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The Potential Sex Determination Genes, Sox9a and Cyp19a, in Walleye Pollock (*Gadus Chalcogrammus*) Are Influenced by Water Temperature

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Abstract: Our aim was to study the relationship between the sex-determining genes, *sox9a* and *cyp19a*, and water temperature in *Gadus chalcogrammus*. We assessed the sex ratio based on the expression levels of *sox9a* and *cyp19a* at different water temperatures (5, 8, 11, and 14 °C) and at different stages of walleye pollock development (embryos, larvae, and juveniles). Next, we used immature walleye pollock to assess *sox9a* expression in males and *cyp19a* and vitellogenin (VTG) expression in females at different water temperatures. Males expressed *sox9a* in the gonadal tissues, while females expressed *cyp19a* in the gonadal tissues and VTG in the blood plasma. In the first experiment, *cyp19a* expression was higher at 5 °C and 8 °C, and *sox9a* expression was higher at 11 and 14 °C. In the second experiment, *sox9a* expression remained relatively stable, but *cyp19a* expression decreased with increasing temperature, decreasing significantly after 14 °C. Similar patterns were also observed for VTG expression. These results indicate that lower water temperatures increase *cyp19a* expression, which increases the female ratio. Higher water temperatures increase *sox9a* expression, which increases the male ratio. Therefore, this study highlights the potential of the sex-determining genes and the influence of water temperature.

Keywords: sex determination; sox9a; cyp19a; walleye pollock; water temperature

1. Introduction

Walleye pollock (*Gadus chalcogrammus*) resides in the coastal waters of Korea and Japan, throughout the Kamchatka Peninsula and the Bering Sea and into the central coast areas of California [1]. The habitat water temperatures (in the Bering Sea) range between 1 and 10 °C, and for fertilized larvae, between 2 and 7 °C [2,3]. Rising water temperatures due to climate change have affected the walleye pollock habitats. Water temperature influences habitat selection, migration, and physiological changes in walleye pollock, including sex determination, reproduction, growth, hormonal changes, and metabolism [4–9]. Environmental factors such as the water temperature are important to sex determination [10,11]. Sex determination happens at an early developmental stage, and sex is determined by a combination of genetic-dependent sex determination (GSD), environment-dependent sex determination (ESD) such as temperature, density, pH, and hypoxia, and endocrine factors [12–14]. In particular, the water

temperature experienced during embryos development has greater influence on sex determination than the genetic makeup [10,15]. After individual sex has been determined, gonadal differentiation in teleosts can lead directly to sexual differentiation in males and females or to development of both in hermaphroditic species [10,16]. Teleost fish possess numerous sex-determination genes. In male fish, sexual differentiation is achieved by the upregulation of doublesex and mab-3-related transcription factor 1 (dmrt1), which is highly conserved. Dmrt1 acts with transcription factor SRY (Sex determining region Y)-box 9a (*sox9a*) in testis formation [17,18]. Members of the SRY-box (Sox)-family are important for male determination because of their roles in testis determination and cartilage formation [19,20]. In female fish, sexual differentiation is achieved by a positive feedback loop of cytochrome P450 aromatase (*cyp19a*), which involves the transcription factor forkhead box protein L2 (foxl2) [21,22]. Cytochrome P450 aromatase (*cyp19a*), a member of the cytochrome P450 superfamily, is important for female determination due to its role in synthesizing estrogens. Estrogens promote ovarian development and maintain female differentiation. *Cyp19a* has been identified in females in different animal phyla. Simpson et al. (1994) found *cyp19a* transcripts in human ovaries, placenta, adipose tissues and the brain [23]. In fish, *cyp19a* is used for steroidogenesis of the gonadal steroids, which plays a critical role in gonadogenesis.

This study aimed to investigate the expression levels of *sox9a* and *cyp19a* and the mechanisms underlying the response of these genes to different water temperatures. Prior to this study, gonadal tissues from adult fish were studied to investigate the induction of *sox9a* and *cyp19a*, gonadal histological analysis, and VTG analysis in blood plasma. They were then divided into two experiments. The water temperatures studied were 5, 8, 11, and 14 °C. The first experiment reported the induction levels of *sox9a* and *cyp19a* in the embryos, larva, and juveniles (approximately 8–12 cm in length) at the different water temperatures. The second experiment investigated the induction levels of sox9a and cyp19a in immature fish (approximately 20–25 cm in length) to study the effects of water temperature in developed gonadal tissues (testis or ovary). The induction levels of vitellogenin (VTG) in female immature fish were also measured based on the VTG induction levels at different water temperatures, previously reported from this laboratory [24]. VTG is an egg yolk precursor that is synthesized in the liver. It is stimulated by estrogen and is rarely induced in males (though the VTG gene is present in male fish) [25]. As mentioned previously, *cyp19a* encodes aromatase to control estrogen synthesis, so correlation between *cyp19a* and VTG expression patterns in different water temperatures were compared.

To date, there have been no studies of sex determination or sex changes in walleye pollock in different water temperatures. Therefore, we suggest a potential mechanism of sexual determination based on water temperature in this report.

2. Materials and Methods

2.1. Animals and Materials

Walleye pollock (*G. chalcogrammus*) were obtained from the East Sea Fisheries Research Institute in Korea. Adult fish (3+ years old) were held in a flow-through tank (10-ton, 4 m diameter and 1 m depth), with an ambient photoperiod and a continuous supply of filtered seawater (10 µm PREFLOW II, Cs Technofil Co. Ltd., Gyeonggi-do, Korea) at 7.5 °C. The fish were fed twice per day. Embryos were obtained from the adult fish and were hatched and grown until the immature fish stage. The sizes of the fish used in the experiment at each developmental stage are shown in Figure 1. Approximately 1000 embryos (Figure 1A) and larvae (Figure 1B), 100 juveniles (Figure 1C), and 50 immature fish (Figure 1D) were grown in 10-ton water tanks at different temperatures (5, 8, 11, and 14 °C). After the eggs hatched, the fish were fed (in order) rotifers, Artemia nauplius, and frozen copepods. The dissolved oxygen (DO) and pH were maintained at 9–10 mg/L and 7.9–8.1, respectively. In experiment 1, the embryos were reared for 5 days, the larva for 30 days, and the juveniles for 180 days. In experiment 2, the immature fish were reared at 7.5 °C for approximately 180 days and

then transferred to the water temperature treatments for ~10 months before the experiment (Figure 1E). The experimental protocol used in this study was reviewed and approved by the National Institute of Fisheries Science-Institutional Animal Care and Use Committee (NIFS-IACUC) per the ethical procedures and scientific care guidelines (approval number: 2018-NIFS-IACUC-16). The fish were sacrificed by an anesthetic overdose (MS-222 (Sigma), 50 ppm), and the heart, brain, kidney, intestine, and liver tissues were collected and stored at −80 °C.

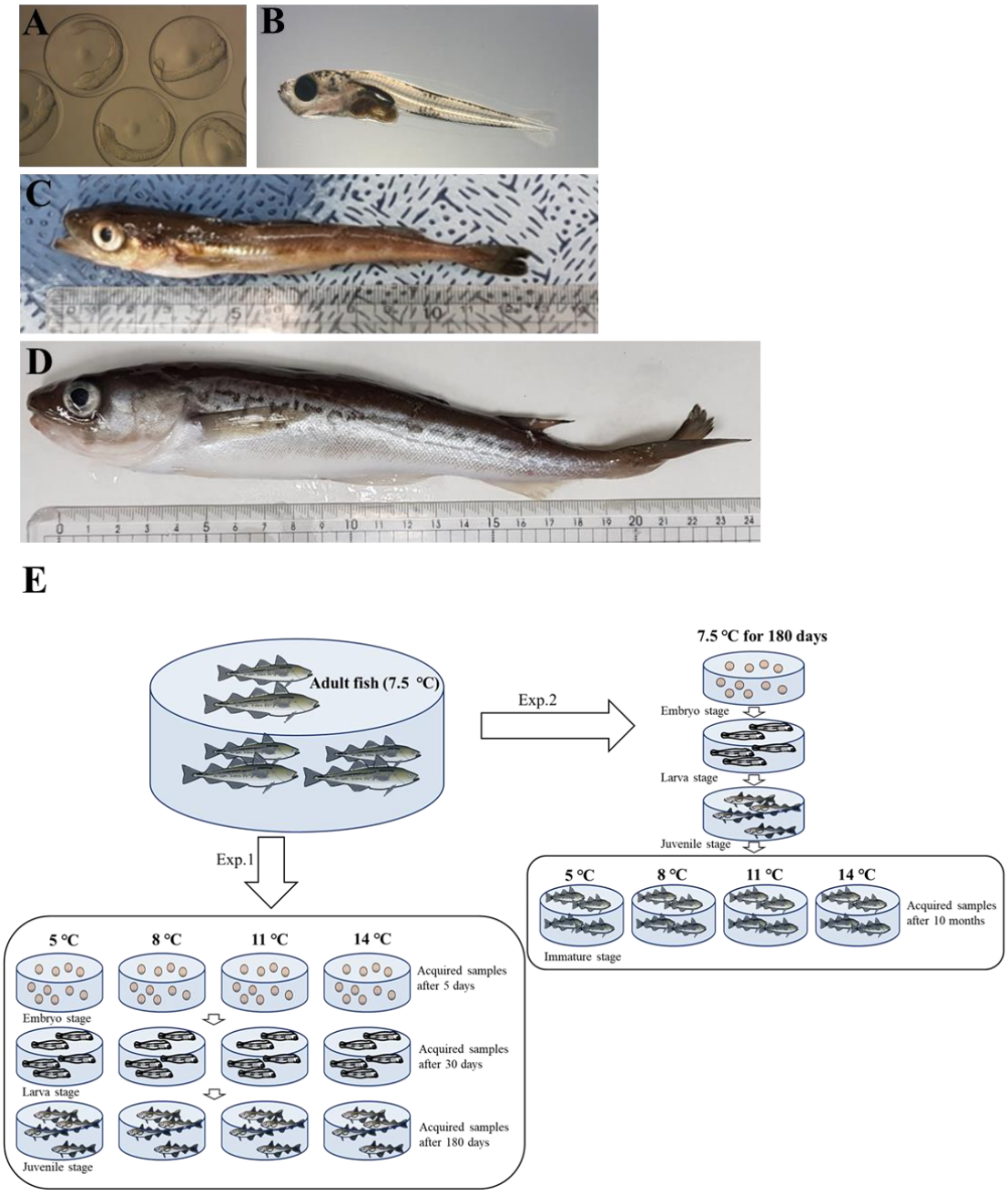

Figure 1. Walleye pollocks used in the experiment. (**A**) Embryos (2.5X 125 h after fertilization); (**B**) Larvae (1.6X; 30 days after hatching); (**C**) Juvenile fish (8–12 cm in length, approximately 180 days); (**D**) Immature fish (20–25 cm in length, 1+ year); (**E**) Schematic of the experimental samples. The fertilized eggs (Experiment 1) obtained from the mature (3+ years) walleye pollocks were raised in different water temperatures (5, 8, 11, and 14 °C). The juveniles (Experiment 2) were raised at 7.5 °C for approximately 180 days before relocation to the 5, 8, 11, and 14 °C treatments, in which they were raised for 10 months.

2.2. Isolation of mRNA Sequence

The reverse transcriptase PCR (RT-PCR) was performed to confirm the mRNA sequences and induction levels of sox9a and cyp19a gonadal tissues (testis or ovary) in adult walleye pollock. The gonadal tissues were homogenized using liquid nitrogen and PYREX® Dounce Homogenizer Tissue Grinders (Thomas Scientific, Swedesboro, NJ, USA), and total RNA was extracted using TRIzol (Invitrogen). Only high-quality RNA (a ratio of 2.0 or higher for the absorbance at 260 nm and 280 nm) was used in the experiment. RNA integrity was assessed by electrophoresis on 1% agarose–formaldehyde gels after staining with ethidium bromide. The single-strand cDNA was synthesized from 5 µg of the total RNA with an Oligo (dT) primer by Avian Myeloblastosis Virus (AMV) Reverse Transcriptase (Promega). PCR was performed with the obtained cDNA (as a template), and 20 µM of the forward primer, reverse primer, Taq DNA polymerase, and 2.5 µM of each dNTP (Enzynomics, Korea). The conserved regions of the zebrafish (*Danio rerio*) *sox9a* (GenBank accession no. NM131643), Atlantic cod (*Gadus morhua*) *sox9a* (Genbank accession no. JN802288), and olive flounder (*Paralichthys olivaceus*) *sox9a* (GenBank accession no. KY924902) were used to design the primers. The *cyp19a* primers were designed from the conserved regions of the Atlantic cod (*Gadus morhua*) *cyp19a* (GenBank accession no. JJN802290), Sweetfish (*Plecoglossus altivelis*) *cyp19a* (Genbank accession no. KF296362), and common carp (*Cyprinus carpio*) *cyp19a* (GenBank accession no. DQ534411). All the primers were designed in the 'Primer 3' program, and Table 1 presents the primer sequences used in the experiments. The PCR conditions were 30 cycles at 94 °C for 30 s, 52 °C for 50 s, and at 72 °C for 1 min. Electrophoresis was used to evaluate the PCR products before insertion into a TA vector (Enzynomics, Korea) and cloning. The PCR products were evaluated by electrophoresis, and the nucleotide sequences were determined on a 3730xl DNA analyzer (Cosmogentec, Korea). The *sox9a* and *cyp19a* fragments were registered in GenBank (GenBank accession no. MT350342 and MT350341, respectively).

Table 1. Primer lists.

Primer Name	Sequence(5′→3′)	Size (bp)
RT-PCR *sox9a* F	GAGACTTCAAGAAGGACG	260 bp
RT-PCR *sox9a* R	CTTCGTTGAGGAGTCTCC	
RT-PCR *cyp19a* F	TGGAGATGGTGATCGCCG	360 bp
RT-PCR *cyp19a* R	CATGGTGAAGTCCACCAC	
RT-qPCR *sox9a* F	GTGCTGAAGGGGTACGACTG	92 bp
RT-qPCR *sox9a* R	TTCATGGGTCTCTTGACGTG	
RT-qPCR *cyp19a* F	AACTGGACCGCATCAACTTC	100 bp
RT-qPCR *cyp19a* R	ATCACCATCTCCAGCACACA	
18s rRNA F	GCTCACCCGCTACTTGGATA	89 bp
18s rRNA R	TCTGATAAATGCACGCATCC	

2.3. Quantitative Real Time-PCR

The quantitative real time-PCR (RT-qPCR) was used to examine changes in *sox9a* and *cyp19a* mRNA expression levels in whole or target tissues. For this experiment, 50 mg of the embryos and larval samples were used. The brain, intestine, liver, kidney, heart, and gonadal tissues from ten juveniles, five immature fish and adult fish from each water tank (chosen randomly) were used for total RNA extraction. First of all, DNase I was treated to remove genomic DNA contamination in total RNA. Each 20 µL reaction, based on the TOPreal™ One-step RT-qPCR Kit (Enzynomics co Ltd., Daejeon, Republic of, Korea), produced a 20 µL reaction that included the SYBR Green with the final concentration of 0.25 µM. Both primers had a concentration of 10 pmol/µL each, along with 50 ng/µL RNA extract. The Chromo4 System (Bio-Rad Inc., Contra Costa, CA, USA) was used to measure the reaction with 30 min at 50 °C, 10 min at 95 °C, 40 cycles of 5 s at 95 °C and 30 s at 60 °C (with endpoint data collection). All samples were tested in triplicate. The primers used for qRT-PCR was designed using 'primer3' program. The primer sequences are shown in Table 1. The $2^{-\Delta Ct}$ method was used for

the two independent tests of relative Ct values. Ct values for reference gene (18s rRNA) were used to normalized expression of target genes.

2.4. Vitellogenin Assay

The correlation between VTG and *cyp19a* relative expression in female fish were investigated in differing water temperatures. Fish (>10 months old, approximately 20 cm) were raised from March to October in tanks with different water temperatures (5, 8, 11, and 14 °C). To distinguish the sex, the fish were dissected. The protocol for the previous experiment was used for VTG detection [24].

2.5. Histological Analysis

The gonadal tissues in adult fish were fixed in 10% neutral buffered formalin for 48 h, dehydrated in a graded alcohol series, embedded in paraffin wax, and sectioned with a Reichert microtome (4 μm). The sections were stained with hematoxylin and eosin (H&E) and examined using an Olympus Provis AX70 microscope equipped with an Olympus Camedia C70/70 camera.

2.6. Statistical Analysis

Statistical analyses were performed in GraphPad Prism (version 8.4.3). Differences between the means were tested by Mann–Whitney or Kruskal–Wallis with post-hoc Dunn's multiple comparison tests were used ($p < 0.05$). All of the data are plotted as mean ± standard deviation (SD), and the appropriate statistical test values are reported. Pearson correlation analysis was used to examine the correlations between VTG and *cyp19a.*

3. Results

3.1. Sox9a and Cyp19a mRNA Expression in the Gonadal Tissues with Vitellogenin

Prior to experiments 1 and 2, the adult fish were investigated for histological analysis of gonadal tissues, RT-PCR for the fragment mRNA sequence (data not shown; GenBank provided in Section 2.2), RT-qPCR of *sox9a* and *cyp19a* in gonadal tissues, and the VTG level in blood plasma, which are shown in Figure 2. Histological analysis of male gonadal tissues showed spermatozoa (Spz) and empty spaces after spermatozoa removal at a mature stage (Figure 2A). The testis is an unrestricted lobule that was spermatozoa-filled with spermatocysts, while the female ovarian follicles is filled with yolk globules and vacuoles and encased in zona radiata at the vitellogenesis stage (pre-spawning condition) (Figure 2B). *Sox9a* was only found in male gonadal tissues while *cyp19a* was only found in female gonadal tissues (Figure 2B). The male gonadal tissue showed only *sox9a* with 260 bp PCR product, while the female gonadal tissue showed only *cyp19a* with 360 bp PCR product. The comparative expression was very high—*sox9a* was expressed at a high level, and *cyp19a* was expressed at a very low level (Figure 2D). In females, an average level of plasma VTG was 434.6 ± 29.18 ng/mL, while in males, the average level of plasma VTG was 1.76 ± 0.43 ng/mL (Figure 2E).

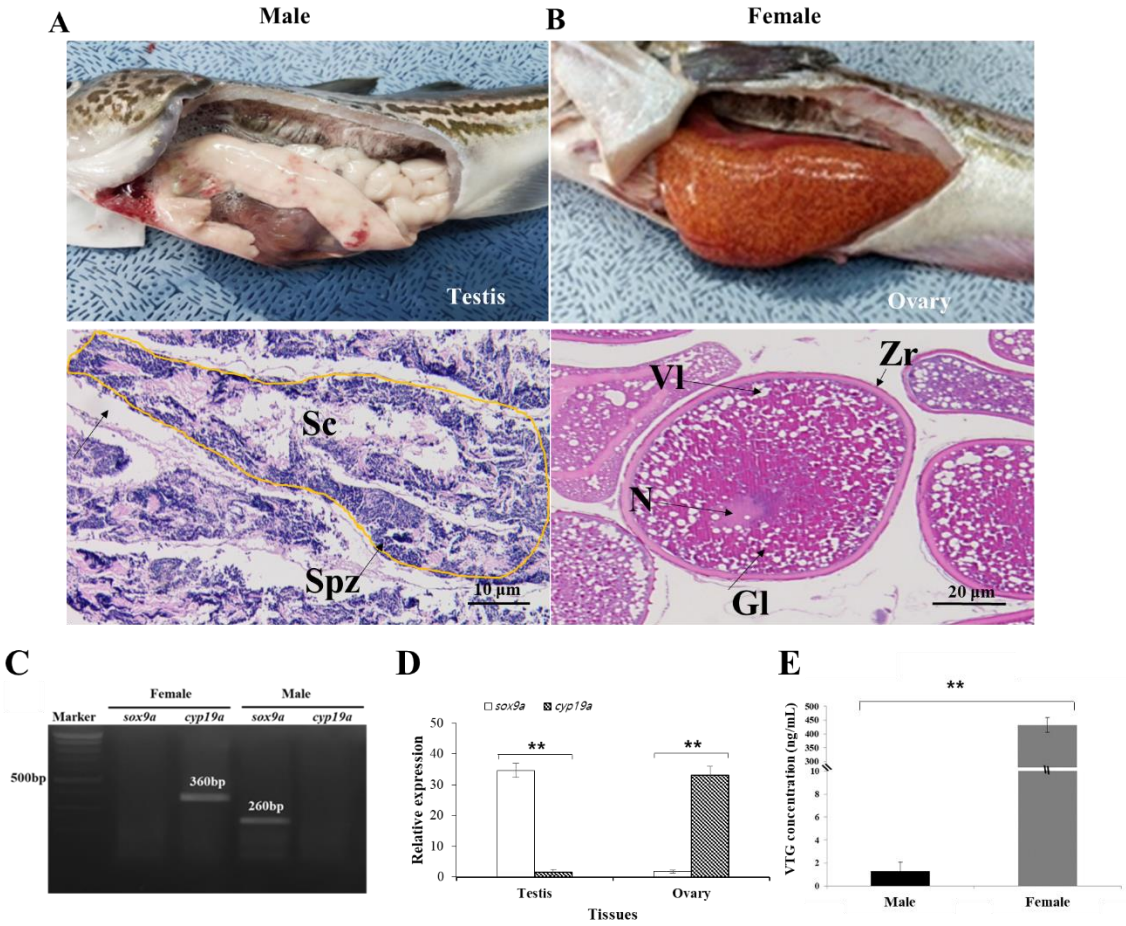

Figure 2. (**A**) Analysis of the gonadal tissues from mature males and their histological analyses; Spz indicates spermatozoa, Sc indicates spermatocysts (yellow), and the arrows represent empty spaces after spermatozoa removal; (**B**) Analysis of the gonadal tissues from mature females and their histological analyses. N indicates nucleus, Zr indicates zona radiata, Gl indicates yolk globules, and Vl indicates vacuoles; (**C**) Analysis of *sox9a* (260 bp) and *cyp19a* (360 bp) expression in mature male and female gonadal tissues; the results were confirmed by 1.2% agarose gel. The DNA marker used was 100 bp Plus DNA Ladder (Bioneer, Korea). (**D**) The relative mRNA expression levels of *sox9a* and *cyp19a* in aldult male and female gonadal tissues. The expression of each sample was relative quantity to the expression of 18s rRNA Values are reported as mean ± SD of the three experiments; (**E**) VTG levels in mature male and female blood (by ELISA). * indicates $p < 0.05$, ** indicates $p < 0.01$.

3.2. Sox9a and Cyp19a mRNA Expression in Embryos and Larvae at Different Water Temperatures

Figure 3A shows the expression of *sox9a* and *cyp19a* in embryos at different water temperatures. *Cyp19a* expression was higher at 5 and 8 °C, while *sox9a* expression was higher at 11 and 14 °C. Figure 3B shows the expression of *sox9a* and *cyp19a* in larvae at different water temperatures. Similar to the embryos, *cyp19a* expression was higher at 5 and 8 °C, and *sox9a* expression was higher at 11 and 14 °C. Figure 3C shows the increment of *sox9a* induction in the embryos compared to *cyp19a*; *sox9a* expression increased with increasing water temperature, and at 14 °C, it was significantly different. Figure 3D shows the increment of *sox9a* induction in larvae compared to *cyp19a*. Similarly, *sox9a* expression increased with increasing water temperature, and at 14 °C, it was significantly different.

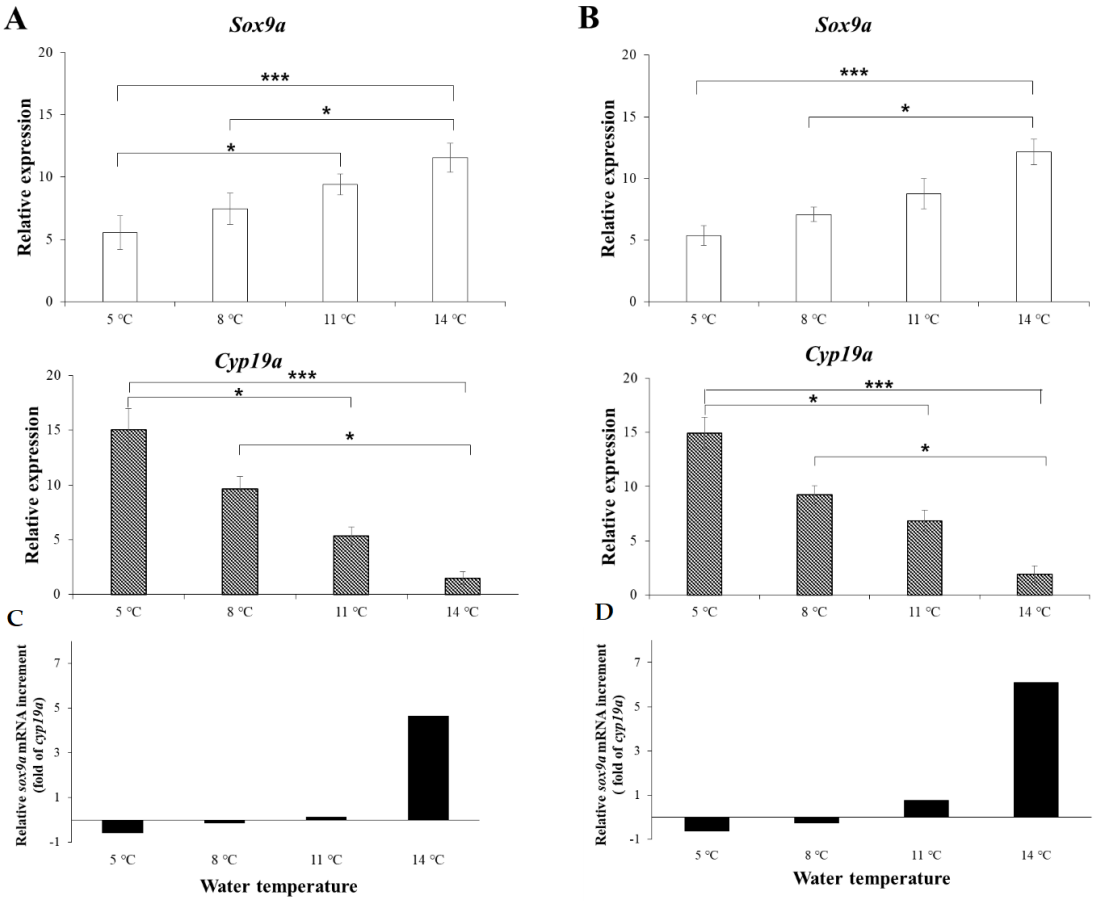

Figure 3. (**A**) *Sox9a* and *cyp19a* mRNA expression levels in the embryos at different water temperatures; (**B**) *Sox9a* and *cyp19a* mRNA expression levels in the larval samples at different water temperatures; (**C**) Induction increments of *sox9a* at the embryos stage compared to *cyp19a*; (**D**) The induction increments of *sox9a* at the larvae stage compared to *cyp19a*. The expression of each sample was relative quantity to the expression of 18s rRNA. Values are reported as mean ± SD of the three experiments. Mann–Whitney tests were used. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

3.3. Distribution of Sox9a and Cyp19a mRNA in Juvenile Fish

The distribution of *sox9a* and *cyp19a* mRNA expression was observed in the tissues of juvenile fish. These tissues were from the brain, intestine, liver, kidney, heart, and gonads (testis and ovary). The relative abundance of mRNAs in the walleye pollock tissues showed distinct differences (Figure 4). The *cyp19a* gene in females was induced at a high level in the brain and gonads and at comparatively lower levels in the intestine, liver, kidney, and heart at 5 °C (Figure 4A). Similarly, the *sox9a* gene in males was induced at a high level in the brain and gonads at 14 °C (Figure 4B). Table 2 shows the number of individuals with high *sox9a* or *cyp19a* expression in the different water temperatures. There were more individuals with high *cyp19a* expression at 5 to 11 °C, whereas at 14 °C, there were more individuals with high *sox9a* expression.

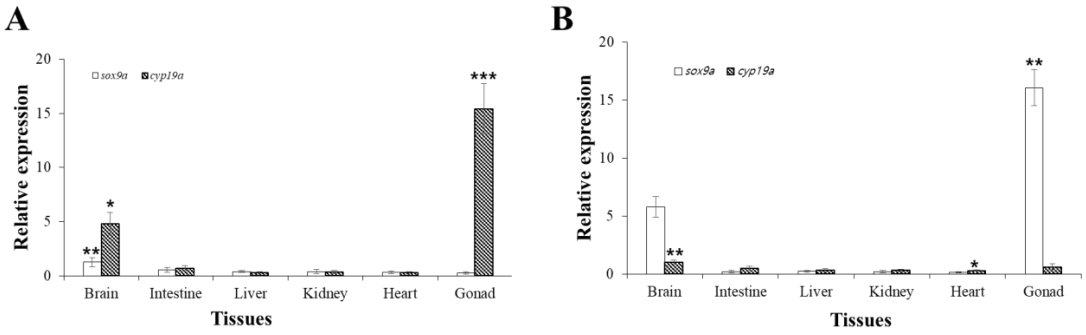

Figure 4. *Sox9a* and *cyp19a* mRNA expression in the juvenile fish samples. (**A**) The sample in females when *cyp19a* induction is greater than *sox9a* induction at 5 °C. (**B**) The sample in males when *sox9a* induction is greater than *cyp19a* induction at 14 °C. Kruskal–Wallis with post-hoc Dunn's multiple comparison tests were used. The expression of each sample was relative quantity to the expression of 18s rRNA Values are reported as mean ± SD of the three experiments. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

Table 2. Number of individuals based on the expression of *sox9a* and *cyp19a* in each water temperature. Individuals were counted between higher expression levels for *sox9a* and *cyp19a*. Gene expression was examined by randomly selecting 10 fish from each tank.

Water Temp / Gene (Number)	5 (°C),	8 (°C),	11 (°C),	14 (°C)
Sox9a	2	4	4	9
Cyp19a	8	6	6	1
Total Number	10	10	10	10

3.4. Sox9a and Cyp19a mRNA Expression with Vitellogenin in Immature Walleye Pollock

As mentioned previously (Section 3.1), it was confirmed that in gonads of adult fish, *sox9a* was only induced in testis, while *cyp19a* was only induced in ovary. Based on this result, the change in the induction levels of *sox9a* and *cyp19a* were investigated immature walleye pollock of male testis and female ovary, respectively. In immature fish, the samples were dissected to identify sex via gonadal tissues. Immature walleye pollocks were raised at different temperatures for 10 months, and the induction of *sox9a* and *cyp19a* mRNA in the gonadal tissues was studied in relation to the VTG induction level in female blood plasma (Figure 5). The water temperature had no significant impact in the *sox9a* induction in testis. For *cyp19a*, there was little difference between 5 and 8 °C, but at 11 °C, the induction level decreased. The *cyp19a* induction level at 14 °C was less than half of the value observed at 5 °C in ovary (Figure 5A). These results indicated that the *sox9a* induction level was similar at all water temperatures, but *cyp19a* induction decreased as the water temperature decreased. VTG induction in female blood plasma was 306.63 ± 28.84 ng/mL at 5 °C, 277.81 ± 17.57 ng/mL at 8 °C, 147.23 ± 18.03 ng/mL at 11 °C, and 25.68 ± 6.58 ng/mL at 14 °C (Figure 5B). The individual distribution between *cyp19a* and VTG were recorded based on different temperatures (Figure 5C). The individuals that were raised at 5 and 8 °C can be found at the top right. The individuals that were raised at 11 °C are found in the middle. The individuals raised at 14 °C formed a group at the bottom left (Figure 5C). The correlation coefficient between *cyp19a* and VTG at different temperatures were highest in 5 °C groups with $R^2 = 0.89019$ (Figure 5D). There was a tendency for the coefficients to decrease as the temperature increased, with $R^2 = 0.8166$ at 8 °C (Figure 5E), $R^2 = 0.5257$ at 11 °C (Figure 5F), and $R^2 = 0.465$ at 14 °C (Figure 5G). This result showed that VTG was similar patterns to *cyp19a*.

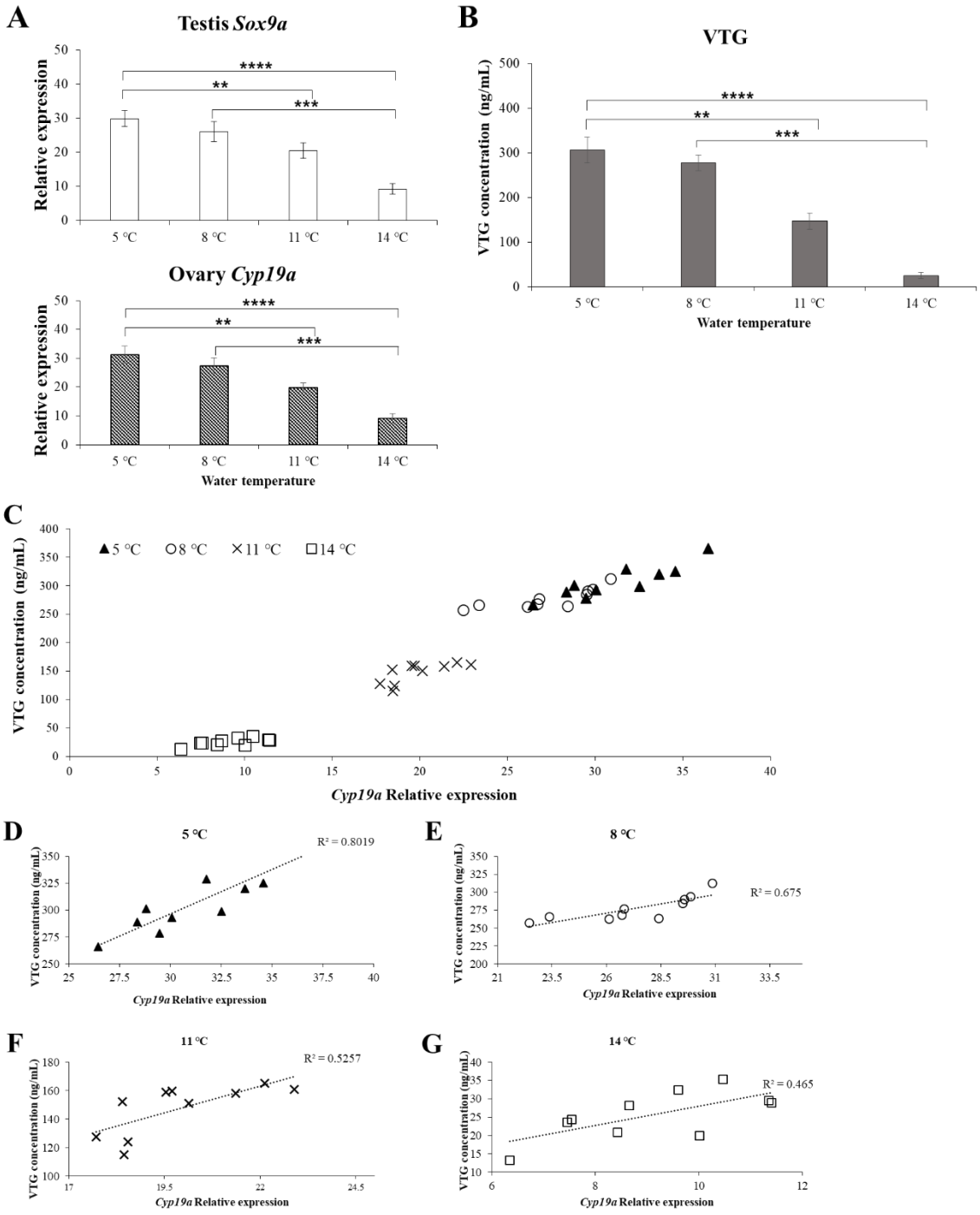

Figure 5. (**A**) *Sox9a* (white bars) and *cyp19a* (shaded bars) mRNA expression in immature walleye pollock gonadal tissues (testis and ovary) at different water temperatures; (**B**) VTG levels in female blood. (**C**) The correlation between *cyp19a* and VTG at different water temperatures. (**D**) The correlation between *cyp19a* and VTG at 5 °C (▲), (**E**) The correlation between *cyp19a* and VTG at 8 °C (○), (**F**) The correlation between *cyp19a* and VTG at 11 °C (X), and (**G**) The correlation between *cyp19a* and VTG at 14 °C (□). The expression of each sample was relative quantity to the expression of 18s rRNA. Kruskal–Wallis with post-hoc Dunn's multiple comparison tests were used. Values are reported as mean ± SD of the three experiments. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p < 0.0001$.

4. Discussion

In this report, we have described the expression patterns of two sex-related genes, *sox9a* and *cyp19a*, in walleye pollock. Consistent with previous experiments, we found *sox9a* in mature male walleye pollock gonadal tissues and *cyp19a* in mature female counterparts. Degani (2014) and Adolfi et al. (2015) suggested that fish *sox9a* is a sex-related gene that is increased in expression in the testes and decreased in the ovaries [26,27]. Other researchers have studied the expression patterns of these genes zebrafish, medaka, and tilapia gonadal tissues during differentiation [28–30]. In several studies, *cyp19a* expression was higher in the ovaries, which was similarly observed in other species, including tilapia, black porgy (*Acanthopagrus schlegelii*), half-smooth tongue-sole (*Cynoglossus semilaevis*), goldfish (*Carassius auratus*), and spotted scat (*Scatophagus argus*) [31–33]. Fish sex determination and differentiation occur during fertilization and in the post-larval stages, and the water temperature plays a vital role in this process (as reported in > 60 different fish species) [10,13,34–36]. In this study, the expression pattern of *sox9a* and *cyp19a* were similar during the embryos and larval stages. At 5 °C, *cyp19a* expression was the highest, and as the temperature increased, *cyp19a* expression decreased. Conversely, *sox9a* expression was high between 11–14 °C. This suggests that increases in water temperature are associated with decreased *cyp19a* expression. Tanaka et al. (2019) raised walleye pollocks in 2, 5, and 8 °C water and reported higher female ratios in the 2 and 5 °C treatments and a higher male ratio in the 8 °C treatment [37]. Fish in our study were raised at 7.5 °C, providing our reasoning for testing at higher temperatures. Despite our use of different water temperatures in juvenile fish, the results were consistent: the female ratio was higher at lower temperatures. To fully investigate the sex ratio, additional studies with more samples are required. Nonetheless, the results of these two independent studies indicate that water temperature is an important factor in walleye pollock sex determination. This is expected, as the sex in walleye pollocks is determined at early developmental stages, and *cyp19a* affects the sex-determination of females. *Sox9a* and *cyp19a* expression in the brain and gonadal tissues of juvenile fish were high. This is significant because the aromatization of androgens into estrogens in the brain is necessary for the regulation of several physiological and behavioral processes. In vitro assays of the brains in rats [38], birds, and fish [39] have also shown aromatase activity in many regions. The gonadal tissue investigation showed that high *cyp19a* expression was associated with female tissues, while high *sox9a* expression was associated with male tissues. For most fish, including walleye pollock, the up-regulation of *sox9a* leads to differentiation into male testis, whereas the down-regulation of *sox9a* leads to differentiation into female ovaries. The opposite patterns have been observed for *cyp19a*; up-regulation leads to differentiation into ovaries and down-regulation leads to differentiation into testes [21]. These results also show that walleye pollock sex is determined early in development, at the embryo or larval stages. Jorgensen et al. (2008) found that *sox9a* in zebrafish is expressed in the undifferentiated gonads, and Nakamoto et al. (2005) and Kobayashi et al. (2008) reported roles for *sox9* in the formation of primitive gonadal tissues in medaka and tilapia [31–33]. Similar observations have been reported in zebrafish and black porgy [40].

Immature walleye pollocks (approximately 20–25 cm in length) were raised in different water temperatures for 10 months, and there were no differences in *sox9a* expression in the male gonadal tissues. However, *cyp19a* expression significantly decreased at 11 °C and even more at 14 °C.

Previously, we studied the expression levels of 17β-estradiol (E2) and VTG at different water temperatures and found that increased water temperature is associated with E2 decreases, which affects the induction of VTG [24]. High temperatures impaired the ovaries in female pejerreyes (*Odontesthes bonariensis*) by inhibiting the follicle-stimulating hormone receptors (fshr) and plasma E2 levels [41]. Anderson et al. (2012 and 2017) found that suppression of plasma E2 and impairment of downstream VTG is common in female *Salmo salar* exposed to heat [42,43]. Studies have also shown that *cyp19a* plays an important and conserved role in the regulation of ovarian E2 [21,44,45]. In this study, VTG induction decreased as the water temperature increased and declined sharply at 14 °C (similar to *cyp19a*). The correlation between *cyp19a* and VTG is 11 and 14 °C lower than 5 and 8 °C, which may be related to the vitellogenesis stage during feminization, so further study is needed.

5. Conclusions

This study shows that sex determination and/or differentiation in walleye pollocks occurs in the embryos and post-larval stages. Higher water temperatures tend to result in a higher male ratio, whereas lower water temperatures lead to a higher female ratio. Higher water temperatures also suppress *cyp19a* expression, which decreases VTG and potentially affects sexual differentiation in female *Gadus chalcogrammus*.

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References

1. Hiatt, T.; Dalton, M.; Felthoven, R.; Fissel, B.; Garber-Yonts, B.; Haynie, A.; Kasperski, S.; Lew, D.; Package, C.; Sepez, J.; et al. Economic status of the ground fish fisheries off Alaska, 2009. In *Stock Assessment and Fishery Evaluation Report for the Ground Fish Fisheries of the Gulf of Alaska and Bering Sea/Aleutian Islands Area*; North Pacific Fishery Management Council: Anchorage, AK, USA, 2010; p. 254.
2. Bakkala, R.G. *Structure and Historical Changes in the Ground Fish Complex of the Eastern Bering Sea*; NOAA Technical Report NMFS: Miami, FL, USA; U.S. Department of Commerce: Washington, DC, USA, 1993.
3. Nakatani, T.; Maeda, T. Thermal effect on the development of walleye pollock eggs and their upward speed to the surface. *Bull. Jpn. Soc. Sci. Fish.* **1984**, *50*, 937–942. [CrossRef]
4. Graham, C.T.; Harrod, C. Implications of climate change for the fishes of the British Isles. *J. Fish Biol.* **2009**, *74*, 1143–1205. [CrossRef]
5. Hinch, S.G.; Cooke, S.J.; Healey, M.C.; Farrell, A.P. Behavioural physiology of fish migrations: Salmon as a model approach Fish Physiology. *Behav. Physiol. Fish.* **2005**, *24*, 239–295.
6. Hari, R.E.; Livingstone, D.M.; Siber, R.; Burkhardt-Holm, P.; Herbert, G. Consequences of climatic change for water temperature and brown trout populations in Alpine rivers and streams Glob. *Chang. Biol.* **2006**, *12*, 10–26. [CrossRef]
7. Jobling, M. Temperature and growth: Modulation of growth rate via temperature change. In *Global Warming: Implications for Freshwater and Marine Fish*; Wood, C.M., McDonald, D.G., Eds.; Cambridge University Press: Cambridge, UK, 1997; pp. 225–253.
8. Salinger, D.H.; Anderson, J.J. Effects of water temperature and flow on adult salmon migration swim speed and delay. *Trans. Am. Fish. Soc.* **2006**, *135*, 188–199. [CrossRef]
9. Sponaugle, S.; Cowen, R.K. Larval supply and patterns of recruitment for two Caribbean reef fishes, *Stegastes partitus* and *Acanthurus bahianus*. *Mar. Freshw. Res.* **1996**, *47*, 433–447. [CrossRef]
10. Devlin, R.H.; Nagahama, Y. Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture* **2002**, *208*, 191–364. [CrossRef]
11. Baroiller, J.F.; D'Cotta, H.; Saillant, E. Environmental effects on fish sex determination and differentiation. *Sex. Dev.* **2009**, *3*, 118–135. [CrossRef]
12. Baroiller, J.F.; D'Cotta, H. Environment and sex determination in farmed fish. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **2001**, *130*, 399–409. [CrossRef]
13. Baroiller, J.F.; Guiguen, Y. Endocrine and environmental aspects of sex differentiation in gonochoristic fish. *EXS* **2001**, *177*, 201.
14. Godwin, J. Social determination of sex in reef fishes. *Semin. Cell. Dev. Biol.* **2009**, *20*, 264–270. [CrossRef] [PubMed]
15. Conover, D.O.; Heins, S.W. Adaptive variation in environmental and genetic sex determination in a fish. *Nature* **1987**, *326*, 496–498. [CrossRef] [PubMed]

16. Koopman, P.; Gubbay, J.; Vivian, N.; Goodfellow, P.; Lovell-Badge, R. Male development of chromosomally female mice transgenic for sry. *Nature* **1991**, *351*, 117–121. [CrossRef] [PubMed]
17. Kent, J.; Wheatley, S.C.; Andrews, J.E.; Sinclair, A.H.; Koopman, P.A. Male-specific role for sox9 in vertebrate sex determination. *Development* **1996**, *122*, 2813–2822. [PubMed]
18. Smith, C.A.; Roeszler, K.N.; Ohnesorg, T.; Cummins, D.M.; Farlie, P.G.; Doran, T.J.; Sinclair, A.H. The avian Z-linked gene dmrt1 is required for male sex determination in the chicken. *Nature* **2009**, *461*, 267–271. [CrossRef]
19. Heule, C.; Salzburger, W.; Böhne. Genetics of sexual development: An evolutionary playground for fish. *Genetics* **2014**, *196*, 579–591. [CrossRef]
20. Piferrer, F.; Ribas, L.; Díaz, N. Genomic approaches to study genetic and environmental influences on fish sex determination and differentiation. *Mar. Biotechnol.* **2012**, *14*, 591–604. [CrossRef]
21. Guiguen, Y.; Fostier, A.; Piferrer, F.; Chang, C.-F. Ovarian aromatase and estrogens: A pivotal role for gonadal sex differentiation and sex change in fish. *Gen. Comp. Endocrinol.* **2010**, *165*, 352–366. [CrossRef] [PubMed]
22. Wang, D.-S.; Kobayashi, T.; Zhou, L.-Y.; Paul-Prasanth, B.; Ijiri, S.; Sakai, F.; Okubo, K.; Morohashi, K.I.; Nagahama, Y. Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with Ad4 binding protein/steroidogenic factor 1. *Mol. Endocrinol.* **2007**, *21*, 712–725. [CrossRef]
23. Simpson, E.R.; Mahendroo, M.S.; Means, G.D.; Kilgore, M.W.; Hinshelwood, M.M.; Graham-Lorence, S.; Amarneh, B.; Ito, Y.; Fisher, C.R.; Michael, M.D.; et al. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocr. Rev.* **1997**, *15*, 342–355.
24. Kim, S.S.; So, J.H.; Maeng, C.H.; Kim, N.-J.D.; Lee, C.J.; Ma, Y.; Yoo, H.K.; Choi, J.; Byun, S.G.; Lim, H.J.; et al. The influence of water temperature on the induction of vitellogenin in walleye pollock *Gadus chalcogrammus*. *Aquaculture* **2019**, *511*, 634195. [CrossRef]
25. Sole, M.; Porte, C.; Barcelo, D. Analysis of the estrogenic activity of sewage treatment works and receiving waters using vitellogenin induction in fish as a biomarker. *Trac Trend Anal. Chem.* **2001**, *20*, 518–525. [CrossRef]
26. Degani, G. Expression of SOX3 and SOX9 genes in gonads of blue gourami. *Adv. Biol. Chem.* **2014**, *4*, 322–330. [CrossRef]
27. Adolfi, M.C.; Carreira, A.C.; Jesus, L.W.; Bogerd, J.; Funes, R.M.; Schartl, M.; Sogayar, M.C.; Borella, M.I. Molecular cloning and expression analysis of dmrt1 and sox9 during gonad development and male reproductive cycle in the lambari fish, *Astyanax altiparanae*. *Reprod. Biol. Endocrinol.* **2015**, *13*, 2. [CrossRef]
28. Nakamoto, M.; Suzuki, A.; Matsuda, M.; Nagahama, Y.; Shibata, N. Testicular type Sox9 is not involved in sex determination but might be in the development of testicular structures in the medaka, Oryzias latipes. *Biochem. Biophys. Res. Commun.* **2005**, *333*, 729–736. [CrossRef]
29. Jorgensen, A.; Morthorst, J.E.; Andersen, O.; Rasmussen, L.J.; Bjerregaard, P. Expression profiles for six zebrafish genes during gonadal sex differentiation. *Reprod. Biol. Endocrinol.* **2008**, *6*, 25. [CrossRef]
30. Kobayashi, T.; Kajiura-Kobayashi, H.; Guan, G.; Nagahama, Y. Sexual dimorphic expression of DMRT1 and Sox9a during gonadal differentiation and hormone-induced sex reversal in the teleost fish Nile tilapia (Oreochromis niloticus). *Dev. Dyn.* **2008**, *237*, 297–306. [CrossRef]
31. Deng, S.P.; Chen, S.L.; Xu, J.Y.; Liu, B.W. Molecular cloning, characterization and expression analysis of gonadal P450 aromatase in the half-smooth tongue-sole, *Cynoglossus semilaevis*. *Aquaculture* **2009**, *287*, 211–218. [CrossRef]
32. Chen, X.W.; Jiang, S.; Gu, Y.F.; Shi, Z.Y. Molecular characterization and expression of *cyp19a* gene in *Carassius auratus*. *J. Fish Biol.* **2014**, *85*, 516–522. [CrossRef]
33. Liu, H.; Mu, X.; Gui, L.; Su, M.; Li, H.; Zhang, G.; Liu, Z.; Zhang, J. Characterization and gonadal expression of FOXL2 relative to *Cyp19a* genes in spotted scat *Scatophagus argus*. *Gene* **2015**, *561*, 6–14. [CrossRef]
34. Ospina-Alvarez, N.; Piferrer, F. Temperature-dependent sex determination in fish revisited: Prevalence, a single sex ratio response pattern, and possible effects of climate change. *PLoS ONE* **2008**, *3*, 2837. [CrossRef]
35. Conover, D.; Kynard, B. Environmental sex determination: Interaction of temperature and genotype in a fish. *Science* **1981**, *213*, 31. [CrossRef] [PubMed]
36. Baroiller, J.F.; Guiguen, Y.; Fostier, A. Endocrine and environmental aspects of sex differentiation in fish. *Cell. Mol. Life Sci.* **1999**, *55*, 910–931. [CrossRef]

37. Tanaka, H.; Nakagawa, T.; Yokota, T.; Chimura, M.; Yamashita, Y.; Funamoto, T. Effects of spawning temperature on the reproductive characteristics of walleye pollock *Gadus chalcogrammus*. *Fish. Sci.* **2019**, *85*, 901–911. [CrossRef]
38. Roselli, C.E. Distribution and regulation of aromatase activity in the rat hypothalamus and limbic system. *Endocrinology* **1985**, *117*, 2471–2477. [CrossRef]
39. Gelinas, D.; Callard, G.V. Immunolocalization of aromatase- and androgen receptor-positive neurons in the goldfish brain. *Gen. Comp. Endocrinol.* **1997**, *106*, 155–168. [CrossRef] [PubMed]
40. Rodriguez-Mari, A.; Yan, Y.L.; Bremiller, R.A.; Wilson, C.; Canestro, C.; Postlethwait, J.H. Characterization and expression pattern of zebrafish Anti-Mullerian hormone (Amh) relative to *sox9a*, sox9b, and *cyp19a*1a, during gonad development. *Gene Expr. Patterns* **2005**, *5*, 655–667. [CrossRef] [PubMed]
41. Soria, F.N.; Strüssmann, C.A.; Miranda, L.A. High water temperatures impair the reproductive ability of the pejerrey fish *Odontesthes bonariensis*: Effects on the hypophyseal-gonadal axis. *Physiol. Biochem. Zool.* **2008**, *81*, 898–905. [CrossRef]
42. Anderson, K.; Pankhurst, N.W.; King, H.; Elizur, A. Effect of thermal challenge on the expression of genes involved in ovarian steroidogenesis in Tasmanian Atlantic salmon (*Salmo salar*). *Aquaculture* **2017**, *479*, 474–478. [CrossRef]
43. Anderson, K.; Swanson, P.; Pankhurst, N.; King, H.; Elizur, A. Effect of thermal challenge on plasma gonadotropin levels and ovarian steroidogenesis in female maiden and repeat spawning Tasmanian Atlantic salmon (*Salmo salar*). *Aquaculture* **2012**, *334–337*, 205–212. [CrossRef]
44. Luckenbach, J.A.; Early, L.W.; Rowe, A.H.; Borski, R.J.; Daniels, H.V.; Godwin, J. Aromatase cytochrome P450: Cloning, intron variation, and ontogeny of gene expression in southern flounder (*Paralichthys lethostigma*). *J. Exp. Zool. A* **2005**, *303*, 643–656. [CrossRef] [PubMed]
45. Matsuoka, M.P.; Nes, V.S.; Andersen, O.; Benfey, T.J.; Reith, M. Real-time PCR analysis of ovary- and brain-type aromatase gene expression during Atlantic halibut (*Hippoglossus hippoglossus*) development. *Comp. Biochem. Physiol. B* **2006**, *144*, 128–135. [CrossRef] [PubMed]