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

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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 17α-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.

Key Words

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

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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 cytchrome 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) [Jakupsstovu 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-methyltilhydrotestosterone or 17α-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 (Forsoksdyrutvalget). 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-
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Determination of Sex in Juveniles

Since visual identification of gonads in early juvenile stages was impossible, we used 
**cypl9ala** expression as a molecular marker of female sex [van Nes et al., 2005; van Nes and Andersen, 2006] using forward primer 5’-GTGCCCCTCAATGAGAAAGA-3’ and reverse primer 5’-TGCCTGCCCTTTGTGCC-3’ (online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000341379).

**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 (Flormega), 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 mRNA 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...
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 ovarially-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
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...
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), 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
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

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**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).
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 miRNAs 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 arbitrage 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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