation non-sequenced 2,
102 Table 1).

103 **Table 1** Summary of the number of samples used for small RNA-seq and qPCR analyses

		Sex			
		small RNA-seq	qPCR	weight (g)	length (cm)
Experiment 1: sex	Male (Matures)	3		1333	47
	Female (Matures)	3		1281	45
	Male (Immatures)	5	5	44	15
	Female (Immatures)	5	5	41	15
Validation non-sequenced 1	Male		5	289	28
	Female		5	398	30
Validation non-sequenced 2	Male		5	279	27
	Female		4	484	32
Experiment 2: stress	Male	9	15	39	14
	Female	11	12	45	15

104

105 *2.1.2. Experiment 2: Stress experiment*

106 Juvenile European Seabass (n = 63, mean weight: 42.1 ± 15.7 g and length: 14.5 ± 1.5 cm) were maintained
107 in a recirculating aquaculture system, with three tanks of 1.5 m^3 each (21.0 ± 0.1 °C and water renewing at
108 1.2 m^3/h), connected to a common biofilter tank. The three tanks were covered with a black tarpaulin
109 ensuring they were visually isolated from the experimenters. The photoperiod (12L/12D) inside the tarpaulin
110 was maintained using a specific a light system: AquaRay miniLED 500, 10000K white (Tropical Marine
111 Center). Fish were fasted 24 h prior the start of the experiment. Beforehand the stress procedure, six fish per

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112 tank (n=18 in total) were quickly sampled and euthanized using benzocaine (150 mg/L). The blood was
113 immediately collected by 3 experimenters, ensuring that all fish were collected within 2 minutes to avoid
114 cortisol rise commonly reported after death (Sadoul and Geffroy, 2019). Thenceforward, 15 fish per tank
115 were quickly placed in a 10L bucket at a density of 300kg/m3 for 2 minutes. Following this confinement
116 challenge, they were placed in 3 recovering tanks (100L tanks, supplied with renewed water from the same
117 original tank) to recover from the stress for 0.5, 1.5 and 6 hours (Fig. 1B). Supplementary individuals were
118 collected for qPCR validation (n = 30) see details in Table 2).

119 **Table 2** Summary of the number of samples used for small RNA-seq and qPCR validation of stress
120 biomarkers

		Stress			
		small RNA-seq	qPCR	weight (g)	length (cm)
Experiment stress	T0	4	12	32	13
	T0.5	5	4	40	14
	T1.5	5	8	36	14
	T6	7	6	38	14

121

122 *Blood sampling*

123 At 0.5, 1.5 and 6 hours following the confinement challenge, a subsample of five fish was collected from
124 each tank (total n=15 per recovering tank), euthanized with a lethal dose benzocaine (150mg/L) and sexed
125 (note that one individual was not sexed). The blood was sampled from the caudal vein thanks to EDTA-
126 coated syringes. After centrifugation (3000 x g, 5 min, 10°C) the plasma was allocated in two new tubes: one
127 part for cortisol quantification (20µL) and the other part for small-RNA sequencing, stocked at -20°C and -
128 80°C, respectively.

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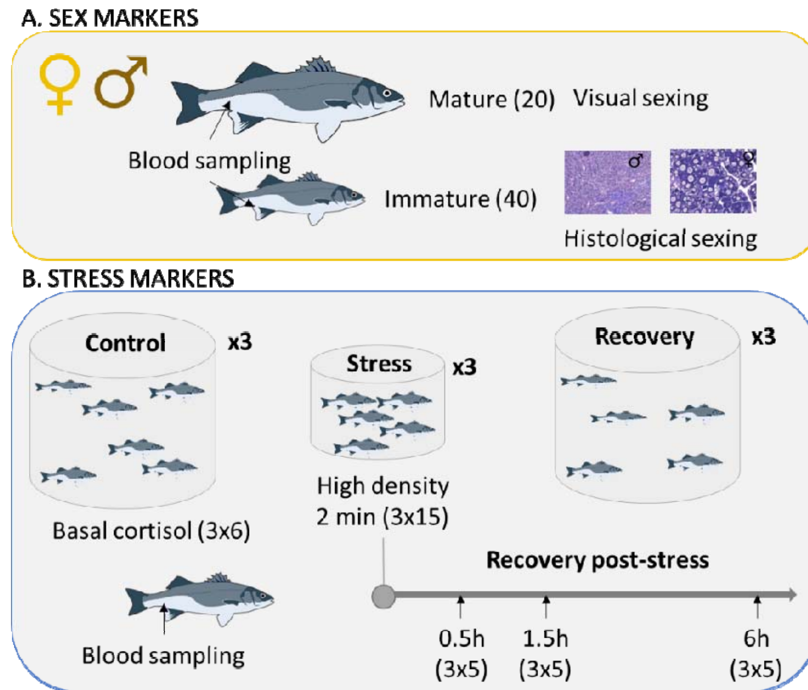

129

130 **Fig. 1** Summary of the two experiments conducted to identify (A) sex and (B) stress markers. The number in
131 brackets represent the number of fish used

132 **2.2. Identification of markers**

133 *2.2.1. Histology*

134 Gonads of immature European seabass were fixed in Bouin's fluid for 6 to 8 h and rinsed in clear water for
135 one hour. Then, they were rinsed in EtOH 70% for several days and placed in a dehydration automate (STP
136 120, MM, France). Each gonad was embedded in paraffin and cut at 5µm sections. Slides were stained using
137 the Masson's trichrome methods (MYREVA SS30, MM, France).

138 *2.2.2. Measurement of stress*

139 Plasmatic cortisol was measured using a Cortisol ELISA kit (Neogen Lexington, KY, USA). According to
140 the supplier, the cross-reactivity of the antibody with other steroids is as follows: prednisolone 47.5%,
141 cortisone 15.7%, 11-deoxycortisol 15.0%, prednisone 7.83%, corticosterone 4.81%, 6β-hydroxycortisol
142 1.37%, 17-hydroxyprogesterone 1.36%. Following manufacturer's instructions, samples or standard (Cortisol
143 standard solution) were added in each well in duplicate and supplemented with the conjugated cortisol
144 enzyme. After one hour of incubation, each well was washed and filled with the substrate. Absorbance was

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145 read at 650 nm with a microplate reader (Synergy HT, BioTek Instrument, VT, USA) after 30 minutes of
146 incubation in the dark. To confirm the repeatability of the experiment, one sample was placed on the three
147 different plates. Parallel displacement curves were obtained for plasma by comparing serial dilutions of
148 pooled plasma (1:1 – 1:250) and the cortisol standard preparation (0.04 – 10 ng/ml). All values are expressed
149 with the standard error.

150 *2.2.3. RNA extractions*

151 Thawed plasma of each fish from the two experiments was homogenized in QIAzol lysis reagent (Beverly,
152 MA, USA) following manufacturer's instructions. The total RNA was resuspended in 15 µL of RNAse free
153 water. MiRNAs were quantified using the smallRNA Analysis kit (DNF-470-0275) on a Fragment Analyzer
154 (Agilent).

155 *2.2.4. Small RNA Sequencing*

156 Following these steps, six matures (over the 20 collected) and ten immatures (over the 40 collected)
157 individuals from first experiment (sex identification) and 21 individuals from the second experiment (stress
158 experiment) were of sufficient quality and quantity, to be further processed (Table 1 and Table 2). Libraries
159 were constructed using the NEXTFLEX Small RNA-seq kit v3 (Perkin Elmer, #NOVA-5132-05). Briefly, a
160 3' Adenylated adapter was ligated to the 3' end of 0,5 ng of microRNA and purified to remove 3' adapter
161 excess. A 5' adapter was ligated to the 5' end of the 3' ligated microRNA. The resulting construction was
162 purified to remove 5' adapter excess. 5' and 3' ligated microRNAs underwent a reverse transcription using a
163 M-MuLV reverse Transcriptase and a RT primer complementary to the 3' adapter. Resulting cDNAs were
164 used as a matrix in a 25 cycles PCR using a pair of uniquely barcoded primers. The resulting barcoded library
165 was size selected on a Pippin HT (SAGE Science) using 3 % agarose cassette (#HTG3004) and aiming for a
166 size range between 147 bp and 193 bp. Once size selected, libraries were verified on a Fragment Analyzer
167 using the High Sensitivity NGS kit (#DNF-474-0500) and quantified using the KAPA Library quantification
168 kit (Roche, ref. KK4824).

169 *2.2.5. MiRNA alignment and quantification*

170 Image analyses and base calling were performed using the NovaSeq Control Software and Real-Time
171 Analysis component (Illumina). Demultiplexing was performed using Illumina's conversion software

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172 (bcl2fastq 2.20). The quality of the raw data was assessed using FastQC from the Babraham Institute and the
173 Illumina software SAV (Sequencing Analysis Viewer).
174 The raw reads were trimmed using Cutadapt (version 3.5) (Martin, 2011) to remove the sequencing adapter
175 (TGGAATTCTCGGGTGCCAAGG) at the 3′-end. Additionally, 4 bases were also trimmed from the 5′-end
176 and 3′-end of the reads as indicated in the manual of NEXTflex Small RNA-Seq Kit v3 from Bio Scientific.
177 Before counting step, samples with a rRNA degradation profile were filtered out. This resulted in 13 and 16
178 immature and mature fish, respectively, for the experiment on sex and 21 fish for the experiment on stress.
179 MiRNA analysis was performed with Prost ! v0.7.60 pipeline (Desvignes et al., 2019). In this pipeline,
180 alignment was performed with BBMap (Bushnell, 2014) version 38.90 to genome of interest : *Dicentrarchus*
181 *labrax* GCA_000689215.1_seabass_V1.0. The miRNA annotation and counting were retrieved by Prost!
182 (parameters in Text S1) from a custom annotation provided in miRBase v21 (available in *Prost!* Github)
183 using *Gasterosteus aculeatus* miRNA sequences (the phylogenetically closest species).

184 **2.3. Validation of markers by qPCR**

185 *2.3.1. cDNA synthesis and quantitative real-time PCR*

186 For the validation of markers by qPCR, total RNA from each of the non-sequenced samples (n = 56
187 individuals for sex and n = 30 individuals for stress; Table 1) were normalized at 10 ng/µL and the reverse
188 transcription of RNA was done following the manufacturer instructions (miRCURY LNA RT Kit, Qiagen).
189 We added 0.5µl of controls UniSp6 and cel-mir-39-3p to the samples as internal reference to check the
190 efficiency of the reverse transcription and PCR amplification, respectively. The reverse transcription reaction
191 was conducted on a total volume of 10 µL containing 5 µL of 2x miRCURY SYBR Green Master Mix, 1 µL
192 of the resuspended primer mix (miRCURY LNA PCR Assay), 3µL of diluted cDNA and 1µL of RNase-free
193 water. Quantitative RT-PCR was performed on a Light Cycler 480 System (Roche Life Science) with the
194 following conditions: 95°C for 2 min, and 45 cycles of 95°C for 10sec, 56°C for 60sec.
195 In addition to the exogenous control (cel-miR-39-3p), we selected one endogenous miRNA based on the
196 small RNA sequencing and that was stable regarding the different conditions: miR-23b-2-3p. Sequences of
197 miRNAs primer are provided in Table S1.

198 *2.3.2 Statistical analysis*

199 Differentially expressed miRNA were identified using one Bioconductor (Gentleman et al., 2004) package:
200 DESeq2 1.32.0 (Love et al., 2014). Data were normalized using the default method for DESeq2 package.

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201 MiRNA with adjusted p-value below 5% (according to the FDR method from Benjamini-Hochberg) were
202 considered significantly differentially expressed between conditions: Sex (Male *vs* Female) or Stress (0 *vs* 0.5
203 *vs* 1.5 *vs* 6 hours).

204 For qPCR experiments, the differences between sex or timing after stress was assessed using the non-
205 parametric Kruskal-Wallis test (Kruskal & Wallis, 1952). For RT-qPCR on non-sequenced samples for
206 sexing (n = 46; Table 1), a linear mixed-effects model was applied to consider the experiment effect. To
207 identify the miRNAs that presented a linear increase (or decrease) in expression over time, following the
208 confinement challenge, we ran a loop in R to automatically detect all miRNAs (normalized counts) that are
209 significantly correlated to the time. For cortisol level analyses, the differences between timing after stress
210 was assessed using the non-parametric Kruskal-Wallis test. A Non-metric Multi-dimensional Scaling
211 (NMDS) approach was used to compare individuals from the different time-points and that presented various
212 cortisol levels. The package vegan v2.6-2 (Oksanen et al., 2013) was used and the construction of the
213 dissimilarity matrix was based on the Bray-Curtis methods. All statistical analyses presented in this section
214 were conducted with the R v 4.1.0 (Core Team, 2020).

215 **2.4. Prediction of target**

216 We first retrieved the 3'UTR sequences from the European seabass genome browser
217 (http://seabass.mpipz.mpg.de/) that is based on the published genome of the European seabass (Tine et al.,
218 2014). To identify miRNA targets, we used the freely available Perl script of TargetScanHuman 8.0.
219 (https://www.targetscan.org/vert_80/) (Agarwal et al., 2015). In order to be the more specific possible, we
220 used a conservative approach, consisting of focusing only on genes presenting a 8-mer sequence in the
221 3'UTR region; hence with an exact match to positions 2-8 of the mature miRNA (the seed + position 8)
222 followed by an 'A'. The "enrichGO" function of the R package ClusterProfiler version 4.4.4 (Yu et al., 2012),
223 with the GO dataset of the European sea bass was used to analyse function profiles of all genes potentially
224 targeted by a given miRNA. A hypergeometric test was performed and enrichment p-value of gene ontology
225 was calculated to find significantly enriched GO terms in the input list of each mirRNA target genes.
226 Enrichment analysis was performed and a P value <0.05 was considered to indicate a statistically significant
227 difference. The "ggplot 2" and "enrichplot" R packages were used to generate the cnetplot. The proportion of
228 clusters in the pie chart was determined by the number of genes.

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229 **3. Results**

230 **3.1. Identification of miRNA differentially expressed between sex**

231 Gonads of juveniles' fish that were used for identification of sex markers were histologically differentiated,
232 allowing to clearly discriminating males from females. Oocytes from ovarian tissues were at the primary
233 growth stage (Fig. S1A), while some spermatocytes, spermatids and spermatozoids were distinguishable in
234 the testis (Fig. S1B).

235 A total of 223 miRNAs, from the 10 samples sequenced (immature individuals), were annotated
236 with *Prost!* (Desvignes et al., 2019). Among these miRNAs, 11 were differentially expressed between
237 immature males and females (Table S2). Eight of these miRNAs were significantly more abundant in
238 females, while the remaining three miRNAs were significantly more abundant in males. From that list of 11
239 miRNAs, we identified three miRNAs potentially acting on the regulation of genes involved in pathways of
240 sexual development (based on the mammalian literature). Specifically, both miR-1388-3p and miR-7132a-5p
241 were up-regulated in the females' plasma (padj = 0.004 and p = 0.0003; Fig. 2A), while miR-499a-5p was
242 more abundant in males' plasma (padj = 0.0006, Fig. 2A). They were thus chosen for further validations by
243 qPCR (Table 1). Both, miR-7132a-5p and miR-499a-5p were validated (Fig. 2B) on the sample sent to
244 sequencing. To test for their consistency, additional immature individuals were analyzed (n = 10 on non-
245 sequenced 1, n = 9 on non-sequenced 2; n = 27 sampled in the stress experiment, Table 1). No significant
246 differences were observed between males and females, even though the miR-499a-5p tended to be higher in
247 males than females for the three set of samples (Fig. S2). We also took advantage of the sequencing of
248 individuals from the stress experiment to perform a DESeq2 analysis on the sex (all stressful conditions
249 mixed; 9 M vs 11 F, Table 1). Only the miR-499-a-5p tended to be more abundant in plasma of males
250 (adjusted p-value = 0.38, non-adjusted p-value = 0.01; Table S3).

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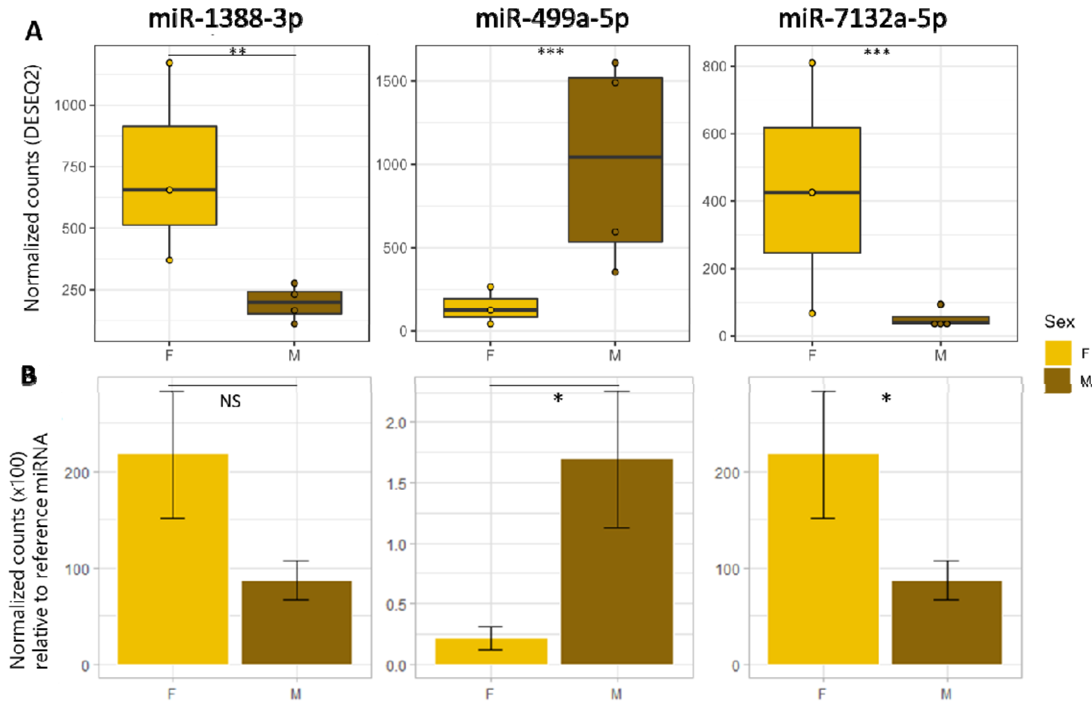

251

252 **Fig. 2** Normalized counts of circulating miRNAs based on A) small RNA-seq and B) RT-qPCR of sequenced
253 samples. Reference miRNA used for RT-PCR relative quantification was the miR-23b-2-3p. NS: non-
254 significant, *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$

255 A total of 441, 692 and 288 genes were predicted as potential target for respectively miR-1388-3p, miR-
256 499a-5p and miR-7132a-5p (Table S4). For each miRNA, potential target genes were classified by their
257 molecular function (MF) and biological processes (BP). On a total of 16368 possible biological processes in
258 the European seabass, we detected only 59 significantly enriched GO for miR-7132a-5p, 103 significantly
259 enriched GO for miR-499a-5p and 97 significantly enriched GO for miR-1388-3p (Table S5). On a total of
260 20503 possible molecular functions in the European seabass, we detected only 17 significantly enriched GO
261 for miR-7132a-5p, 32 significantly enriched GO for miR-499a-5p and 28 significantly enriched GO for miR-
262 1388-3p (Table S5). Within those GO of molecular functions and molecular process, many of them are
263 involved in epigenetic regulation, mostly regarding histone methylation. Only the miR-499a-5p presented
264 potential target genes in a GO directly related to sexual development: "Androgen receptor binding" (Table
265 S5).

266 **3.2. Dynamics of circulating miRNAs after an acute stress**

267 The basal level of cortisol at T0 was 2.4 ±1.0 mg/ml (n=18; Fig. 3A). We observed a significant increase of
268 cortisol at 0.5h (86.2 ±8.1 ng/ml, n=15, p-value = 1.4×10^{-7}) and 1.5h (126.0 ±25.7 ng/ml, n=15, p-value =

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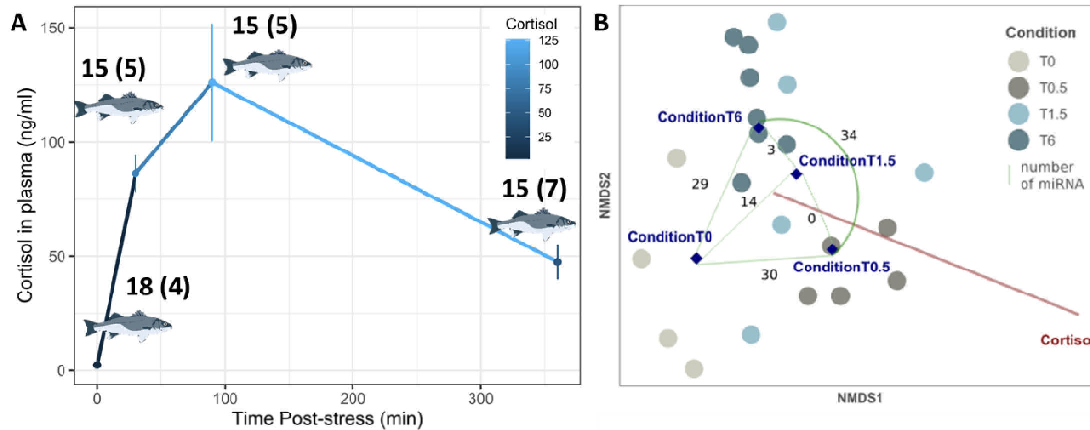
269 9.7 x 10^{-9}) following the confinement stress. Six hours after de stress, cortisol level decreased, though the fish
270 did not return to their basal level (47.5 ±7.5ng/ml; Fig. 3A).



271

272 **Fig. 3** (A) Cortisol (ng/mL) quantified in plasma of European seabass after an acute stress of confinement
273 and (B) Non-metric multidimensional scaling (NMDS) of miRNAs differentially expressed between various
274 time points after confinement stress. Numbers in brackets corresponded to the number of samples used for
275 sequencing. On NMDS, points represent individuals, diamonds represent the centroid value for each
276 condition and the red line represent the fitted cortisol value.

277 A total of 257 miRNAs, from the 21 samples sequenced, were annotated with *Prost!*. Results from a 2-
278 dimensional NMDS analysis yielded a stress level < 0.2 (stress = 0.11) and a noteworthy discrimination of
279 control individuals from individuals of the T0.5 condition (Fig. 3B). The DESEQ2 analyses allowed to detect
280 30 miRNAs differentially expressed between T0 and T0.5; 14 miRNAs differentially expressed between T0
281 and T1.5; 29 miRNAs differentially expressed between T0 and T6 and 34 miRNAs differentially expressed
282 between the T0.5 and the T6 conditions (Fig. 3B). Interestingly, the Venn diagram identified one miRNA
283 (miR-200a-3p) as common for all comparison and was thus chosen for further qPCR validation (Fig. S3). We
284 also purposely chose four other miRNAs from the above-described list. This choice was based on the fact
285 that two of them: miR-155-5p and miR-205-1-5p (Fig. 4) followed the cortisol production dynamic, while
286 another: miR-143-3p, presented an opposite pattern (Fig. 4). Indeed, the number of normalized counts
287 between T0 and T0.5 significantly increased for miR-155-5p; miR-200a-3p and miR-205-1-5p whereas it
288 decreased for the miR-143-3p (Fig. 4). Additionally, miR-223-3p was also selected because it presented
289 positive linear correlation with cortisol production over time, and its expression was 2-fold higher at 6 hours
290 post-stress compared to T0 (padj = 0.013; Fig. 4). Mir-155-5p, miR-200a-3p and miR-205-1-5p significantly
291 increased in the 30 minutes following the confinement challenge (padj = $9{,}81.10^{-8}$; $5{,}65.10^{-13}$ and $9{,}03.10^{-12}$,
292 respectively).

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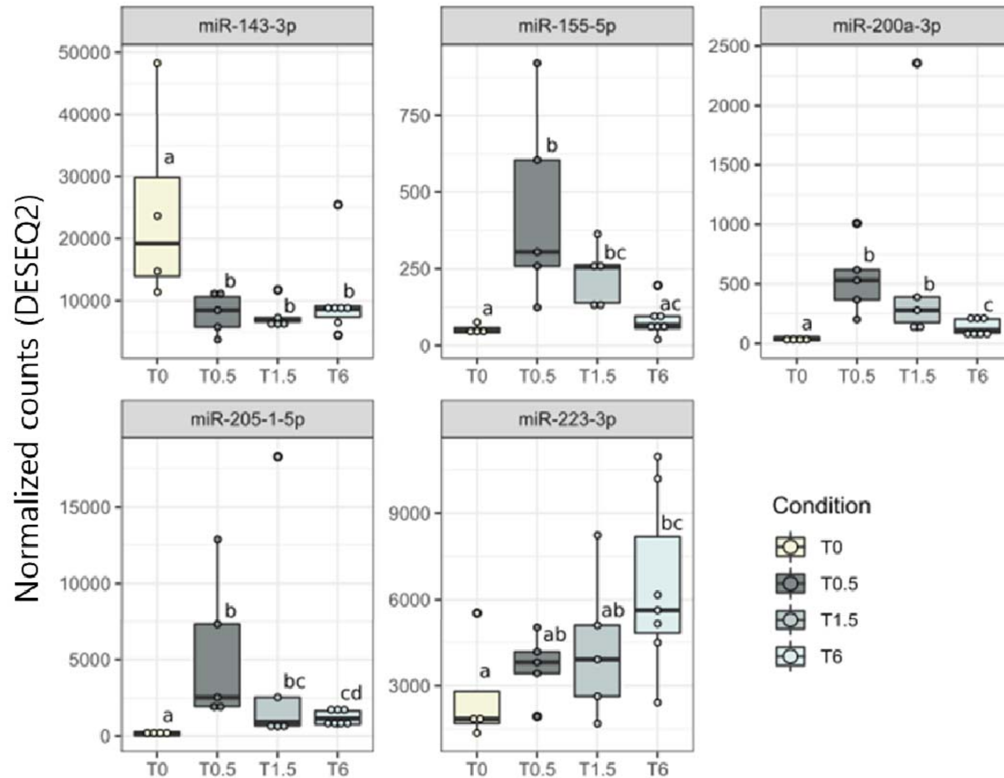
293



294

295 **Fig. 4** Normalized counts of miRNAs differentially expressed in plasma of European seabass after exposure
296 to a confinement stress. Different letters reveal a significant difference. The color code is associated with
297 timing of sampling after the acute stress.

298 The profile of expression observed with small RNA-sequencing and RT-qPCR was similar for miR-
299 155-5p, miR-143-3p, miR-200a-3p and miR-223-3p but not for miR-205-1-5p (Fig. S4). To ensure that the
300 miRNAs selected were effective markers, they were also tested by RT-qPCR but on other individuals (non-
301 sequenced) from the same experiment. Regarding miR-143-3p, miR-200a-3p and miR-205-1-5p, they
302 presented the same profile on RT-qPCR to that observed by sequencing (Fig. 5). This was especially true for
303 miR-205-1-5p that increased significantly at 1.5 hour following the stressful challenge (p-value = 0.006).
304 However, previous observations of the expression of miR-223-3p were not confirmed by RT-qPCR on those
305 different samples (Fig. 5). In addition, the quantity of miR-155-5p was two low on these new samples for

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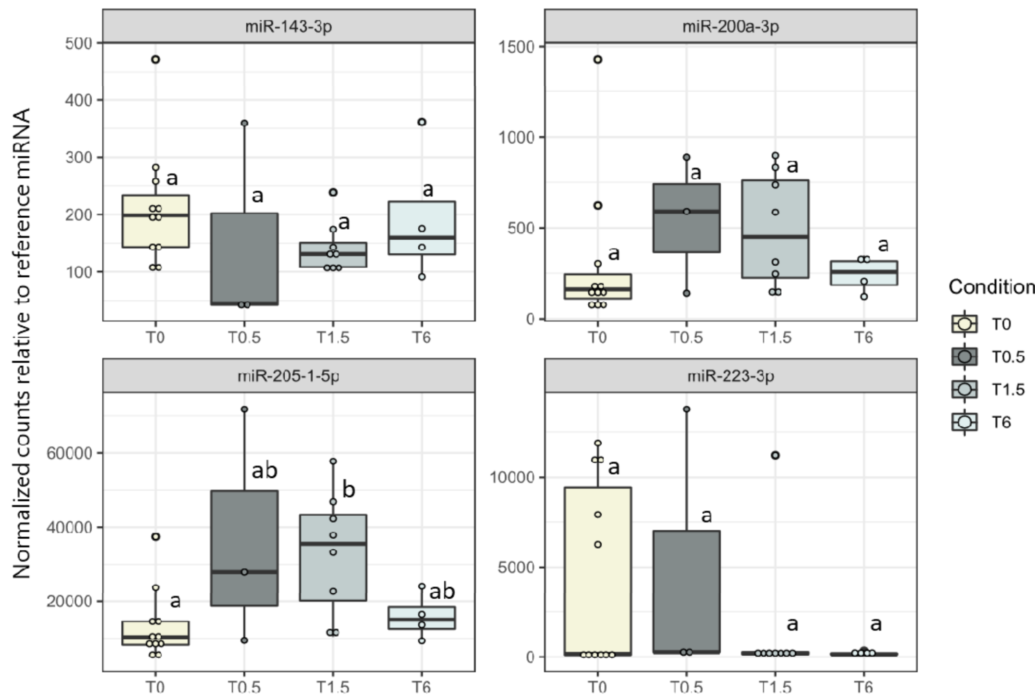
306 being correctly interpreted (i.e. very high CTs), and was thus discarded from the analysis.



307

308 **Fig. 5** Relative expression profile of four miRNAs in plasma of European seabass after a confinement stress.
309 RT-qPCR were done on non-sequenced samples collected at T0 (n=6), T0.5 (n=10), T1.5 (n=6) and T6
310 (n=8). Reference miRNAs used for RT-PCR relative quantification was miR-23b-2-3p. Different letter reveal
311 a significant differences. The color code is associated with timing of sampling after the acute stress.

312

313 We focused only on those five miRNAs for the GO analysis. A total of 785, 739, 1146, 961 and 907 genes
314 were predicted as potential target for respectively miR-155-5p, miR-143-3p, miR-200a-3p, miR-223-3p and
315 miR-205-1-5p (Table S4). For each miRNA, potential target genes were classified by their molecular
316 function (MF) and biological processes (BP). On a total of 16368 possible biological processes in the
317 European seabass, we detected only 118 significantly enriched GO for miR-155-5p, 70 significantly enriched
318 GO for miR-143-3p, 155 significantly enriched GO for miR-200a-3p, 116 significantly enriched GO for
319 miR-223-3p and 138 significantly enriched GO for miR-205-1-5p (Table S6). On a total of 20503 possible
320 molecular functions in the European seabass, we detected only 28 significantly enriched GO for miR-155-5p,
321 23 significantly enriched GO for miR-143-3p, 26 significantly enriched GO for miR-200a-3p, 33
322 significantly enriched GO for miR-223-3p and 19 significantly enriched GO for miR-205-1-5p (Table S6).
323 Interestingly many pathways related to the primary (i.e. involving monoamine neurotransmitters and

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corticosteroids release) and secondary (i.e. metabolism, hydromineral balance and cardiovascular functions) response to stress were pinpointed in the GO analysis (Table S6, Fig. 6).

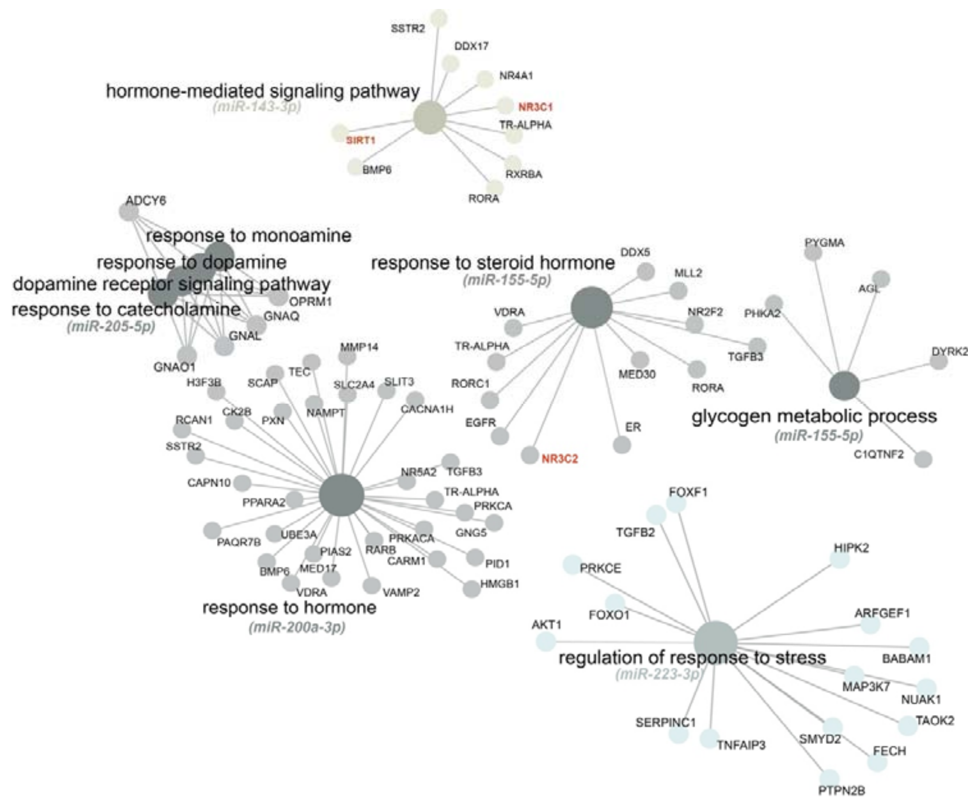

Fig. 6 Key pathways of genes potentially targeted miR-143-3p, miR-205-1-5p, miR-155-5p, miR-200a-3p and miR-223-3p. The different colors are associated with the level of expression of each miRNA at each time point. See Fig. 4 and Fig. 5 for the description of colors code used.

For instance, within those GO of biological process, many of them are involved in the stress response: “hormone-mediated signaling pathways” (including key genes such as glucocorticoid receptor, nr3c1 and sirtuin, sirt1) for miR-143-3p; “response to steroid hormones” (including the mineralocortoid receptor, nr3c2) for miR-155-5p; “response to monoamines, catecholamine, dopamine” for miR-205-1-5p; “response to hormones” for miR-200a-3p and “regulation of response to stress” for miR-223-3p (Fig. 6). We also identified potential target genes involved in 1) behavioural response :”regulation of behaviour”, “social behaviour”; “regulation of locomotion” 2) regulation of blood pressure : “regulation of heart rate”, “angiogenesis”, “blood vessel development”, “heart development”; and 3) energy balance: “glycogen metabolic process”, “response to lipid”, “lipid modification”, “response to glucose”, “energy reserve metabolic process”, “response to insulin”, “negative regulation of protein metabolic process” (Table S6, see Fig. S5 for a detailed list of selected GO of biological processes, miRNA per miRNA).

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341 **4. Discussion**

342 In this study, we investigated the potential of miRNAs as biomarker of sex and stress on the basis of a simple
343 blood collection on the European seabass. We divided the research in two parts: searching for (i) sex markers
344 and (ii) acute stress markers. Various miRNAs were modulated by the fact of being male or female, or by an
345 acute stress.

346 Here, we identified eleven miRNAs interesting for sexing immature European seabass, three of them being
347 strongly differentially expressed between sexes miR-1388-3p, miR-7132a-5p and miR-499a-5p. Among
348 them, the miR-499a-5p, more detected in males, was likely the more interesting because of the similar profile
349 obtained by RT-qPCR. In human, high level of circulating miR-499a is associated with early detection of
350 breast cancer in women, while low levels were detected in cells of men with prostate cancer (Y. Chen et al.,
351 2021; Kabirizadeh et al., 2016), suggesting a conserved role in steroid regulation. A recent study on zebrafish
352 detected significantly more miR-499-5p in testis than in ovaries (van Gelderen et al., 2022). Yet, studies on
353 other fish species have identified that miRNAs from the miR-499 family are involved in slow muscle
354 phenotype determination (Duran et al., 2020) and the miR-499a-5p is key in regulating *Sox6* (Sex
355 determining region Y-box 6) activity (Nachtigall et al., 2015; X. Wang et al., 2011). Interestingly, in the
356 European seabass, the 3'UTR sequence of sox6 also have two possible 8-mer recognition sites for miR-499a-
357 5p and *sox6* is significantly more expressed in the gonads of females experiencing molecular sex
358 differentiation, compared to the gonads of males (Geffroy et al., 2021). This would support the possible
359 repressive action of miR-499a-5p on *sox6*, though the exact mechanisms still remain to be established.
360 Finally, the genes *kdm1a* (Lysine-specific histone demethylase 1A) and *prkcb* (Protein kinase C beta type)
361 belonging to the GO "Androgen receptor binding" are also possible target for miR-499a-5p, supporting a
362 possible role in sexual development. More studies are necessary to really depict the role of miR-499a-5p and
363 understand why this miRNA is more detected in males' plasma compared to females' plasma.

364 Regarding miR-1388-3p (overexpressed in females of our experiment), this miRNAs was also more detected
365 in ovary of *Paralichthys olivaceus* relative to testis (Yang et al., 2018). Furthermore, miR-1388-5p is
366 involved in the regulation of the Spin-1 gene, which is important for hormone control, gametogenesis, and
367 oocyte meiotic resumption in rainbow trout (F. Wang et al., 2020). Finally, the miR-7132a-5p (overexpressed
368 in females of our experiment), was found to be up-regulated *Cynoglossus semilaevis* in pseudomales (ZW)
369 compare to males (ZZ) (Zhao et al., 2021). In addition, the -3p strand of the miR-7132a was found to be up-

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370 regulated in the juveniles' gonads of the common carp exposed to atrazine (F. Wang et al., 2019). It should
371 be noted that none of the miRNAs tested in qPCR (in individuals that were not sequenced) were detected as
372 significantly different between males and females, which might be due to the low quality of the RNAs used.

373 Regarding fish, our experiment corroborates observations of other studies conducted on gonads (miR-499-5p
374 and miR-1388-5p), even though we were astonished to not identify other "usual suspects" involved in sexual
375 development such as miR-202, which is highly sex-specific in the gonads of many other fish species (Geffroy
376 et al., 2016; Juanchich et al., 2016; Qiu et al., 2018; Shen et al., 2023; J. Zhang et al., 2017) or statistically
377 differentially expressed between the two gonads (Gay et al., 2018; J. Zhang et al., 2017). This might simply
378 transduce that the miRNAs detected in the plasma does not specifically mirror what happen in other organs.
379 In that sense, the recent study of Cardona and colleagues compared the miRNA repertoire of various body
380 fluids or tissues of the rainbow trout and highlighted a large differences in the identity of the miRNAs
381 observed in plasma compared to ovarian fluids (Cardona et al., 2021). Another explanation could be that
382 miRNAs found in the plasma at a specific moment transduce a punctual physiological state at this moment,
383 rather than a long-term phenotype (e.g., sex). This would explain why miRNAs related to stress were more
384 reliably found in qPCR compared to those related to sex.

385 For the stress experiment, we first characterized the stress response of our fish, when exposed to
386 confinement. This was highly challenging since the European seabass has often been regarded as a species
387 with relatively high basal cortisol levels compared to other species (Alfonso et al., 2023). When considering
388 individuals encompassing the same mass range (20-60 mg) the basal level varied between 14 and 80 ng/mL
389 in other studies using a comparable ELISA quantification method (Cerqueira et al., 2020; Tsalafouta et al.,
390 2015). Here, the level of cortisol was as low as 2 ng/ml, ensuring that we successfully obtained a reliable
391 basal level of unstressed fish. To our knowledge, our study is the first to report a long term (6 hours) miRNA
392 production dynamic following an acute stress in a fish species. A recent study described that 10 miRNAs
393 were modulated one hour following an air exposure challenge in rainbow trout, in distinct non-lethal
394 biological matrices (water, mucous and plasma ; (Ikert et al., 2021). Here, the confinement stress affected the
395 expression of 14 and 30 miRNAs at 0.5 and 1.5 hours post-stress, respectively. However, none of the
396 miRNAs detected were similar to that of the Ikert et al. (2021) study. We could thus not tease out a specific
397 effect of the stress applied or of the species studied, as pinpointed in a recent review (Raza et al., 2022). For
398 instance, miR-210-3p has been associated with hypoxic stress in the rainbow trout (Cardona et al., 2022) and

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399 miR-276b-3p was shown to be upregulated following a salinity stress challenge in *Portunus trituberculatus*
400 (X. Chen et al., 2019). Among miRNAs that we observed differentially expressed during stress recovery, we
401 purposely chosen to focus on miR-155-5p, miR-143-3p, miR-200a-3p, miR-223-3p and miR-205-1-5p to
402 conduct the RT-qPCR validation part. This choice was mainly based on their potentially interesting targets
403 (discovered thanks to TargetScan) following a stress. At the physiological level, the stress response is
404 conducted by the central nervous system, and led to a secretion of neurotransmitters and stress hormones that
405 constitute the first stress response (Schreck & Tort, 2016). The second response involve the secretion of
406 energetic metabolites (glucose, lactate..), the modulation of osmoregulation and immune response, while the
407 third response lead to drastic changes in performances (decrease of growth, disease resistance, behavioral
408 change ..). Interestingly, the GO analysis allowed us to provide some hypothesizes regarding the down-
409 regulation of key genes involved in the first two stages of the stress response. For instance, we identified the
410 glucocorticoid receptor (*gr* or *nr3c1*) as potential target of miR-143-3p *in silico*. Such a link would make
411 sense since 1) miR-143-3p is highly expressed at T0 compared to other time-points, supporting a possible
412 production of GR post-stress and 2) gain- and loss-function approaches in GR and miR-143-3p confirmed
413 that the glucocorticoid receptor was indeed a target of miR-143-3p in humans (L. Zhang et al., 2020). This
414 support a specific role of miR-143-3p in the primary stress response. MiR-155-5p and miR-200a-5p,
415 observed in the mid-intestine, are mainly involved in the response to an oxidative stress of the Wuchang
416 bream, *Megalobrama amblycephala* (Song et al., 2021). It is worth noting that the mineralocorticoid receptor
417 (mr or nr3c2), a potent receptor of cortisol (Prunet et al., 2006), is a potential target of miR-155-5p in our *in*
418 *silico* analyses, supporting its possible role in the regulation of stress response. The miR-223 was also
419 associated to the modulation of oxidative stress response in the Nile tilapia (Tang et al., 2013). We observed
420 that the miR-205-1-5p was the only one to show a statistical and steadily increase of its relative expression
421 following the confinement stress. Our predictive analysis, using TargetScan Human, allowed to detect several
422 pathways related to stress such as “regulation of response to stress”, energy balance, such as “response to
423 insulin”, “lipid modification” as well as “angiogenesis” and “negative regulation of blood circulation”. In
424 human model, miR-205-5p regulates the VEGFA-angiogenesis (Oltra et al., 2020), supporting a role in the
425 secondary stress response. In fish studies, miR-205-5p was also detected following stressful events like heat
426 stress or hypoxia (Lai et al., 2016; Liu et al., 2022).
427 Challenges reported in diagnostic of human cancer are linked to technical issues and individual-related
428 parameters that could influence the presence/absence of circulating miRNA (Tiberio et al., 2015). In this

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429 review, authors reported the various technical parameters (hemolysis, anticoagulant used, extraction method,
430 miRNA measurement, data normalization) that could explain the differences in studies outcomes. Here, we
431 also detected many limitations that are likely explained by differences in the quality of the samples, since for
432 those of high-quality, small RNA-seq was confirmed by qPCR, but not for samples of apparently lower
433 quality. Finally, a major difference between our study and the vast majority of the above-mentioned studies is
434 that most of the other authors worked on tissues to identify the modulation of miRNA content in response to
435 a stress or a physiological status. In fishes, miRNAs have been identified in several matrices, including non-
436 lethal ones such as plasma, mucus, water and feces. Here we focused on plasma, that directly reflect the
437 physiological state of an individual and this opens new perspectives of use and application to follow natural
438 and captive livestock. Following the stressful challenge, we identified several key miRNAs that are readily
439 released in blood (increasing in the 30 min after stress event) and return to their basal level in few hours.
440 However, the information gathered highlighted that this method is likely too precise to provide cues on the
441 sex, as it will rather indicates a physiological state linked to the gonadal development stage.

442

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450 **Conflict of interest**

451 The authors declare no conflict of interest.

452 **Data accessibility**

453 All data generated or analyzed during this study are included in this article and supplementary information.

454 **Author Contribution**

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457 Bidet, Mathilde Estevez-Villar, Xavier Mialhe; Writing original draft, Camille Houdelet, Benjamin Geffroy;
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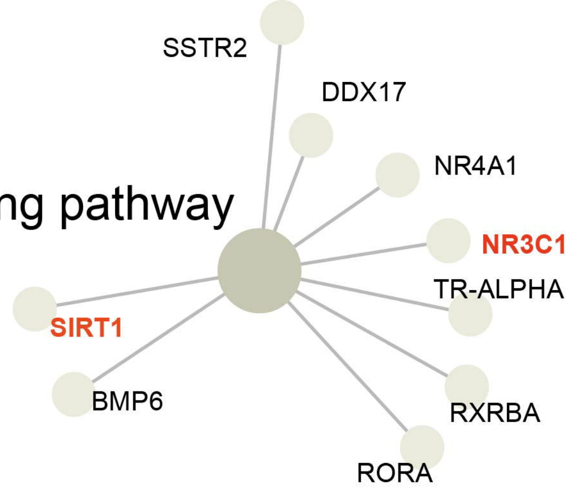
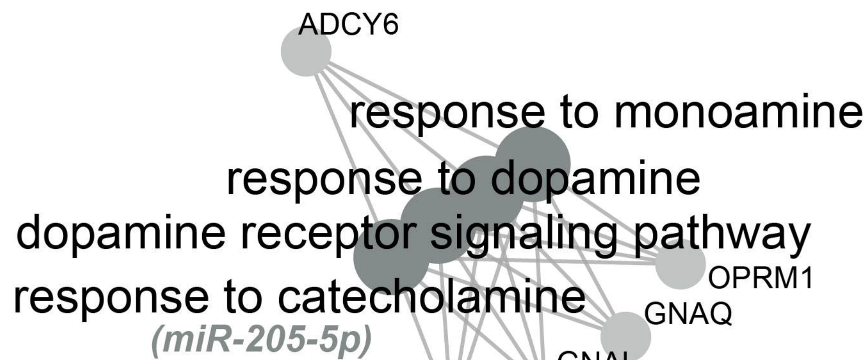
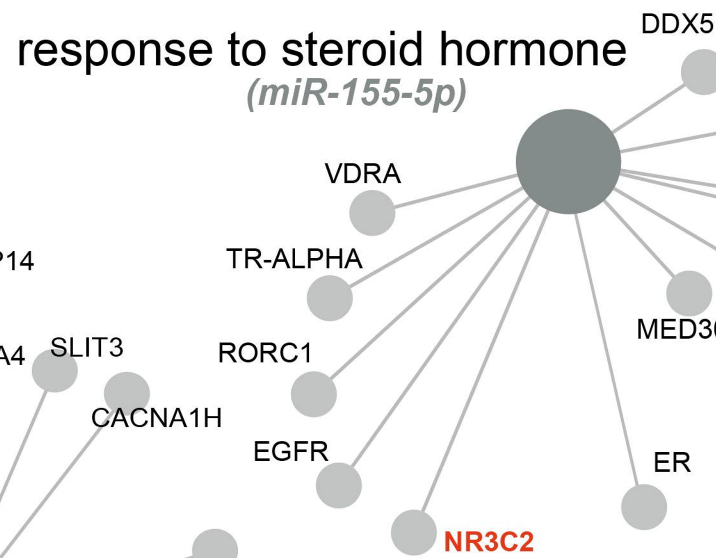
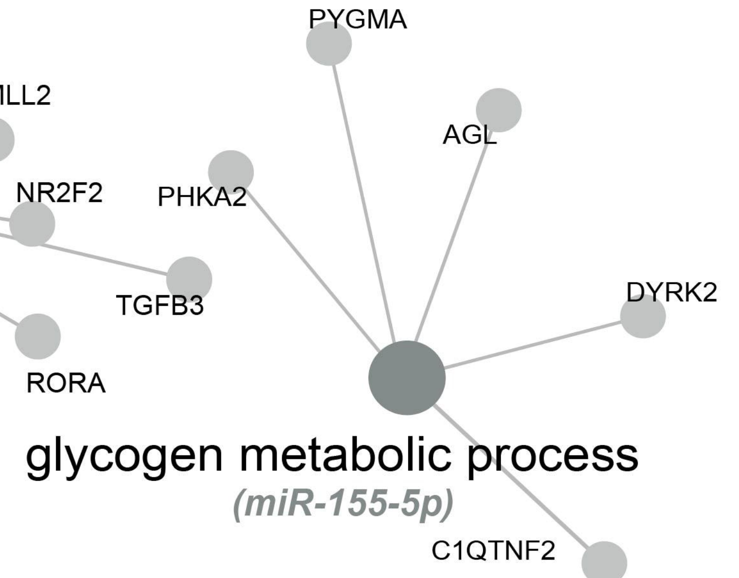
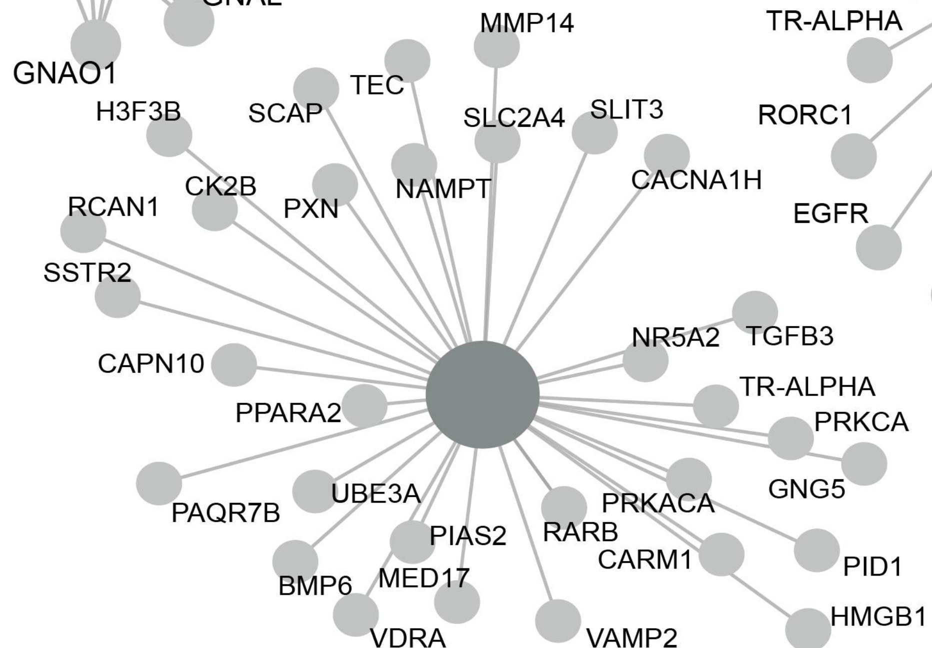
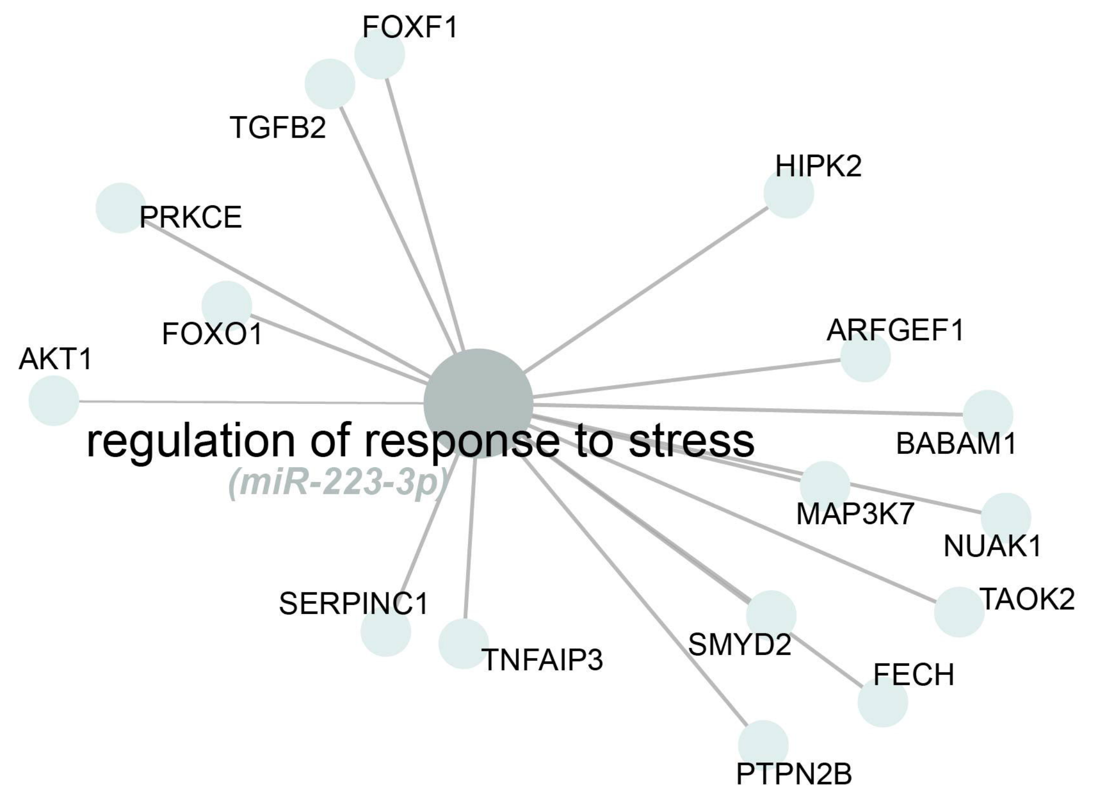

hormone-mediated signaling pathway
(miR-143-3p)
SSTR2
DDX17
NR4A1
NR3C1
TR-ALPHA
SIRT1
BMP6
RXRBA
RORA


ADCY6
response to monoamine
response to dopamine
dopamine receptor signaling pathway
response to catecholamine
(miR-205-5p)
OPRM1
GNAQ
GNAL
GNAO1


response to steroid hormone
(miR-155-5p)
DDX5
MLL2
NR2F2
TGFB3
VDRA
TR-ALPHA
RORC1
EGFR
NR3C2
ER
MED30
RORA


PYGMA
AGL
PHKA2
DYRK2
glycogen metabolic process
(miR-155-5p)
C1QTNF2


MMP14
TEC
SCAP
H3F3B
SLC2A4
SLIT3
NAMPT
CACNA1H
CK2B
PXN
RCAN1
SSTR2
CAPN10
PPARA2
NR5A2
TGFB3
TR-ALPHA
PRKCA
PAQR7B
UBE3A
PRKACA
GNG5
PIAS2
RARB
CARM1
PID1
BMP6
MED17
VDRA
VAMP2
HMGB1
response to hormone
(miR-200a-3p)


FOXF1
TGFB2
HIPK2
PRKCE
FOXO1
AKT1
ARFGEF1
BABAM1
regulation of response to stress
(miR-223-3p)
MAP3K7
NUAK1
TAOK2
SERPINC1
TNFAIP3
SMYD2
FECH
PTPN2B