Circulating small non-coding RNAs provide new insights into vitamin K nutrition and reproductive physiology in teleost fish.

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Abstract

Background: Vitamin K (VK) is a fat-soluble vitamin better known for its essential role in blood coagulation, but its involvement in a range of other biological processes is not yet fully understood.

Methods: Here we explore the potential role of VK in reproduction in a fish teleost model species, the Senegalese sole (*Solea senegalensis*), showing male reproductive dysfunction by means of biochemistry, transcriptomic and bioinformatics analysis. **Results:** Fish fed with dietary VK supplementation had increased testosterone plasma levels and lower sperm DNA fragmentation in males. Using a transcriptomic approach, small non-coding RNAs (sncRNAs) from blood plasma were sequenced and several differentially expressed sncRNAs (2 piRNAs and 7 miRNAs transcripts) were associated with nutritional status and/or better sperm quality. Bioinformatic analyses of predicted mRNAs targeted by sncRNAs revealed that they may be involved in blood coagulation and gonadotropin-releasing hormone pathways, among other processes. **Conclusions:** Present results suggest an unexpected and complex regulation of the nutritional status and reproductive performance at the level of the whole organism through circulating sncRNAs.

General significance:

This work provide evidence for the usefulness of circulating sncRNAs as reliable and less-invasive physiological biomarkers in fish nutrition and reproduction that might be applied to evaluate another physiological traits.

Keywords: fat soluble vitamins; phylloquinone; small non-coding RNAs; sperm quality; reproduction; Senegalese sole *Solea senegalensis*

1. Introduction

Fat-soluble vitamins have pleiotropic roles in vertebrate development and homeostasis throughout the entire life span. Although during the last decades a detailed knowledge on the metabolism and nutritional requirements of fat-soluble vitamins (vitamin A, D, E and K) has been documented in mammalian [1] and fish [2] species, the full range of biological processes and molecular pathways in which vitamins participate are still far from being fully understood.

Since the 1970's, vitamin K (VK) has been largely known for its role in blood coagulation as a co-factor for the γ-carboxylation of different clotting factors (reviewed in [3]). Other VK-dependent proteins involved in different biological processes (e.g. tissue mineralization and vascular tissue development and homeostasis) have been discovered since then [4], expanding the known VK-related biological processes. The transcriptional activation of several genes through VK binding to the pregnane X receptor (Pxr; also known as the steroid and xenobiotic receptor), mostly known as a master regulator of xenobiotic cholesterol and bile acid metabolisms [5], further expanded our notion of the broader physiological roles where VK might participate. Today, VK is known to be involved in skeletogenesis [6] and redox homeostasis [7,8]. Additional evidence has also suggested that VK is involved in sphingolipid [9] and glucose metabolism [10], brain development and cognitive capacities [9,11,12], pathological calcification and inflammation [13,14,15], angiogenesis [16], and reproduction [10,17,18]. With regards to the particular role of VK in reproduction, while the transactivation of PXR in Leydig cells increased the expression of several genes involved in steroidogenesis [19], rats fed VK deficient diets showed decreased expression of genes related with the biosynthesis of cholesterol and steroid hormones, as well as reduced concentration of testosterone in plasma and testis [20]. Similar results regarding the role of menaquinone-4 (MK-4, a particular VK metabolite) in testis and testosterone were also reported by [17]. Although its metabolism (reviewed in [21]), recycling system (reviewed in [22,23]) and functions in key tissues and organs

has been characterized, there is no single biomarker that is considered a gold-standard measure of VK status [24,25]. Thus, the identification of the different molecular pathways by which VK acts on these biological processes still needs to be determined [24,26] in order to identify suitable biomarkers for defining optimal VK dietary requirements at the organismal level.

Evidence of VK metabolism, function, and dietary requirement in fish species has been acquired in recent years [2,4,27]. Indeed, we and other authors have already characterized the physiological effects of VK nutritional status and its action in molecular pathways using fish species as vertebrate models [28-34]. However, although VK deficiency in parental fish does not affect the egg hatching rate or its survival [35], little else is known about the role of VK in fish gametogenesis and its underlying mechanisms. Senegalese sole (*Solea senegalensis*) is a marine flatfish species previously used as a model in ecotoxicology studies [36] and the origin of body asymmetry [37]. This species has a main spawning season from March and July, although a secondary spawning season can occur after summer. Nevertheless, males reared in captivity (F1) show a reproductive disorder [38-40], being unable to display the species-specific courtship behavior [41] and having low volume and quality of sperm [38].

Over the last two decades, evidence has been obtained describing the key role of non-coding RNAs (ncRNAs) in gene expression and translation regulation, as well as in transposon activity in a diverse set of biological functions in multicellular organisms [42-44]. Cells transport intracellular microRNAs (miRNAs) into the extracellular environment either encapsulated within extracellular vesicles and/or associated with lipoproteins or RNA-binding proteins such as those from the AGO family (reviewed in [45-47]). The expression patterns of ncRNAs in body fluids have been found to be highly correlated with disease states and other physiological conditions in humans [45,47]. In humans, miRNAs have been suggested to be relevant biomarkers in dietary and lifestyle intervention studies (reviewed in [48]). Furthermore,

several differentially expressed (DE) miRNAs found in blood plasma were recently associated with sex differentiation in the tongue sole (*Cynoglossus semilaevis*) [49].

The present study is aimed at (i) evaluating the effect of VK nutritional supplementation on fish reproduction by monitoring levels of sexual hormones in blood plasma; (ii) evaluating sperm production in volume and quality (viability and DNA fragmentation); and (iii) identifying circulating small ncRNAs (sncRNAs) in Senegalese sole males associated with a particular dietary VK supplementation and/or reproductive outcome. This is the first report linking circulating sncRNAs with vertebrate nutritional status and reproductive capacity, thereby providing new insights into the underlying molecular mechanisms at an organismal level. Furthermore, the present results provide evidence for the usefulness of circulating sncRNAs as reliable and less-invasive physiological biomarkers in fish.

2. Materials and methods

2.1. Ethical statement

All experiments complied with the ARRIVE guidelines [50] and were performed according to 2010/63/EU of the European Parliament and Council, guideline 86/609/EU of the European Union Council and Portuguese legislation (Decreto-Lei 113/2013) for animal experimentation and welfare. All the persons involved in the experiments have a FELASA class C permit for animal experimentation.

2.2. Vitamin K levels in experimental diets and its proximate composition

Two isolipidic and isonitrogenous semi-dry fish feeds were developed by Sparos Lda (http://www.sparos.pt/index.php/en/; Olhão, Portugal) with equal proximate composition of crude protein (62.9 %), crude fat (14.8%), crude fiber (1.1%), crude ash (10.2%) and total phosphorus content (1.5%). While the Control diet was not supplemented with phylloquinone (VK_1), an enriched VK_1 diet (VK+ diet) was supplemented with 1250 mg kg^{-1} of VK_1. Nominal values of the dietary VK content in

formulated feeds were confirmed through HPLC with fluorescence detection analysis (Silliker-Mérieux NutriSciences, France; https://www.merieuxnutrisciences.com/es/en; Vila Nova de Gaia, Portugal).

2.3. Broodstock management

Senegalese sole F1 breeders (863.64 ± 183.54 g wet weight) were obtained from Aquacria, Group Sea8 (Aveiro; Portugal), shipped to Ramalhete Experimental Station (Faro, Portugal), sexed, PIT-tagged, and acclimatized for 2 months prior to the start of the experiment. Breeders were stocked into 3 m^3 indoor flat-bottom round fiber glass tanks, each one containing 16 fish (10♀:6♂). Water exchange was kept at 0.5 m^3/h and constantly aerated (dissolved oxygen > 5 mg L^{-1}). Photoperiod was simulated with a clock system according to environmental conditions in the area (37°00′22″N, 7°58′03″W). Temperature and salinity varied according to external conditions (from 14.2 ± 3.1 to 20.5 ± 5.2 °C; and mean salinity of 36.3 ± 0.8). Broodstocks were fed *ad libitum* with experimental inert diets on a daily basis.

2.4. Fish sampling

After one month feeding on experimental diets, fish were continuously sampled from March to July (21 days between sampling points). Fish were mildly anaesthetized with 300 ppm 2-phenoxyethanol (Sigma-Aldrich). Samples of blood (up to 2 ml) were collected from each fish by puncturing the caudal vein with a single-use needle (20 G) and deposited in a 2 ml Eppendorf tube, both previously treated with cold buffered sodium citrate (3.8% in 0.01 M PBS). After 15 min at 4ºC, plasma was separated from other blood components through differential centrifugation (15 min at 3500 rcf) and collected. 100 µL of plasma were sampled to perform sex hormone quantification and stored at -20 °C until steroid extraction. The remaining plasma was re-centrifuged at 3000 rcf for 5 min (to avoid cell debris contamination) and 500 µL of supernatant

plasma has been collected, snap-frozen in liquid nitrogen, and stored at -80 ºC until RNA isolation and analysis.

2.5. Gonadal maturation assessment

Females were examined for gonad maturation assessment according to [51]. Apparent ovary size was scored from I to IV after visual examination of external abdominal swelling and abdominal palpation. At the extremes, a score of I was given to females with no apparent swelling and slightly detectable ovary, while those showing maximum abdominal swelling and very patent ovary running longitudinally along the entire length of the visceral cavity were scored IV. In males, specimens were considered to be spermiating when at least 5 µL of sperm was extracted. The spermiating condition was checked by gently pressing of testes on the fish blind side, after urogenital pore was dried. Sperm was collected with a syringe without a needle and samples were stored on ice until further analysis. Urine contaminated samples were discarded. The percentage of spermiating males was determined.

2.6. Steroid immunoassays

Levels of 11-Ketosterone (11-KT), testosterone (T) and estriol (E_3, 16α-hydroxy-17β-Estradiol) were assessed by the respective enzyme-linked immunosorbent assay kits from Cayman Chemicals (Lansing, Minnesota).

2.7. Sperm quality analysis

2.7.1. Concentration

The sperm volume collected was measured using micropipettes. Cell concentration was determined using the CASA system (ISAS Proiser, Spain) as described in [52]. Sperm concentration was assessed in a Makler chamber using a phase-contrast microscope (Nikon 200, Japan) with a 10 x negative contrast objective and a digital camera (Basler A312f C-mount, Germany) set for 50 fps. The settings for CASA

software were previously adapted for this species. Prior to analysis, sperm was diluted 10-fold in a non-activating medium (1% NaCl, ~300 mOsm Kg^{-1}) and 5 µL of diluted sperm was used for measuring the cell concentration.

2.7.2. Cell viability

Propidium Iodide (PI; Sigma-Aldrich) was added at 1 µg/ml final concentration to detect dead cells. Immediately after, samples were acquired in a flow cytometer (FACSCalibur, BD Biosciences, CA, USA) adjusted for blue excitation (488 nm) line for the detection of PI (670/30). Flow cytometer settings were previously adjusted using a positive control (100% dead cells). Data analysis was performed applying Weasel 3.1 free software. A total of 75,000 events were counted for each sample and the percentage of viable (PI non-stained) cells was recorded.

2.7.3. *Detection of DNA fragmentation*

An alkaline comet assay was performed according to the method described by [53] with slight modifications. Briefly, 10 µl of semen were diluted in a non-activating solution (Ringer) to obtain a final concentration of approximately 10^6 spermatozoa per ml. Cells were embedded in 0.5% agarose prepared in 0.1 M PBS and placed in agarose pre-coated slides. The slides were placed in a coplin jar containing lysis solution (2.5 M NaCl, 100 mM Na2-EDTA, 10 mM Tris, 1% Triton X-100, 1% Lauril sarcosine) at 4°C for 1 h. For electrophoresis, the slides were placed in an electrophoresis cube (sub-Cell GT, Bio-Rad, Portugal) filled with approximately 1.5 L of electrophoresis solution (0.3 M NaOH, 1 mM Na2-EDTA). Electrophoresis was conducted at 25 V and 300 mA at 4 °C for 10 min. After electrophoresis, the slides were drained and washed in neutralizing solution (0.4 M Tris) at 4°C for 5 min (this step was performed twice). The slides were drained, fixed in pure methanol and stored at 4°C until further observation. For visualization, PI (1mg mL^{-1}) was added to stain DNA and observe the comets. A total of 100 cells were photographed (Nikon DS-Ri1, Japan) and analyzed in the two slides

performed by sample. The percentage of tail DNA fragmentation was determined using the KOMET 6.0 software (Andor Technology, Ireland).

2.8. *Small non-coding RNAs analysis*

2.8.1. Isolation, libraries preparation, and sequencing

Small non-coding RNAs (sncRNAs) were isolated from blood plasma samples using the miRNeasy Serum/Plasma Kit (Qiagen, Germany) following the manufacturer's instructions, and assessment of RNA quality and quantity was performed in a 2200 TapeStation Nucleic Acid system using High Sensitivity RNA ScreenTapes (Agilent, USA). Library preparation of 11 samples were performed using NEBNext® Multiplex Small RNA Library Prep Set for Illumina (New England BioLabs Inc, USA) following manufacturer protocol, and libraries size, purity, and concentration were evaluated on a High Sensitivity D1000 ScreenTape. Normalized libraries were grouped in two pools, which were then sequenced (single-end, 76 cycles) on two MiSeq flow cells using the *v3* kit (Illumina, San Diego, CA). All sequencing data were submitted to the NCBI SRA database (accession number SRP135777).

2.8.2. Bioinformatic analysis

Raw reads were processed using the Small RNA-Seq analysis module in CLC Genomics Workbench (version 5.5.1, CLC bio, Aarhus, Denmark). Sequenced reads were first checked for quality control (QC), >85% of base being above Q30; and adapter (CTGCTGTACGGCCAAGGCC) trimmed. Small non-coding RNAs annotation was performed by comparison to the miRBase v21.0 (www.mirbase.org/), piRBase v1.0 (*regulatoryrna.org/*), and Rfam v12.0 (http://rfam.xfam.org/) databases. Only small non-coding RNAs with at least 5 counts and with maximum one mismatch were considered. sncRNAs counts were normalized by total counts per library.

2.8.3. Relative gene expression analysis

Transcript levels of some sncRNAs found to be differentially expressed (DE) by RNA-Seq in blood plasma among different experimental groups (males fed Control versus VK+ diets and males showing low versus high sperm DNA fragmentation) was confirmed by qPCR. 25 ng of sncRNAs previously isolated with miRNeasy Serum/Plasma Kit were polyadenylated and reverse-transcribed using NCode miRNA First-Strand cDNA Synthesis kit (Invitrogen) according to the manufacturer's instructions. PCR amplifications were achieved using sncRNA-specific primers (Supplementary Table 1) and NCode SYBR miRNA qRT-PCR kit (Invitrogen). Relative sncRNA expression was calculated using the ΔΔ*Ct* method following MIQE guidelines, including a calibrator sample within each plate [54]. Relative sncRNA expression was normalized using a housekeeping sncRNA. Three different housekeeping sncRNAs, *piR-41105, piR-33988,* and *miR*-99, were previously tested for their stability using Bestkeeper software [55]. The most stable among our experimental samples was *piR-41105* (0.88 vs 0.93 and 0.92, respectively) and therefore it was used for relative gene expression normalization in the qPCR studies.

2.8.4. mRNA target prediction

An assembled *Solea senegalensis* transcriptome (v.4.1; http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/) was used to predict mRNAs targeted by DE sncRNAs. To explore potential targeted mRNAs, the corresponding 5' and 3' UTR regions and the coding sequence (CDS), were considered. Potential mRNA binding sites for sncRNAs were identified using RNAhybrid [56]. An energy threshold of ≤−26 kcal mol^{-1} and a strict seed matching (no G:U allowed) in 2-8 nucleotides (nt) from the miRNA 5' end and in 16-24 nt from the piRNA 5' end was applied. Seeding regions (2-8 and 16-24 nt) lengths were considered based on previous studies on miRNAs [57,58] and piRNAs [59]. Gene ontology (GO), overrepresentation (Binomial test; $P < 0.05$), and pathway analysis of predicted

mRNAs was done using the Panther (http://www.pantherdb.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) platforms.

2.9. Statistical analysis

Results are given as mean and standard deviation. All data were checked for normality (Kolmogorov–Smirnov test) and homoscedasticity of variance (Bartlett's test). Data on sperm concentration, viability, DNA fragmentation and qPCR gene expression results from Control versus VK+ males were compared by means of Student's t-test. Data on 11-KT, T and E_3 levels in blood plasma were compared by means of Student's t-test within each sampling time or by one-way analysis of variance (ANOVA) in animals fed with Control or VK1+ diets during the experimental trials. In this particular case, when significant differences were detected, the Tukey multiple-comparison test was used to detect differences among experimental groups. Statistical analysis of differential expression on whole sequenced sncRNAs with CLC Genomics Workbench was performed with Baggerley's test - FDR *P*-value correction. In all statistical analyses, the level of significance was set at $P < 0.05$. All the statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software, Inc.), except those of the NGS data, which were performed with the CLC Genomics Workbench.

3. Results and Discussion

The VK content of Control diet was found to be 0.4 ± 0.01 mg VK_1 kg^{-1}, while the VK+ diet contained 1085.15 ± 5.73 mg VK_1 kg^{-1}. Differences between nominal and real dietary contents have been already found (reviewed in [27]). In contrast to the reported low correlation between nominal and real dietary VK content when menadione (synthetic form of VK and the most extensively used source of VK in commercial diets) was used [60,61] (Roy and Lall, 2007; Graff et al., 2010), probably due to its high instability; present and previous work [30] has shown a higher correlation of dietary VK contents when using VK_1 as its main source.

3.1. *The effects of dietary VK supplementation on gonadal maturation and hormonal profile are time- and gender-dependent*

During the experimental period, coincident with this species reproduction season, no natural spawning was observed regardless of the dietary VK content. Previous studies have shown that one of the limitations of rearing Senegalese sole in captivity is the lack of spawning of viable fertilized eggs from F1 breeders [39,62,63]. Nevertheless, changes in both the maturation of the female's gonads and the percentage of spermiating males were observed during the present trial (Fig. 1). Regardless the dietary VK supplementation, female Senegalese sole showed a decreasing gonad maturation score from April onwards (from 3.16 ± 0.5 to 0.9 ± 1.02) in agreement with previous studies [64]. Nevertheless, this decrease was statistically significant one month earlier in females fed with the VK+ diet than in those fed on Control diet (Fig. 1a). In the males (Fig. 1b), no spermiating fish were found in March from both Control and VK+ supplemented groups. A higher percentage of spermiating males fed with VK+ were found in April and at the end of May when compared those fed with Control diets (66.67 vs 33.33 % and 66.67 vs 16.67 %, respectively). Similar results on the percentage of spermiating males (60-69%) were previously reported [38,65].

Hormonal plasma levels were found to be significantly affected by nutritional VK supplementation (Fig. 2). The profile of testosterone (T) levels in females was different when fed on Control versus VK+ diets (Fig. 2a). Control females showed a decreasing testosterone level during the experimental trial (from 1.32 ± 0.25 to 0.71 ± 0.19 ng mL^{-1}), being significantly reduced in July. Comparable results of T in blood plasma, with highest values in spring and lowest at the end of the summer were reported [40,64,66]. In contrast, females fed on the VK+ diet exhibited an increasing level of plasma T towards the end of the trial (from 1.1.9 ± 0.16 to 2.15 ± 0.32 ng mL^{-1}), being significantly different to the T levels of the female fish fed with the Control diet.

In males, plasma T levels during the experimental trial (Fig. 2b) were similar in value and profile to previous studies [40,67], decreasing after March and April (between 3.02 ± 0.38 and 3.55 ± 1.58 ng mL^{-1}), when the highest values were detected. Differences in T levels were detected between dietary treatments only in April, where VK+ males showed higher T levels than those fed with the Control diet (3.23 ± 0.43 and 1.83 ± 0.44 ng mL^{-1}, respectively). Our results confirm the evolutionary conservation of the previously reported stimulatory effect of dietary VK supplementation on T plasma levels in other vertebrate species [17,20].

To further understand the effects of dietary VK supplementation on hormonal regulation, estriol (E_3) and 11-ketosterone (11-KT) levels were also evaluated at specific sampling times (Fig. 3), when major differences in T levels between dietary treatments were found in females (June and July) and males (March-May). Firstly, E_3 levels decreased between June and July, being significantly lower in Senegalese sole females fed with VK+ diet when compared to those from the Control females in July (8.7 ± 1.39 and 35.41 ± 15.02 ng μL^{-1}, respectively; Fig. 3a). Estriol is a product of estradiol and estrone metabolism [68]. Although it has a less potent estrogen receptor [69], present results indicate an altered metabolism of estrogens that might have implications on the reproductive system of females. Secondly, plasma levels of 11-KT (Fig. 3b), an important hormonal mediator of fish spermatogenesis [70,71], remained constant from March to May in males from the Control group (ranging from 3.08 ± 1.11 to 7.33 ± 5.07 ng mL^{-1}), but were significantly increased (21.96 ± 8.12 ng mL^{-1}) during March in fish fed on the VK+ diet. These seasonal profiles were in agreement with previous characterizations in Senegalese sole male's plasma [38,40,67]. A positive correlation between T and 11-KT plasma levels were also observed during pre-spawning stages in males of other fish species [72]. Courtship display in males like the zig-zagging courtship behavior in male three-spined stickleback fish (*Gasterosteus aculeatus*) are typically mediated by androgens, and particularly by 11-KT (reviewed in [73]). Thus, dietary VK supplementation might represent an additional nutritional

approach contributing to overcome the reported lack of courtship in Senegalese sole F1 males [39].

3.2. *Sperm quality of F1 Senegalese sole males is improved by dietary VK supplementation*

To determine if VK supplementation affects the quantity and quality of Senegalese sole sperm, another constrain in the Senegalese sole F1 male's reproduction [38], the percentage of fluent males (%), sperm concentration (M ml^{-1}), viability (%) and tail DNA fragmentation (%) were compared between males after a six months feeding (July) with the Control and VK+ diets (Table 1). Dietary VK supplementation increased values of sperm concentration (502 ± 210 vs 684 ± 457 M ml^{-1}), and quality in terms of viability (89.6 ± 7.2 vs 91.2 ± 6.6 %), but the differences were not statistically significant. A wide variability in sperm concentration has been demonstrated in Senegalese sole F1 males during the year, with spermiating and non-spermiating males occurring each month [38,65]. Since percentages of 30-60% [65,74] or 60-80% [38] of sperm cell viability were previously registered in this flatfish species, present results (between 89-92%) might reflect the substantial improvement of the rearing conditions during recent years [39]. Nevertheless, and importantly, the VK+ diet significantly reduced sperm DNA fragmentation (46.6 ± 5.4 % in the Control group vs 34.7 ± 8.7 % in the VK+ group). Our recorded values fall within the normal range of DNA fragmentation in Senegalese sole sperm during the year [38,65]. DNA integrity has been shown to strongly correlate with fertilization and early-embryo developmental success (reviewed in [75]). Oxidative stress is potentially one of the main mechanisms responsible for sperm DNA strand fragmentation [76]. VK, in addition to acting as a co-factor for the γ-carboxylation of the VK-dependent proteins and a ligand for PXR, is known for its antioxidant role against reactive oxygen species (ROS) through various mechanisms [77]. Increased levels of dietary VK increased the antioxidant capacity of juvenile Jian carp (*Cyprinus carpio* var. Jian), and increased the activities of several enzymes (e.g. superoxide dismutase,

glutathione-S-transferase, glutathione peroxidase and glutathione reductase) involved in redox status maintenance [78]. Several enzymes involved in the VK metabolism are also involved in antioxidant activity, including UbiA prenyltransferase domain-containing protein 1 (Uiad1; [29]) and VK epoxide reductase (VKOR) complex subunit 1 (Vkorc1) and Vkorc1-like protein 1 (Vkorc1l1; [23]). Expression of *Vkorc1l1* is known to promote the elimination of intracellular ROS and prevent oxidative damage in the presence of VK [7], and it has been found in the testis of several mammalian species [79] and the Senegalese sole (Fernández et al., unpublished results). Recently, hydrogen peroxide (H_2O_2), a potential inductor of DNA damage, has been shown to be widespread in Senegalese sole spermatozoa, and particularly in the nucleus [80]. Nevertheless, whether the specific mechanism by which dietary VK supplementation decreased sperm DNA fragmentation involves Vkorc1l1, and more generally how dietary VK increases the plasma level of steroid sex hormones (T and 11-KT), remains to be uncovered.

The present results remark the wide biological roles of VK and their evolutionarily conservation. The identification and development of biomarkers has been the main objective for determining the optimal VK dietary requirements under normal and pathological conditions (extensively reviewed in [18,25,26]). Typically, biomarkers used to assess VK nutritional status include i) prothrombin time and partial thromboplastin time tests, ii) plasma concentration and activity of blood coagulation factors, and iii) circulating or urinary concentration of the under-carboxylated form of VK-dependent proteins and/or VK metabolites (reviewed in [25,26]). However, and in contrast to other nutrients, there is no single biomarker that is considered a gold-standard measure of VK status in an integrative manner [25,26]. SncRNAs in circulation might be new, reliable and integrative biomarkers of VK status.

3.3. Small non-coding RNAs from male Senegalese sole blood plasma

Through RNA-Seq analysis, a wide set of sncRNAs were identified in the blood plasma of male Senegalese sole (Table 2; Fig. 4), including miRNAs (9.18 %), P-element–induced wimpy testis (PIWI) interacting RNAs (piRNAs; 45.59 %), transfer RNAs (tRNAs; 13.49 %), and ribosomal RNAs (rRNAs; 1.05 %). The expression of miRNAs in a large set of tissues has been involved in different biological process in fish species (reviewed in [81]). However, only one recent study has previously reported the presence of different sncRNAs in the fish blood plasma [49], where miRNAs were the dominating fraction of sncRNAs in exosomes. Here, piRNAs were found as the most commonly sncRNAs represented in blood plasma when they are either encapsulated into extracellular vesicles or associated to RNA-binding proteins. Furthermore, present results increase (in 150 new miRNAs) the number of miRNAs already described in Senegalese sole [82], and the number of miRNAs found in the blood plasma of flatfish species [49] (see Supplementary Table 2).

3.3.1 Circulating piRNAs are associated with dietary VK supplementation in male Senegalese sole

A comparative analysis of the sncRNAs found in blood plasma from male Senegalese sole when fed with Control versus VK+ diet, identified 2 DE piRNAs associated to dietary VK supplementation (Table 3). While piR-675//676//4794//5462 (piR-675_var) expression in males fed with VK+ diet was ~2 fold higher than its expression in the Control diet fed fish; piR-74614 was barely expressed (4.35 ± 2.35 reads per total reads) in males fed with VK+ diet and was not found in males fed with the Control diet. Differences in expression values of these piRs were confirmed by qPCR, being similarly regulated as f by NGS analysis (Fig. 5). This is the first report associating the gene expression level of a piRNA in blood plasma with a nutritional supplementation.

Anti-Miwi crosslinking immunoprecipitation coupled with high-throughput sequencing data (CLIP-seq) suggested different pairing signatures to piRNAs induce the cleavage of the targeted mRNA [83,84]. A different base-pairing signature in

piRNAs loaded on Aubergine, acting as an adhesive trap to capture mRNAs in the germ plasm, was described by [59]. In any case, although locations of piRNA-mRNA pairing were enriched in UTR regions, interactions were also found in the CDS [59,83,84]. Here, transcripts encoding 45 known proteins were predicted to be targeted by piR-675_var and 92 by piR-74614 using RNAhybrid platform and considering the UTR and CDS regions from the Senegalese sole transcriptome [85], a minimum energy threshold of -30 kcal mol^{-1} and a strict seed matching (no G:U allowed) in 16-24 nt from the piRNA 5' end. A GO molecular function analysis of the predicted mRNA targets showed that binding and catalytic activities (40 and 32.9 %, respectively; Fig. 6a) might be regulated. The main biological processes associated with these predicted targets are cellular and metabolic processes, biological regulation and response to stimulus (28.8, 19.9, 11.6 and 11.6 %, respectively; Fig. 6b). Among the 37 pathways represented, 2-arachidonoylglicerol biosynthesis, p38 MAPK, interleukin signaling, angiogenesis, and Wnt signaling were particularly overrepresented (Fig. 6c). Different genes involved in phospholipid (*inpp5jb, xpr1a*, and *plcb3*), sphingolipid (*lpin3*), and glicerophospholipid (*cept1b* and *lpin3*) metabolism and/or signaling were predicted to be targeted by both piR-74614 and piR-675_var. Lipins (Lpin1, 2 and 3) are known to regulate fatty acid metabolism, particularly the conversion of phosphatidic acid to diacylglycerol (DAG), critical for homeostasis of cellular lipid stores and membranes (reviewed in [86]). Cept1 has been found to act as a dual-specificity enzyme, catalyzing the *de novo* synthesis of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from DAG, which are the two major membrane phospholipids found in eukaryotic cells [87]. The hypothetical targeting of *cept1b* and *lpin3* by the two DE piRNAs might have important implications for lipid metabolism since both act sequentially on *de novo* synthesis of PC and PE (Supplementary figure 1). Genes targeted by piR-74614 were found to be related to development and signaling of the central nervous system (*slc6a16a*, *six6b*, *timp2a*, *slc6a2*, *atxn1a*, *pcdh2ab8*, *elavl4*, *trim46a* and *Lrfn2b*), and blood coagulation (*gata2a* and *f2rl2*). The role of VK in blood coagulation has been

largely reported in different studies [3]. More recently, several articles have suggested that VK has an impact on angiogenesis [16,28], brain development and cognitive capacities [9,34], and regulating lipid metabolism, particularly that of sphingolipids [9]. As phospholipid and cholesterol composition determines the fluidity of the plasma membrane, their modulation has a direct impact on sperm physiology and functionality [75]. Indeed, improved sperm quality in zebrafish has been reported for diets enriched in PE content [88].

3.3.2 Different miRNAs and piRNAs from blood plasma are associated with Senegalese sole sperm quality

A lower sperm DNA fragmentation in Senegalese sole was associated with an enriched dietary VK content. A comparative analysis focusing on the spermiating males revealed an additional 7 miRNAs and one piRNA to be DE between males with low and high sperm DNA fragmentation (Table 4). While miR-146a-1//164a-2//146a-3 and miR-146a were found down-regulated by almost 2-fold, let-7g, let-7e(18nt), let-7a1, let-7a-3//7a-2//7a-1, let-7e(23nt) and piR-675_var were found to be up-regulated (1.83, 1.5, 1.44, 2.16, 2.02 and 2.96 fold, respectively) in fish showing low sperm DNA fragmentation when compared to those with high sperm DNA fragmentation, and some confirmed by qPCR analysis (Fig. 7). Previous works found these sncRNAs to be involved in processes where VK plays an important role. Let-7a, let-7e and miR-146a have been previously associated with testis maturation in vertebrate organisms [89], including fish [90,91,92], consistent with the role of VK in reproduction through the synthesis of T [17,20, present work]. *In vitro* miR-146a “knock-down” improved osteogenesis [93], while dietary VK supplementation has been shown to improve skeletal development in Senegalese sole [30]. An inflammatory response suppression has been found to be mediated by miR-146a [94] and higher expression of miR-146a was associated with atherosclerotic arteries [95]. Also, let-7g has been reported to prevent atherosclerosis [96], while let-7e is known as an important regulator of endothelial function and

inflammation, and might be a key molecule on the development of atherosclerosis [97]. Although some controversy remains, several studies have suggested that VK can suppress the production of different inflammatory cytokines, known as one underlying mechanisms of atherosclerosis [98]. Present results expand the numbers and types of circulating sncRNAs (miRNAs plus piRNAs) associated with sperm quality, suggesting that its quantification from blood plasma might represent a new, reliable and less-invasive procedure to evaluate fish physiological condition, as previously suggested for miRNAs in human nutrigenomics studies (reviewed in [48]).

Similar to piRNAs, miRNA-mRNA interaction has been demonstrated at CDS and UTR regions, but in contrast to piRNAs, a well-known base-pairing signature for miRNAs:mRNAs interaction has been reported [57,58]. While the length of the seed region alone does not influence mRNA translational repression [57], the minimum free energy (MFE) has been found a more accurate predictor of positive miRNAs:mRNAs interaction [99]. Here, RNAhybrid algorithm with a MFE threshold of ≤−26 kcal mol^{-1} was applied to reduce the rate of false positives of predicted mRNA targets by DE miRNAs. While 2342 protein-coding genes were predicted to be targeted by the up-regulated sncRNAs (let-7g, let-7e(18nt), let-7a1, let-7a-3//7a-2//7a-1, let-7e(23nt) and piR-675_var) when comparing blood plasma from males showing low and high sperm DNA fragmentation, 157 were found to be targeted by down-regulated sncRNAs (miR-146a and miR-146a_var), with 32 being targeted by both up- and down-regulated sncRNAs (Fig. 8a). Taking into account that other widely used algorithms have predicted more than 3,000 targeted transcripts for a single miRNA [95], the numbers of targeted transcripts here predicted seem to be more conservative and therefore, likely to reduce the presence of false positives.

A GO molecular function analysis of predicted targets showed that most target genes are involved in binding and catalytic activity categories (362 and 336 genes representing 37.3 and 34.6 % of functionally annotated, respectively). Only two predicted gene targets, namely *glutathione reductase* and mitochondrial *thioredoxin*

reductase 2, were classified within the antioxidant activity category (Fig. 8b). Nevertheless, both genes are known to form part of the machinery that maintains cellular redox homoeostasis [100], with glutathione reductase being one of the main enzymes responsible for the detoxification of ROS in fish sperm [101]. Although cellular and metabolic processes (with 611 and 438 gene targets, respectively) were the most abundantly represented biological processes, 13 gene targets were found to be associated with reproduction (Fig. 8c), being particularly involved in cell cycle (*host cell factor C1a*, *non-SMC condensin II complex subunit D3*, *gamma-tubulin complex component 2* and *4*, and *DNA mismatch repair protein Msh2*), testis development (*doublesex and mab-3 related transcription factor 2a*), and/or hormonal synthesis/regulation (*STARD3 N-terminal-like* and *LIM domain only 4 like*). The cell-cycle *Msh2* gene is involved in DNA mismatch repair and implicated in male infertility [102] and finding it to be targeted by a DE sncRNA in this study is consistent with our results showing differences in sperm DNA fragmentation in male Senegalese sole fed with different dietary VK content. *LIM domain only 4 like*, which regulates glycoprotein hormone α-subunit (αGsu) synthesis, is a common subunit for the heterodimeric hormones Lh, Fsh and Tsh [103] and the synthesis of αGsu, Lh and Fsh has been found to be one of the main overrepresented pathways by predicted mRNA targets when comparing males with high or low sperm DNA fragmentation (see below).

Several pathways were found overrepresented when the predicted mRNA targets of the DE sncRNAs were categorized (Fig. 8d). Among them, blood coagulation was again found to be overrepresented (Supplementary figure 2). However, Wnt signaling, Gonadotropin-releasing hormone (GnRH) receptor, Inflammation mediated by chemokine and cytokine signaling, Integrin signalling and Huntington disease pathways were the most abundantly represented, with more than 20 targeted genes each. Wnt signaling has also been found enriched in testis during the transition of salmon into puberty [92], being some of Wnt signaling-related genes reported to be significantly regulated during gametogenesis in rainbow trout and Swiss mice

[104,105]. Among them, *wnt2*, *fzd3*, *lrp5* and *ctbp1* were predicted to be targeted by DE sncRNAs in the present study. With regards to GnRH receptor signaling, among the 28 predicted gene targets of DE sncRNAs, 14 were specifically associated with the signaling process between cell membrane receptors and gonadotropins expression and secretion (Supplementary figure 3). It is thought that GnRH can act centrally to mediate sexual behavior, as well as increasing gonadal steroids via the hypothalamic–pituitary–gonadal axis to elicit courtship behavior [73]. Present results identifying GnRH receptor signaling as a target of the sncRNAs DE in blood plasma are consistent with i) the effectiveness of hormonal manipulations with Fsh, Lh and GnRH to improve sperm production and quality in fish species reared/maintained in captivity [106], and Senegalese sole in particular [38], ii) with the recently suggested altered secretion of Lh as a potential cause of the low reproductive performance of F1 Senegalese sole males [40], and iii) the lack of courtship behavior in these males [41]. Therefore, the present association of particular DE sncRNAs in blood circulation found in male Senegalese sole fed on different VK dietary contents and different degrees of sperm DNA fragmentation, unexpectedly suggests an additional and complex regulatory mechanism of the reproductive performance of the whole organism in addition to the widely known hormonal signaling mechanisms (reviewed in [107]).

The discovery of extracellular secretion of miRNAs suggested the existence of an intercellular and inter-organ communication system in the body [46]. While circulating miRNAs that are bound solely to proteins are apparently non-specific remnants of a physiological activity on specific cells and cell death, there are increasing evidence that cells selectively encapsulate miRNAs into extracellular vesicles to be secreted outside the cell [46,47]. Whether the ncRNA content of extracellular vesicles can be taken up by recipient cells via membrane receptors, vesicle fusion, endocytosis, or remain attached to the plasma membrane activating specific signaling pathways, remains to be characterized [108]. Although the biological consequence of released ncRNAs on distant sites is still unknown, the present work has not only expanded the

association of circulating miRNAs on sex determination [49] to sperm quality in fish species, but also revealed new sncRNAs (in particular piRNAs) associated with VK nutritional status and sperm quality.

4. Conclusions

Dietary VK supplementation in a marine fish species with a reproductive disorder has been shown to regulate the plasma levels of different steroid hormones (T, 11-KT and estriol), and, particularly, to improve sperm quality by reducing DNA fragmentation. While sequencing of circulating sncRNAs identified several transcripts directly associated with VK nutritional status and sperm quality, a bioinformatic analysis of predicted mRNA targets revealed an additional and complex mode of regulation of the main underpinning molecular pathways (e.g. Wnt and GnRH signaling) of fish gametogenesis by VK at organismal level. Although the biological consequences of released ncRNAs on distant sites remains to be elucidated, the present results suggest that circulating sncRNAs are potential biomarkers of nutritional and reproductive conditions. Therefore, its quantification might be an innovative, less-invasive and more ethical procedure to monitor fish physiology.

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Authorship

All authors have made substantial contributions regarding the conception and design of the study (IF, EC and PG), acquisition of data and analysis (IF, JF, VR, MK, CO, MR, CC, EC and PG), interpretation of data (IF, JF, EC and PG), drafting the article (IF, JF, EC and PG) and final approval of the version submitted (IF, JF, VR, MK, CO, MR, JD, CC, MLC, EC and PG.)

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Figure captures:

Figure 1. Gonad maturation score (mean ± standard deviation) in females (a) and percentage of spermiating males (b) of Senegalese sole fed with Control (grey bars) and VK supplemented (black bars) diets throughout experimental trial. Fish were fed diets containing 0 (Control diet) or 1250 mg phyloquinone (VK+ diet) Kg^{-1} during 6 months. *Different letters* (*a* or *b*, for fish fed with Control diet; and *x* or *y* for fish fed with VK+ diet) denote significant differences among sampling points along experimental trial within each experimental group (One way ANOVA). Significance $P < 0.05$; n=10 for females and n=6 for males. *May-A*, start of May; *May-B*, end of May.

Figure 2. Testosterone plasma levels (mean ± standard deviation) in ng mL^{-1} from females (a) and males (b) of Senegalese sole fed with Control (grey bars) and VK supplemented (black bars) diets throughout experimental trial. Fish were fed diets containing 0 (Control diet) or 1250 mg phyloquinone (VK+ diet) Kg^{-1} during 6 months. *Asterisk* denotes significant differences between experimental groups at each specific sampling point (Student's t-test); while *different letters* (*a,b* and/or *c* for fish fed with Control diet; and *x* or *y* for fish fed with VK+ diet) indicate when significant differences were found among sampling points along the experimental trial within each

experimental group (One way ANOVA). Significance $P < 0.05$; n=10 for females and n=6 for males. *May-A*, start of May; *May-B*, end of May.

Figure 3. Estriol (a) and 11-ketosterone (b) levels (mean ± standard deviation) in plasma (ng μL^{-1}) from females and males of Senegalese sole, respectively. Control (grey bars) and VK supplemented (black bars) fish were fed diets containing 0 (Control diet) or 1250 mg phyloquinone (VK+ diet) Kg^{-1} during 6 months. *Asterisk* denotes significant differences between experimental groups at each specific sampling point (Student's t-test). *Different letters* (*x* or *y*) denote significant differences between sampling points along experimental trial within each experimental group (Student's t-test in females and One way ANOVA in males). Significance $P < 0.05$; n=10 for females and n=6 for males.

Figure 4. Percentage of transcripts corresponding to unannotated or the main different annotated categories of small non-coding RNAs (sncRNAs): piRNAs, tRNAs, miRNAs, rRNAs, snRNAs and snoRNAs; found in Senegalese sole male's blood plasma.

Figure 5. Relative gene expression levels (mean ± standard deviation) of identified differentially expressed small non-coding RNAs (sncRNAs) in blood plasma from Senegalese sole males fed with vitamin K supplemented diet (VK+ diet) over fish fed with Control diet (a and b). Differentially expressed sncRNAs regarding dietary VK content: piR-675//676//4794//5462 (piR-675_var; a) and piR-74614 (piR-74614; b). *Asterisk* denotes significant differences between experimental groups (Student's t-test). Significance $P < 0.05$; n=5 in males fed with Control diet and n=6 in males fed with VK+ diet. Gene expression of piR-41105 was used for normalizing gene expression values.

Figure 6. Gene Ontology (GO) analysis of predicted mRNAs targeted by sncRNAs differentially expressed in blood plasma from Senegalese sole males fed with vitamin K

supplemented diet (VK+ diet) over fish fed with Control diet. Pie chart of GO molecular function (a) and GO biological process (b), and list of overrepresented GO pathways of the predicted mRNAs targeted by sncRNAs (c). GO and overrepresentation pathway analyses of predicted mRNAs were done with Panther (http://www.pantherdb.org/).

Figure 7. Relative gene expression levels (mean ± standard deviation) of identified differentially expressed small non-coding RNAs (sncRNAs) in blood plasma from Senegalese sole males' showing low (fed with VK+ diet) or high (fed with Control diet) sperm DNA fragmentation (a, b and c). Differentially expressed sncRNAs regarding sperm DNA fragmentation: piR-675//676//4794//5462 (piR-675_var; a), miR-146a (b) and Let-7g (c). *Asterisk* denotes significant differences between experimental groups (Student's t-test). Significance $P < 0.05$; n=4 or n=3 in males with high (fed with Control diet) or low DNA fragmentation (fed with VK+ diet), respectively. Gene expression of piR-41105 was used for normalizing gene expression values.

Figure 8. Venn diagram, Gene Ontology (GO) and overrepresentation of predicted mRNAs targeted by differentially expressed (DE) sncRNAs from blood plasma of Senegalese sole males showing low and high sperm DNA fragmentation. Venn diagram of predicted mRNAs targeted by down- and up-regulated sncRNAs with the number of targeted mRNAs in parenthesis (a). List of GO molecular functions represented by predicted mRNAs targeted by DE sncRNAs with the number and the percentage of genes included in each category (b). Pie chart of GO biological process of predicted mRNAs targeted by DE sncRNAs (c). List of overrepresented GO pathways of predicted mRNAs targeted by DE sncRNAs showing the number of genes, the fold enrichment and the P value (d). Note that the mRNAs have been predicted to be targeted by sncRNAs down- or up-regulated in blood plasma from fish showing low (fed with VK+ diet) or high (fed Control diet) sperm DNA fragmentation. GO and

overrepresentation pathway analyses of predicted mRNAs were done with Panther (http://www.pantherdb.org/).

Supplementary figure 1. Glycerophospholipid metabolism KEGG pathway. Red boxes indicate predicted targeted genes by DE piRNAs (piR-675_var and piR-74614) found up-regulated in blood plasma from Senegalese sole fish fed VK+ versus Control diet. E.C. 3.1.3.4: *Phosphatidate phosphatase Lpin3*; E.C. 2.7.8.1 and E.C. 2.7.8.2: *Choline/ethanolamine phosphotransferase 1* (*Cept1*). Modified from Kanehisa laboratories 2017.

Supplementary figure 2. Blood coagulation KEGG pathway. Red boxes indicate predicted targeted genes by DE sncRNAs found in blood plasma from fish showing different sperm DNA fragmentation (fish with low DNA fragmentation (fed with VK+ diet) with respect to fish with high DNA fragmentation (fed with Control diet)). Modified from Kanehisa laboratories 2017.

Supplementary figure 3. GnRH signaling KEGG pathway. Red boxes indicate predicted targeted genes by DE sncRNAs found in blood plasma from fish showing different sperm DNA fragmentation (fish with low DNA frag. (fed with VK+) with respect to fish with high DNA frag. (fed with Control diet)). Modified from Kanehisa laboratories 2015.

Table 1. Comparison of reproductive performance and quality of sperm production in Senegalese sole males after six months feeding (July) with Control or vitamin K supplemented diets.

Variable:	Control	VK+
Mean sperm concentration (M mL^{-1})[a]	502 ± 210	684 ± 457
Viability (%)	89.6 ± 7.2 %	91.2 ± 6.6 %
Tail DNA fragmentation (%)	46.6 ± 5.4 %	34.7 ± 8.7 %*

[a] M, Millions; * Significantly different Student's t-test, $P < 0.05$. n= 6 males from each dietary treatment

Table 2. Summary of the sequenced small non-coding RNAs in circulation in Senegalese sole males fed with Control or vitamin K supplemented (VK+) diets. Their reproductive performance regarding its quality (DNA fragmentation (comet assay tail)) is also indicated.

Feed	Fish code	Total raw reads:	After trimming		miRNAs		piRNAs		Rfam		DNA** %
			# reads	%	# reads	%	# reads	%	# reads	%	
Control	A4-C1	2251523	1925861	85.5	282260	14.6	736326	38.2	290792	15.1	48.7
	A4-C2	3241198	2851602	87.9	63877	2.2	1355469	47.5	485711	17.0	44.2
	A4-C3	2373932	2055535	86.5	157148	7.6	927912	45.1	328547	15.9	43.2
	A4-C4	3430247	2961920	86.3	470582	15.8	1125129	37.9	413438	13.9	-
	A4-C4	3226574	2769521	85.8	383746	13.8	1192258	43.0	339097	12.2	54.9
VK+	A3-VK+1	2501824	2226287	88.9	40721	1.8	1377331	61.8	352748	15.8	32.8
	A3-VK+2	3808392	3355598	88.1	202663	6.0	1676143	49.9	463159	13.8	-
	A3-VK+3	3367948	2944000	87.4	205245	6.9	1553338	52.7	366508	12.4	-
	A3-VK+4	2594449	2259005	87.1	125741	5.5	995753	44.1	411787	18.2	-
	A3-VK+5	4252594	3764525	88.5	257937	6.8	1798461	47.7	533119	14.1	43.7
	A3-VK+6	3606143	3060114	84.8	559062	18.2	1235078	40.3	400580	13.1	26.5

M*, Millions per ml; ** DNA tail fragmentation.

Table 3. Name, sequence, length, resource, match type, mismatches, mean and standard deviation and FDR Q-value of differentially expressed small non-coding RNAs from blood plasma of Senegalese sole males fed with vitamin K supplemented (VK+; n = 6) versus Control diets (n = 5).

Name	Sequence	nt	Resource	Match type	Mismatches	Control		VK+		Q-value
						Mean*	SD	Mean*	SD	
piR-675//676//4794//5462	GCAUUGGUGGUUCAGUGGUAGAAUUCUCGG	30	*Danio rerio*	Variant	1	239.49	49.91	423.14	83.14	0.007
piR-74614	GCAUUGGUGGUUCAGUGGUAGAGUUC	26	*Danio rerio*	Exact	0	0	0	4.35	2.35	0.007

*Normalized values by totals.

Table 4. Name, sequence, length, resource, match type, mismatches, mean and standard deviation and FDR Q-value of differentially expressed small non-coding RNAs from blood plasma of Senegalese sole males with sperm showing low (n = 3) versus high DNA fragmentation (n = 4).

Name	Sequence	nt	Resource	Match type	Mismatches	Low DNA (VK+) fragmentation		High DNA fragmentation		Q-value
						Mean*	SD	Mean*	SD	
let-7g	UGAGGUAGUAGUUUGUAU	18	*Oryzias latipes*	Mature 5' sub	0	959.4	78.06	525.59	103.63	< 0.0001
miR-146a-1//146a-2//146a-3	UGAGAACUGAAUUCCAUAGAUA	22	*Salmo salar*	Mature 5' sub variant	1	812.5	169.77	1401.26	152.08	0.002
let-7e(18nt)	UGAGGUAGUAGAUUGAAU	18	*Oryzias latipes*	Mature 5'	0	933.2	26.07	625.46	123.34	0.002
miR-146a	AUCUAUGGGCUCAGUUCUUUU	21	*Oryzias latipes*	Precursor	0	121.0	44.79	237.53	16.19	0.018
let-7a-1	UGAGGUAGUAGGUUGUAUAGU	21	*Oryzias latipes*	Mature 5'	0	25408	2201.25	17678.1	2712.05	0.027
let-7a-3//7a-2//7a-1	UGAGGCAGUAGGUUGUAUAGUU	22	*Tetraodon nigroviridis*	Mature 5' variant	1	184.5	32.42	85.77	30.28	0.029
let-7e(23nt)	UGAGGUAGUAGAUUGAAUAGUUU	23	*Tetraodon nigroviridis*	Mature 5' super variant	1	1582.6	293.44	784.68	219.51	0.047
piR-675//676//4794//5462	GCAUUGGUGGUUCAGUGGUAGAAUUCUCGG	30	*Danio rerio*	Variant	1	675.67	61.18	228.69	50.46	0.0003

*Normalized values by totals. VK+, individuals fed dietary vitamin K supplemented diets

Figure 1

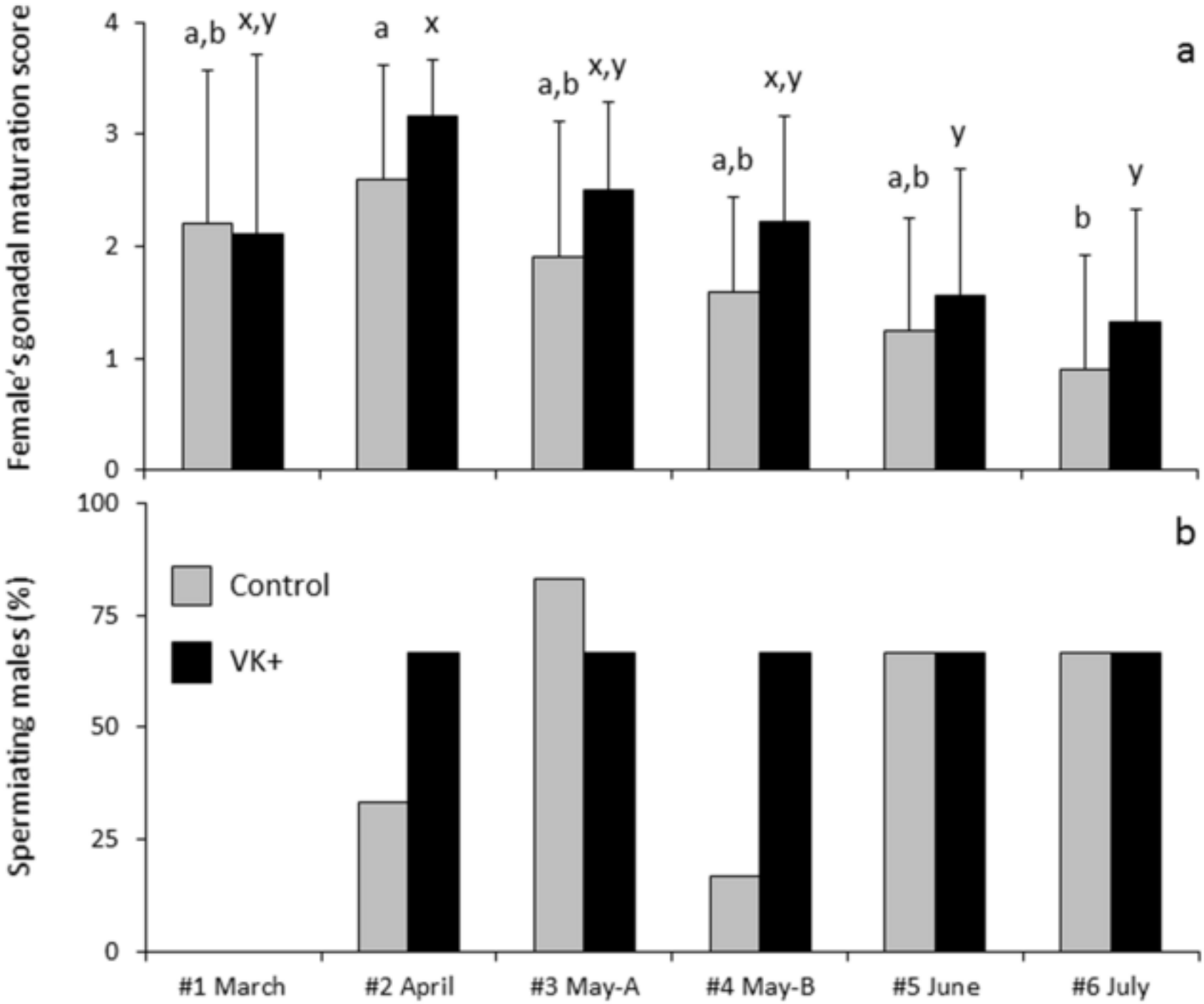

Female's gonadal maturation score
4
3
2
1
0
a,b
x,y
a
x
a,b
x,y
a,b
x,y
a,b
y
b
y
a
Spermiating males (%)
100
75
50
25
0
Control
VK+
b
#1 March
#2 April
#3 May-A
#4 May-B
#5 June
#6 July

Figure 2

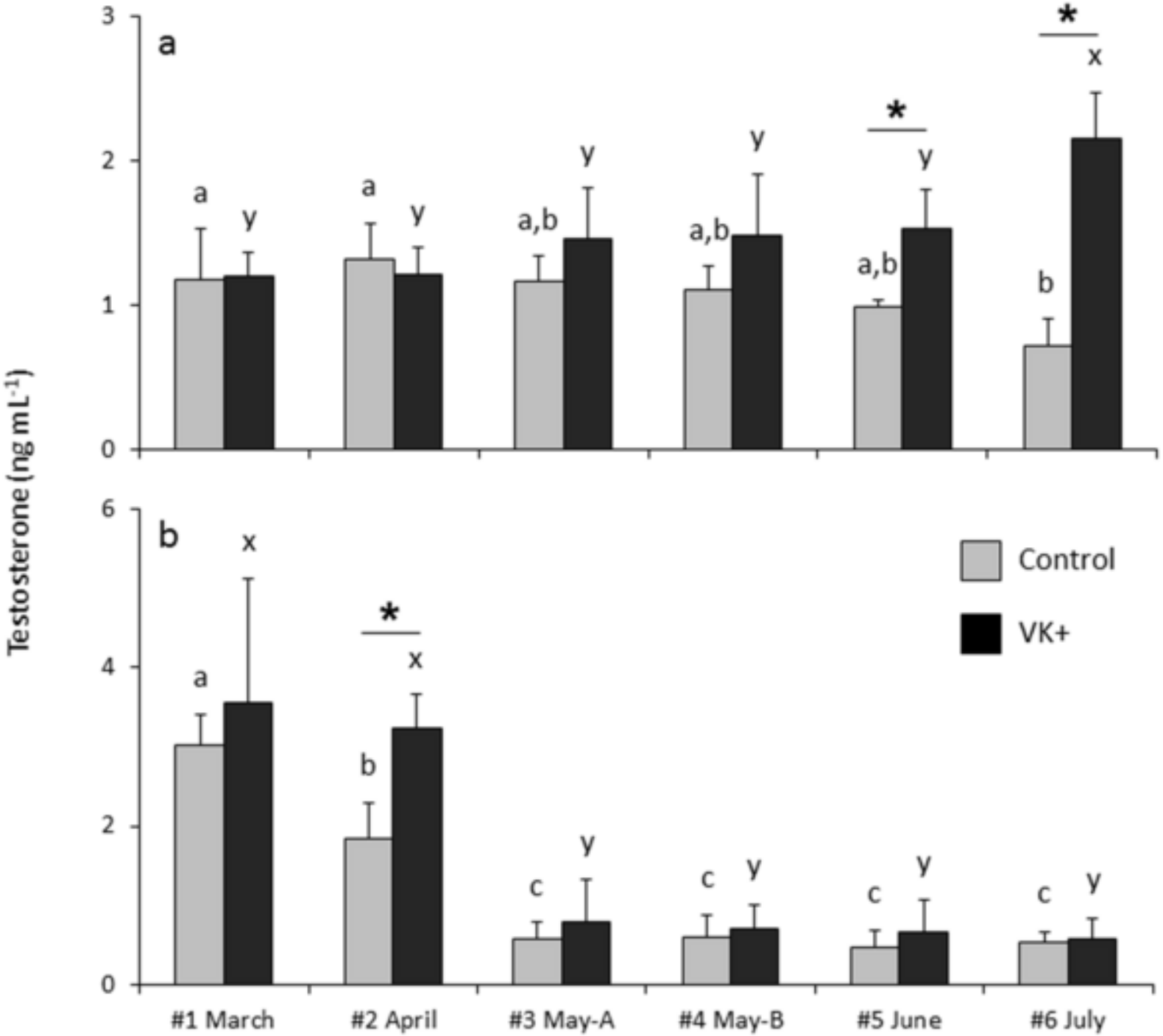

a
b
Testosterone (ng mL-1)
Control
VK+
#1 March
#2 April
#3 May-A
#4 May-B
#5 June
#6 July

Figure 3

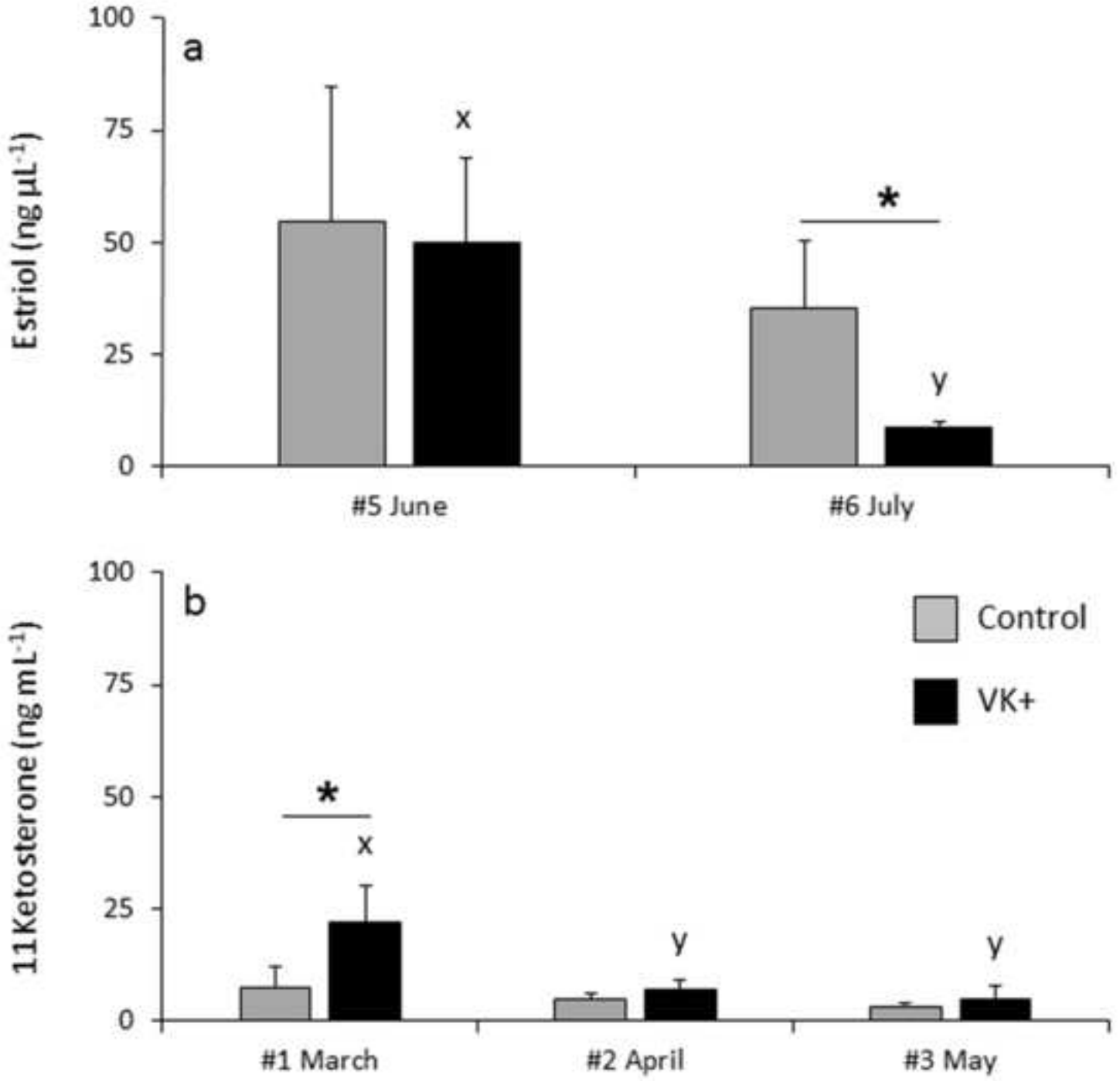

a
Estriol (ng µL-1)
100
75
50
25
0
x
y
*
#5 June
#6 July
b
11Ketosterone (ng mL-1)
100
75
50
25
0
*
x
y
y
Control
VK+
#1 March
#2 April
#3 May

Figure 4

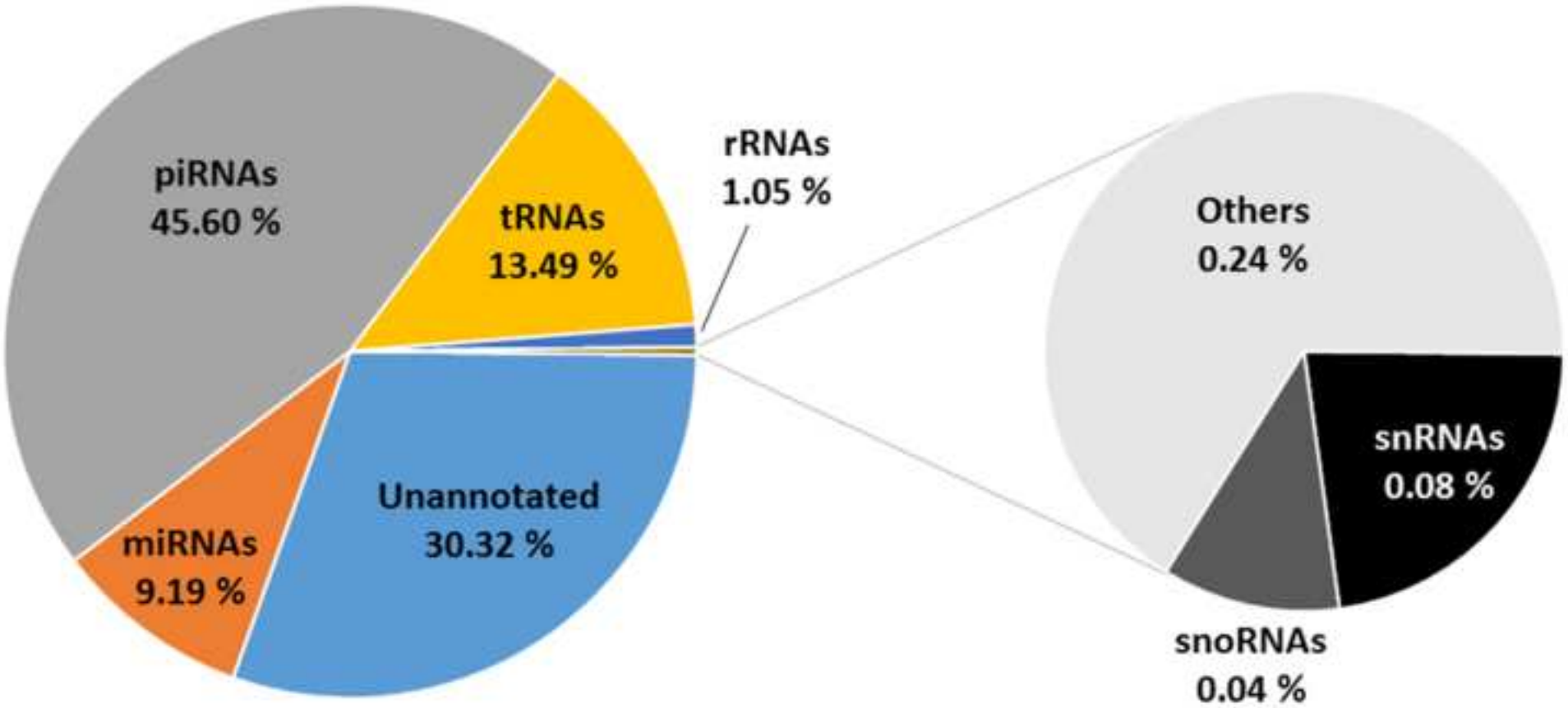

piRNAs
45.60 %
tRNAs
13.49 %
rRNAs
1.05 %
Others
0.24 %
snRNAs
0.08 %
snoRNAs
0.04 %
Unannotated
30.32 %
miRNAs
9.19 %

Figure 5

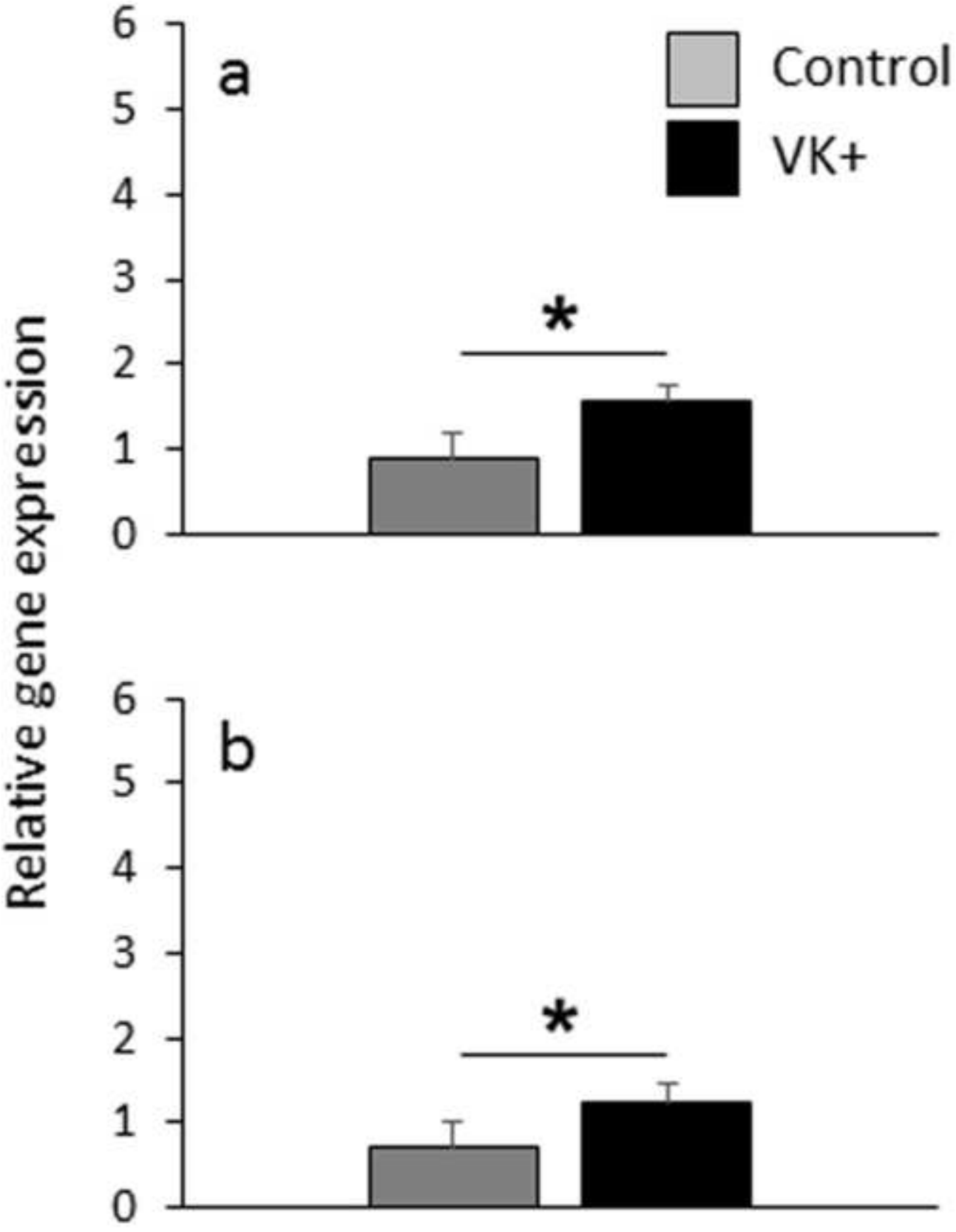
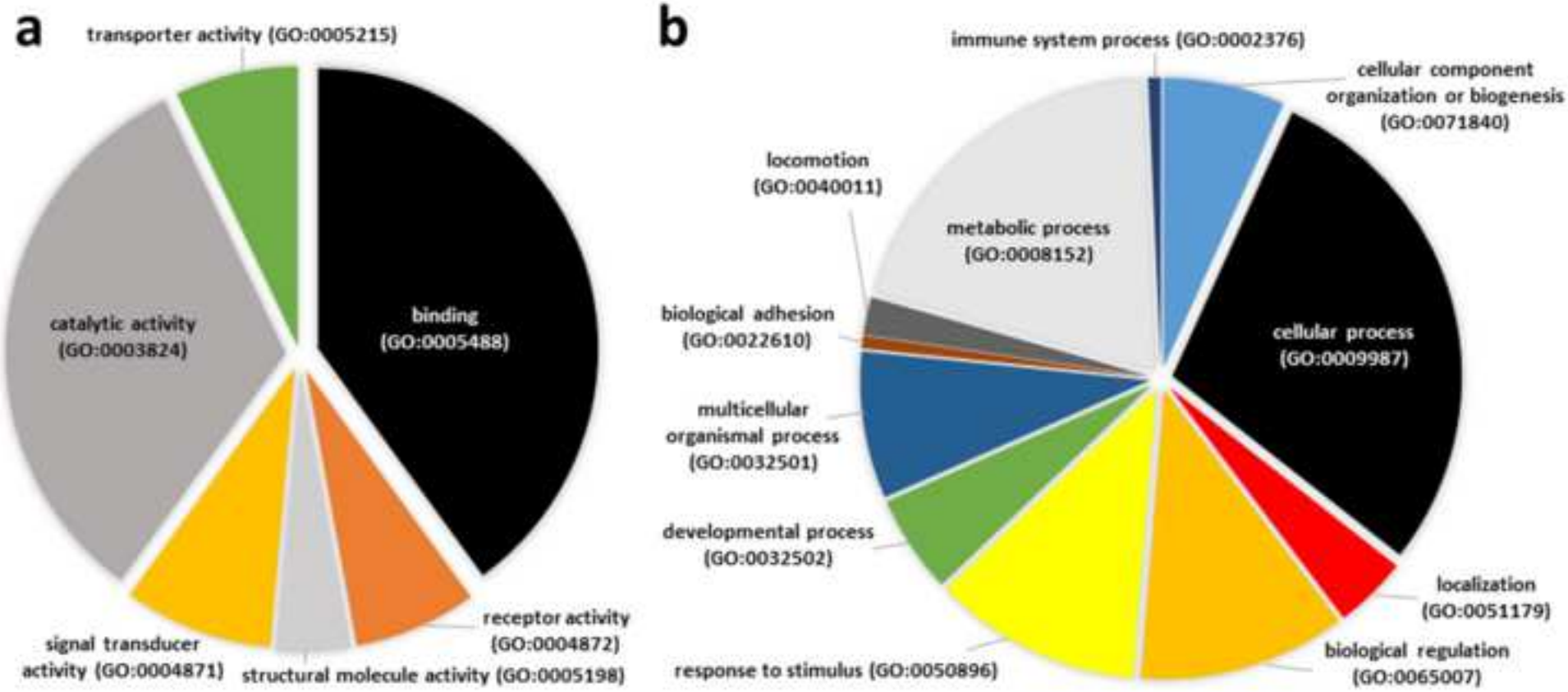


Figure 6



c

PANTHER Pathways	Nº targeted genes	Nº genes ref. transcriptome	Fold enrich.	P value
2-arachidonoylglycerol biosynthesis (P05726)	1	4	32.82	0.03
p38 MAPK pathway (P05918)	2	30	8.75	0.02
Interleukin signaling pathway (P00036)	2	42	6.25	0.04
Angiogenesis (P00005)	3	101	3.9	0.04
Wnt signaling pathway (P00057)	4	158	3.32	0.03

Figure 7

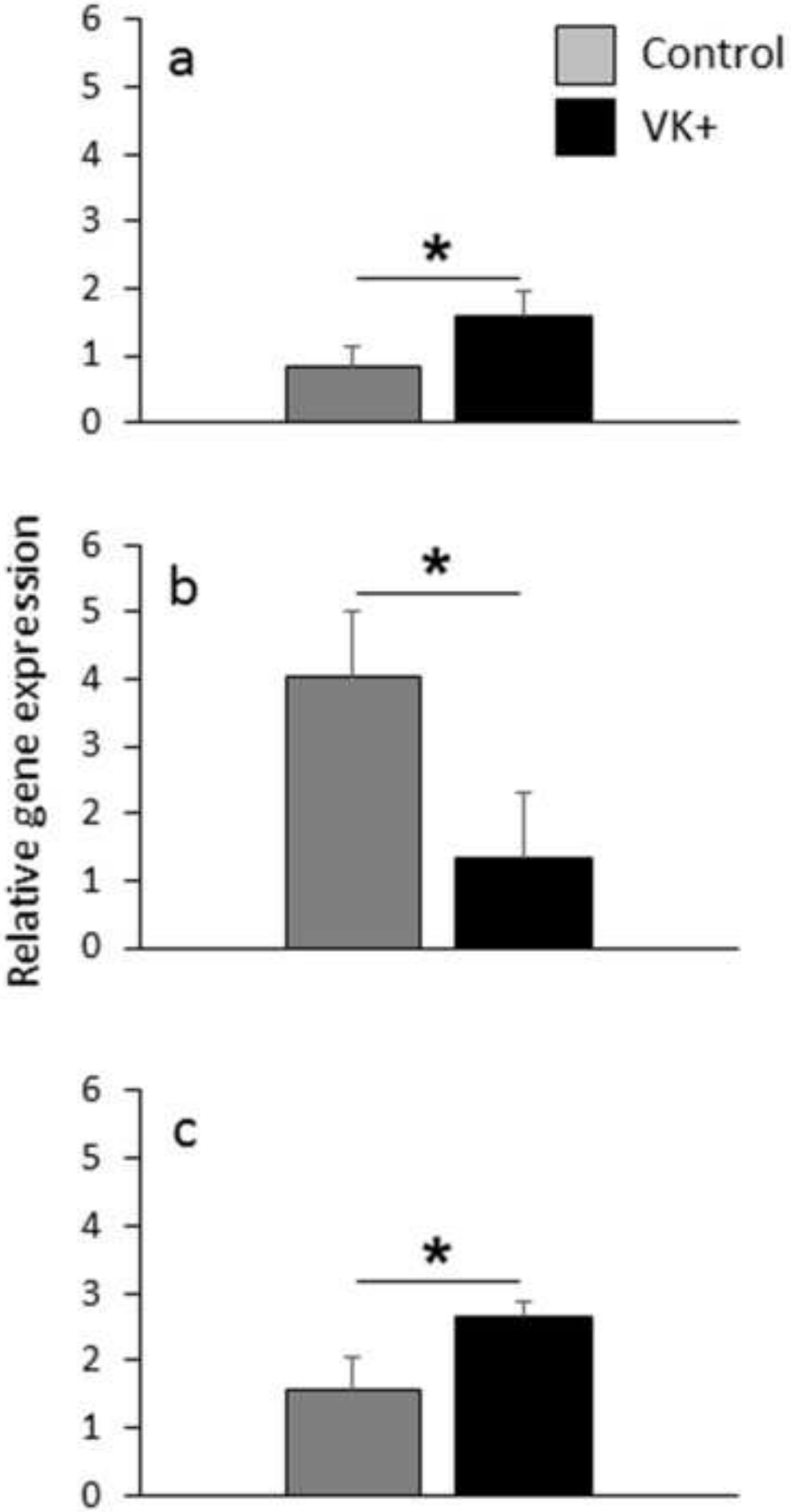
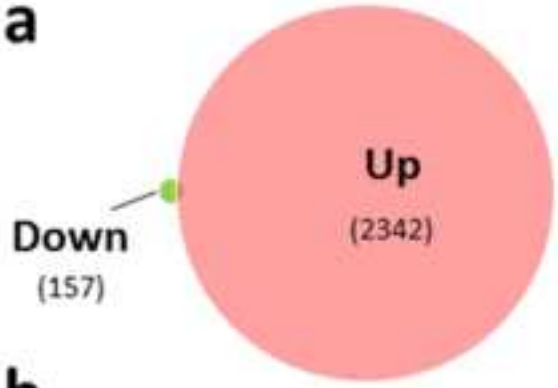


Figure 8

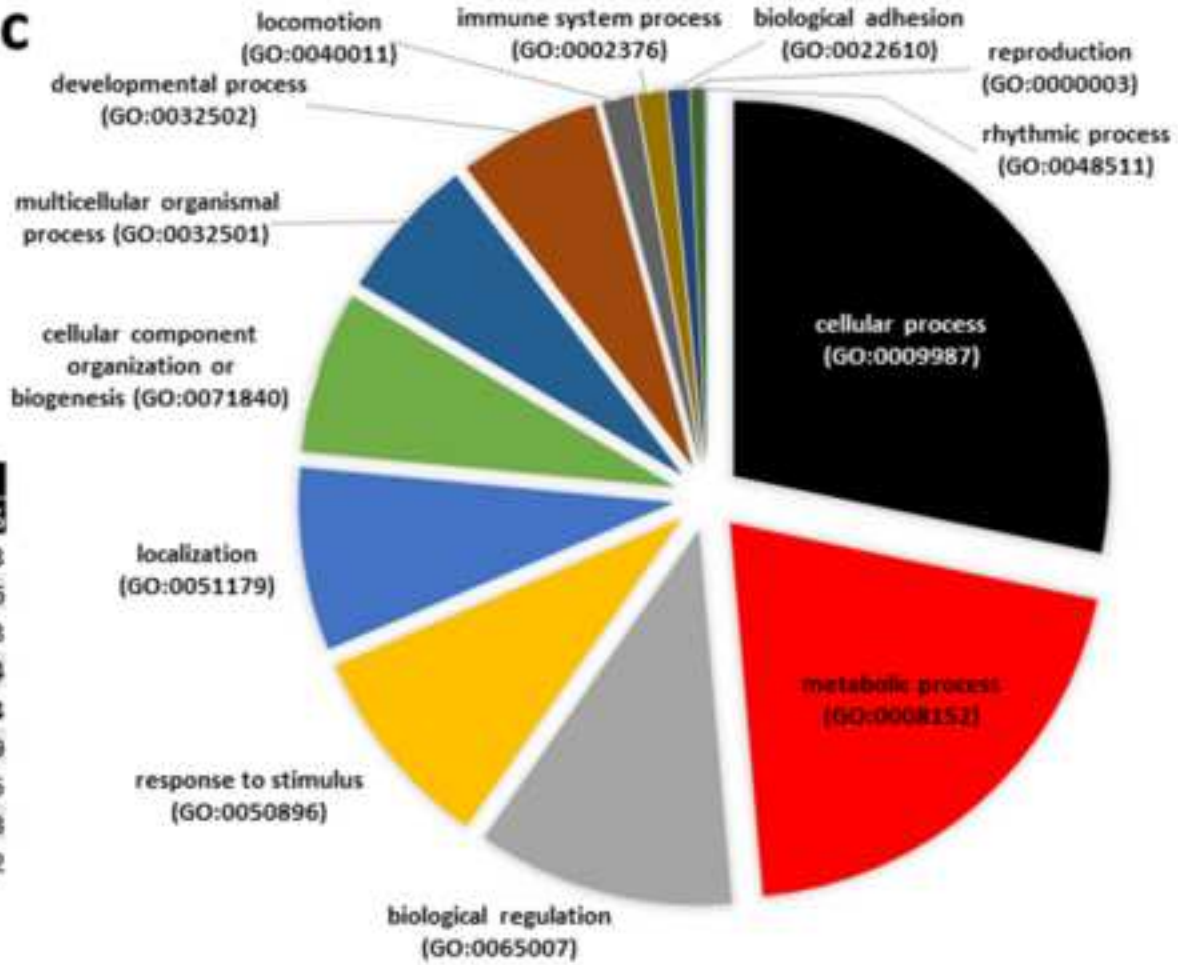
a



b

Molecular function	Genes	
	Nº	%
Binding (GO:0005488)	362	37,3
Catalytic activity (GO:0003824)	336	34,6
Transporter activity (GO:0005215)	81	8,3
Receptor activity (GO:0004872)	72	7,4
Signal transducer activity (GO:0004871)	72	7,4
Structural molecule activity (GO:0005198)	38	3,9
Translation regulator activity (GO:0045182)	5	0,5
Channel regulator activity (GO:0016247)	3	0,3
Antioxidant activity (GO:0016209)	2	0,2

c



d

Panther pathways	Nº genes	Nº genes ref. transcriptome	Fold enrich.	P value
Wnt signaling pathway (P00057)	30	158	1,51	0,019
Gonadotropin-releasing hormone receptor pathway (P06664)	28	143	1,56	0,016
Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	24	127	1,51	0,034
Integrin signalling pathway (P00034)	21	103	1,63	0,023
Huntington disease (P00029)	20	85	1,88	0,066
Interleukin signaling pathway (P00036)	10	42	1,9	0,042
Blood coagulation (P00011)	8	23	2,77	0,010
5-Hydroxytryptamine degredation (P04372)	5	14	2,85	0,033
MYO signaling pathway (P06215)	2	2	7,97	0,027
ALP23B signaling pathway (P06209)	2	2	7,97	0,027

Supplementary table 1. Small non-coding RNA (sncRNA) name and primer sequences.

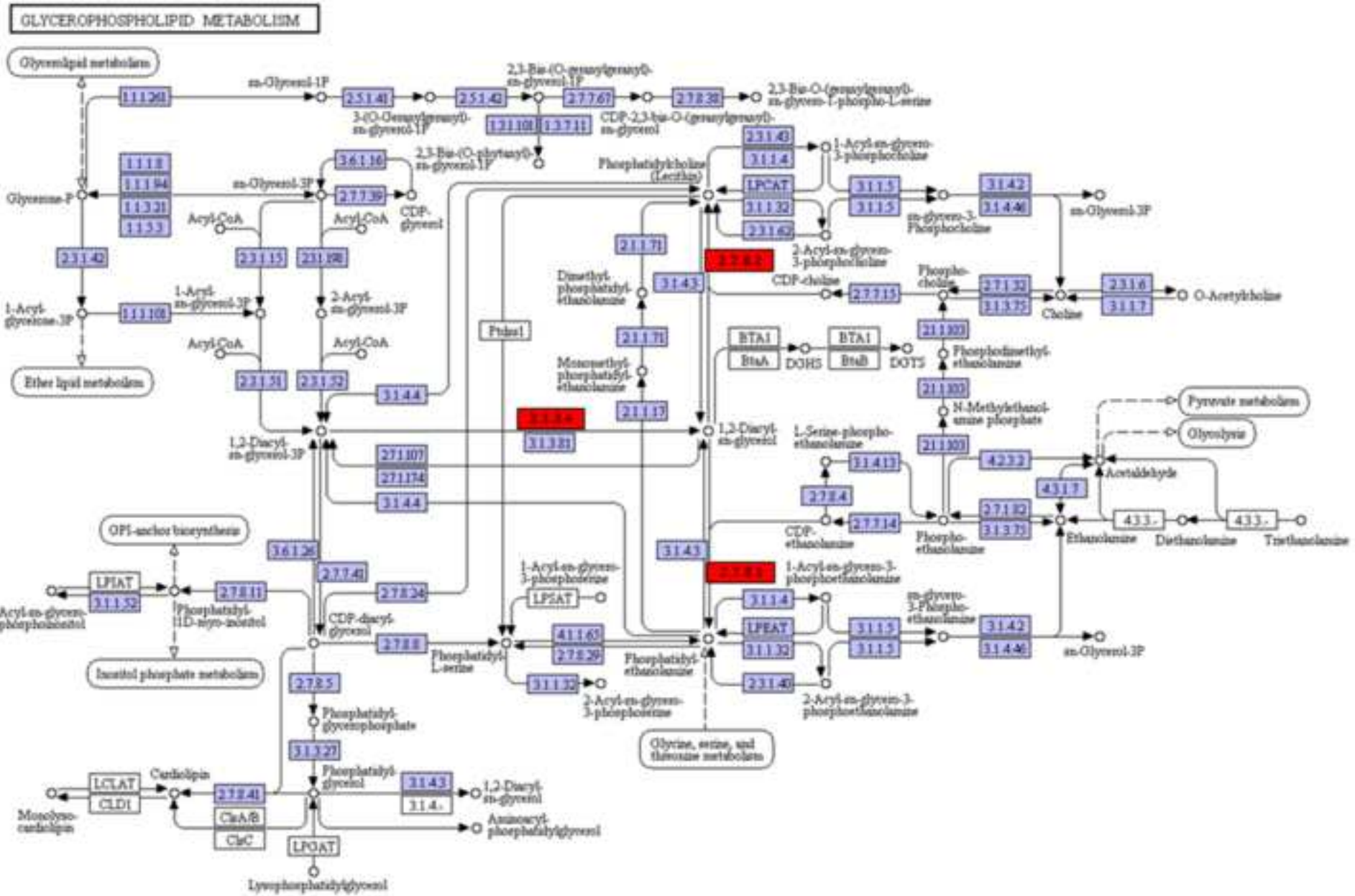
sncRNA name	5’ to 3’ nucleotide sequences (Forward primer)*
piR-675_var	GGTGGTTCAGTGGTAGAATTCTCGG
piR-74614	GCATTGGTGGTTCAGTGGTAGAGTTC
mir-146a	ATCTATGGGCTCAGTTCTTTT
Sse-let-7g	GGGGTGAGGTAGTAGTTTGTAT
piR-41105	GTTTCCGTAGTGTAGTGGTTATCACCTTC
piR-33988	GCTAGCGGTTAGGATTCCT
mir-99	AACCCGTAGATCCGATCTTGA

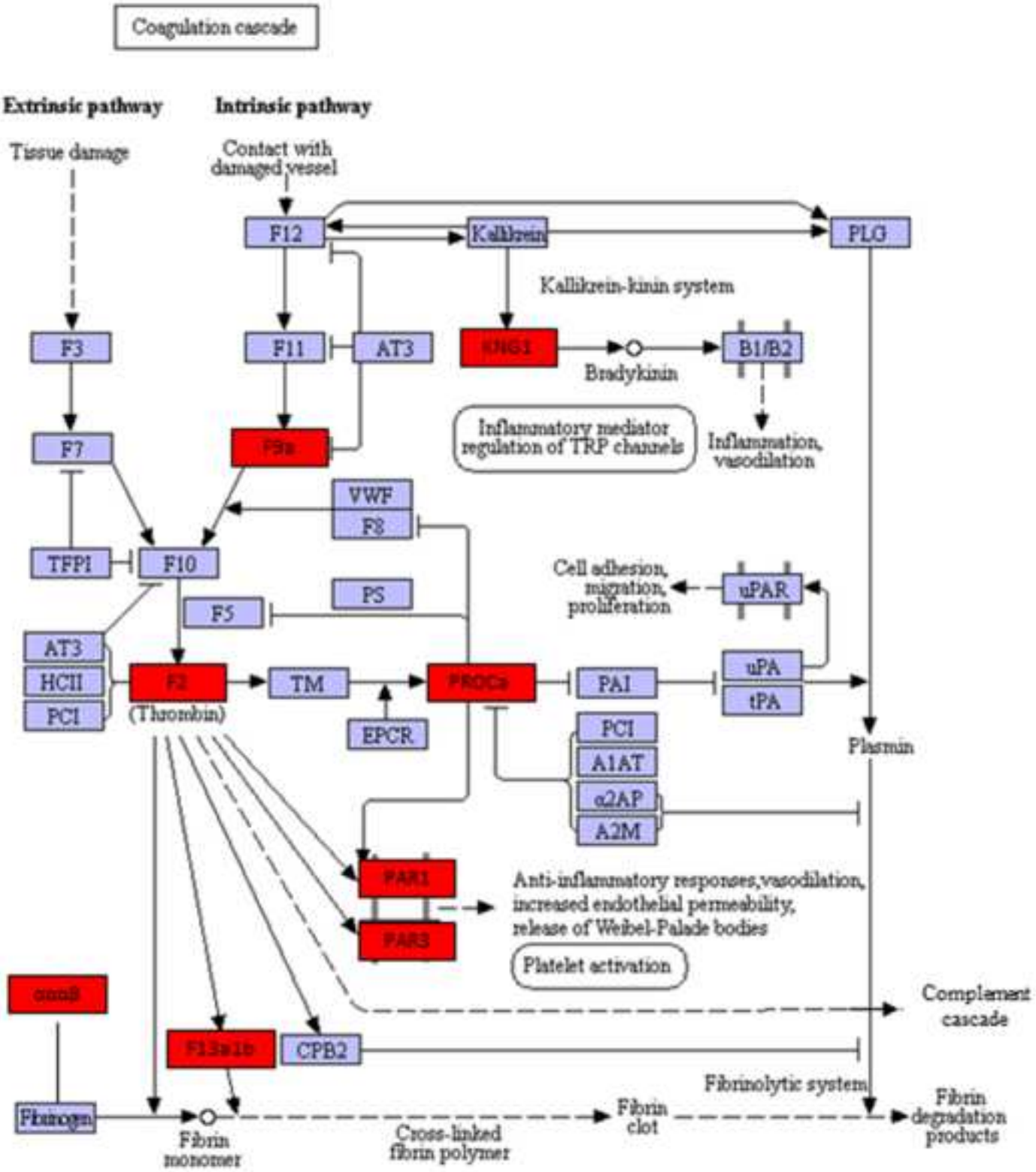
*Reverse primer supplied with NCode miRNA First-Strand cDNA synthesis and qRT-PCR kit (Invitrogen)

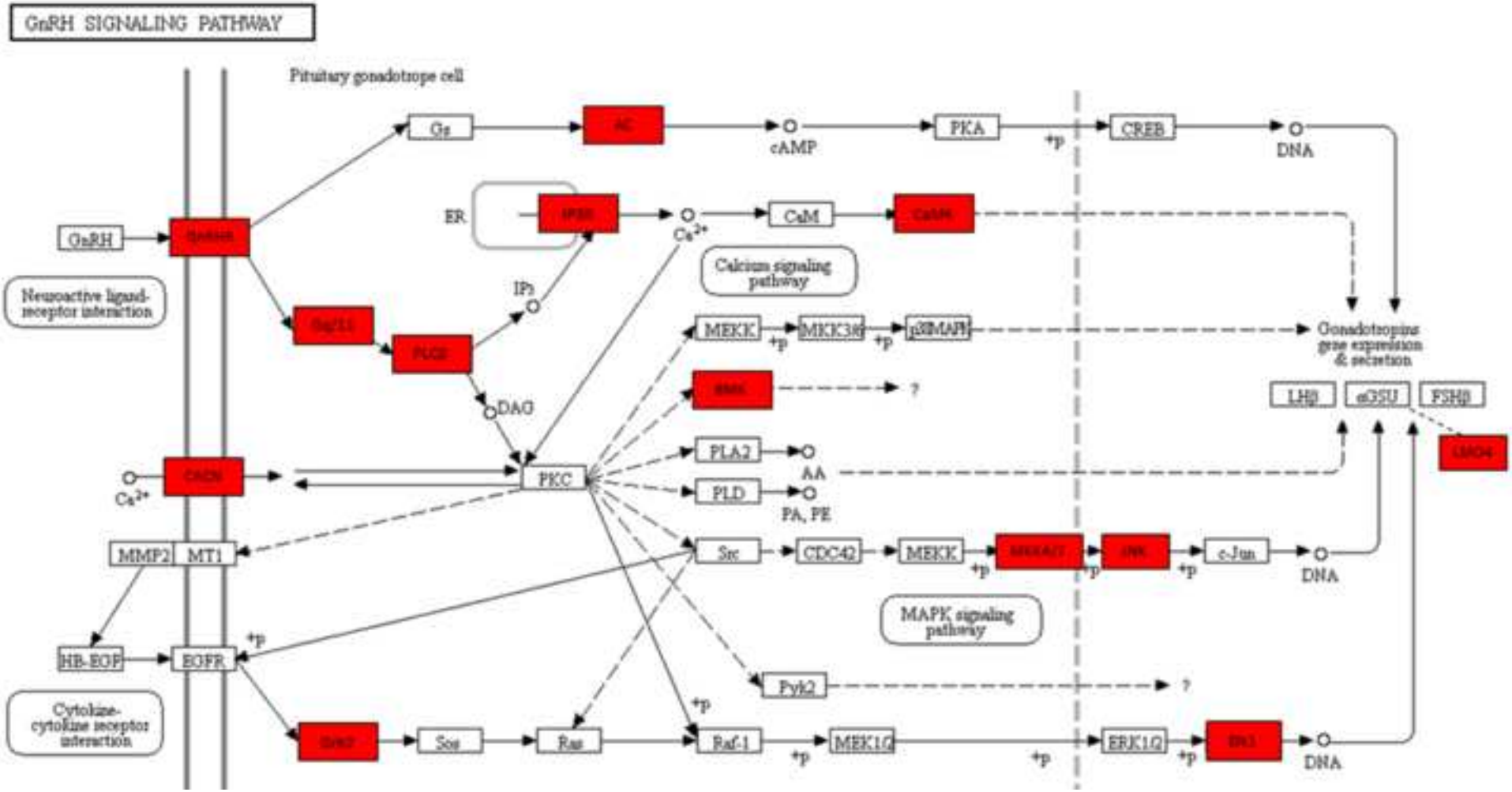
Supplementary Table 2. Comparison of the number of total, in common and newly identified miRNAs found in research works performed with the same fish species (Senegalese sole), same sampled liquid biopsy, and/or gender.

Number	Campos et al., 2014	Sun et al., 2017	Sun et al., 2017 #	Present work
(iso)-miRs identified	320	342	326	369
miRNAs identified*	320	342	326	246
common miRNAs*	96	57	56	-
new miRNAs* identified	150	189	190	-
Comparison within	**Species**	**Liquid biopsies**	**Fish gender**	**-**

Only miRNAs perfectly matched, with a read count ≥ 5 and independently of the species to which was annotated were considered.*, miRNAs unequivocally annotated; #, miRNAs identified in males.


GLYCEROPHOSPHOLIPID METABOLISM
Glycerolipid metabolism
Glycerone-P
sn-Glycerol-1P
sn-Glycerol-3P
1-Acyl-glycerone-3P
1-Acyl-sn-glycerol-3P
2-Acyl-sn-glycerol-3P
Acyl-CoA
CDP-glycerol
1,2-Diacyl-sn-glycerol-3P
Ether lipid metabolism
2,3-Bis-(O-geranylgeranyl)-sn-glycerol-1P
3-(O-Geranylgeranyl)-sn-glycerol-1P
2,3-Bis-(O-phytanyl)-sn-glycerol-1P
CDP-2,3-bis-O-(geranylgeranyl)-sn-glycerol
2,3-Bis-O-(geranylgeranyl)-sn-glycerol-1-phospho-L-serine
Phosphatidylcholine (Lecithin)
1-Acyl-sn-glycero-3-phosphocholine
2-Acyl-sn-glycero-3-phosphocholine
sn-glycero-3-Phosphocholine
CDP-choline
Phospho-choline
Choline
O-Acetylcholine
Dimethyl-phosphatidyl-ethanolamine
Monomethyl-phosphatidyl-ethanolamine
Ptdss1
BTA1
BtaA
BtaB
DOHS
DOTS
Phosphodimethyl-ethanolamine
N-Methylethanol-amine phosphate
1,2-Diacyl-sn-glycerol
1-Serine-phospho-ethanolamine
CDP-ethanolamine
Phospho-ethanolamine
Ethanolamine
Dethanolamine
Triethanolamine
Acetaldehyde
Pyruvate metabolism
Glycolysis
OPI-anchor biosynthesis
1-Acyl-sn-glycero-3-phosphoinositol
Phosphatidyl-1D-myo-inositol
Inositol phosphate metabolism
CDP-diacyl-glycerol
Phosphatidyl-L-serine
1-Acyl-sn-glycero-3-phosphoserine
2-Acyl-sn-glycero-3-phosphoserine
Phosphatidyl-ethanolamine
1-Acyl-sn-glycero-3-phosphoethanolamine
2-Acyl-sn-glycero-3-phosphoethanolamine
sn-glycero-3-Phospho-ethanolamine
Glycine, serine, and threonine metabolism
Phosphatidyl-glycerophosphate
Phosphatidyl-glycerol
Cardiolipin
Monolyso-cardiolipin
1,2-Diacyl-sn-glycerol
Aminoacyl-phosphatidylglycerol
Lysophosphatidylglycerol
LPCAT
LPSAT
LPEAT
LPIAT
LCLAT
CLD1
ClaA/B
ClaC
LPGAT


Coagulation cascade
Extrinsic pathway
Intrinsic pathway
Tissue damage
Contact with damaged vessel
F12
Kallikrein
PLG
Kallikrein-kinin system
F3
F11
AT3
KNG1
B1/B2
Bradykinin
Inflammatory mediator regulation of TRP channels
Inflammation, vasodilation
F7
F9a
VWF
F8
TFPI
F10
PS
Cell adhesion, migration, proliferation
uPAR
F5
AT3
HCII
PCI
F2
(Thrombin)
TM
PROCa
PAI
uPA
tPA
EPCR
PCI
A1AT
α2AP
A2M
Plasmin
PAR1
PAR3
Anti-inflammatory responses,vasodilation, increased endothelial permeability, release of Weibel-Palade bodies
Platelet activation
Complement cascade
F13a1b
CPB2
Fibrinolytic system
Fibrinogen
Fibrin monomer
Cross-linked fibrin polymer
Fibrin clot
Fibrin degradation products


GnRH SIGNALING PATHWAY
Pituitary gonadotrope cell
Gs
AC
cAMP
PKA
CREB
DNA
GnRH
ER
IP3
Ca2+
CaM
Calcium signaling pathway
Neuroactive ligand-receptor interaction
MEKK
MKK3/6
p38MAPK
Gonadotropin gene expression & secretion
DAG
?
LHB
αGSU
FSHB
PLA2
AA
PKC
PLD
PA, PE
Ca2+
MMP2
MT1
Src
CDC42
MEKK
JNK
c-Jun
DNA
MAPK signaling pathway
HB-EGF
EGFR
+p
Pyk2
Cytokine-cytokine receptor interaction
Sos
Ras
Raf-1
MEK1/2
ERK1/2
DNA