Genomics 114 (2022) 266–277

Contents lists available at ScienceDirect

Genomics

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

Sex-biased dynamics of three-spined stickleback (*Gasterosteus aculeatus*) gene expression patterns

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ARTICLE INFO

Keywords:
Three-spined stickleback
Gender
Gene expression
Transcriptome
RNAseq
miRNA

ABSTRACT

The study of the differences between sexes presents an excellent model to unravel how phenotypic variation is achieved from a similar genetic background. Sticklebacks are of particular interest since evidence of a heteromorphic chromosome pair has not always been detected. The present study investigated sex-biased mRNA and small non-coding RNA (sncRNA) expression patterns in the brain, adipose tissues, and gonads of the three-spined stickleback. The sncRNA analysis indicated that regulatory functions occurred mainly in the gonads. Alleged miRNA-mRNA interactions were established and a mapping bias of differential expressed transcripts towards chromosome 19 was observed. Key players previously shown to control sex determination and differentiation in other fish species but also genes like *gapdh* were among the transcripts identified. This is the first report in the three-spined stickleback demonstrating tissue-specific expression comprising both mRNA and sncRNA between sexes, emphasizing the importance of mRNA-miRNA interactions as well as new presumed genes not yet identified to have gender-specific roles.

1. Introduction

Studies of differences between the sexes continue to shed light on the mechanisms underlying phenotypic variation despite a similar genetic background. In particular, fish species are considered of great importance in this regard since they exhibit an extensive variety in the biology of sexes, including hermaphroditic species (synchronic, protogynous, or protandrous) and gonochoristic species with either distinct sex chromosomes or sex-determining genes [1]. The mechanisms underlying these processes as well as genes and regulators with important roles during sex-determination are not yet completely identified. Some of the mammalian sex determination and differentiation genes have also been identified in teleosts but still, their functions are not fully clarified. Studying sex-specific transcriptomes may identify gender-specific gene expression profiles contributing to our understanding of sex-determination (e.g. [2,3]). Studies in mammalian species showed that RNAseq analysis of genes mapping on the Y chromosomes facilitated sex identification at early stages [4]. Similarly investigating the sex-specific transcriptomes may unravel novel ways of determining sex in teleosts with distinct sex-specific chromosomes or hot spots. Therefore, for numerous model and non-model fish species, the gonad transcriptome has been investigated. Several studies have focused on differentially expressed genes during testis development, e.g., the yellow catfish, (*Pelteobagrus fulvidraco*) [5], or during ovary development, e.g., in the striped bass (*Morone saxtilis*) [6] while others have described the difference between adult testis compared to ovaries, e.g., greater Amberjack (*Seriola dumerili*) [7], fugu (*Takifugu rubripes*) [8], and in the spotted knifejaw (*Oplegnathus punctatus*) [9]. Gonad development in teleosts has been shown to be influenced by a broad range of genetic and environmental (external) factors, which may also be species-specific [10,11]. Besides the gonads, it has been shown that brain and adipose tissues present gender-specific gene expression profiles [12]. Regarding the brain, suppression of the hypothalamus–gonadal axis affects sex determination, while pituitary hormones are essential for regulating sex differentiation [13–15]. On the other hand, adipose tissue has been broadly recognized across species to be a multi-faceted organ, with sex-dependent differences regarding metabolic and endocrine functions [10]. However, only little is known about sex-specific differences at the molecular level regarding the regulation and deposition of adipose tissue [17]. Regarding teleosts, it has been shown that some sex steroids can be produced by interrenal cells and adipose tissue [16,18]. Sticklebacks are of particular interest since they belong to the group of

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https://doi.org/10.1016/j.ygeno.2021.12.010
Received 4 May 2021; Received in revised form 17 November 2021; Accepted 5 December 2021
Available online 18 December 2021

teleosts where evidence of a heteromorphic chromosome pair has not always been detected and sex determination varies within the stickleback genus. The black-spotted stickleback (*Gasterosteus weatlandi)* for example, has been shown to encompass sex chromosomes while others, such as the three-spined stickleback, lack cytologically distinguishable sex chromosomes [19]. Nevertheless, by applying microsatellite markers, it has been proposed that the locus for sex determination resides on the chromosome (Chr.) 19 in three-spined, while in the nine-spined stickleback (*Pungitus pungitus*), genes located in Chr.12 are considered to determine the sex [19,20]. Besides sex-determining regions or genes, some conserved genes, as shown in mammals, may also play important roles in sex determination and differentiation [21]. In mammals, sexual determination is found to be stable, and sex-specific genes act together to form distinct sex phenotypes [22]. In teleosts, this process is very diverse and plastic, and thus the need to unravel novel sex-related genes persists.

The three-spined stickleback (*Gasterosteus aculeatus*) belongs to the well-studied teleost fish species, native in fresh, brackish, or even saltwater, and has been a model organism for years in a broad range of biological research fields (e.g. [23–26]). It is a gonochoristic species, for which, during the mating season, sexual dimorphism has been observed [24,25]. It has also been shown that regulatory mechanisms involved in the endocrine system and the major histocompatibility complex (MHC) encompass important roles during mating [29]. Furthermore, in the three-spined stickleback, noticeable levels of sexual dimorphism in brain size have been demonstrated which may have significant effects on neural development among the sexes [30]. Both genomic and transcriptomic data are available for the three-spined stickleback [23,28,31], but information on sex-specific transcriptomes is still lacking. Besides the expression of protein-coding genes, a significant portion of the genome undergoes transcription but is not translated. These transcripts have been classified as non-coding RNAs (ncRNAs) and were long thought to have no functional role [33,34]. Today it has been widely accepted that ncRNAs have key cellular functions, and a broad range of studies have been published and are underway to unravel their ability to modulate gene expression. In particular, micro RNAs (miRNA) are a class of ncRNAs that have documented roles in the regulation of biological processes through their modulation of protein-coding gene expression [35,36]. In the present study, we present the dynamics and alleged interaction of gene and snc RNA expression across three tissue types (gonads, adipose tissues, and brain) of the three-spined stickleback to explore possible gender-specific expression and regulation patterns.

2. Results

2.1. mRNA sequencing

We performed RNA-seq from three tissues (gonads, adipose tissue, and brain) from three individuals (biological replicates) each of males and females, resulting in a total of 18 libraries that produced ~543 million reads. After quality trimming, ~508 million reads (~ 94%) remained for downstream analysis. The majority of the trimmed reads (average ~ 76%) were successfully mapped to the stickleback genome (Supplemental file S1). The most abundant transcripts after read-normalization are illustrated in Fig. 1, where most replicates, except for M1-adipose tissue, identified the same most-abundantly expressed genes: For the brain, *cytochrome c oxidase subunit* II was the most expressed transcript, whereas for adipose tissue *trypsin-1*, for the female gonads, *apolipoprotein Eb* and for the male gonads, *heat shock protein 90 alpha* and gonadal soma derived factor 2.

2.2. Differential gene expression analysis among all tissues

Pairwise comparison of male and female tissues resulted in three datasets. Transcripts with *p*-values adjusted according to the Benjamini-Hochberg procedure (padj) < 0.05 and with log2 fold change $\geq |1|$ were applied in the present study to define transcripts as differentially expressed (Supplemental file S2). The generated scree plot determined that the first four components described most of the data, and thus the components for the multi-dimensional scaling (MDS) plot (Fig. 2a). The MDS plot of the differentially expressed transcripts demonstrated that the samples grouped by tissue types (Fig. 2b). Mapping of transcripts to the three-spined stickleback genome showed that for all female tissues, most differentially expressed genes mapped to Chr. 19 (Fig. 2c, Supplemental file S3). Enrichment analysis of genes on Chr. 19 identified the GO terms nucleosome assembly as well as DNA-templated transcription and initiation among the most significant (Fig. 2d).

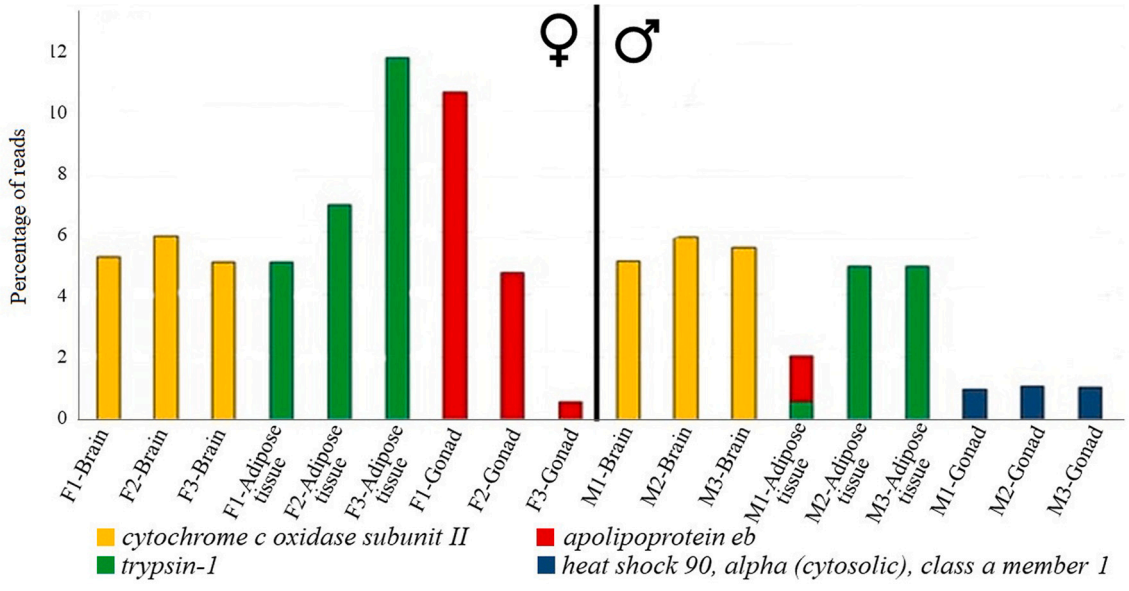

Fig. 1. The proportion of the most abundant sequence reads of the normalized reads from in all tissues. The y-axis represents the percentage of reads associated with the sequence having the highest count for each sample. The colour-codes are presenting the corresponding gene, i.e. yellow: *cytochrome c oxidase subunit II*, red: *apolipoprotein eb*, green: *trypsin-1,* and blue: *heat shock 90, alpha, class a member*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

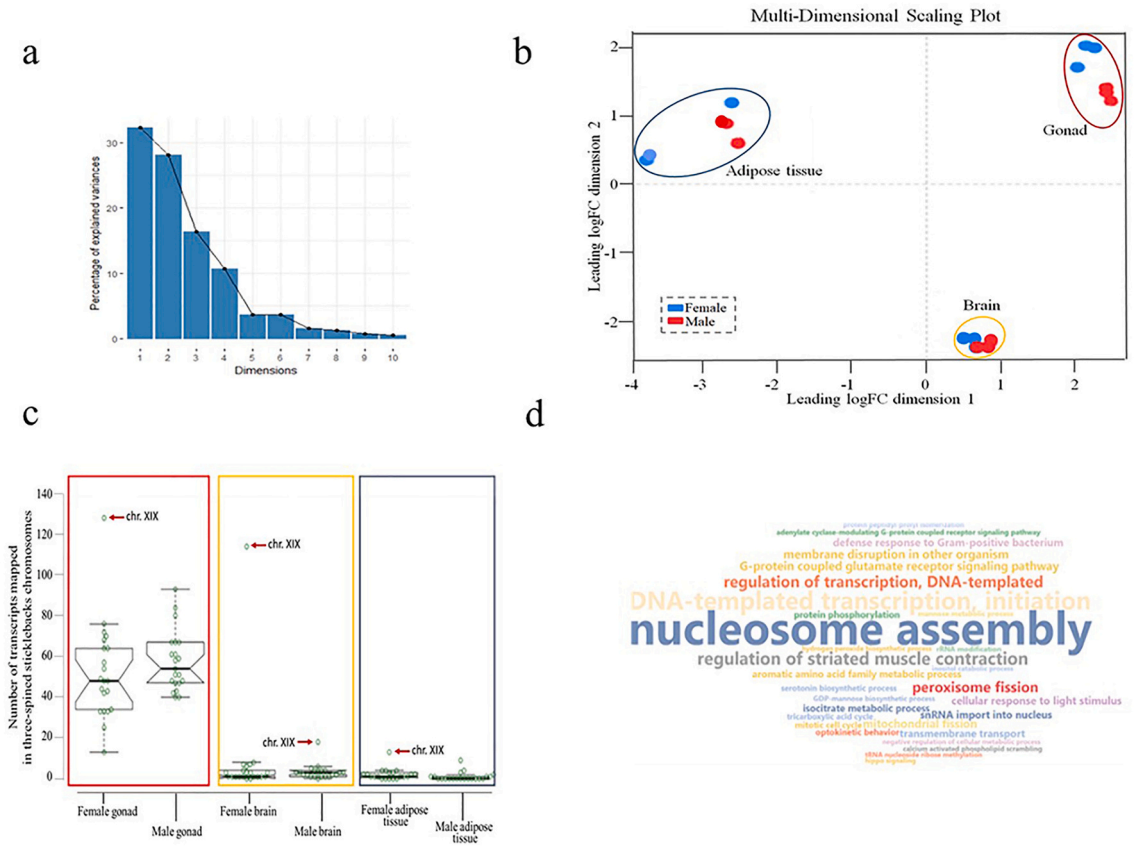

Fig. 2. **a.** Scree plot of all differentially expressed genes. The first four principal components with a cumulative percentage of variance explained >95%. **b.** MDS plot of the tissues studied, grouped with the biological replicates and tissue types. **c**. Box-Whisker Plot of differential expressed transcripts mapped onto the chromosomes. Chr. 19 is dominant in all tissues originating from a female three-spined stickleback. DEG defined as padj <0.05 and FC > 1. **d**. Enrichment analysis of all transcripts located on Chr.19 and retrieved via Ensembl BioMart database (two tail, FDR 0.05, reduced to most specific FDR 0.05 word cloud with p-value).

2.3. Differential gene expression analysis between female and male tissues

Differentially expressed genes between male and female gonads, brains, and adipose tissue are illustrated in the form of volcano plots in Fig. 3a. Among the three tissue types, as expected, most differentially expressed genes were found between the male and female gonads. A total of 2306 transcripts were found to be differentially expressed, with the male-biased transcripts being 1208 while female-biased transcripts amounted to 1098 transcripts. Enrichment analysis resulted in testis and ovary-specific GO terms (Fig. 3b, c). Among both were genes known to play an important role either in sex determination or sex differentiation, i.e., *dmrt1, dmrt3a, gtf3ab, cyp19a1a*.

The comparison of male and female brains revealed a total of 236 transcripts being differentially expressed (69 male-biased and 167 female-biased transcripts). Concerning male and female adipose tissue, only 67 transcripts were differentially expressed, with 22 transcripts being up-regulated in male adipose tissue and 45 transcripts being up-regulated in female adipose tissue.

Subsequently, the 2306 differentially expressed transcripts in the gonads were mapped onto the three-spined genome (Supplemental file S2). A total of 189 transcripts (~8%) mapped to Chr. 19, with 128 found in higher abundance in the female gonads and 61 in the male gonads. Out of the 236 differentially expressed transcripts in the brain, 132 (~55%, male-biased 18, female-biased 114) mapped to Chr. 19, and out of 67 differentially expressed transcripts in adipose tissue, 16 mapped to Chr. 19 (~24%, male up-regulated 3, female up-regulated 13).

2.4. Common genes differentially expressed among gonads, brain, and adipose tissue

In total, 51 genes were jointly differentially expressed in both gonads and brains, including the well-known housekeeping gene *gapdh*. In particular, 35 were commonly up-regulated in the female gonads and brains while and 12 were in the male gonads of both tissues. The remaining four transcripts had no concerted expression in the two tissues (Supplemental file 2). Between gonad and adipose tissues, 11 genes were found to be regulated in common. Common to all gonad, adipose, and brain were five genes: Four were down-regulated in female tissues (*apoptosis inhibitor 5, ELKS/RAB6-interacting/CAST family member 1a, ELKS/Rab6-interacting/CAST family member 1-like, Protein Z, vitamin K-dependent plasma glycoprotein a)* and one was up-regulated, again in female tissues *(eukaryotic translation initiation factor 4, gamma 2b,*). Between adipose and brain, only three genes were shared: Up-regulated in female tissues *were adenosine deaminase-like* and *zona pellucida sperm-binding protein 3-like,* whilst *napsin A aspartic peptidase (napsa)* was down-regulated (Fig. 4, Supplemental file S2).

2.5. Small RNA sequencing

Small RNA sequencing of all samples resulted in ~350 million reads with an average of 21 million reads per sample. Out of these reads, 95% were successfully mapped to the stickleback genome. Annotation identified a distinct distribution of the main types of ncRNA [ribosomal RNA (rRNA), miRNA, small nucleolar RNA (snoRNA), and small nuclear (snRNA)] within the three tissues studied, with the gonads having the highest proportion of rRNA transcripts. Among the three other types,

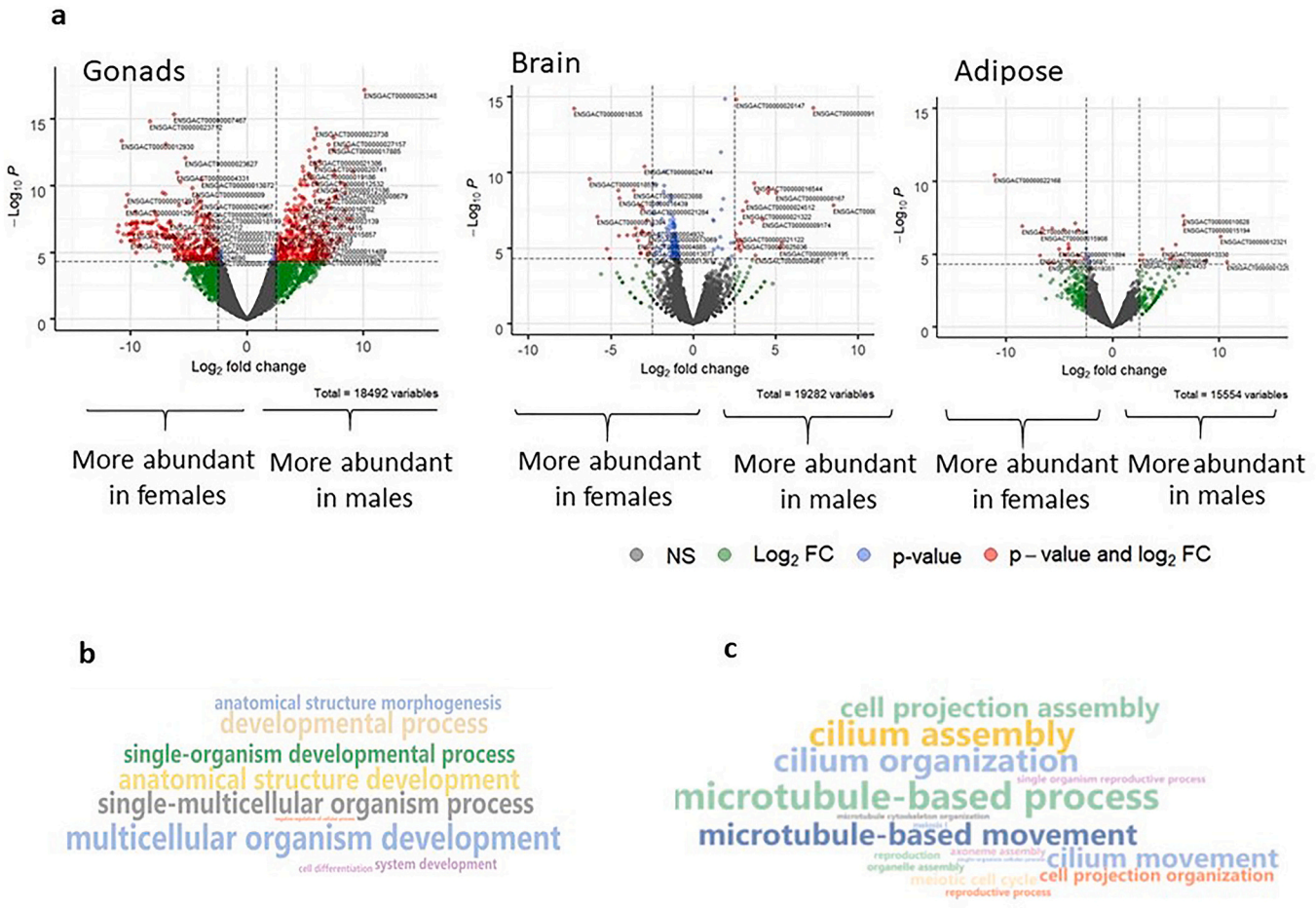

Fig. 3. **a**Volcano plots of female-male mRNA expression comparisons in gonads, brain, and adipose tissue. Grey dots correspond to transcripts that did not pass the *p*-values and log2FC thresholds. Green and blue dots correspond to transcripts that pass either only the log2FC (log2FC > |2.5|) or the p-value (p-value <0.005) threshold respectively. Red dots correspond to transcripts which meet both criteria ((log2FC > |2.5| and p-value p-value <0.005). **b**. Enrichment analysis of the female-biased transcripts resulted in ovary-specific biological process GO terms. **c**. Enrichment analysis of the male-biased transcripts resulted in testis-specific biological process GO terms. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

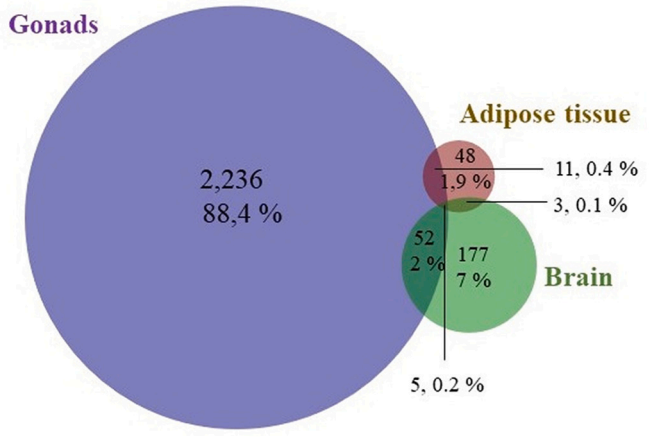

Fig. 4. Venn diagram illustrating common differentially expressed genes among gonads, adipose tissue, and brain.

miRNA dominated with ~56 million reads (~16%) resulting in 3434 unique reads obtained out of all tissues within the present work (Fig. 5).

2.6. *Differential sncRNA expression analysis between female and male tissues*

When comparing sncRNA expression between males and females, sncRNAs with *p*-values below 0.005 were considered differentially expressed (Fig. 6a). In male and female gonads, a total of 169 sncRNAs were found to have significantly different abundance, including 27 miRNAs (Fig. 6b). Seven sncRNAs identified as miRNAs in three-spined stickleback GamiRdb were subsequently annotated through miRBase. Of particular interest is the miRNA ENSGACT00000028186, which had high similarity with the western gorilla (*Gorilla gorilla*) miR-202 [37] and thus was annotated in the present study as miR-202. It is known that several miRNAs are highly conserved across even evolutionary distant species. Between mammals and fish, 90 families of miRNAs have been identified to be highly conserved and thus suggest that they may have also significant biological functions [38]. Most of the sncRNAs (24) annotated as miRNAs were more highly expressed in male gonads. Among the male-biased miRNAs, the miR-7 family was highly represented. Only three sncRNAs (miR-455, miR-2188, and miR-727) were more highly expressed in female gonads (Supplemental file S4). In the brain, only two sncRNA were detected to be differentially expressed: one identified as snoRNA and the other as snRNA. In adipose tissue, in total

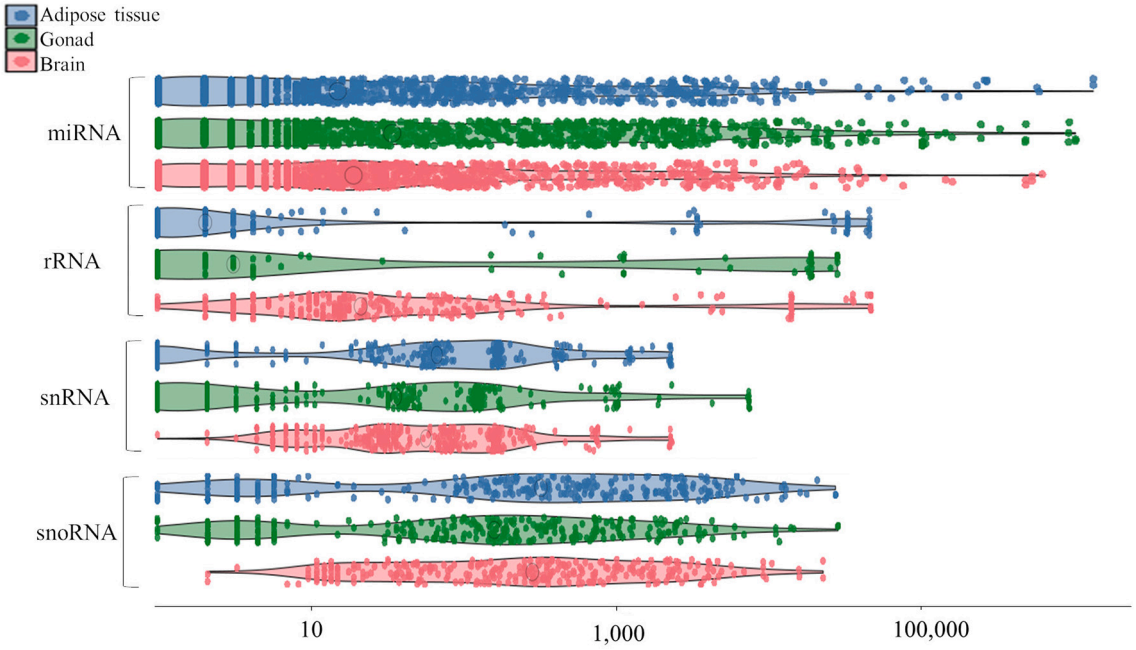

Fig. 5. Summary of three-spined stickleback female and male sncRNA read counts (x-axis) in adipose tissue, brain, and gonads presented as a violin plot reflecting the data distribution. Data are shown as jittered dots and the median of the data is indicated as an open circle. The values are plotted on a log10 scale.

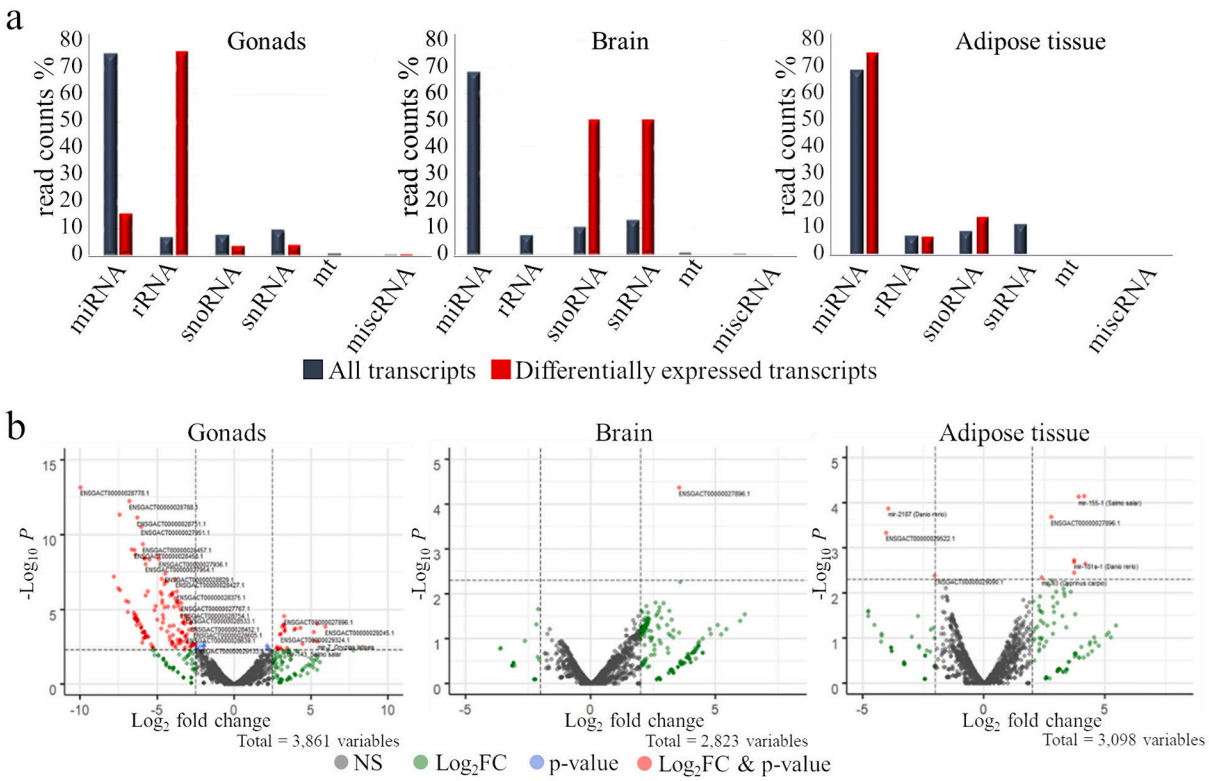

Fig. 6. **a**. Sequencing biotype distribution of all (in blue) and of differentially expressed (in red) sncRNAs identified in the present study. **b**. Volcano plots of ncRNA expression in gonads, brain, and adipose tissue. Grey dots correspond to transcripts that did not pass the *p*-values and log2FC thresholds. Green and blue dots correspond to transcripts that pass either only the log2FC (log2FC > |2.5|) or the p-value (p-value <0.005) threshold respectively. Red dots correspond to transcripts which meet both criteria (log2FC > |2.5| and p-value p-value <0.005). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

13 sncRNAs were differentially expressed between males and females: 10 out of them were identified as miRNAs (Supplemental file S4). Three miRNAs (miR-146a, miR-155, and miR-7a) were differentially expressed in common between adipose tissue and gonads, being in both cases more highly expressed in males.

2.7. Putative miRNA – mRNA interaction

To assess the putative interactions between miRNAs and mRNAs identified in this study, we examined the 3′UTR of all available three-spined stickleback transcripts for miRNA target sites. In total 52,772 transcript ids were obtained, for which in 19,597 cases (~37%) no 3′UTR was available. For those transcripts with significantly different abundance between female and male gonads (2306), the available 3′UTR sequences amounted to 851 (~37%). Applying RNAhybrid, a total of 1750 putative targets sites were identified for 13 differentially expressed and annotated miRNAs (mfe < −25) (Supplemental file 5). Putative miR-202 targets being down-regulated in male gonads within the present study amounted to 51 transcripts (Table 1) out of which 36 were mapped to 10 gene ontology (GO) terms comprising locomotion and biological adhesion (Fig. 7). Putative targets were identified for an additional three male-biased miRNAs (miR-146, miR-7, and mir-143). Conversely, three miRNAs showed higher expression in females than males (miR-2188, miR-455, and miR-727). Identified miRNAs were also previously reported to be involved in reproduction (Table 2), and two, i.e. miR-146a and miR-455 are also well-known to be active during an immune response.

3. Discussion

The present study examines extensive gender-specific transcriptome profiles for three three-spined stickleback tissues. The first focus was to profile mRNA transcripts across the three tissues and to determine the particularities of the differentially expressed genes. We further investigated the sncRNA profiles of the three tissues to detect sex-linked sncRNAs and their putative targets.

Concerning the transcriptome profiles, for each of the samples, on average, 28 million trimmed reads were generated (Supplemental file S1), out of which about 21 million reads mapped to the three-spined stickleback genome. The sequencing depth required for DEG analysis is defined by the aim of the experiment. A broad range of RNA studies supports that a smaller amount of reads and more biological replicates are a more effective strategy to obtain higher accuracy in large-scale DEG studies (e.g. [39]). In the present study, the purpose was to evaluate the transcriptome profiles between male and female individuals in three tissue types and, according to the Encode RNAseq standards [40], a modest depth of sequencing and a minimum number of two replicates are required. The obtained read count and replicate numbers in the present study are in concordance with those standards.

First insights into the gender-specific transcriptome have been achieved by the detection of the most highly expressed genes of the tissues under study prior to DEG analysis (Fig. 1). In the brain for both genders, *cytochrome c oxidase* (COX) *subunit II* was found to be the most expressed gene. Organs with an augmented need of oxidation such as the heart, liver, kidney, skeletal muscles, and in particular, the brain are highly dependent on the normal functioning of COX [41,]. Also, in adipose tissue, both genders had the same most highly-expressed gene (i.e., *trypsin-1*) in nearly all replicates. In the gonads, the most highly expressed gene in females was *apolipoprotein Eb*, while in males it was the *heat shock protein 90*. Hsp90, in particular, has been shown to interact with steroid hormone receptors, signaling kinases, and various transcription factors. Although in the present study, *hsp90 alpha* has been found to be the highest expressed gene in the male gonads, it was not detected to be at a significantly higher expression level compared to the female gonads. In the Japanese flounder, however, *hsp90 alpha* has been found to be up-regulated in the male gonads [42]. It has also been

Table 1
miR-202 putative targets found in the present study to be differentially expressed between male and female gonads.

PutativeTargets of miR-202	Gene	log2 Fold Change	Chr.
ENSGACT00000010539	naalad2	−6749	Group I
ENSGACT00000019537	acadl	−5572	Group I
ENSGACT00000017464	DMWD	−2672	Group I
ENSGACT00000014534	nxn	−1928	Group I
ENSGACT00000013065	gfm1	−1694	Group I
ENSGACT00000020746	adma	−1845	Group II
ENSGACT00000021034	si:ch211 − 122f10.4	-1,76	Group II
ENSGACT00000018776	si:ch211-14a17.6	−9757	Group III
ENSGACT00000017985	tfa	−5843	Group III
ENSGACT00000023023	aqp1a.1	−3,11	Group III
ENSGACT00000020313	gpx4b	−1711	Group III
ENSGACT00000026059	napepld	−3606	Group IV
ENSGACT00000026070	gene: ENSGACG00000019687	−2,13	Group IX
ENSGACT00000014800	sult6b1	−2913	Group VI
ENSGACT00000005564	nid1a	−1759	Group VI
ENSGACT00000025680	YBX2	−4518	Group VII
ENSGACT00000027329	gene: ENSGACG00000020622	−3063	Group VII
ENSGACT00000025468	slc7a7	−1642	Group VII
ENSGACT00000009974	gene: ENSGACG00000007500	−5212	Group X
ENSGACT00000005702	gene: ENSGACG00000004315	−3735	Group X
ENSGACT00000012398	ccdc106b	−3028	Group X
ENSGACT00000016192	cacng1b	−3922	Group XI
ENSGACT00000007277	aldh3b1	−3171	Group XI
ENSGACT00000019423	hsd17b14	−1959	Group XI
ENSGACT00000008295	ndufa4l2a	−2039	Group XII
ENSGACT00000008224	gene: ENSGACG00000006172	−1528	Group XIII
ENSGACT00000021374	zgc:73226	−3537	Group XIV
ENSGACT00000021022	egfl7	−1675	Group XIV
ENSGACT00000004109	nrn1la	−4606	Group XIX
ENSGACT00000010018	lamb1a	−3403	Group XIX
ENSGACT00000005280	nell2a	−2944	Group XIX
ENSGACT00000014854	uevld	−2319	Group XIX
ENSGACT00000008114	gene: ENSGACG00000006110	−2202	Group XIX
ENSGACT00000004360	tcp11l1	−1961	Group XIX
ENSGACT00000016142	lgmn	−2431	Group XV
ENSGACT00000013718	lbh	−2069	Group XV
ENSGACT00000006666	manba	−1948	Group XV
ENSGACT00000012234	si:ch211 − 15d5.11	-1852	Group XV
ENSGACT00000006557	hat1	−5167	Group XVI
ENSGACT00000004721	pdzk1	−4227	Group XVI
ENSGACT00000015526	gene: ENSGACG00000011676	−4346	Group XVII
ENSGACT00000006301	gene: ENSGACG00000004759	−3,07	Group XVII
ENSGACT00000004277	ctsa	−2066	Group XVII
ENSGACT00000009136	gene: ENSGACG00000006882	−4266	Group XVIII
ENSGACT00000006156	gene: ENSGACG00000004657	−3099	Group XVIII

(*continued on next page*)

Table 1 (*continued*)

PutativeTargets of miR-202	Gene	log2 Fold Change	Chr.
ENSGACT00000014136	ntd5	−3006	Group XX
ENSGACT00000017461	si:ch211 − 271d10.2	−2769	Group XX
ENSGACT00000017553	rab25b	−2576	Group XX
ENSGACT00000004815	grhl2a	-2405	Group XX
ENSGACT00000011865	dgat1b	−1881	Group XX
ENSGACT00000007108	apodb	−3232	Group XXI

reported that *hsp90 alpha* may have a distinct role in human aging and in male fertility [43]. Concerning *apolipoprotein Eb*, which was found not only to be the highest expressed gene in the female gonads but also to be significantly higher expressed, only little is known about sex-specific expression. In zebrafish, it has been shown that apolipoprotein Eb, together with *estrogen receptor 2a* (esr2a), a gene essential for female reproduction [44], are involved in zebrafish fins and scale development [45]).

To generate an MDS plot of differentially expressed genes between males and females in at least one of the tissues, a scree plot was calculated and four degrees of freedom (Fig. 2a) were selected followed by the 2D illustration for MDS illustration (Fig. 2b). Clearly, but not surprisingly, the three tissue types form separate groups, with the brain samples clustering tighter together than the other tissue samples. Gonad tissues and adipose tissues are separated on dimension one, while the

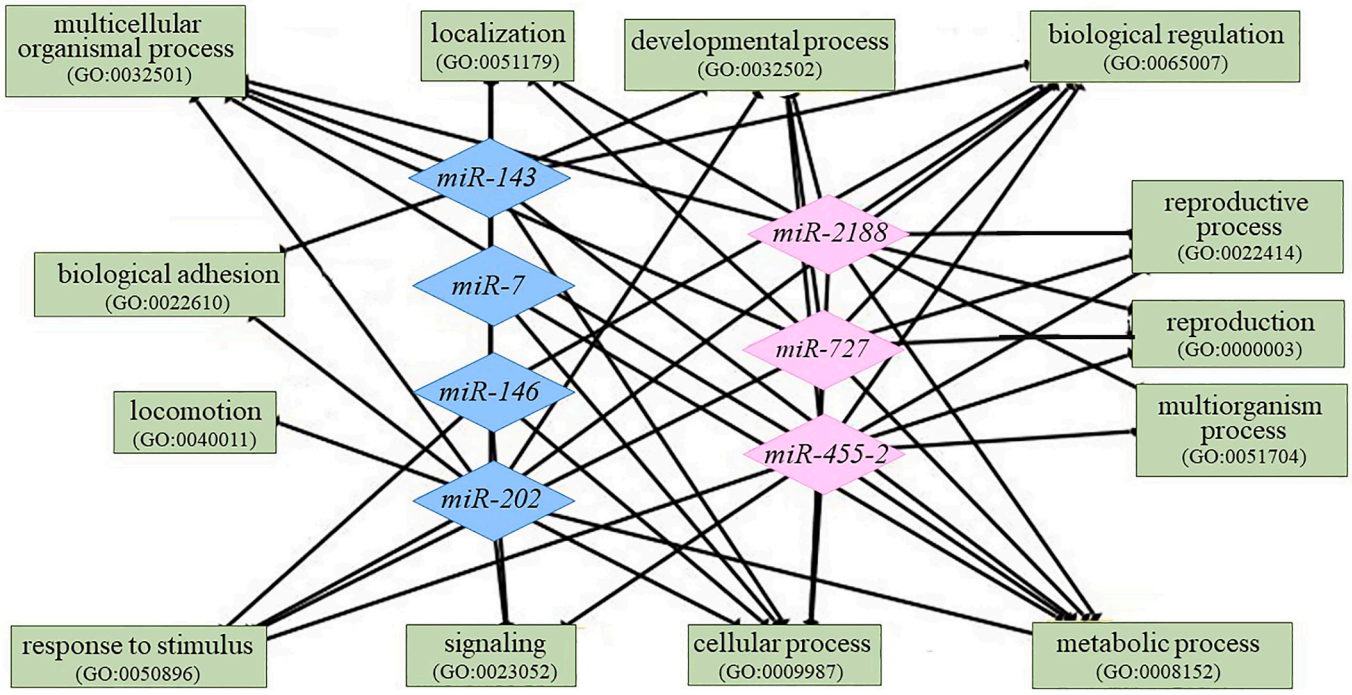

Fig. 7. miRNA-GO network analysis. Diamonds represent miRNA nodes, in blue miRNAs in higher abundance in males, in violet colour miRNAs in higher abundance in female gonads. Green boxes represent biological process GO terms. Edges indicate the presumed inhibitory effect of miRNAs on target genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Gonad miRs for which putative target genes were detected with a mfe-value < −25.

miRNA	Development stage/tissue	Reference	DE in the present study
miR-143	Vitellogenic follicles, Ovary, Testis	Zayed et al.[86]; Bizayehu et al.[87]; Ma et al.[88]; Jing et al.[93]; Presslauer et al.[90]	up in male
ENSGACT (0)$_6$28186 miR-202	Vitellogenic follicles, Ovary, Testis	Zayed et al.[86]; Bizayehu et al.[87]; Ma et al.[88]; Jing et al.[93]; Juanchich et al. [89]; Tao et al. [91]; Qiu et al.[92]	up in male
miR-146a	Ovary, Testis	Reza et al.[35] (mammalian)	up in male
miR-181a	Ovary, Testis	Juanchich et al.[89]; Presslauer et al.[90]	no differential expression
miR-181b	Ovary	Juanchich et al.[89]	no differential expression
miR-2188	n/a	n/a	up in female
miR-455	n/a	n/a	up in female
miR-727	Ovary	Xiao et al.[70]	up in female
miR-7	Testis	Jing et al.[93]	up in male
miR-7a	n/a	n/a	up in male
miR-7b	Testis	Wang et al.[9]	up in male
let-7a-1	Ovary, Testis	Bizayehu et al.[87]; Ma et al.[88]; Wang et al.[9]	up in male

brain separates from both on dimension two, which can be interpreted such that the differences between adipose tissue and gonads are bigger than the difference of differentially expressed genes in the brain. Mapping of all differentially expressed genes to the stickleback genome revealed a bias towards Chr. 19 in female gonads, adipose tissues as well as brain (Fig. 2c). Among them, transcripts related to the reproduction processes, such as *wee2* [46], which is involved in the normal oocyte maturation processes, *hsd17b12a,* whose human homologue (*hsd17b12*) participates in the hormonal synthesis pathway and the ovarian function [47], *plcz1,* whose mutations has been associated with male infertility [48], *mafb*, which is involved in the formation of male urethra [49] and *ribc2* which is related to the motile cilia function [50]. *Wee2* and *hsd17b12a* were found to be up-regulated in the female gonads while *plcz1, mafb,* and *ribc* were up-regulated in the male gonads. In fact, Chr. 19 in three-spined stickleback has been suggested to comprise the region controlling sex determination and has been shown to have the characteristics of a nascent Y chromosome [19].

Most DEG between the genders was detected as expected between the gonad tissues followed by the brain and finally the adipose tissue. The number of DEGs may vary among studies due to different thresholds applied but also due to different amounts of generated transcripts. The number of DEG between testis and ovary in the present study are amounting to 2306 transcripts (~9% of the total read number obtained) with the applied threshold of padj. 0.05 and FC > |1|. Higher numbers were reported in the sharp snout sea bream (*Diplodus puntazzo*) [51] and greater Amberjack (*S. dumerili*) [7] with DEG between gonads of ~19% and ~16% respectively. On the other hand, in turbot, only 4% were differentially expressed between male and female gonads [52]. Regarding sex differentiation in non-gonadal tissues such as brain and adipose tissue only little information is available for teleosts. In mammals, however, this has been thoroughly discussed (e.g. [53]). The main focus in teleosts has been in brain sexual dimorphism at both the physiological and gene expression level. A low proportion of differentially expressed genes between male and female whole brain tissues have previously been reported in several other fish species, such as zebrafish [54,55], the sharp snout seabream [51], the tropical gar (*Atractosteus tropicus*) [56], and in the bluehead wrasses (*Thalassoma bifasciatum*) [57]. On the other hand, higher numbers have been stated in the red porgy brain (*Pagrus pagrus*) [58]. Still, the brain has important functions in the development of hormonal pathways influencing sexual phenotypes. Therefore, future investigations to generate a brain gene expression atlas but separating the different brain regions will be of great significance. Accordingly, enrichment analysis of differentially expressed genes in male and female brains and adipose tissue did not result in any significantly enriched GO terms. Only in gonads did enrichment analysis reveal enriched GO terms for male- and female-biased expression (Fig. 3b, c). GO terms including "cilium assembly" and "microtubule-based process" dominated the male-biased enrichment analysis. The cilium is a particular eukaryotic organelle and is constrained by the extrusion of the cytoplasmic membrane. It contains a regular longitudinal array of microtubules that are attached to a centriole [59]. Cilia are present all over the male reproductive system either with a wide range of ciliary extension diversity including motile sperm flagella as well as non-motile sensory primary cilia. Primary cilia have also been suggested to serve as biosensors of the male reproductive tract [60].

Several genes were identified that were DE between males and females in more than one of the tissues studied. Among the studied tissues, the gonads and the brain shared the highest common number of differentially expressed genes (51 genes), including *gapdh1*. *Gapdh* has been frequently used as a housekeeping gene in quantitative real-time PCR studies. However, during the last years, studies have shown the presence of two *gapdh* paralogs in teleosts, with different expression patterns during early development [61]. In the present study, higher abundance in the female gonads and the female brains of the three-spined stickleback were detected. The same patterns were found in the sharp snout sea bream (*Puntazzo puntazzo*) [62] and the greater amberjack (*S. dumerili*) [7]. Gender-specific effects of *gapdh* in gonads were also shown after the exposure to 17α-ethinylestradiol (EE2) in the fathead minnow [63]. The study showed that *gapdh* is down-regulated by estrogen in males but not in females, also supporting the alleged different roles in males compared to females in teleost gonads.

Similar to mRNA, we generated for sncRNA analysis on average 21 million reads per sample. According to Sun et al., [64] a minimum sequence depth of 10 million reads per sample is required to obtain a reasonable capture for expressed miRNAs, which has been achieved in the present work. Annotation showed that among the three tissues, the gonads had the highest amount of rRNA transcripts (Fig. 5). This was expected since it has been shown that especially in the female gonads, the amount of 5 s rRNA is high [62,65]. Concerning differentially expressed miRNAs among the three tissues, the highest numbers were detected when comparing male and female gonads [27] (Fig. 6a), while significant differences in miRNA expression in the brain were not detectable in the present study (Fig. 6b), and only 10 miRNAs were differentially expressed in adipose tissue. It has been reported that regardless of the species, distinct miRNAs in the ovary are highly expressed and thus implement essential roles in ovarian function. Among those miRNAs are the let 7-family, miR-21, miR-99, miR-125b, mirR-126, mirR-143, miR-145, and miR-199b [33]. All the above-mentioned miRNAs were detected in the present work, with let-7a and miR-143 being in significantly higher abundance in the male gonads (Supplemental file 4). Let-7a and miR-143 are conserved in the mature stage of five important commercial fish species with functions in ovary development and testis maturation, while in yellowfin seabream (*Acanthopagrus latus*) gonads both miRNAs were among the most abundant ones in testis [66]. Let-7a has been thoroughly studied in mice where it performs important regulatory functions during spermatogonial differentiation via the retinoic acid signaling pathway [67]. Similar to let-7a, the miR-7 family has been shown to be involved in the teleost testis development [32]. Representatives of the miR-7 family were also found in higher abundance in the testis of the three-spined stickleback in the present study.

Among the sex-biased miRNAs, a male-biased expression of miR-202-3p was found. In mice, it was reported that miR-202-3p and -5p are highly expressed in testis, i.e., spermatogonial stem cells (SSCs). The authors further showed that miR-202-3p might act as a gatekeeper of SSC differentiation [68]. On the other hand, in medaka, it has been shown that miR-202-5p regulates the early steps of oogenesis and that the lack of miR-202 may reduce female fecundity [69]. Since the significance of miR-202 during reproduction has been widely shown also in other teleost fish species (Table 2), we identified putative targets for miR-202-3p mapping to 13 gene ontology (GO) terms (Supplemental File 5, Fig. 7). Putative targets have also been detected for a further six miRNAs i.e. miR-143, miR-7, miR-146, miR-2188, miR-455-2, and miR-727 (Fig. 7). The latter, miR-727 has been detected in Nile tilapia at higher expression levels in mature females than males, while miR-455 has been shown to be higher expressed in the male testis [70]. This conflicting expression may indicate that although miRNAs are highly conserved, they may exert different functions in different organisms. On the other hand, miR-2188 and miR-146a have been found to be mainly involved in immune response in several teleost species [71–73].

It has been shown that mRNAs may be regulated by various miRNAs and thus may cooperate in controlling the regulation of target genes [74,75]. Target search revealed alleged targets for seven miRNAs, three with higher abundance in the females' gonads and four with higher abundance in the male gonads. miRNAs with presumed targets obtained within the present work and found in other teleost species to be gender-specific expressed are listed in Table 2. GO analysis of targets with high mRNA and low miRNA expression, or vice versa is illustrated in Fig. 7. The three miRNAs with higher abundance in the female gonads are uniquely involved in the GO terms "reproduction", "reproductive process" and "multi-organism process" while the miRNAs with higher

abundance in males were uniquely assigned to the GO terms "biological adhesion", and "locomotion".

One of the best-studied genes essential for maintaining testis identity in a broad range of teleost species, *dmrt1*, has been found to be targeted by miR-455 as well as miR-2188-5p. Accordingly, we found both miRs in higher abundance in females, while *dmrt1* was detected to be more highly expressed in male gonads than in female gonads in our stickleback data. Another well-known gene influencing the sex steroid ratio is *cyp19a*. *Cyp19a* irreversibly converts androgens into estrogen and may be influenced by temperature [76]. Inhibition of *cyp19a1a* expression causes masculinizing effects and thus is considered to take a leading role in the sex ratio of vertebrates. In the present work, *cyp19a1a* was also found to be in higher abundance in the female gonads. FishTargetScan 6.2 [77,78] presumes that miR-181b is a regulator of *cyp19a1a*. The 3′UTR of the three-spined stickleback *cyp19a1a* gene was obtained within the present work but was not found in the Ensembl database. Our analysis did not predict an interaction between miR-181b and the *cyp19a1a* gene, although the mfe value for miRNA-mRNA interaction was close to the thresholds set in the present analysis. Establishing a suitable threshold of free energy turns out not to be obvious since up until today data from evaluated miRNA-mRNA complexes are still scarce [79], thus the interaction between miR-181b and the cyp19a1a gene may be worthy of further investigation.

In summary, we present a transcriptomic analysis comprising mRNA as well as sncRNA of sex-specific differences in the three-spined stickleback. In three tissues, we define the most highly expressed and DEGs that characterize this model species and identify possible interactions between the coding and non-coding genome responsible for sexual differentiation. In particular, our study adds further weight to earlier observations that Chr. 19 is the principal locus of sex determination in the three-spined stickleback.

4. Material and methods

4.1. Sampling

Female and male three-spined stickleback gonad, brain, and adipose tissue were collected in three replicates ($n = 3$) following the European Union Directive 2010–63-EU on the protection of animals used for scientific purposes. The fish originated from the long-established colony of the Centre for Environment, Fisheries and Aquaculture Science (Cefas) Weymouth laboratory, UK. These samples used for analysis were collected from control fish that were part of different investigations in which the kidney and the liver were the target tissues, thereby applying the three Rs principle. The housing and care of the three-spined stickleback colony has been as described in Blaker et al., 2022 [52]. At the time of sampling, groups of about 100 individuals of mixed sex were maintained in 100 L tanks at 17 ± 1C, 12hL:12hD photoperiod. Fish were fed to satiation twice daily with frozen mosquito larvae. Sampling took place when the sticklebacks were 8 months old (1.5 ± 0.2 g). The sex of the fish was assigned during dissection by macroscopical examination of the gonads. Before dissection fish were anesthetized in buffered tricaine mesylate (MS-222) solution, weighed, measured, and killed by a schedule 1 method and according to the UK Home Office legislation. Samples were immediately removed and flash-frozen in liquid nitrogen and stored at −80 °C until they were dispatched in dry ice to the Institute of Marine Biology, Biotechnology, and Aquaculture at the Hellenic Centre for Marine Research in Crete, Greece.

4.2. Total RNA extraction

According to the manufacturer's instructions, the total RNA of all samples was extracted using Nucleospin miRNA kits (Macherey-Nagel, Düren, Germany). In brief, tissue was disrupted with mortar and pestle in liquid nitrogen and homogenized in lysis buffer by passing lysate through a 23-gauge (0.64 mm) needle five times. RNA quantity was estimated by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), while the quality was evaluated by agarose (1%) gel electrophoresis, as well as capillary electrophoresis (Agilent 2100 Bioanalyzer) using RNA Nano Bioanalysis chip (Agilent Technologies, Santa Clara, CA 95051, USA). Samples with a 28 s:18 s of 2:1 and an RNA integrity number value >8 were used for library construction.

4.3. Library preparation and sequencing

Eighteen mRNA libraries (three libraries per tissue and gender) were prepared using the Truseq stranded mRNA library preparation kit (Illumina, 5200 Illumina Way, San Diego, CA 92122 USA) according to the manufacturer's instructions, with multiplexed adapter ligation. Library size was checked on a Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA 95051, USA), and concentration was quantified by qPCR, using the Kappa Library Quantification kit (Kappa Biosystems, Wilmington, MA 01887, USA).

For small RNA library preparation *NEBNext® Small RNA* Library Prep Set for Illumina was used (New England Biolabs Inc., 240 County Road Ipswich, England). All libraries were sequenced on an Illumina HiSeq 2500 with 150 bp paired-end reads (PE) for mRNA libraries and 50 bp single-end reads (SE) for small RNA libraries.

4.4. Data acquisition

All sequencing reads generated for each library were quality checked using FastQC (v0.11.5) [80]. Adaptor sequences, as well as low-quality nucleotides, were removed, applying the standard protocol for trimming NGS data Trimmomatic (v. 0.32) [81] with the parameters phred33, minlen:36, slidingwindow:4:15, and trailing:3. Cleaned sequence reads were mapped to the three-spined stickleback's genome (Gasterosteus_aculeatus.BROADS1.dna.toplevel.fa, http://ftp.ensembl.org/pub/release-76/fasta/gasterosteus_aculeatus/dna/) using CLC workbench [82] and mirDeep2. For downstream analysis, read counts were transcripts per million (TPM). SncRNAs with less than five reads across all samples were discarded.

4.5. Annotation

Annotation of the assembled transcriptome was carried out by i) mapping reads to the genome of stickleback with default parameters, ii) submission to blastx search against the nr database of NCBI with *E*-value $<1 \times 10^{-8,}$ and iii) using Blast2GO platform with default parameters [83]. Blast2GO platform was also used for gene ontology (GO) terms assignment. Small RNA transcripts were annotated applying mirDeep2 software and the publicly available database GAmiRdb: http://bio.kuleuven.be/apps/gamirdb/index.php#top.

4.6. Putative miRNA – mRNA interaction

To investigate putative miRNA-mRNA interactions three-spined stickleback 3′UTR sequences were extracted using the Ensemble tool BioMart. For genes known to be involved in the steroid hormone biosynthesis pathway, but for which the 3′UTR sequence was not available via the Ensemble database, transcripts detected in the present study were examined to retrieve the 3′UTR of the genes of interest. Obtained 3′UTRs and miRNAs found in different abundances between the male and the female gonads were submitted to RNAhybrid vs2.1.2. The threshold was set to the minimum free energy (mfe) of < -25.

4.7. Differential expression and data evaluation

The generated mRNA count matrix served as the input file for differential expression analysis, following the EdgR pipeline integrated with SARTools [84]. After pairwise comparison, transcripts with *p*-values adjusted according to the Benjamini-Hochberg procedure (padj)

≤ 0.05 and log2fold change ≥ |1| between male and female tissues were considered as differentially expressed. MDS and volcano plots were generated in R3.5.1 [85]. Differentially expressed miRNAs were detected applying CLC workbench [82]. SncRNAs with less than five reads across all samples were discarded and sncRNA with p-values <0.005 and logFC >2 were considered to be differentially expressed between male and female tissues.

4.8. Enrichment analysis

Two-tailed enrichment analysis with default parameter (filter value: 0.05 filter mode: FDR, two-tailed) was applied. The transcriptome downloaded via the Ensemble BioMart served as the reference dataset, while the differential expressed transcripts detected in the gonads, the brain, and the adipose tissue after comparison of females and males were defined as the test set.

Data availability

All reads generated in the present study from Illumina sequencing were submitted to the Sequence Read Archive (SRA) of NCBI under the BioProject accession number PRJNA723504.

Author contribution

E.K. carried out RNA extractions and evaluation contributed to the analysis and writing. G.D.G generated the libraries and performed Illumina sequencing, E.A. conceptualization of sampling, and contributed to drafting the manuscript. E.S. conceived and designed the study, carried out the analysis, and drafted the manuscript.

Funding

This work was supported by the Ministry of Education and Religious Affairs, under the Call “ARISTEIA I" of the National Strategic Reference Framework 2007–2013 (ANnOTATE), co-funded by the EU and the Hellenic Republic through the European Social Fund.

Declaration of Competing Interest

None.

Acknowledgments

This research was supported in part through computational resources provided by IMBBC (Institute of Marine Biology, Biotechnology, and Aquaculture) of the HCMR (Hellenic Centre for Marine Research). Funding for establishing the IMBBC HPC has been received by the MARBIGEN (EU Regpot) project, LifeWatchGreece RI, and the CMBR (Centre for the study and sustainable exploitation of Marine Biological Resources) RI.” The authors would like to thank Dr. Ioanna Katsiadaki from Cefas Weymouth laboratory, UK, for providing us with the fish tissues. Sequencing was performed by the Norwegian Sequencing Centre (www.sequencing.uio.no), a national technology platform hosted by the University of Oslo and Oslo University Hospital and supported by the “Functional Genomics” and “Infrastructure” programs of the Research Council of Norway and the Southeastern Regional Health Authorities.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2021.12.010.

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