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Sex-Biased miRNA Expression in Atlantic Halibut *(Hippoglossus hippoglossus)* Brain and Gonads

T.T. Bizuayehu[a] J. Babiak[a] B. Norberg[b] J.M.O. Fernandes[a] S.D. Johansen[a, c]
I. Babiak[a]

[a]Faculty of Biosciences and Aquaculture, University of Nordland, Bodø, [b]Institute of Marine Research, Austevoll Research Station, Storebø, and [c]Department of Medical Biology, Faculty of Health Sciences, University of Tromsø, Tromsø, Norway

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Atlantic halibut · Fadrozole · Methyltestosterone · miRNA · Next-generation sequencing · Sex differentiation · Sex reversal · Teleost

Abstract
The role of miRNA in fish sexual development is not elucidated yet. We profiled miRNAs in gonads and brains of Atlantic halibut using SOLiD sequencing technology. We found tissue- and sexually dimorphic expression of several miRNAs, including miR-29a, miR-34, miR-143, miR-145, miR-202-3p, miR-451, and miR-2188. miR-9 and miR-202 were abundant in brain and gonads, respectively. In the next step, we selected some miRNAs showing differential expression patterns between sexes and performed RT-qPCR on 3 age groups: juveniles, 3-year-, and 5-year-olds. In brains, miR-451 was significantly down-regulated in juveniles compared to adults. let-7a, miR-143, and miR-202-3p were up-regulated in gonads of mature males compared to immature females at the same age. We investigated the effect of suppressing aromatase cytochrome P450 enzyme on miRNA expression at the onset of sex differentiation through masculinization with Fadrozole or 17-α-methyltestosterone. We found significant differences in miRNA expression between masculinized individuals and untreated controls. miR-202-3p was significantly down-regulated in female juveniles compared to male juveniles. The expression levels of let-7a and miR-451 were restored after termination of the masculinization treatment. Our data give a first insight into miRNA involvement in sexual development in teleosts.

microRNAs (miRNAs) are small (~18–26 nt) regulatory, non-coding RNAs involved mostly in mRNA degradation and post-transcriptional repression. A number of mature miRNA sequences are conserved in organisms phylogenetically as divergent as amphibians, birds, fish and mammals. miRNAs have been implicated in a number of developmental processes, such as maternal transcript clearance (miR-430) and axial patterning (miR-196) during embryonic development [Giraldez et al., 2006; He et al., 2011]. They have also been shown as important part of the regulatory elements in neuronal differentiation (miR-9, -124, -3099) and spermatogenesis (let-7) in mammals and sex differentiation in chicken (e.g. miR-202) [Ahn et al., 2010; Bannister et al., 2011; Tong et al., 2011; Yuva-Aydemir et al., 2011].

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Igor Babiak
Faculty of Biosciences and Aquaculture
University of Nordland, PO Box 1490
NO–8049 Bodø (Norway)
Tel. +47 755 17 922, E-Mail igor.babiak@uin.no

In vertebrates, a number of miRNAs show a temporal and spatial pattern of expression, such as miR-9 and miR-124, which are abundant in brain [Kapsimali et al., 2007], while miR-202 is found abundantly in gonadal tissues [Bannister et al., 2009, 2011]. Recently, we reported on the miRNA expression profile during early development in Atlantic halibut, *Hippoglossus hippoglossus* [Bizuayehu et al., 2012], the largest flatfish of the Atlantic Ocean with high commercial value, but tissue distribution of miRNA expression has not been explored yet in this species.

In fishes, master sex-determining genes have been found so far in Patagonian pejerrey *(Odontesthes hatcheri)* [Hattori et al., 2012], medaka [Weltzien et al., 2002; Imsland and Jonassen, 2005; Matsuda et al., 2007] and Salmonidae (Yann Guiguen, pers. commun.). Gonadal cytochrome P450 aromatase is the most important effector of the sex-regulatory pathway in fish species, as it converts testosterone to estrogens [Guiguen et al., 2010]. High expression of the gonadal aromatase gene *(cyp19a1a)* and high enzyme activity in gonads are associated with femaleness. Expression of aromatase and estrogen receptor genes has been studied in Atlantic halibut [van Nes and Andersen, 2006]. Aromatase has brain and gonadal isoforms. Gonadal *cyp19a1a* expression in halibut is detectable as early as at hatching stage [van Nes et al., 2005]. Atlantic halibut has an XY sex-determining system, with heterogametic males and homogametic females [Tvedt et al., 2006]. In histological sections, germ cells are first visible at approximately 10 mm fork length (L_F), at the yolk-sac stage. Then they migrate towards the posterior of the abdominal cavity where the presumptive gonad elongates at 21–29 mm L_F, at the metamorphosis stage. Gonadal sex differentiation occurs by 38 mm L_F, which coincides with the post-metamorphic settled stage [Hendry et al., 2002]. At this stage, the ovarian cavity and mitotic oogonia are visible, but the presumptive testis remains undifferentiated. The first distinguishable spermatogonial cells can be identified at 74 mm L_F [Hendry et al., 2002]. In the wild, males mature after 4 years (total length, T_L, 55 cm and body weight, BW, 1.7 kg), while females reach sexual maturation after 7 years (average T_L = 112–115 cm and BW = 18 kg) [Jákupsstovu and Haug, 1988]. Masculinization treatment applied prior to gonad differentiation can alter the phenotypic sex of genetic females, resulting in gonadal differentiation towards testes. This can be achieved in halibut through administration of either a poorly convertible testosterone analogue, such as 17α-methyldihydrotestosterone or 17-α-methyltestosterone (MT), or an aromatase inhibitor, such as Fadrozole (FA) [Hendry et al., 2003; Babiak et al., 2012].

The regulatory roles of miRNAs in teleost reproductive development have not been investigated extensively. An effort has been reported to identify and characterize miRNAs during sex differentiation and sexual maturation in rainbow trout [Juanchich et al., 2011]. Based on mammalian models, miRNAs can play a role in sex differentiation and sexual development in teleosts. In mammals and birds, several studies have been reported on the regulatory modulations of miRNAs during ovarian development and spermatogenesis [Bannister et al., 2009; Papaioannou and Nef, 2010; Tong et al., 2011].

The primary objective of this study was to analyze miRNA expression in brain and gonad of Atlantic halibut males and females. We performed deep sequencing using SOLiD technology. Emphasis was given on the search for sex- and/or tissue-specific expression. Selected miRNAs that showed differential expression patterns were further profiled in 3 age groups (juveniles, 3-year-, and 5-year-old individuals) using quantitative real-time PCR (RT-qPCR). Also, we investigated miRNA expression in juveniles subjected to masculinization treatment at the onset of sex differentiation. We found sex- and/or tissue-biased expression of several miRNAs, some of them showing a differing pattern from that observed in other vertebrates so far. Our results are the first miRNA characterization in a reproductive system in teleosts and suggest possible roles of miRNAs in teleost sexual development.

Materials and Methods

Husbandry, Treatments and Sampling

Experiments were conducted strictly according to the guidelines of the National Animal Research Authority, Norway (Forsøksdyrutvalget). Fish were reared at the University of Nordland, Bodø, Norway, and the Institute of Marine Research, Austevoll Research Station, Storebø, Norway, under standard aquaculture procedures [Mangor-Jensen et al., 1998; Babiak et al., 2006]. Samples were obtained from 3 age groups: 2-month-old juveniles (n = 10), 3-year-old (n = 12), and 5-year-old individuals (n = 12). The 3 sex developmental stages represented (1) onset of gonadal formation [Hendry et al., 2002], (2) immature individuals (3-year-old males, average T_L = 36.2 cm, BW = 0.98 kg; 3-year-old females, T_L = 37.5 cm, BW = 1.02 kg; and 5-year-old females, T_L = 73.75 cm, BW = 3.18 kg) and (3) mature males (5-year-old males, T_L = 68.17 cm, BW = 2.36 kg) at stage IV testis [Weltzien et al., 2002]. Females at 5 years were immature, in a primary oocyte stage.

The sex reversal experiment has been described previously [Babiak et al., 2012]. In brief, Atlantic halibut post-metamorphic larvae (average T_L = 30 mm), before gonadal differentiation [Hendry et al., 2002], were fed for 6 weeks a formulated diet supplemented with either 5 mg/kg feed MT, a synthetic androgen ana-

logue poorly convertible by aromatase cytochrome P450 enzyme, or 100 mg/kg feed FA, an aromatase inhibitor; an untreated control group was also created. Samples were collected at the end of the 6-week treatment period (6W) and 2 weeks after the end of the treatment period, at 8 weeks (8W). Samples were snap-frozen in liquid nitrogen and stored at –80°C.

RNA Extraction

For deep sequencing, total RNA was extracted from brains and gonads of 3-year-old male (n = 6) and female (n = 6) Atlantic halibut. At this age both sexes were immature. Equal portions of RNA were pooled within sexes. For RT-qPCR, skin, muscle, gut and kidney tissues were collected from 5-year-old males (n = 6) and females (n = 6). Based on a previous study that showed the location of an emerging gonad in halibut [Hendry et al., 2002], which could not be visually distinguished yet in our study, we cut a posterior part of the visceral cavity, containing part of the guts and kidney, and including the surrounding muscle and skin tissues from 6W (n = 10) and 8W (n = 10) juveniles to analyze gonadal expression. To discriminate the expression of selected miRNAs in the gonads, we extracted RNA from gut, kidney, muscle and skin separately, and performed RT-qPCR.

RNA was extracted using TRIzol Reagent (Invitrogen) by homogenizing the whole brain or gonadal tissues. RNA quantity and integrity was checked on a Bioanalyzer (Agilent Technologies).

Deep Sequencing and Data Analysis

Small RNA libraries were prepared as previously described [Bizuayehu et al., 2012] with 15 cycles of PCR amplification. Sequencing of small RNAs was performed using the SOLiD 3 system on a quadrant of a slide with 50-nt read length.

Sequence reads were analyzed using the RNA2map pipeline (SOLiD Software Development Community). All parameters for the pipeline and normalization methods were put as previously described [Bizuayehu et al., 2012]. Mapping of all small RNAs in this study was performed de novo to find uncharacterized miRNAs in the studied tissues.

To identify tissue-abundant miRNAs, the geometric mean of normalized counts in a gonad was divided by the geometric mean of normalized counts in a brain to identify abundant miRNAs in gonad and vice versa to identify brain-abundant miRNAs. miRNAs having more than 5-fold difference between the tissues and at least 1,000 read counts in one of the tissues were considered as highly expressed. These identified miRNAs were further compared to other species by blasting our sequences to miRBase 18 databases (www.mirbase.org) and searching through previously published results. miRNAs were considered as abundant when the ratio between normalized read counts in gonadal or brain tissues had at least 2-fold difference between the sexes.

Determination of Sex in Juveniles

Since visual identification of gonads in early juvenile stages was impossible, we used *cyp19a1a* expression as a molecular marker of female sex [van Nes et al., 2005; van Nes and Andersen, 2006] using forward primer 5′-GTGCCCGTCAATGAGAAAGA-3′ and reverse primer 5′-TGACTGCCGCCTTGTGC-3′ (online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000341378).

RT-qPCR

We performed separate RT-qPCR analyses for age groups and the sex reversal experiment. For age groups, RT-qPCR was done on brain and gonadal tissues of 6 individuals from each sex (2-month-old juveniles, 3-year-, and 5-year-old fish). For the sex reversal study, brain and gonadal tissues of 5 individuals from each treatment group and 10 individuals (5 males and 5 females) from untreated groups were quantified.

miRNA RT-qPCR data normalization can be done using the mean value of the panel of miRNAs, but in case of few miRNAs, reference genes shall be selected by testing their stability in a given experiment [Mestdagh et al., 2009]. For normalization, we tested multiple genes for each experiment separately using 2 detection chemistries. The final normalized data is not affected as far as the same detection chemistry is used for a single gene in a given experiment. The investigated miRNAs and 2 endogenous reference small RNA (U6 and 5S RNA) quantifications were done using TaqMan MicroRNA assays (Applied Biosystems). To compare the suitability of reference genes we also tested 3 endogenous mRNAs: β-actin *(Actb)*, eukaryotic translation elongation factor 2a *(Eef2)*, and β2-tubulin *(Tubb2)*, which have been used in RT-qPCR expression analyses of different developmental stages in Atlantic halibut [Fernandes et al., 2008], and 1 exogenous reference gene, luciferase *(Luc)* RNA (Promega), added at the concentration of 1 pmol/μg of total RNA. Reverse transcription of mRNA was performed using QuantiTect reverse transcription kit (Qiagen) with primer mix following the manufacturer's instructions. We used SYBR green I (Roche) for detection of the 3 mRNAs and the exogenous gene.

The RT-qPCR was performed on LightCycler 480 (Roche) using white 96-well plates (Roche). For small RNA probes, the PCR cycle conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. SYBR green-based detections were done under the thermal cycle conditions of 95°C for 15 min, followed by 45 cycles of 95°C for 15 s, 60°C for 20 s and 72°C for 20 s.

Selection of Reference Genes

The suitability of reference genes for normalization of RT-qPCR data was evaluated using Normfinder, which considers the variation between sample groups [Andersen et al., 2004]. The best 2 reference gene combinations were chosen for each experiment separately.

Among the 6 tested reference genes, for the 3 developmental stages, *Luc* and *Tubb2* were the best combination of genes for normalization of data obtained from gonads, whereas *Luc* and *Actb* were the best combination for brain samples.

We also performed similar reference gene tests for the sex reversal experiment. U6 and *Eef2* were found as the best combination of reference genes for gonadal samples, whereas U6 and *Luc* were the best reference for brain samples.

Statistical Analysis

Statistical differences were computed by a relative expression software tool, REST [Pfaffl et al., 2002], using 5,000 iterations. Five-year-old males were used as a reference point for age groups. Similarly, 6W and 8W males were used for the sex reversal study as a reference point for 6W and 8W treatment groups, respectively. Values with $p < 0.05$ were considered as significant. Considering the inclusion of other tissues during the total RNA extractions

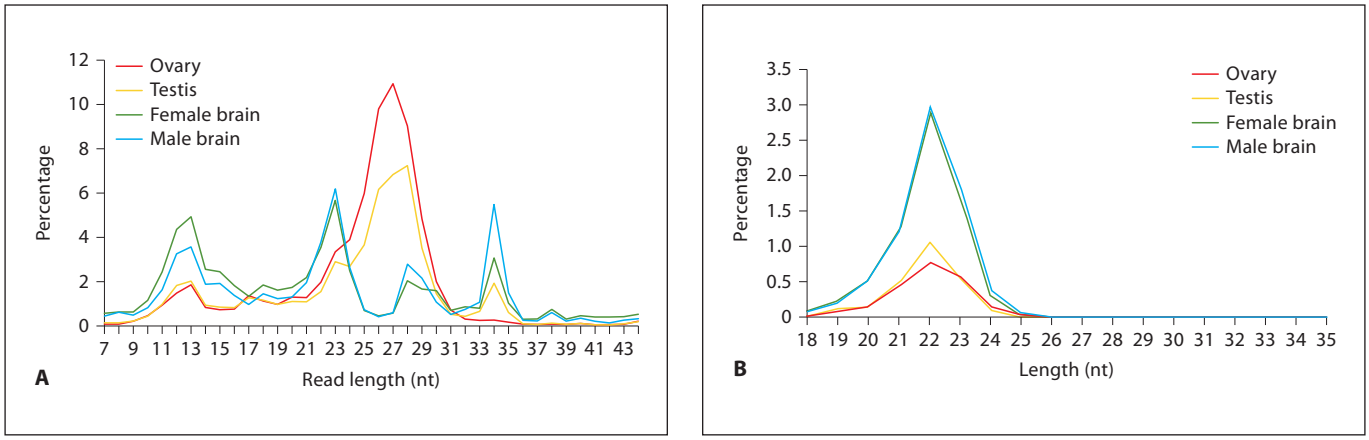

Fig. 1. The size distribution of SOLiD libraries (**A**) and miRNAs (**B**) in brain and gonadal tissues of 3-year-old Atlantic halibut. Sequences >44 nt are not shown for purpose of visualization; their frequency was 40, 32, 44, and 45% in ovary, testis, female brain, and male brain, respectively.

from a juvenile gonad, we assumed that comparing RT-qPCR results between juveniles and adults is meaningless. Thus, no statistical differences were inferred between juveniles and adults. Since we had no mature females, we were cautious in the interpretation of the significance differences between mature males and females of the same age.

Results

Small RNA Libraries from Atlantic Halibut Brain and Gonads

The number of usable reads in brains and gonads exceeded 12 millions. Gonadal libraries were enriched with 26–28-nt small RNAs, whereas the brain tissues were enriched with miRNA fractions (21–24 nt). Except for ovarian tissue, abundant small RNA fractions of size 34 nt were observed in the datasets (fig. 1A). The frequency of miRNAs was 2.8% in ovary, 2.9% in testis, 9.9% in female brain, and 11.8% in male brain (online suppl. fig. 2). Length distribution of mapped miRNA reads showed a peak at 22 nt in both sexes and tissues (fig. 1B). The percentage of unmapped reads was over 67% (online suppl. fig. 2).

Identification and Expression of Conserved miRNAs

Although the diversity and number of miRNA reads in the brains were by far higher than in the gonads (online suppl. table 1), considerable numbers of identical miRNAs were found in both tissue types. We found 150, 151, 169 and 168 conserved miRNAs in the ovary, testis, female brain and male brain, respectively (online suppl. table 2). Among miRNAs that exceeded 500 normalized counts, 7 sex-biased miRNAs in brain (miR-9-3p, -124, -129-3p, -137, -153b, -212, and -219) and a single sex-biased miRNA in gonads (miR-202-3p) were found (fig. 2A, online suppl. table 3 and fig. 3).

Thirty-one miRNAs in gonads had at least 2-fold difference in expression between sexes; these included 17 ovary-enriched (such as miR-15a, -29a, -34, -143, -202-3p, and -733) and 14 testis-enriched miRNAs (such as let-7a, miR-1, -24, -126, -146a, and -301c) (fig. 2B, online suppl. fig. 3). Three miRNAs in female brain (miR-130a, miR-451, and miR-2188) and 4 miRNAs in male brain (miR-7a, miR-219, miR-137, and miR-724) were expressed 2-fold higher than in the opposite sex (fig. 2C, online suppl. fig. 3).

At least 2-fold difference in the expression pattern between the sexes was found among some miRNA families. For instance, among let-7 and miR-103 families, let-7a, e, f and g, and miR-103 showed higher expression in testis, while let-7h and i, and miR-107b were highly expressed in ovary. In some miRNAs the guide and the passenger strands had sex-biased expression patterns, for instance miR-126 and miR-210 families had higher expression of the passenger strand in testis but of the guide strand in ovary.

Based on the deep sequencing results, we selected some miRNAs that showed differences between sexes to investigate their expression in the 3 age groups, namely juveniles, 3-year-old immature males and females, and 5-year-old mature males and immature females. Since visual identification and separate extraction of RNA from

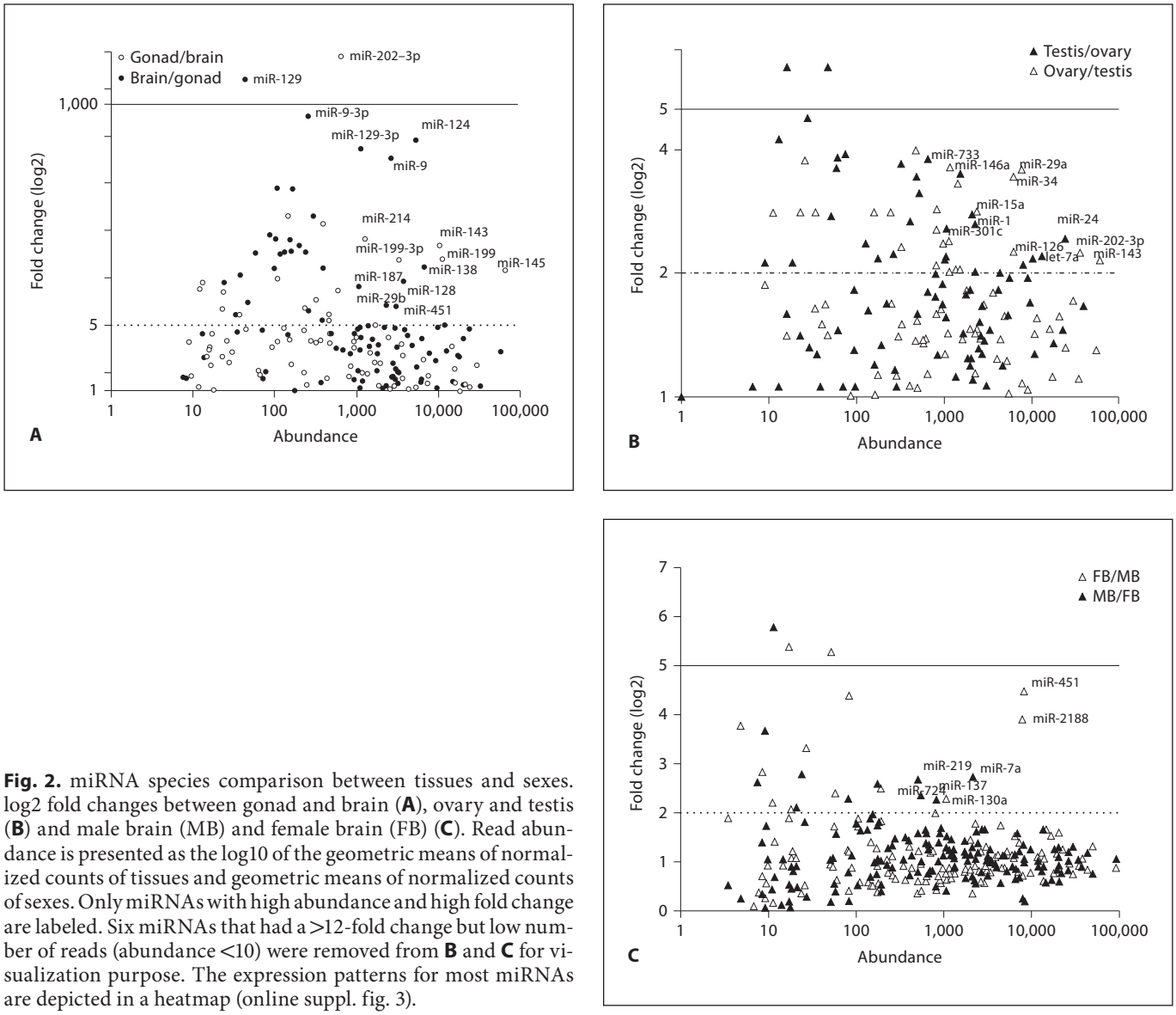

Fig. 2. miRNA species comparison between tissues and sexes. log2 fold changes between gonad and brain (**A**), ovary and testis (**B**) and male brain (MB) and female brain (FB) (**C**). Read abundance is presented as the log10 of the geometric means of normalized counts of tissues and geometric means of normalized counts of sexes. Only miRNAs with high abundance and high fold change are labeled. Six miRNAs that had a >12-fold change but low number of reads (abundance <10) were removed from **B** and **C** for visualization purpose. The expression patterns for most miRNAs are depicted in a heatmap (online suppl. fig. 3).

early juvenile gonads was impossible, we examined the expression of let-7a, miR-19b, miR-24, miR-143, miR-145, and miR-202-3p in neighbor tissues, i.e. gut, kidney, muscle, and skin. miR-143 and miR-145 were expressed in all the examined tissues, while the remaining miRNAs showed no expression (online suppl. fig. 4).

We found significant differences among age groups and between sexes in let-7a, miR-9, miR-143, miR-145, miR-202-3p and miR-451 expressions (fig. 3A, B). The expression of miR-143 and miR-145 was higher in immature males than in immature females. miR-202-3p showed significantly higher expression in immature males compared to mature males (fig. 3A). miR-202-3p expression was higher in male juveniles compared to female juveniles. In brain tissue, miR-9 showed higher expression in immature males compared to juveniles and mature males, while miR-451 showed higher expression in 3-year-old females compared to juveniles and 5-year-old females (fig. 3B).

miRNA Expression in Brains and Gonads of Halibut Subjected to Masculinization

We found significant differences among masculinized and control groups in let-7a and miR-202-3p expression

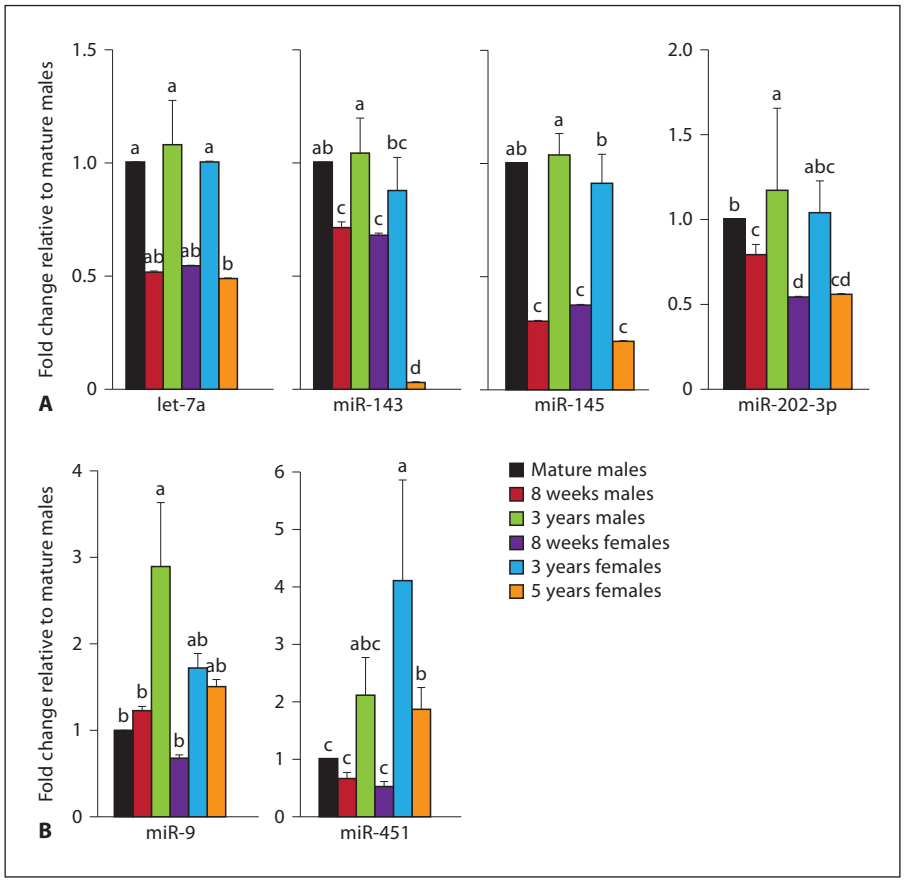

Fig. 3. The expression of selected miRNAs in 3 age groups using RT-qPCR. The fold changes in gonads (**A**) and brains (**B**) are calculated relative to mature males. Normalizations were performed using the geometric mean of *Luc* and *Tubb2* for gonadal samples, likewise *Luc* and *Actb* for brain samples. The sample sizes were 5 for juvenile males and 6 for the rest of the groups. The error bars and different letters denote standard deviations and significant differences ($p < 0.05$), respectively.

patterns. let-7a was significantly ($p < 0.05$) up-regulated in FA-treated fish at 2 weeks after the treatment (8W) compared to expression at the end of the treatment (6W; fig. 4A). The expression of miR-202-3p was significantly down-regulated in control females compared to control males (6W, $p < 0.05$). Significant down-regulation of miR-202-3p was also found in 6W females compared to control 8W males ($p = 0.01$) and 8W females ($p < 0.01$), FA ($p < 0.01$) and MT ($p < 0.001$) groups (fig. 4A). The expressions of miR-19b and miR-24 were not influenced by either masculinization treatment.

In contrast to miR-124, which showed similar expression in brain tissue ($p > 0.05$), miR-451 was differentially expressed among the treatment groups (fig. 4B). The expression of miR-451 was significantly down-regulated in the MT group compared to that of 6W control male and female groups ($p < 0.05$). FA-treated fish at 8W had significantly lower expression of miR-451 than FA-treated fish at 6W ($p < 0.05$).

Discussion

Deep Sequencing of Atlantic Halibut Brain and Gonadal Small RNA

Deep sequencing provides unprecedented detail of miRNAs expressed in a given sample. To our knowledge, this is the first report on sexually dimorphic expression of miRNA in the reproductive system of a teleost animal using deep sequencing data further supported with RT-qPCR analysis. Our SOLiD data contained various species of small RNAs clustered in different size ranges. Size distribution of the reads was similar to that of Atlantic halibut developmental stage profiles [Bizuayehu et al., 2012]. Uncharacterized very small miRNAs (11–13 nt) have been found also in humans [Schulte et al., 2010]. Reads of ~22 nt corresponded to miRNAs. Considerable enrichment in small RNAs of 26–29 nt in gonads (fig. 1A) corresponds to piRNAs, which have been implicated in germ cell maintenance and transposon silencing [Houwing et al., 2007; Ro et al., 2007]. Given the fact that limited genomic resources are currently available for Atlantic halibut, large

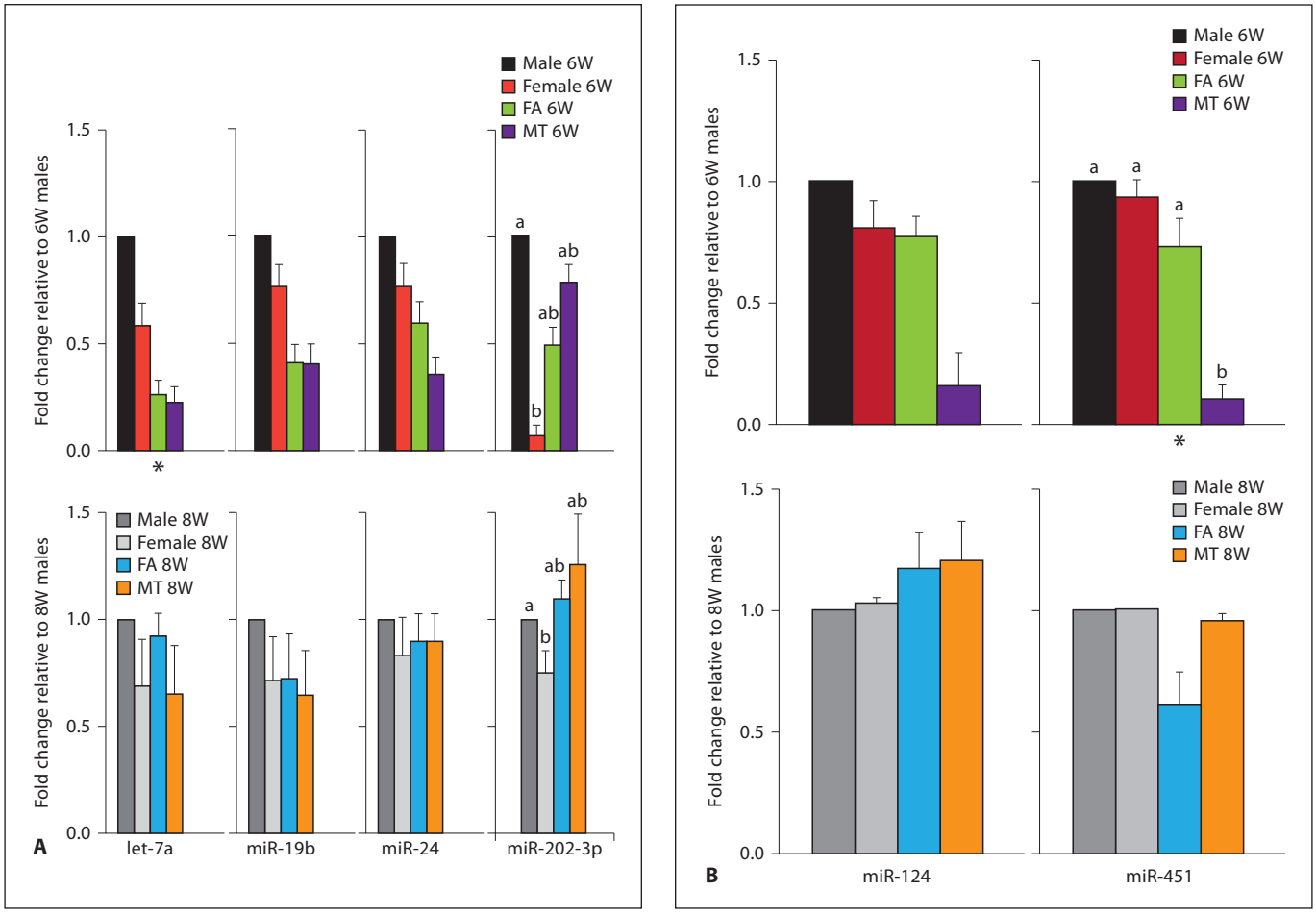

Fig. 4. The expression of miRNAs in masculinized and control post-metamorphic Atlantic halibut using RT-qPCR. The fold changes in gonads (**A**) and brains (**B**) are calculated relative to control males for the 6W and 8W groups separately to compare treatment effects in a given time point. For comparison of time effect within a given treatment, fold changes are calculated relative to control 6W males (detailed description of the groups is given in Materials and Methods). Normalizations were performed using the geometric mean of U6 and *Eef2* for gonadal samples (n = 5 for each group, except for miR-202-3p where n = 10 for each group), likewise U6 and *Luc* for brain samples (n = 5 for each group). Error bars denote standard deviations, the different letters show significant differences in expression between groups within a given sampling time, and asterisks represent significant differences between sampling time within the same treatment ($p < 0.05$).

numbers of reads remained unmapped. However, a large proportion of deep sequencing data remains uncharacterized or discarded because of sequencing errors in model species [Morin et al., 2008; Ling et al., 2011]. SOLiD sequencing technology has an advantage over other similar technologies in discriminating single nucleotide polymorphisms from sequencing errors by interrogating each nucleotide in 2 different ligation steps. However, in the absence of a genome database, a single color space error can propagate to the subsequent color calls, and in consequence the number of mapped reads could considerably be reduced in non-conserved genomic regions. Thus, we assume that the high number of unmapped reads in our datasets can be attributed to the lack of Atlantic halibut genome data and to the stringent criteria of mapping.

Sex-Biased Expression of Conserved miRNAs

Brain and gonad, organs quite different in cellular origin and structure, shared a considerable number of miRNAs. Of the top 27 most expressed miRNAs in brain and gonads, 7 miRNAs (miR-19b, -24, -99, -100, -125b, -125c, and -130c) showed similar expression in both tissues. The remaining miRNAs were expressed preferentially in one of the tissues (online suppl. table 3). miR-124

and miR-9 were the most abundant miRNAs in Atlantic halibut brain. Previously they were found enriched in mammalian brain [Sempere et al., 2004; Linsen et al., 2010; Ling et al., 2011]. Other miRNAs, such as let-7a, miR-103 and miR-107, were also expressed abundantly, though not exclusively, in Atlantic halibut brain. miR-2188 showed sex-biased expression in Atlantic halibut brain; it was also found in songbirds brain [Warren et al., 2010], zebrafish brain, eye, gills and skin [Soares et al., 2009] and clawed frog skin [Armisen et al., 2009], but has not previously been reported as sex-biased. We found expression of miR-212 and miR-219 in Atlantic halibut brain but not in gonads; contrary, in mammals, their expression has been reported in mouse ovary (online suppl. table 4). These differences could result from the non-conserved binding sites at the 3′ UTR of target mRNAs in different species [Gao, 2010]. Abundantly expressed gonad-biased miRNAs in Atlantic halibut were limited to miR-202 and miR-202-3p, previously reported in gonads of frog, chicken, mouse and rat [Armisen et al., 2009; Bannister et al., 2009, 2011; Chiang et al., 2010; Linsen et al., 2010], suggesting the conserved roles of miRNAs among similar tissues of distantly related species, probably targeting orthologous genes.

Preferential expression of one of the duplexes (-5p or -3p) of mature miRNA has been observed in different tissues of mouse [Chiang et al., 2010]. In chicken, sex-dependent differential regulation of the 2 arms of miR-202 has been reported [Bannister et al., 2009]. Similarly, several miRNA families were differentially expressed in 2 regions of mouse brain [Juhila et al., 2011]. In the present study, we found differential expression of miRNAs within families and miRNA arms between tissues and sexes in a teleost, which could result from target preference of miRNAs as it has been previously shown in mammals [Juhila et al., 2011]. Alternatively, it can be an effect of variation in post-transcriptional processing and in vivo accumulation or stability of miRNAs [Bail et al., 2010].

In this study, let-7a, miR-143, and miR-145 showed similar expression patterns, with significant up-regulation in sexually mature males compared to females and immature males (fig. 3A). Functions of these miRNAs in sexual maturation have not been reported in fish yet. miRNAs are important in testis development and spermatogenesis in mammals [Hayashi et al., 2008; Papaioannou et al., 2009; Papaioannou and Nef, 2010]. let-7a participates in defining testis-derived germline stem cells in humans [Jung et al., 2010]. In mice, let-7 families play a role in repressing genes involved in spermatogonial proliferation and promote spermatogonial differentiation through retinoic acid signaling [Tong et al., 2011]. miR-143 and miR-145 inhibit cell proliferation and differentiation in mammals [Gammell, 2007; Sirotkin et al., 2010]. In addition, miR-145 has been shown in double-negative feedback loops involving *OCT4*, a pluripotency factor essential in human embryonic stem cell self-renewal [Xu et al., 2009]. miR-143 may promote apoptosis in germinal epithelium cells. In malignant cells, miR-143 has been shown in targeting *BCL2* (a gene that blocks physiological apoptosis) and other cell cycle regulators [Ugras et al., 2011]. One of the features of spermatogenesis is incomplete cytokinesis, which is observed in apoptotic germ cells [Print and Loveland, 2000]. Apoptosis is a part and parcel of fish spermatogenesis, although not as intensive as in mammals [Schulz et al., 2010]. In mice, the first wave of spermatogenesis is accompanied by a wave of apoptosis, observed also throughout the adult stage, to maintain an optimal ratio between Sertoli cells and germ cells [Rodriguez et al., 1997]. Apoptosis is under the control of several negative and positive regulators, *BCL2* is one of those that shield programmed cell death [Furuchi et al., 1996], and a misexpression of this gene has been implicated in abnormal spermatogenesis [Furuchi et al., 1996; Rodriguez et al., 1997]. Taken together, the significant up-regulation of let-7a, miR-143, and miR-145 in sexually mature Atlantic halibut males suggests that these miRNAs are a component of the spermatogenesis regulatory pathway that may arbitrate germ cell fate through controlling proliferation and apoptosis. The balance between spermatogonial self-renewal and differentiation can be vital, especially for seasonally reproducing organisms like Atlantic halibut.

We found that miR-9 was up-regulated and miR-451 was down-regulated in male brain compared to female brain in 3-year-old halibut (fig. 3B). miR-9 has been implicated in neuronal progenitor cell proliferation and migration, neuronal differentiation, in restricting the extent of midbrain and hindbrain boundary, and brain plasticity [Delaloy et al., 2010; Gao, 2010; Yuva-Aydemir et al., 2011]. miR-451 is involved in erythroid homeostasis [Pase et al., 2009]. Although a single miRNA, for instance miR-9, has multiple targets in various functional pathways [Gao, 2010; Yuva-Aydemir et al., 2011], the biological significance of the observed sex-biased expression of miR-9 and miR-451 is unknown.

miRNAs and Sex Differentiation in Atlantic Halibut

Gonadal expression of miRNA has been reported in various species. For example, miR-202-3p has been found abundantly in gonads of mouse and *Xenopus* [Ro et al., 2007; Armisen et al., 2009]. miR-202-3p was up-regulated

in chicken testis compared to ovary [Bannister et al., 2009, 2011]. Our data on Atlantic halibut, a teleost vertebrate, are in agreement with these findings. All these studies suggest that gonadal expression of miR-202-3p is conserved among divergent vertebrates.

Manipulation of estrogen has been used to elucidate the biological roles of miRNAs [Bannister et al., 2011]. Previously it has been shown that expression of miR-451 was reduced in growth hormone-treated rat liver [Cheung et al., 2009]. In the present study, let-7a and miR-451 expressions were restored after FA and MT delivery was terminated (fig. 4A, B). This indicates that the expression of these 2 miRNAs has been reversibly suppressed by hormonal modulation, namely by increasing androgen level.

In the current study the lack of discernible difference in the expression of miR-202-3p between masculinized fish and control females, contrary to the difference between control males and females, could result from the presence of genetic females in masculinized groups [Babiak et al., 2012]. Alternatively, it could be the effect of FA and MT administration on the expression of miR-202-3p, but no significant difference 2 weeks after termination of the treatment was found. Androgens and estrogens have been implicated in miRNA biogenesis, processing and regulation in mammals [Shi et al., 2007; Castellano et al., 2009; Cochrane et al., 2011]. As discussed above, the direct regulatory role of the neuroendocrine system in gonadal sex differentiation and the up-regulation of miR-202-3p in 3-year- and 5-year-old halibut males may suggest that this miRNA likely responds to hormonal signaling in a teleost vertebrate.

In conclusion, this study provides a first insight into miRNA structure in a reproductive system of teleosts, namely in brain and gonadal tissues, using deep sequencing followed by RT-qPCR analyses. We found sex-biased expression of several miRNAs in different age groups as well as in masculinized individuals. Our findings indicate the possible regulatory modulation of miRNAs in sex differentiation and sexual maturation of Atlantic halibut in particular, and in fish in general.

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References

Ahn HW, Morin RD, Zhao H, Harris RA, Coarfa C, et al: MicroRNA transcriptome in the newborn mouse ovaries determined by massive parallel sequencing. Mol Hum Reprod 16:463–471 (2010).

Andersen CL, Jensen JL, Orntoft TF: Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245–5250 (2004).

Armisen J, Gilchrist MJ, Wilczynska A, Standart N, Miska EA: Abundant and dynamically expressed miRNAs, piRNAs, and other small RNAs in the vertebrate *Xenopus tropicalis*. Genome Res 19:1766–1775 (2009).

Babiak I, Ottesen O, Rudolfsen G, Johnsen S: Quantitative characteristics of Atlantic halibut, *Hippoglossus hippoglossus* L., semen throughout the reproductive season. Theriogenology 65:1587–1604 (2006).

Babiak J, Babiak I, van Nes S, Harboe T, Haugen T, Norberg B: Induced sex reversal using an aromatase inhibitor, fadrozole, in Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture 324–325:276–280 (2012).

Bail S, Swerdel M, Liu H, Jiao X, Goff LA, et al: Differential regulation of microRNA stability. RNA 16:1032–1039 (2010).

Bannister SC, Tizard MLV, Doran TJ, Sinclair AH, Smith CA: Sexually dimorphic microRNA expression during chicken embryonic gonadal development. Biol Reprod 81:165–176 (2009).

Bannister SC, Smith CA, Roeszler KN, Doran TJ, Sinclair AH, Tizard ML: Manipulation of estrogen synthesis alters mir202* expression in embryonic chicken gonads. Biol Reprod 85: 22–30 (2011).

Bizuayehu T, Lanes C, Furmanek T, Karlsen B, Fernandes J, et al: Differential expression patterns of conserved miRNAs and isomiRs during Atlantic halibut development. BMC Genomics 13:11 (2012).

Castellano L, Giamas G, Jacob J, Coombes RC, Lucchesi W, et al: The estrogen receptor-α-induced microRNA signature regulates itself and its transcriptional response. Proc Natl Acad Sci USA 106:15732–15737 (2009).

Cheung L, Gustavsson C, Norstedt G, Tollet-Egnell P: Sex-different and growth hormone-regulated expression of microRNA in rat liver. BMC Mol Biol 10:13 (2009).

Chiang HR, Schoenfeld LW, Ruby JG, Auyeung VC, Spies N, et al: Mammalian microRNAs: experimental evaluation of novel and previously annotated genes. Genes Dev 24:992–1009 (2010).

Cochrane DR, Cittelly DM, Richer JK: Steroid receptors and microRNAs: relationships revealed. Steroids 76:1–10 (2011).

Delaloy C, Liu L, Lee J-A, Su H, Shen F, et al: MicroRNA-9 coordinates proliferation and migration of human embryonic stem cell-derived neural progenitors. Cell Stem Cell 6: 323–335 (2010).

Fernandes J, Mommens M, Hagen O, Babiak I, Solberg C: Selection of suitable reference genes for real-time PCR studies of Atlantic halibut development. Comp Biochem Physiol B Biochem Mol Biol 150:23–32 (2008).

Furuchi T, Masuko K, Nishimune Y, Obinata M, Matsui Y: Inhibition of testicular germ cell apoptosis and differentiation in mice misexpressing Bcl-2 in spermatogonia. Development 122:1703–1709 (1996).

Gammell P: MicroRNAs: recently discovered key regulators of proliferation and apoptosis in animal cells. Identification of miRNAs regulating growth and survival. Cytotechnology 53:55–63 (2007).
Gao F-B: Context-dependent functions of specific microRNAs in neuronal development. Neural Dev 5:25 (2010).
Giraldez AJ, Mishima Y, Rihel J, Grocock RJ, Van Dongen S, et al: Zebrafish mir-430 promotes deadenylation and clearance of maternal mRNAs. Science 312:75–79 (2006).
Guiguen Y, Fostier A, Piferrer F, Chang CF: Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. Gen Comp Endocrinol 165: 352–366 (2010).
Hattori RS, Murai Y, Oura M, Masuda S, Majhi SK, et al: A Y-linked anti-müllerian hormone duplication takes over a critical role in sex determination. Proc Natl Acad Sci USA 109: 2955–2959 (2012).
Hayashi K, Chuva de Sousa Lopes SM, Kaneda M, Tang F, Hajkova P, et al: MicroRNA biogenesis is required for mouse primordial germ cell development and spermatogenesis. PLoS One 3:e1738 (2008).
He XJ, Yan YL, Eberhart JK, Herpin A, Wagner TU, et al: miR-196 regulates axial patterning and pectoral appendage initiation. Dev Biol 357:463–477 (2011).
Hendry CI, Martin-Robichaud DJ, Benfey TJ: Gonadal sex differentiation in Atlantic halibut. J Fish Biol 60:1431–1442 (2002).
Hendry CI, Martin-Robichaud DJ, Benfey TJ: Hormonal sex reversal of Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture 219:769–781 (2003).
Houwing S, Kamminga LM, Berezikov E, Cronembold D, Girard A, et al: A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in zebrafish. Cell 129:69–82 (2007).
Imsland AK, Jonassen TM: The relation between age at first maturity and growth in Atlantic halibut *(Hippoglossus hippoglossus)* reared at four different light regimes. Aquaculture Res 36:1–7 (2005).
Jákupsstovu S, Haug T: Growth, sexual maturation, and spawning season of Atlantic halibut, *Hippoglossus hippoglossus*, in Faroese waters. Fisheries Res 6:201–215 (1988).
Juanchich A, Klopp C, Guiguen Y, Bobe J: MicroRNAs expression during fish oogenesis and sex differentiation. 9th International Symposium on Reproductive Physiology of Fish, vol 4, p 80 (Cochin, India 2011).
Juhila J, Sipila T, Icay K, Nicorici D, Ellonen P, et al: MicroRNA expression profiling reveals miRNA families regulating specific biological pathways in mouse frontal cortex and hippocampus. PLoS One 6:e21495 (2011).
Jung YH, Gupta MK, Shin JY, Uhm SJ, Lee HT: MicroRNA signature in testes-derived male germ-line stem cells. Mol Hum Reprod 16: 804–810 (2010).
Kapsimali M, Kloosterman WP, de Bruijn E, Rosa F, Plasterk RHA, Wilson SW: MicroRNAs show a wide diversity of expression profiles in the developing and mature central nervous system. Genome Biol 8:16 (2007).
Ling KH, Brautigan P, Hahn C, Daish T, Rayner J, et al: Deep sequencing analysis of the developing mouse brain reveals a novel microRNA. BMC Genomics 12:176 (2011).
Linsen S, de Wit E, de Bruijn E, Cuppen E: Small RNA expression and strain specificity in the rat. BMC Genomics 11:249 (2010).
Mangor-Jensen A, Harboe T, Shields RJ, Gara B, Naas KE: Atlantic halibut, *Hippoglossus hippoglossus* L., larvae cultivation literature, including a bibliography. Aquaculture Res 29: 857–886 (1998).
Matsuda M, Shinomiya A, Kinoshita M, Suzuki A, Kobayashi T, et al: *Dmy* gene induces male development in genetically female (XX) medaka fish. Proc Natl Acad Sci USA 104:3865–3870 (2007).
Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, et al: A novel and universal method for microRNA RT-qPCR data normalization. Genome Biol 10:R64 (2009).
Morin RD, O'Connor MD, Griffith M, Kuchenbauer F, Delaney A, et al: Application of massively parallel sequencing to microRNA profiling and discovery in human embryonic stem cells. Genome Res 18:610–621 (2008).
Papaioannou MD, Nef S: MicroRNAs in the testis: building up male fertility. J Androl 31: 26–33 (2010).
Papaioannou MD, Pitetti J-L, Ro S, Park C, Aubry F, et al: Sertoli cell Dicer is essential for spermatogenesis in mice. Dev Biol 326:250–259 (2009).
Pase L, Layton JE, Kloosterman WP, Carradice D, Waterhouse PM, Lieschke GJ: Mir-451 regulates zebrafish erythroid maturation in vivo via its target *gata2*. Blood 113:1794–1804 (2009).
Pfaffl MW, Horgan GW, Dempfle L: Relative expression software tool (RESTR) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:e36 (2002).
Print CG, Loveland KL: Germ cell suicide: new insights into apoptosis during spermatogenesis. Bioessays 22:423–430 (2000).
Ro S, Song R, Park C, Zheng H, Sanders KM, Yan W: Cloning and expression profiling of small RNAs expressed in the mouse ovary. RNA 13:2366–2380 (2007).
Rodriguez I, Ody C, Araki K, Garcia I, Vassalli P: An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. EMBO J 16: 2262–2270 (1997).
Schulte JH, Marschall T, Martin M, Rosenstiel P, Mestdagh P, et al: Deep sequencing reveals differential expression of microRNAs in favorable versus unfavorable neuroblastoma. Nucleic Acids Res 38:5919–5938 (2010).
Schulz RW, de Franca LR, Lareyre JJ, Legac F, Chiarini-Garcia H, et al: Spermatogenesis in fish. Gen Comp Endocrinol 165:390–411 (2010).
Sempere L, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V: Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol 5:R13 (2004).
Shi XB, Xue L, Yang J, Ma AH, Zhao J, et al: An androgen-regulated miRNA suppresses Bak1 expression and induces androgen-independent growth of prostate cancer cells. Proc Natl Acad Sci USA 104:19983–19988 (2007).
Sirotkin AV, Lauková M, Ovcharenko D, Brenaut P, Mlynček M: Identification of microRNAs controlling human ovarian cell proliferation and apoptosis. J Cell Physiol 223:49–56 (2010).
Soares A, Pereira P, Santos B, Egas C, Gomes A, et al: Parallel DNA pyrosequencing unveils new zebrafish microRNAs. BMC Genomics 10:195 (2009).
Tong MH, Mitchell D, Evanoff R, Griswold MD: Expression of Mirlet7 family microRNAs in response to retinoic acid-induced spermatogonial differentiation in mice. Biol Reprod 85:189–197 (2011).
Tvedt HB, Benfey TJ, Martin-Robichaud DJ, McGowan C, Reith M: Gynogenesis and sex determination in Atlantic halibut *(Hippoglossus hippoglossus).* Aquaculture 252:573–583 (2006).
Ugras S, Brill E, Jacobsen A, Hafner M, Socci ND, et al: Small RNA sequencing and functional characterization reveals microRNA-143 tumor suppressor activity in liposarcoma. Cancer Res 71:5659–5669 (2011).
van Nes S, Andersen Ø: Temperature effects on sex determination and ontogenetic gene expression of the aromatases *cyp19a* and *cyp19b*, and the estrogen receptors *esr1* and *esr2* in Atlantic halibut *(Hippoglossus hippoglossus).* Mol Reprod Dev 73:1481–1490 (2006).
van Nes S, Moe M, Andersen O: Molecular characterization and expression of two *cyp19* (p450 aromatase) genes in embryos, larvae, and adults of Atlantic halibut *(Hippoglossus hippoglossus).* Mol Reprod Dev 72:437–449 (2005).
Warren WC, Clayton DF, Ellegren H, Arnold AP, Hillier LW, et al: The genome of a songbird. Nature 464:757–762 (2010).
Weltzien FA, Taranger GL, Karlsen O, Norberg B: Spermatogenesis and related plasma androgen levels in Atlantic halibut (*Hippoglossus hippoglossus* L.). Comp Biochem Physiol A Mol Int Physiol 132:567–575 (2002).
Xu N, Papagiannakopoulos T, Pan GJ, Thomson JA, Kosik KS: MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells. Cell 137:647–658 (2009).
Yuva-Aydemir Y, Simkin A, Gascon E, Gao FB: MicroRNA-9 functional evolution of a conserved small regulatory RNA. RNA Biol 8: 557–564 (2011).